

Basic Light and Scanning Electron Microscopic Methods for Taxonomic Studies of Ciliated Protozoa

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SUMMARY

The following methods for taxonomic studies of ciliated protozoa are described in detail: live observation, supravital staining with methyl green-pyronin, dry silver nitrate impregnation, wet silver nitrate impregnation, silver carbonate impregnation, protargol impregnation (three procedures), and scanning electron microscopy. Familiarity with these methods (or modifications) is an absolute prerequisite for successful taxonomic work. No staining method is equally appropriate to all kinds of ciliates. A table is provided which indicates those procedures which work best for certain groups of ciliates. A second table relates to the structures revealed by the procedures. Good descriptions usually demand at least live observation, silver nitrate and protargol or silver carbonate impregnation. Some instructions are provided for distinguishing mono- and dikinetids as well as ciliated and non-ciliated basal bodies in silvered ciliates. The brilliancy of the silver preparations has unfortunately recently tempted some taxonomists to neglect live observation. However, many important species characters cannot be seen or are changed in silvered specimens. I thus consider all species descriptions based exclusively on silver slides as incomplete and of doubtful value for both α -taxonomists and ecologists. Especially the latter are usually not trained to correlate the silvered structures with the live appearance of the cell.

Introduction

The micrographs of various freshwater, marine and soil ciliates published by my colleagues and myself during the past two decades, most based on the few methods described here, have been widely accepted as being of a high standard. All methods are modifications of techniques which initially did not work well in our own laboratory, either because they were incompletely described or too complicated. This paper provides detailed step-by-step directions for the methods used in our laboratory. For other techniques the reader is referred to the literature cited and to the methodological guides by Kirby [25], Lee et al. [30] and Dragesco and Dragesco-Kernéis [9].

Structures Revealed by the Methods Described and Interpretation of Silver Stains

There is no single method which can reveal all details necessary for a good description of a ciliate. Likewise, no

staining method is equally appropriate to all kinds of ciliates. Good descriptions usually demand, as is evident from tables 1 and 2, at least live observation, silver nitrate and protargol or silver carbonate impregnation. The brilliancy of the silver preparations has unfortunately recently tempted some taxonomists to neglect live observation. However, many important species characters cannot be seen or are changed in silvered specimens such that species descriptions based exclusively on silver slides are incomplete and of doubtful value for α -taxonomists and ecologists. Especially the latter are usually not trained to correlate the silvered structures with the live appearance of the cell. Whenever it is possible, important and/or unusual details (e.g., species characters) should be documented not only by accurate drawings but also by photographs.

Several species (e.g., in the genera *Pseudoprorodon* and *Stentor*) persistently withstand our methods, their infraciliature and/or silverline system impregnates poorly or not at all. Further improvements of existing methods or new techniques should therefore be developed. Stimulating ideas can be found in the papers of J. Gelei [19, 20], G. Gelei [18] and Horváth [23].

Table 1. Structures revealed by the methods described

Structures revealed	live observation	methyl green-pyronin	dry silver nitrate	wet silver nitrate	silver carbonate	protargol	SEM
Shape (Figs. 2, 10, 13, 16, 21, 24, 28–32, 34)	++	–	+/-	++	–	+	++
Nuclear apparatus (Figs. 5, 12, 31, 32)	+	++	–	–	++	++	–
Contractile vacuole	++	–	–	–	–	–	–
Excretory pore, cytopyge (Figs. 5, 21, 22)	+/-	–	+/-	++	+/-	+/-	+
Colour	++	–	–	–	–	–	–
Mucocysts (Figs. 8, 14, 17)	+	++/-	+/-	+/-	++/-	+/-	+/-
Trichocysts, toxicysts (Fig. 37)	++	++/-	+/-	+/-	++/-	+/-	+/-
Cortical granules (Fig. 38) ¹	++/-	++/-	–	–	+/-	+/-	–
Infraciliature (basal bodies and cilia; Figs. 1–6, 10, 12, 15, 16, 18, 21–25, 28–36)	+/-	–	+	+	++	++	+
Silverline system (Figs. 7, 15, 19, 20, 23, 26, 35, 36)	–	–	++	++/-	–	–	–
Cortical fibres (Figs. 5, 6, 11, 12, 27) ²	–	–	–	–	++	++/-	–
Cytoplasmic fibres (e.g., myonemes, pharyngeal rods and fibres; Figs. 6, 12, 25)	+/-	–	–	–	++/-	++/-	–
Surface ornamentation (Figs. 9, 34)	+/-	–	–	–	–	–	++
Symbiotic algae	++	–	–	–	–	+/-	–

¹ “Cortical granules” include various extrusive (especially mucocysts or mucocyst-like extrusomes) and non-extrusive structures. They are most prominent and important (and often non-extrusive) in several hypotrich ciliates (Fig. 38).

² Two main types can be distinguished: those associated with the basal bodies (e.g., kinetodesmal fibres, transverse and postciliary microtubular ribbons; Figs. 5, 6, 25, 27) and those located in the cortical-cytoplasmic boundary (e.g., the “infraciliary lattice” in several peniculines and prorodontids; Fig. 11).

+ = good, ++ = excellent, +/-, ++/- = sometimes, i.e., depends on species, – = not revealed or poorly preserved.

The methods described here work not only with ciliates but also with many heterotrophic and autotrophic flagellated protists.

Usually, silver staining is undertaken to reveal the infraciliature (= ciliary pattern) and the silverline system (= lines revealed by *silver nitrate*, connecting basal bodies and other cortical organelles such as extrusomes and the cytopyge). Extrusomes, various structures associated with the basal bodies of the cilia (e.g., parasomal sacs, microtubular ribbons) and other cortical fibrillar networks (e.g., the infraciliary lattice) are sometimes also impregnated. This may render interpretation of silver stains difficult. It is beyond the scope of this paper to discuss this problem in detail; in fact, interpretation is different for each method (and even of slight variations!) and almost for each higher systematic category. Thus, I must refer the reader to some key-references [12, 14, 18, 19, 21, 22, 27, 38, 43, 50]. The differentiation of mono- and dikinetids must be mentioned because this is of basic importance for successful taxonomic work.

Cilia and their basal bodies may be arranged singly (monokinetids) or in pairs (dikinetids); the basal bodies may be ciliated or non-ciliated (e.g., [16]). All silver methods described here reveal at least the basal bodies (cilia are usually not impregnated with the dry and wet silver nitrate methods!), *but not each black or brown granule is a basal body of a cilium!* Depending on the

procedure used, other structures which have a similar size as the basal bodies are also impregnated (e.g., parasomal sacs, extrusomes). Thus, the arrangement and the state of the basal bodies cannot be unequivocally ascertained with silver methods and transmission electron microscopy is necessary (SEM is insufficient as it often does not reveal non-ciliated basal bodies!). The following procedure provides a rather reliable differentiation of mono- and dikinetids and of ciliated and non-ciliated basal bodies. (i) Study cells carefully *in vivo*, preferably with interference contrast. With some experience it is easy to see whether or not cilia are arranged in pairs. (ii) Overstain cells with protargol and/or silver carbonate. In overimpregnated cells cilia are usually clearly recognizable. (iii) Non-ciliated basal bodies and/or parasomal sacs are often slightly smaller than ciliated basal bodies. (iv) Study the electron microscopic literature related to the species or group of species under investigation and try to correlate the staining structures with ultrastructural features.

Methods

Observing Living Ciliates

Many physical and chemical methods have been described for retarding the movement of ciliates in order to observe structural details (e.g., [25, 32, 34, 42]). Chemical immobilization (e.g.,

Table 2. Methods for studying the infraciliature and the silverline system of certain groups of ciliates (systematics after Corliss [7])

Taxa	dry silver nitrate	wet silver nitrate	silver carbonate	protargol
Karyorelictida (e.g., <i>Trachelocerca</i> , <i>Loxodes</i> ; usually difficult to impregnate)	—	+/-	+	+
Archistomatina (e.g., <i>Alloiozona</i> , <i>Didesmis</i>)	?	+	+	+
Prostomatina (e.g., <i>Holophrya</i> , <i>Metacystis</i> , <i>Prorodon</i> , <i>Urotricha</i> , <i>Coleps</i> ; Fig. 11)	+	++	++	+
Haptorida (e.g., <i>Enchelys</i> , <i>Spathidium</i> , <i>Didinium</i> , <i>Dileptus</i> ; Figs. 2, 3, 9)	+/-	+	+	++
Pleurostomatida (e.g., <i>Amphileptus</i> , <i>Loxophyllum</i> ; Fig. 4)	+/-	—	+	++
Trichostomata (e.g., <i>Plagiopyla</i> , <i>Spirozoona</i> , <i>Balantidium</i> , <i>Paraisotricha</i> ; special method available [49])	+	+	+	+
Entodiniomorphida (e.g., <i>Ophryoscolex</i> , <i>Cycloposthium</i>)	?	+	+	+
Colpodids (e.g., <i>Colpoda</i> , <i>Woodruffia</i> , <i>Bursaria</i> , <i>Bryometopus</i> ; Figs. 13–18)	+	++	++	+
Synhymeniidae (e.g., <i>Nassulopsis</i> , <i>Chilodontopsis</i>)	+	++/-	++	+/-
Nassulina (e.g., <i>Nassula</i> , <i>Furgasonia</i> ; Figs. 5–8)	+/-	+	++	—
Microthoracina (e.g., <i>Microthorax</i> , <i>Leptopharynx</i>)	++	+	+/-	++
Cyrtophorida (e.g., <i>Chilodonella</i> , <i>Trochilia</i> ; Fig. 12)	++	+	+	++
Chonotrichida (e.g., <i>Spirochona</i> ; few data available)	—	+	?	++
Rhynchodida (e.g., <i>Ancistrocoma</i> , <i>Sphenophrya</i>)	++	+	?	+
Apostomatida (e.g., <i>Hyalophrysa</i> , <i>Ascophrys</i>)	?	+	?	+
Suctoria (adults; Fig. 37)	+	—	—	+
Suctoria (swarmers)	+	+	++	++
Tetrahymenina (e.g., <i>Tetrahymena</i> , <i>Colpidium</i> , <i>Glaucoma</i> ; Figs. 21–23)	++	++	++	++
Ophryoglenina (e.g., <i>Ophryoglena</i> , <i>Ichthyophthirius</i> ; Fig. 24)	+	+	++/-	+
Peniculina (e.g., <i>Paramecium</i> , <i>Urocentrum</i> , <i>Lembadion</i> ; Figs. 10, 26–28)	++	++	++	+/-
Scuticociliatida (e.g., <i>Uronema</i> , <i>Loxocephalus</i> , <i>Biggaria</i> , <i>Pleuronema</i> ; Figs. 25, 29)	+	++	+	++
Astomatida (e.g., <i>Anoplophrya</i> , <i>Hoplitophrya</i>)	++	+/-	+	+
Peritrichida (e.g., <i>Vorticella</i> , <i>Opercularia</i> , <i>Trichodina</i> ; Figs. 19, 20)	++	+/-	+	++
Heterotrichida (e.g., <i>Blepharisma</i> , <i>Metopus</i> , <i>Stentor</i> , <i>Folliculina</i>)	—	+	++	++
Odontostomatida (e.g., <i>Epalxella</i> , <i>Saprodinium</i>)	+	?	?	+
Oligotrichida (e.g., <i>Halteria</i> , <i>Strombidium</i> , <i>Tintinnidium</i>)	—	+/-	++/-	++
Hypotrichida (Euplotidae and Aspidiscidae excluded; Figs. 30–33, 38)	—	+/-	+/-	++
Hypotrichida (Euplotidae and Aspidiscidae; Figs. 34–36)	++	++	+/-	++

+ = recommended, ++ = highly recommended, +/-, ++/- = sometimes useful, depends on species, — = not recommended.

nickel sulfate) or physical slowing down by increasing the viscosity of the medium (e.g., methyl cellulose) are, in my experience, usually unsuitable. These procedures often change the shape of the cell or cause premortal alterations of various cell structures. The following simple method is therefore preferable: place about 0.5 ml of the raw sample on a slide and pick out (collect) the desired specimens with a micropipette under a compound microscope equipped with a low magnification (e.g., objective 4:1, ocular 10×). If specimens are large enough they can be picked out from a petri dish under a dissecting microscope. Working with micropipettes, the diameter of which must be adjusted to the size of the specimens, requires some training.

Transfer the collected specimens, which are now in a very small drop of fluid, onto a slide. Apply small dabs of vaseline (Petroleum jelly) to each of the four corners of a coverslip. Place this coverslip on the droplet containing the ciliates. Press on the vaselined corners with a mounted needle until ciliates are held firmly between slide and coverslip (Fig. 1A–D). As the pressure is increased the ciliates gradually become less mobile and more transparent. Hence, first the location of the main cell organelles (e.g., nuclear and oral apparatus, contractile vacuole) and then the details (e.g., extrusomes, micronucleus) can easily be observed under low (100–400×) and high (oil immersion objective) magnification.

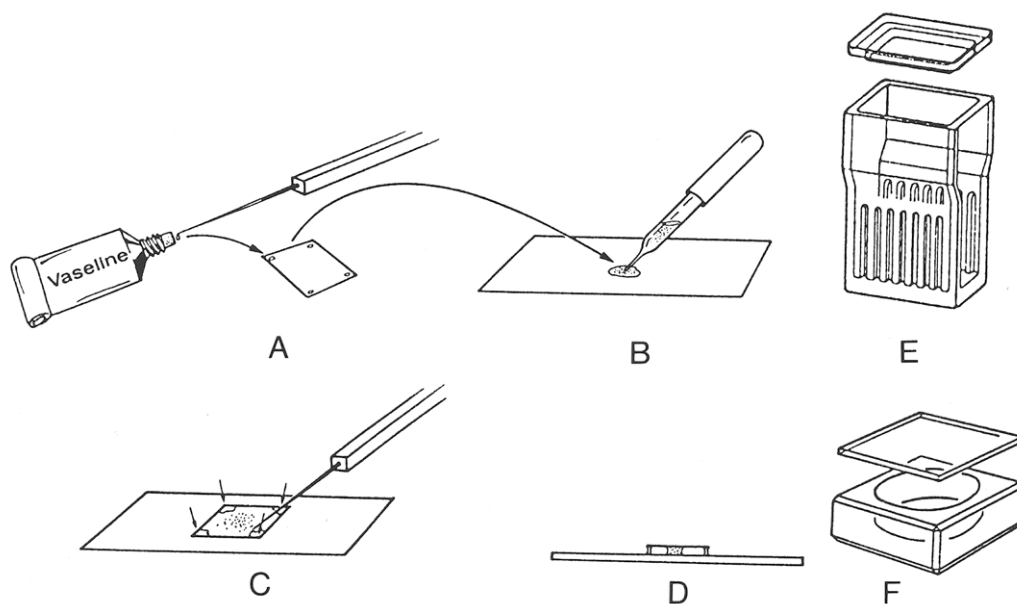


Fig. 1A–D. Preparation of slides for observing living ciliates (from [9]). – Fig. 1E. Staining jar for 8 and 16 (back to back) slides, respectively. – Fig. 1F. Watchglass for the protargol procedure according to Wilbert [46] and the cleaning of ciliates for scanning electron microscopy.

The shape of the cells is of course altered by this procedure. Therefore, specimens taken directly from the raw culture with a large-bore (opening ~ 1 mm) pipette must first be investigated under low magnification ($100\text{--}400\times$). Many species are too fragile to withstand handling with the micropipette and coverslip trapping without deterioration. Investigation with low magnification also requires some experience but it guarantees that undamaged cells are recorded. Video-microscopy is very useful at this point of investigation, especially for the registration of the swimming behaviour.

A compound microscope equipped with differential interference contrast is best for observing ciliates. If not available, use bright-field or phase-contrast; the latter is only satisfactory for very flat species.

Staining Procedures

Although there are numerous methods for staining ciliates, most of the older procedures (e.g., hematoxylin; see [25] for an excellent compilation of protocols) have been outdated by silver impregnation techniques and electron microscopy. Various silver stains are available, but all need some experience and are usually individually modified. However, familiarity with the four silver methods described below is an absolute prerequisite for the description of ciliates. These are thus described in great detail in order also to give even beginners a fair chance to obtain good slides.

Apart from silver impregnation, various other staining techniques are useful for taxonomic work with ciliates, especially the Feulgen nuclear reaction and supravital staining with methyl green-pyronin in order to reveal, respectively, the nuclear apparatus and the mucocysts.

Simple, viz. molecular formulae are given for the chemicals used, since usually only these are found in the catalogues of the suppliers (e.g., Merck). In a laboratory manual it is thus convincing to use this style too, instead of the more correct constitutional or structural formulae.

Four plates of selected micrographs should show the user examples of excellent preparations. There are two ways to do this: either to use a few species and show them treated with all methods described or to select many species to give the reader an impression of the diversity. I decided the second way as it is

possibly more convincing for beginners. However, two species (*Colpidium colpoda*, Figs. 21–23; *Paramecium caudatum*, Figs. 10, 26, 27) are shown prepared with three, and several others with two different methods.

I. Feulgen nuclear reaction. Descriptions of this method are to be found, e.g., in Lee et al. [30] and Dragesco and Dragesco-Kernéis [9]. The Feulgen reaction reveals the nuclear apparatus very selectively. I use it occasionally for α -taxonomic purposes because the nuclear apparatus usually stains well with protargol. As protargol often stains various small cytoplasmic inclusions, too, some caution is necessary, especially with multimicronucleate species. If in doubt, use the Feulgen reaction or an other nuclear staining method, such as that described by Larsen [29].

II. Supravital staining with methyl green-pyronin. This simple method was described by Foissner [13]. It is an excellent technique for revealing the mucocysts of most ciliates (those of tetrahymenids, however, usually do not stain). Mucocysts are stained deeply and very selectively blue or red, and can be observed in various stages of swelling because the cells are not killed instantly. The nuclear apparatus is also stained. Examples: Figs. 8, 17, 38.

Procedure (after Foissner [13])

1. Pick out desired ciliates with a micropipette and place the small drop of fluid in the centre of a slide.
2. Add an equally sized drop of methyl green-pyronin and mix the two drops gently by swivelling the slide.
Remarks: If ciliates were already mounted under the coverslip then add a drop of the dye at one edge of the coverslip and pass it through the preparation with a piece of filter paper placed at the other end of the coverslip.
3. Place a coverslip with vaselined corners on the preparation.
Remarks: Observe immediately. Cells die in the stain within 2 minutes. Mucocysts stain very quickly and many can be observed at various stages of swelling. To reveal the nuclear apparatus, cells should be fairly strongly squashed (= flattened). The preparation is temporary. After 5–10 minutes the cytoplasm often becomes heavily stained and obscures other details.

Reagents

1 g methyl green-pyronin (Merck)

ad 100 ml distilled water

This solution is very stable and can be used for years

III. The "dry" silver nitrate methods. Because of the numerous problems with the basic dry Klein [26, 28] technique, Foissner [11] and others (e.g., [20, 41]) introduced some improvements. The dry methods ("dry" because cells are air-dried and not chemically fixed before being treated with silver nitrate) provide preliminary information on the somatic and oral infraciliature (= ciliary pattern) and are often best for revealing the silverline system (= lines revealed by silver nitrate and connecting basal bodies and other cortical organelles such as extrusomes and the cytophyge). Although the results vary highly, the method is worthwhile because it is quick and often produces excellent preparations, which can be well documented since the cells are flattened during dehydration. Only cortical structures are revealed. Examples: Figs. 15, 19, 20, 23, 26, 35, 36.

Procedure (after Foissner [11] and recent experience)

1. Take 5–10 clean slides and spread a very thin layer of albumen over the middle third of each with a finger-tip. Dry for at least 1 minute.

Remarks: The egg-albumen (remove germinal disk! do not add glycerol) must have been kept open in a wide-necked flask for at least 20 hours; fresh albumen is often less satisfactory. It can be used for 2–3 days if the flask is subsequently sealed; do not, however, stir before use, but skim the albumen from the surface with a finger-tip. To facilitate spreading breathe on slide so that a film of condensation is produced on which the albumen can glide. The albumen layer must be very thin and uniform and should not cover cells.

2. Place a drop of fluid containing the ciliates on the albumized slide, spread with a needle (do not touch albumen layer!) and dry preparation at room temperature.

Remarks: Even single specimens can be placed on the albumized slide with a micropipette. If necessary enrich ciliates by gentle centrifugation or by leaving sample to settle for a few hours, after which time oxygen depletion induces many ciliates to move to the water surface. The amount and chemical composition of the fluid with which the ciliates are air-dried as well as temperature and humidity greatly influence the results. Therefore, 5–10 slides should usually be prepared simultaneously to vary these parameters, e.g. by washing cells with different amounts of distilled water or fresh culture medium. Washing cells with distilled water or spreading the drop to a very thin film is especially recommended with saline fluids, e.g. seawater, sewage, and soils. Temperature and humidity are easily varied using an ordinary hair-dryer.

3. Apply some drops of silver nitrate to the dried material for about 1 minute.

Remarks: Do not touch albumen layer with the pipette. The reaction time does not influence the results; a few seconds are adequate.

4. Wash slides for about 3 seconds under distilled water and re-dry.

Remarks: Wash gently! Apply water current from the top third of the tilted slide so that water runs gently over the dried material. Leave slides tilted during drying.

5. Pre-develop dried slide by exposing it for 5–60 seconds to a 40–60 watt electric bulb at a distance of 3–10 cm.

Remarks: Time and distance influence intensity of impregnation (see also next step).

6. Apply a few drops of developer to the dried preparation for about 30–60 seconds.

Remarks: The pre-development (step 5), the composition of the developer, and the material itself influence impregnation intensity, and quality. The best ratio of these parameters must be evaluated in pilot experiments. If the developer is well adjusted, the albumen around the dried fluid turns brown-black; if the developer is too strong, the albumen appears black (add some component A [see Reagents] and/or reduce pre-developing time); if the developer is too weak, the albumen appears brownish (add some components B and/or C and/or increase pre-development time).

7. Pour developer off slide, rinse gently in tap water for 5–10 seconds and immerse in fixative (sodium thiosulfate).

8. Remove slide from fixative, rinse gently in tap water for 5–10 seconds and immerse in 96–100% ethanol.

Remarks: Fixative must be thoroughly removed, otherwise crystals are formed in the alcohol and remain on the slide, causing the impregnation to fade with time. Do not wash too long and do not use distilled water, otherwise cells swell and eventually detach from the slide! Use ethanol as denaturated alcohol may contain substances which cause fading of preparations. Preparations usually fade within a few weeks when the silver nitrate is reduced only by sunlight or a UV-lamp.

9. Transfer slides to fresh 100% alcohol for 3 minutes and air-dry again. Mount in synthetic neutral mounting medium (e.g., Eukitt, Euparal).

Remarks: Slides should be tilted during drying. Mounting medium should be of medium viscosity. The preparation is stable.

Reagents

- a) Silver nitrate solution (long term stability in brown flask)

1 g silver nitrate (AgNO_3)

ad 100 ml distilled water

- b) Developer (stable for about 1–3 days; must be renewed as soon as it turns dark brown or shows crystals; mix components in the sequence indicated)

20 ml solution A

1 ml solution B

1 ml solution C

Solution A (this is an ordinary developer for negatives; dissolve ingredients in the sequence indicated; stable for years in brown bottle)

1000 ml hot tap water (about 40 °C)

10 g boric acid (H_3BO_3)

10 g borax ($\text{Na}_2\text{B}_4\text{O}_7$)

5 g hydroquinone ($\text{C}_6\text{H}_6\text{O}_2$)

100 g anhydrous sodium sulfite (Na_2SO_3)

2.5 g metol = methylamino-phenol-sulfate =
($\text{CH}_3\text{NHC}_6\text{H}_4\text{OH}$)₂ · H_2SO_4

Solution B (this is a concentrated photographic paper developer; dissolve ingredients in the sequence indicated; stable for about 6 months in brown bottle; soon turns brown [oxidizes], which, however, does not influence its activity)

100 ml distilled water

0.4 g metol = methylamino-phenol-sulfate =
($\text{CH}_3\text{NHC}_6\text{H}_4\text{OH}$)₂ · H_2SO_4

5.2 g anhydrous sodium sulfite (Na_2SO_3)

1.2 g hydroquinone ($\text{C}_6\text{H}_6\text{O}_2$)

10.4 g sodium carbonate (Na_2CO_3)

10.4 g potassium carbonate (K_2CO_3)

0.4 g potassium bromide (KBr)

Solution C (stable for several years)

10 g sodium hydroxide (NaOH)

ad 100 ml distilled water

- c) Fixative for impregnation (stable for several years)
 25 g sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$)
 ad 1000 ml distilled water

IV. The "wet" silver nitrate methods. The first wet ("wet" because cells are chemically fixed before being treated with silver nitrate) method was described by Chatton and Lwoff [4, 5]. The technique became well known after Corliss [6] published the version in use in the Paris laboratory of Fauré-Fremiet. It works well with many different kinds of ciliates, especially with hymenostomes (e.g., *Tetrahymena*, *Paramecium*, *Cyclidium*), prorodontids (e.g., *Prorodon*, *Urotricha*), most colpodids (e.g., *Colpoda*, *Bresslauides*) and some hypotrichs (e.g., *Euplotes*). Less convincing results are usually obtained with peritrichs (e.g., *Vorticella*), heterotrichs (e.g., *Spirostomum*, *Metopus*), oligotrichs (e.g., *Halteria*) and most hypotrichs (e.g., *Oxytricha*, *Urostyla*). The wet methods provide valuable information on the somatic and oral infraciliature as well as the silverlines, which are, however, often rather faintly stained. The shape of the cells is usually well preserved, which is of advantage to the investigation but makes photographic documentation difficult. As with the dry methods, only cortical structures are revealed. Examples: Figs. 7, 10, 16, 28, 29. Several modifications have been described (e.g., [3, 17, 31, 40]). Roberts and Caston [40] investigated the variables of this method in detail.

Procedure (after Corliss [6] and personal experience)

Several slides should be prepared simultaneously from the same material. If only few specimens are available, these must be handled with micropipettes during steps 1–7 (difficult task!); for ample material a centrifuge may be used. Until dehydration (step 15), keep all solutions cold (about 5 °C) as warming detaches the gelatin layer from the slide. The method is not simple and requires experience. Since some steps must be done very quickly it is necessary to be well organized.

1. If possible, concentrate ciliates by gentle centrifugation (the fixative is expensive) or collect individual ciliates and drop them into the fixative.
2. Drop ciliates into Champy's fluid and fix them for 1–30 minutes.
Remarks: The ratio of material to fixative should be at least 1:1, better 1:2. The fixation time apparently does not influence the results greatly. I usually fix for about 10 minutes. Fixation should be carried out in a fume hood since osmic acid fumes are highly toxic.
3. Remove fixative by centrifugation or micropipette and postfix in Da Fano's fluid for at least 5 minutes. Continue this replacement until the solution is the colour of Da Fano's fluid (2–3 times are usually enough).
Remarks: Material can be stored in Da Fano's fluid for years.
4. Place a very clean, grease-free slide on a hot-plate (35–45 °C).
Remarks: The slides must be absolutely grease-free (clean with alcohol and flame); even commercial pre-cleaned slides must be cleaned with an alcohol-moist cloth.
5. Place a small piece (about 2–4 mm in diameter) of gelatin in centre of the warmed slide and allow to melt.
Remarks: Gelatin should have been stored in the refrigerator for at least one week before use. Fresh gelatin often causes cloudy silver precipitates.
6. Quickly add an equal sized or smaller drop of concentrated specimens to the molten gelatin and remove slide from hot-plate.
Remarks: Mix organisms thoroughly into the gelatin using a mounted needle.

7. Quickly remove excess fluid under the dissecting microscope with a warmed micropipette until ciliates remain just nicely embedded in a thin gelatin layer.

Remarks: Steps 6 and 7 must be done quickly to avoid hardening and/or desiccation of the gelatin; if gelatin solidifies during the procedure return the slide to the hot-plate for a few seconds. Excess fluid can be removed only if ciliates are large. For small (< 100 µm) species it is more convenient to spread the drop over the slide until the gelatin layer has the appropriate thickness. If drop does not spread well the slide is not grease-free. The gelatin layer must be very thin to allow the silver nitrate to pass through. Material should be well concentrated. If too much Da Fano's fluid has been used or remains, precipitations develop or the gelatin detaches.

8. Immediately transfer slide to a cold, moist chamber (e.g., a covered petri dish with damp filter paper covering its bottom). Leave for about 5 minutes until gelatin has hardened.

Remarks: Gelatin must be hardened (check with the tip of mounted needle under dissecting microscope if in doubt) but must not desiccate and/or freeze. Desiccated or frozen slides are of poor quality. Harden gelatin in refrigerator or by placing the moist chamber on an ice block.

9. Flush slide in cold distilled water for 3–10 seconds.

Remarks: This step is essential and determines the quality and intensity of the impregnation. If the gelatin is washed too long, the impregnation may become too faint; if it is insufficiently washed coarse silver precipitations cover the gelatin. It is recommended that at least 4 slides, washed 3, 5, 7 and 10 seconds, respectively, be prepared.

10. Immediately transfer slide to cold silver nitrate solution for 30–60 minutes.

Remarks: Keep silver nitrate solution cold, as warming melts and detaches the gelatin from the slide. 30 minute impregnation usually suffices. Prolonged immersion intensifies impregnation only slightly and may cause darkening of cytoplasmic inclusions. Gelatin layer becomes slightly milky in the silver nitrate solution. A distinct milky coat indicates that too much Da Fano's fluid has been used and/or remained (step 9!).

11. Flush slide thoroughly with cold distilled water for 1–3 minutes.

12. Immediately submerge slide in 1–4 cm cold distilled water in a white-bottomed dish, usually a large petri dish lined with white paper is used. Irradiate for 10–30 minutes using sunlight or an ultraviolet source (< 254 nm) placed 10–30 cm above slides until gelatin turns golden brown.

Remarks: Tilt dish gently back and forth and change water after 2–3 minutes of irradiation to avoid silver precipitation. Take care that water remains cold, especially when reduction is performed with sunlight. Reduction with sunlight often produces clearer slides.

13. Check the intensity of impregnation after about 10 minutes of irradiation using a compound microscope. Continue irradiation for another 10–20 minutes if impregnation is still too faint.

Remarks: The infraciliature should stand out dark brown against the light brown coloured gelatin and the unstained cytoplasm. A rusty brown coloured gelatin indicates that too much Da Fano's fluid remained (step 9!).

14. Transfer slides to chilled 30% and then 70% alcohol (isopropanol or ethanol) for 10 minutes each.

Remarks: If necessary continue irradiation.

15. Complete dehydration by 2 transfers at least 10 minutes long through 100% alcohol (isopropanol or ethanol) at room temperature.

Remarks: Gelatin hardens, the alcohol need not be chilled.

Dehydrate thoroughly to avoid milky "water spots" in the mounted slides.

16. Clear by 2 transfers of at least 10 minutes through xylene.
Remarks: A prolonged stay in xylene (e.g., 2 days) sometimes produces extremely clear preparations.
17. Mount in synthetic neutral mounting medium.
Remarks: Do not dry slides between steps 16 and 17! Mounting medium should be rather viscous to avoid air-bubbles being formed when solvent evaporates during drying. If air-bubbles develop in the mounted and hardened slide, re-immersion in xylene for some days until the coverslip drops off. Remount using a more viscous medium and remove possible sand grains protruding from the gelatine. Usually, some air-bubbles are found immediately after mounting; these can be pushed to the edge of the coverslip with a finger or mounted needle. The preparation is stable.

Reagents

- a) Champy's fixative (prepare shortly before use; 9 ml of the fluid usually suffice for 1–2 fixations; use fume hood)
7 parts (3.5 ml) 1% aqueous chromic acid (CrO_3)
7 parts (3.5 ml) 3% aqueous potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$)
4 parts (2.0 ml) 2% aqueous osmium tetroxide (OsO_4)
- b) Da Fano's fluid (stable for several years; large amounts can thus be prepared)
900 ml distilled water (or sea-water, without additional NaCl, for marine ciliates)
10 g cobalt nitrate ($\text{Co}(\text{NO}_3)_2 \cdot 6 \text{H}_2\text{O}$)
100 ml formalin (HCHO ; commercial concentration, about 37%)
10 g sodium chloride (NaCl)
- c) Gelatin (may be used as long as not colonized by bacteria or fungi; fresh molten gelatin must be clear and yellowish in colour).
2 g powdered gelatine
0.005 g sodium chloride (NaCl)
20 ml distilled water
Mix these components and melt gelatin in a water bath, stirring frequently. Pour mixture into sterilized flask and store at least one week in refrigerator before use.
- d) Silver nitrate solution (may be used for several preparations, i.e. for about 40 slides if these are made on the same day; used solutions which are older than 1 day may cause problems)
3 g silver nitrate (AgNO_3)
ad 100 ml distilled water

The following materials must be prepared on the day preceding the preparation:

- a) Salinated gelatin
- b) Osmium tetroxide (takes about 10 hours to dissolve)
- c) Chill a moist chamber, a large petri dish (step 12), the silver nitrate solution, distilled water and alcohol (30%, 70%) in appropriate amounts.

V. *The silver carbonate methods.* Because of the large quantity of cells needed for the basic Fernández-Galiano [10] technique, Augustin et al. [2] proposed a modification which requires only few specimens and may also yield permanent slides. Although the results are highly variable with all modifications, the method is worthwhile because it works very quickly and often produces excellent impregnations, especially with hymenostomes (e.g., *Tetrahymena*, *Paramecium*), prorodontids (e.g., *Prorodon*, *Urotricha*), colpodids (e.g., *Colpoda*, *Bryometopus*, *Platyophrya*) and heterotrichs (e.g., *Stentor*). Fixation is by formalin, which means that the shape of the cells is poorly preserved and even

destroyed (cell bursts) in some species (e.g., most hypotrichs). The cells swell strongly during the preparation process but become very soft and are thus easily flattened between the slide and the coverslip. This makes photographic documentation easy but may result in interpretation errors. The silver carbonate methods reveal the infraciliature and certain cortical and cytoplasmic structures, especially the kinetodesmal fibres and the nuclear apparatus. Several other modifications have been suggested [48, 49]. The silverlines in most cases do not stain. Examples: Figs. 5, 6, 11, 18, 27.

Procedure (after Augustin, Foissner and Adam [2])

1. Place 1 droplet (about 0.05 ml) of a rich ciliate culture or even single specimens on a slide.
Remarks: Slide need not be grease-free. Its middle third should be delimited by lines drawn with a greasy finger-tip or a wax crayon to prevent solutions from spreading over the whole slide.
2. Add 1–2 drops of formalin (about 4%) and fix for 1–3 minutes. Mix organisms with formalin by circular motions of the slide.
Remarks: The duration of this step may greatly influence the results. Species with a firm pellicle (or resting cysts) usually need to be fixed longer (3 minutes or more) than those with a more fragile pellicle (1 minute or less). Some species cannot be fixed well with formalin and cells may even burst. For these fixation with osmium tetroxide vapours (place inverted slide with ciliates for about 1 minute over a 4% osmium tetroxide solution in a fume hood) is sometimes useful. Fix as usual with formalin after osmium treatment.
3. Add 1–3 drops of Fernández-Galiano's fluid to the fixed ciliates, without first washing out the formalin, and mix by circular motions of the slide for 10–60 seconds.
Remarks: The amount of Fernández-Galiano's fluid needed depends on many unpredictable factors (e.g., amount and concentration of fixative, size of drops, kind of species, composition of sample fluid). 1–3 drops usually work well. The same holds for the reaction time (10–60 seconds). The trial and error method must frequently be used to obtain best results!
4. Place slide on a pre-heated (60–80 °C) hot-plate and leave until the drop, which will be rather large, turns golden brown (like cognac). This usually takes 2–4 minutes and the slide must be kept in constant circular motion during this time. As soon as the drop appears cognac-coloured, check impregnation with the compound microscope. Replace the slide on the hot-plate if impregnation is still too faint; if it is already too dark repeat procedure, starting with step 1, but vary amount of Fernández-Galiano's solution and/or impregnation time etc.
Remarks: The correct impregnation time depends on many factors which are difficult to control (size of drops, temperature, kind of species ...). The amount of pyridine and silver carbonate in the Fernández-Galiano fluid is especially important. Add some drops of pyridine and/or silver carbonate solution to the Fernández-Galiano fluid if impregnation is repeatedly too faint, i.e. cannot be intensified by prolonged heating. Fix ciliates in 2–3 drops of formalin instead of 1–2 drops if impregnation is too faint. Ciliates from old cultures, ion-rich fluids (e.g., sewage, soil) or anaerobic biotopes frequently impregnate poorly. For these impregnation sometimes improves if they are washed prior to fixation (fluid from sample and distilled water 1:1).
5. Interrupt impregnation by removing the slide from the hot-plate and by adding 1 drop of fixative (sodium thiosulfate).
Remarks: The preparation is now ready. Augustin et al. [2] describe a method for obtaining permanent slides. Their

quality is, however, often not as good as with wet (fresh) preparations, which are thus usually preferred for investigation and photography. Pick out the well impregnated specimens with a micropipette, place them on a clean slide and cover with a coverslip. For good pictures the drop with the selected specimens should be very small so that cells are compressed between the slide and the coverslip. Excess fluid may be removed from the edge of the coverslip using a piece of filter paper. The impregnation need not be fixed with sodium thiosulfate if the investigation is undertaken immediately. The impregnation is stable for some hours when stored in a moist chamber. I recommend that the cells be compressed between the slide and coverslip immediately after the impregnation since silver precipitates may occur with time in the reaction fluid.

Reagents

- a) Fixative for organisms (stable for a long time)
0.1 ml formalin (HCHO; commercial concentration, about 37%)
ad 10 ml distilled water
- b) Fernández-Galiano's fluid (prepare immediately before use; components can be stored and must be mixed in the sequence indicated. The mixture must be slightly milky. If stored in brown flask it can be used for some hours. Keep away from sunlight. Make up a fresh fluid when no more impregnation can be achieved)
0.3 ml pyridine (C₅H₅N; commercial concentration)
2–4 ml Rio-Hortega ammoniacal silver carbonate solution
0.8 ml proteose-peptone solution
16 ml distilled water
- c) Fixative for impregnation (stable for several years)
2.5 g sodium thiosulfate (Na₂S₂O₃)
ad 100 ml distilled water
- d) Rio-Hortega ammoniacal silver carbonate solution. Preparation (the ratios are important!): 50 ml of 10% aqueous silver nitrate solution are placed in a flask; 150 ml of 5% aqueous sodium carbonate (Na₂CO₃) are added little by little under constant stirring; add 25% ammonia (NH₃), drop by drop, until the precipitate dissolves, being careful not to add an excess; finally add distilled water up to a total volume of 750 ml. The solution is stable for several years.
- e) Proteose-peptone solution (long term stability if not colonized by bacteria and/or fungi; discard dull solutions)
96 ml distilled water
4 g proteose-peptone (bacteriological; sprinkle powder on the surface of the water and allow to dissolve without stirring)
0.5 ml formalin (HCHO; for preservation)

VI. Protargol methods. Protargol methods are indispensable for descriptive research of ciliates. The first procedures were provided by Kirby [24], Moskowitz [36], Dragesco [8] and Tuffrau [44, 45] and many more modifications were subsequently proposed [1, 33, 35, 37, 46, 47, 50]. Here, the 3 variations which produce good results in our laboratory are described. These procedures work well with most ciliate species (some, however, only rarely impregnate well, e.g., *Loxodes*, *Paramecium*) but require at least 20 specimens. Contrary to the silver carbonate method, a single specimen cannot usually be handled successfully. Depending on the procedure used, protargol can reveal many cortical and internal structures, such as basal bodies, cilia, various fibrillar systems, nuclear apparatus. The silverlines, however, never impregnate. The shape of the cells is usually well preserved in permanent slides, which is an advantage for the investigation but makes photographic documentation more difficult. However, pictures as clear as those taken from wet silver carbonate

impregnations can be obtained with the Wilbert modification if the cells are photographed prior to embedding in the albumen glycerol.

Procedure A (after Foissner [15] and recent experience)

Most of our preparations are done using this procedure, which is described here for the first time in detail. The quality of the slides is usually adequate but frequently not as good as with the Wilbert modification. The latter demands more material and experience; inexperienced workers may easily lose all the material. As in all protargol methods, the procedure is rather time consuming and complicated. Experiments with students showed that beginners have a fair chance of obtaining good slides (Fig. 31). A centrifuge may be used for step 2; staining jars (Fig. 1E) are necessary for steps 6–16.

1. Fix organisms in Bouin's or Stieve's fluid for 10–30 minutes.

Remarks: The fixation time has little influence on the quality of the preparation within the limits given. Ratio fixative: sample fluid should be at least 2 : 1. Pour ciliates into fixative using a wide-necked flask in order to bring organisms in contact with the fixative as quickly as possible. Both fixatives work well but may provide different results with certain organisms. Stieve's fluid may be supplemented with some drops of 2% osmium tetroxide for better fixation of very fragile ciliates, e.g., the hypotrich *Urosoma*. This increases the stability of the cells but usually reduces their impregnability.

2. Concentrate by centrifugation and wash organisms 3–4 times in distilled water.

Remarks: There are now 2 choices: either to continue with step 3 or transfer the material through 30–50–70% alcohol into 70% alcohol (isopropanol or ethanol) where it remains stable for several years. Transfer preserved material back through the graded alcohol series into distilled water prior to continuing with the next step. Impregnation may be slightly modified in preserved material.

3. Clean 8 slides (or less if material is very scarce) per sample. The slides must be grease-free (clean with alcohol and flame).

Remarks: Insufficiently cleaned slides may cause the albumen to detach. Mark slides on back if several samples are prepared together. I use staining jars with 8 sections so that I can work with 16 slides simultaneously by putting them back to back (Fig. 1E).

4. Put 1 drop each of albumen-glycerol and concentrated organisms in the centre of a slide. Mix drops with a mounted needle and spread over the middle third.

Remarks: Use about equally sized drops of albumen-glycerol and suspended (in distilled water) organisms to facilitate spreading. The size of the drops should be adjusted so that the middle third of the slide is covered after spreading. Now remove sand, grains, etc. The thickness of the albumen layer should be equal to that of the organisms. Some thicker and thinner slides should, however, also be prepared because the thickness of the albumen layer greatly influences the quality of the preparation. Cells may dry out and/or shrink if the albumen layer is too thin; if it is too thick it may detach or the cells become impossible to study with the oil immersion objective.

5. Allow slides to dry for at least 12 hours (overnight) at room temperature.

Remarks: Slides may be allowed to dry for up to 24 hours but no longer if quality is to be maintained. Oven-dried (2 hours at 60 °C) slides are usually also of poorer quality.

6. Place slides in a staining jar (Fig. 1E) filled with 95% alcohol (isopropanol or ethanol) for 20–30 minutes. Place a staining jar with protargol solution into an oven (60°C).
Remarks: Slides should not be transferred through an alcohol series into concentrated alcohol as this causes the albumen layer to detach! Decrease hardening time to 20 minutes if albumen is already rather old and/or not very sticky.
7. Rehydrate slides through 70% alcohol and 2 distilled water steps for 5 minutes each.
8. Place slides in 0.2% potassium permanganate solution. Remove first slide (or pair of slides) after 60 seconds and the others at 15 second intervals. Collect slides in a staining jar filled with distilled water.
Remarks: Bleaching is by permanganate and oxalic acid (step 9). The procedure described above is necessary because each species has its optimum bleaching time. The sequence in which slides are treated should be recorded as the immersion time in oxalic acid must be proportional to that in the permanganate solution. The albumen layer containing the organisms should swell slightly in the permanganate solution and the surface should become uneven. If it remains smooth, the albumen is too sticky and this could decrease the quality of the impregnation. If the albumen swells strongly, it is possibly too weak (old) and liable to detach. Use fresh KMnO_4 solution for each series.
9. Quickly transfer slides to 2.5% oxalic acid. Remove first slide (or pair of slides) after 160 seconds, the others at 20 second intervals. Collect slides in a staining jar filled with distilled water.
Remarks: Same as for step 8! Albumen layer becomes smooth in oxalic acid.
10. Wash slides 3 times in distilled water for 3 minutes each.
11. Place slides in warm (60°C) protargol solution and impregnate for 10–15 minutes at 60°C.
Remarks: Protargol solution can be used only once.
12. Remove staining jar with the slides from the oven and allow to cool for 10 minutes at room temperature.
Remarks: In the meantime organize 6 staining jars for developing the slides: distilled water – distilled water – fixative (sodium thiosulfate) – distilled water – 70% alcohol – 100% alcohol (isopropanol or ethanol).
13. Remove the first slide from the protargol solution and drop some developer on the layer of albumen. Move slide gently to spread developer evenly. As soon as the albumen turns yellowish, pour off the developer, dip slide into the first 2 distilled water steps for about 2 seconds each and stop development by submerging the slide in the fixative (sodium thiosulfate), where it can be left for 5–10 minutes.
Remarks: Now control impregnation with the compound microscope. The impregnation intensity is sufficient if the infraciliature is just recognizable. The permanent slide will be too dark if the infraciliature is distinct at this stage of the procedure! The intensity of the impregnation can be controlled by the concentration of the developer and the time of development. 5–10 seconds usually suffice for the diluted developer! Some species (e.g., most microthoracids) must be treated with undiluted developer. Development time increases with bleaching time. Therefore commence developing with those slides which were in the bleaching solutions for 60 and 120 seconds, respectively. The thinner the albumen layer, the quicker the development.
14. Collect slides in the fixative (sodium thiosulfate) and transfer to distilled water for about 5 minutes.
Remarks: Do not wash too long; the albumen layer is very fragile and detaches easily!
15. Transfer slides to 70%–100% alcohol for 5 minutes each.
16. Clear by two 10 minute transfers through xylene.
17. Mount in synthetic neutral mounting medium.
Remarks: Same as for step 17 of the wet silver nitrate method!

Reagents

- a) Bouin's fluid (prepare immediately before use; components can be stored)
 - 15 parts saturated, aqueous picric acid ($\text{C}_6\text{H}_3\text{N}_3\text{O}_7$; preparation: add an excess of picric crystals to, e.g., 1 litre of distilled water; shake solution several times within a week; some undissolved crystals should remain; filter before use)
 - 5 parts formalin (HCHO ; commercial concentration, about 37%)
 - 1 part glacial acetic acid (= concentrated acetic acid; $\text{C}_2\text{H}_4\text{O}_2$)
- b) Stieve's fluid (slightly modified; prepare immediately before use; components can be stored)
 - 38 ml saturated, aqueous mercuric chloride (dissolve 60 g HgCl_2 in 1 litre of boiling distilled water)
 - 10 ml formalin (HCHO ; commercial concentration, about 37%)
 - 3 ml glacial acetic acid (= concentrated acetic acid; $\text{C}_2\text{H}_4\text{O}_2$)
- c) Albumen-glycerol (2–4 month stability)
 - 15 ml egg albumen
 - 15 ml concentrated (98%–100%) glycerol ($\text{C}_3\text{H}_8\text{O}_3$)

Pre-treatment of the egg albumen and preparation of the albumen-glycerol: Separate the white carefully from the yolk and embryo of 3 eggs (free range eggs are preferable to those from battery chickens, whose egg white is less stable and sticky). Shake the white by hand (do not use a mixer!) for some minutes in a narrow-mouthed 250 ml Erlenmeyer flask until a stiff white foam is formed. Allow the flask to stand for about 1 minute. Pour the viscous rest of the egg white in a second Erlenmeyer flask and shake again until it is stiff. Repeat until most of the egg white is either stiff or becomes watery; usually 4–6 Erlenmeyer flasks of foam are obtained. Leave all flasks undisturbed for about 10 minutes. During this time a glycerol-like fluid percolates from the foam. This fluid and the white from the last flask are collected and used. Add an equal volume of concentrated glycerol and a small thymol crystal ($\text{C}_{10}\text{H}_{14}\text{O}$) for preservation to the mixture. Mix by shaking gently and pour mixture into a small flask. Leave undisturbed for 2 weeks. A whitish slime settles at the bottom of the flask. Decant the clear portion, discard slime and thymol crystal. A "good" albumen-glycerol drags a short thread when touched with a needle. The albumen is too thin (not sticky enough) or too old if this thread is not formed. Fresh albumen which is too thin may be concentrated by leaving it open for some weeks so that water can evaporate. If the albumen is too sticky, which may cause only one side of the organisms to impregnate well, it is diluted with distilled water or old, less sticky albumen to the appropriate consistency. The preparation of the albumen-glycerol must be undertaken with great care because much depends on its quality. Unfortunately, all commercial products which I have tried detach during impregnation.
- d) 0.2% potassium permanganate solution (stable for about 1 day)
 - 0.2 g potassium permanganate (KMnO_4)
 - ad 100 ml distilled water
- e) 2.5% oxalic acid solution (stable for about 1 day)
 - 2.5 g oxalic acid ($\text{C}_2\text{H}_2\text{O}_4 \cdot 2 \text{H}_2\text{O}$)
 - ad 100 ml distilled water

- f) 0.4% protargol solution (stable for about 1 day)
100 ml distilled water
ad 0.4 g protargol
Remarks: Use light-brown "protargol for microscopy" (e.g., Merck's "Albumosesilber für die Mikroskopie" or "Proteinate d'Argent", Roques, Paris, France). Some dark-brown, cheaper products do not work! Sprinkle powder on the surface of the water and allow to dissolve without stirring; use a wide-mouthed bottle for solving the protargol.
- g) Developer (mix in sequence indicated; sodium sulfite must be dissolved before hydroquinone is added)
95 ml distilled water
5 g sodium sulfite (Na_2SO_3)
1 g hydroquinone ($\text{C}_6\text{H}_6\text{O}_2$)
Remarks: This recipe yields the stock solution which is stable for some weeks and should be used undiluted for certain ciliates (step 13). Usually, however, it must be diluted with tap water in a ratio of 1:20 to 1:50 to avoid too rapid development and one-sided impregnation of the organisms. Freshly prepared developer is usually inadequate (the albumen turns greenish instead of brownish). The developer should thus be prepared from equal parts of fresh and old (brown) stock solutions. Take great care with the developer as its quality contributes highly to that of the slides. If the developer has lost its activity (which is not always indicated by a brown colour!) the silver is not or only insufficiently reduced and the organisms stain too faintly. A fresh developer should therefore be prepared for each "impregnation week" and some old developer kept. Fresh developer can be artificially aged by adding some sodium carbonate (Na_2CO_3). However, better results are obtained with air-aged solutions, i.e., by a developer which has been kept uncovered for some days in a wide-mouthed bottle. It first turns yellowish, then light brown (most effective) and later dark brown and viscous (at this stage the developer has lost most of its activity but is still suitable for artificial aging of fresh developer = 1:1 mixture mentioned above).
- h) Fixative for impregnation (stable for several years)
25 g sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{ H}_2\text{O}$)
ad 100 ml distilled water

Procedure B (after Wilbert [46] and personal experience)

This modification produces excellent results but demands much experience. I manipulate large cells with micropipettes in a watch-glass (Fig. 1F), whereas the centrifuge is used for steps 1–4, 7, 8 if cells are smaller than about 150 μm . The watch-glass method is used when there are only a few specimens of larger cells; thus an attempt is worthwhile even if only 20 cells are available. The organisms are very soft after development and fixation and are thus easily compressed between slide and coverslip, which greatly facilitates photographic documentation. Examples: Figs. 12, 22, 25, 32, 33.

1. Fix organisms as described in protargol procedure A (Foissner's modification).
2. Wash and, if so desired, preserve organisms as described in protargol method A (Foissner's modification).
Remarks: Wash cells either in the centrifuge (small species) or in a watch-glass (Fig. 1F). To change fluids allow cells to settle on bottom of watch-glass and remove supernatant with a micropipette under the dissecting microscope; concentrate cells in the centre of watch-glass by gentle swirling.
3. Transfer organisms with a small amount of distilled water to an at least tenfold quantity of sodium hypochlorite (NaClO) and bleach for about 3 minutes.
Remarks: This is the critical step in this modification. If bleaching is too strong or too weak all is lost: cells either

dissolve or do not impregnate well. Systematic investigations showed that not the bleaching time but the amount of active chloride in the sodium hypochlorite and the pre-treatment of the cells (fixation method, fresh or preserved material) are decisive for the quality of the preparation. Different species need different concentrations. Unfortunately, the concentration of active chloride in the commercial products varies (10–13%) and is dependent on the age of the fluid. It is thus impossible to provide more than only a few guidelines: 100 ml distilled water + 0.2–0.4 ml NaClO (if product is fresh and cells were not stored in alcohol) or 100 ml distilled water + 0.5–1.6 ml NaClO (if product is older and cells were stored in alcohol). The transparency of the cells under the dissecting microscope may serve as a further indicator: fixed, unbleached cells appear dark and opaque, whereas accurately bleached cells are almost colourless and rather transparent (depends, however, also on size and thickness of the cell). Thus, increase the concentration of sodium hypochlorite stepwise if cells appear too dark with the recommended concentrations. We routinely start with 3 different hypochlorite concentrations if enough material is available.

4. Wash organisms at least 3 times with distilled water and finally once in the protargol solution.
Remarks: Wash thoroughly, especially when fluids are changed with micropipettes, because even the slightest traces of the sodium hypochlorite disturb the impregnation.
5. Transfer to 1% protargol solution and impregnate for 10–20 minutes at 60 °C.
Remarks: This and the next step should be carried out in a watch-glass even for material which is otherwise manipulated with the centrifuge. The impregnation time depends on the kind of material and the degree of bleaching. Check the progress of impregnation every 3–4 minutes under the compound microscope by picking out a few cells with the micropipette under the dissecting microscope; add these to 1 drop of developer. Dilute developer and/or interrupt development by adding a little fixative (sodium thiosulfate) if impregnation is strong enough.
6. Remove most of the protargol solution with a micropipette and add some drops of developer to the remainder containing the organisms.
Remarks: Fresh, undiluted developer is usually used (but see step 5). Control development in compound or dissecting microscope. As soon as the infraciliature becomes faintly visible, development must be stopped by adding a few drops of sodium thiosulfate. Judging the right moment is a question of experience; the permanent slide will be too dark if the infraciliature is very distinct at this stage of the procedure!
7. Stabilize the impregnation by 2 approximately 5 minute transfers through sodium thiosulfate.
Remarks: The developer need not be removed before fixation. For small species this and the next step can be carried out in a centrifuge. Larger species must be manipulated with micropipettes because cells become very fragile and would be damaged in a centrifuge. Cells are very soft at this stage and can thus be easily compressed and photographed. Transfer some of the more darkly impregnated specimens with a very small amount of the fixative onto a clean slide using a micropipette and cover with a coverslip. Organisms are usually flattened by the weight of the coverslip; excess fluid may be removed from the edge of the coverslip with a piece of filter paper.
8. Wash very thoroughly in distilled water (3 times with the centrifuge; 7–10 times in watch-glass with micropipettes). Finally remove as much of the water as possible.
Remarks: Even the slightest traces of the fixative destroy the impregnation within a few days or weeks.

9. Smear a moderately thick layer of albumen-glycerol on a clean slide with a finger. Drop impregnated, washed cells on the albumized slide with a large-bore pipette (opening ~ 1 mm) and dry preparation for at least 2 hours.
Remarks: The cells are too fragile to be spread with a needle. With much care it is possible to orientate cells using a mounted eyelash. Commercial albumen-glycerol can be used.
10. Harden albumen by two 10 minute transfers through concentrated alcohol (isopropanol or ethanol).
Remarks: This and the next step are best carried out in staining jars. The albumen layer turns milky and opaque.
11. Clear by two 5 minute transfers through xylene.
Remarks: The albumen layer turns transparent.
12. Mount in synthetic neutral mounting medium.
Remarks: Same as for step 17 of the wet silver nitrate method!

Reagents

If not stated otherwise, the same reagents like in the first protargol procedure (Foissner's modification) are to be used.

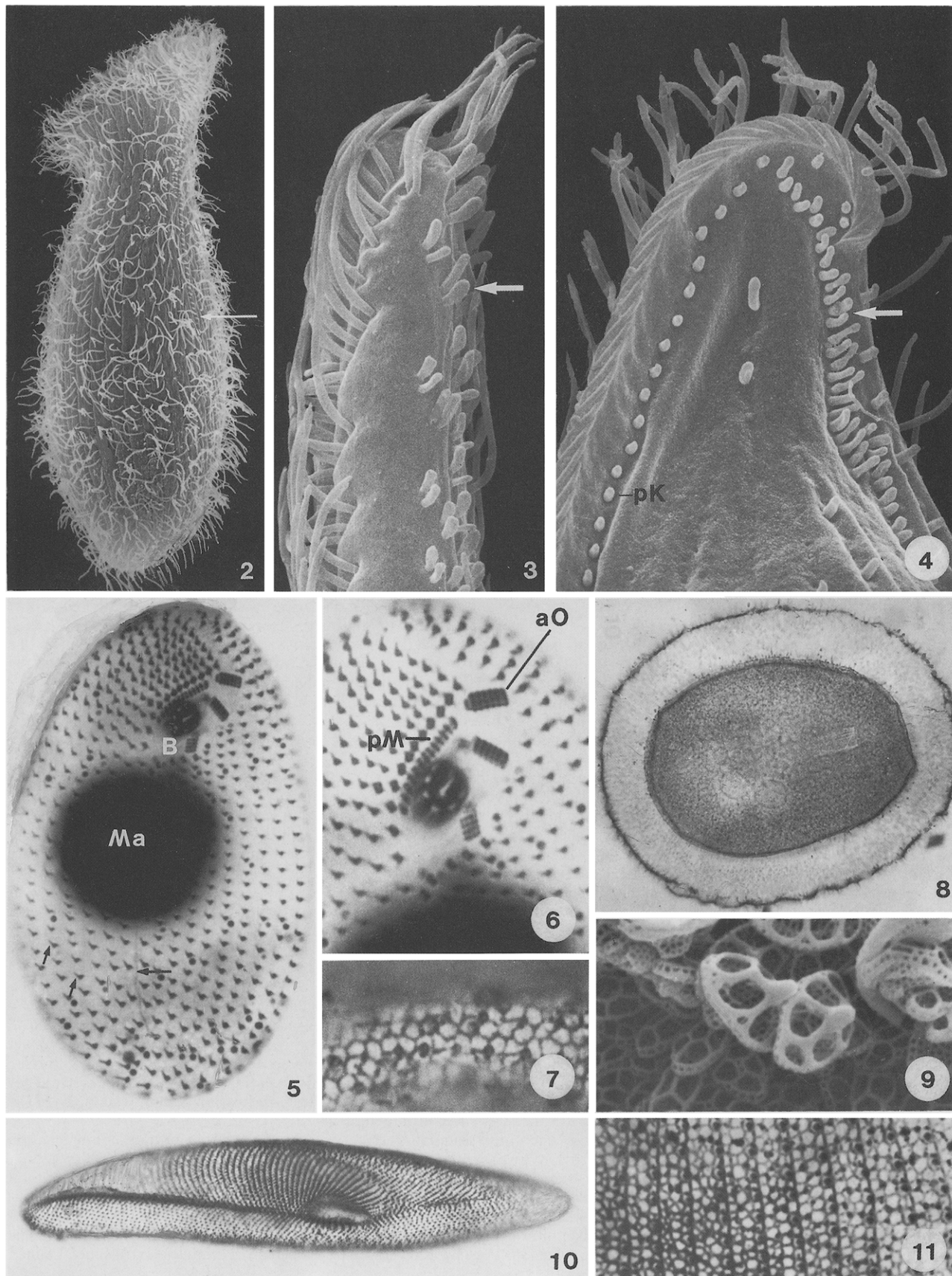
Procedure C for few specimens

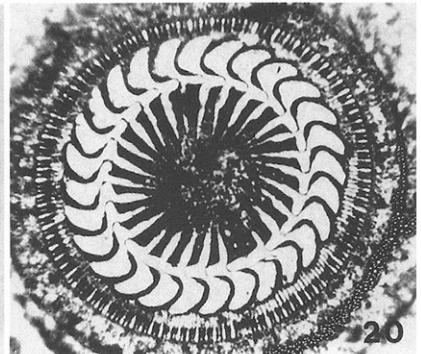
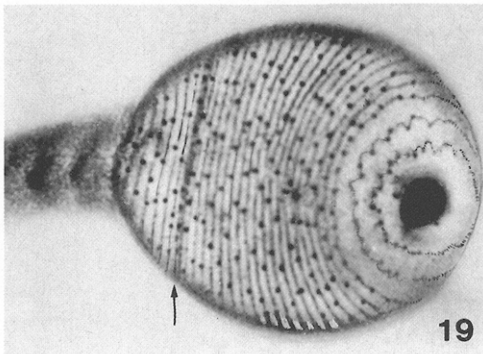
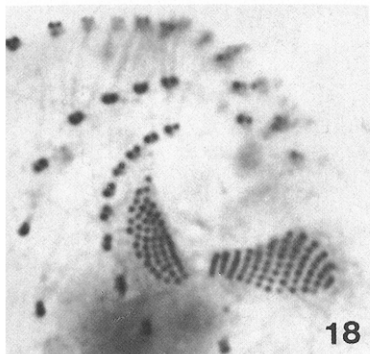
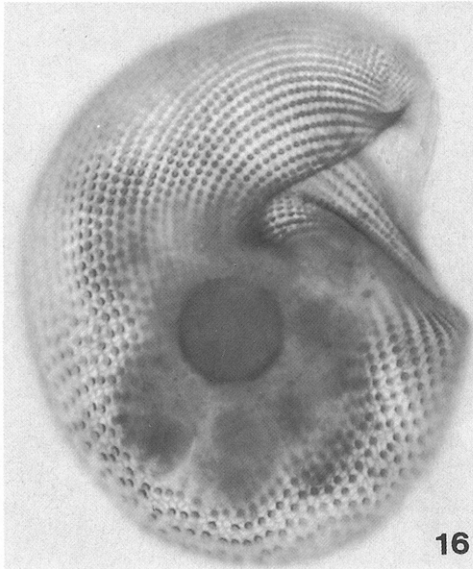
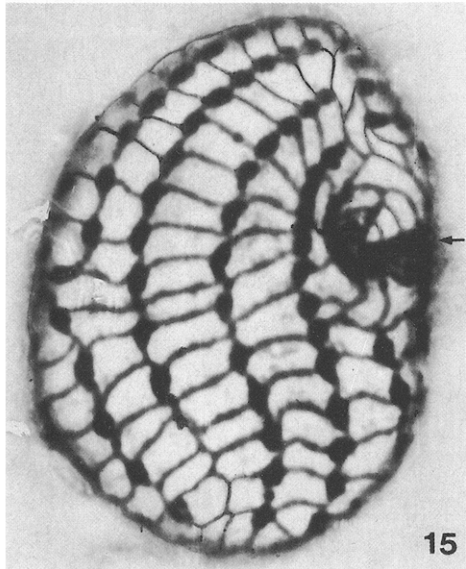
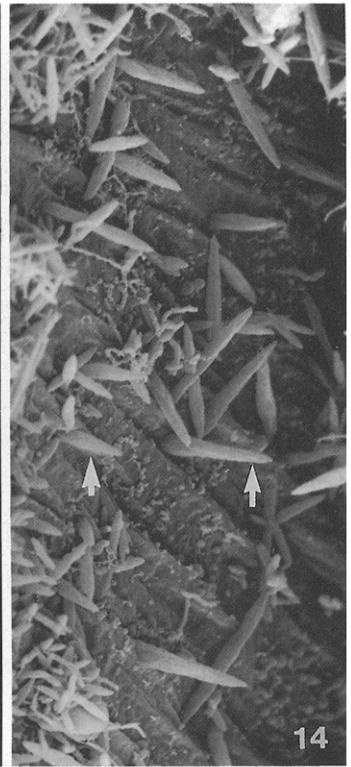
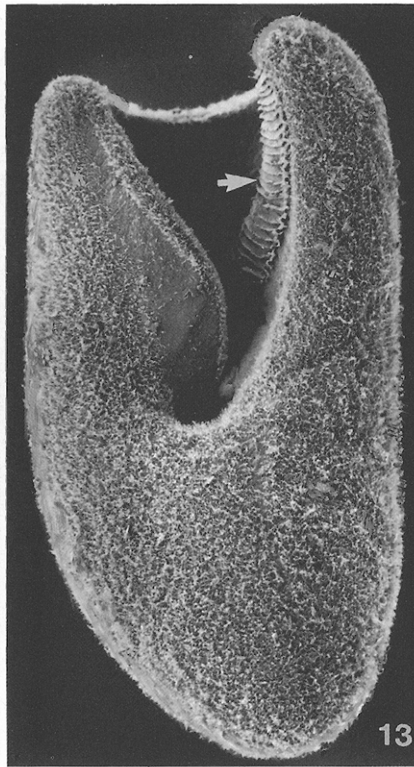
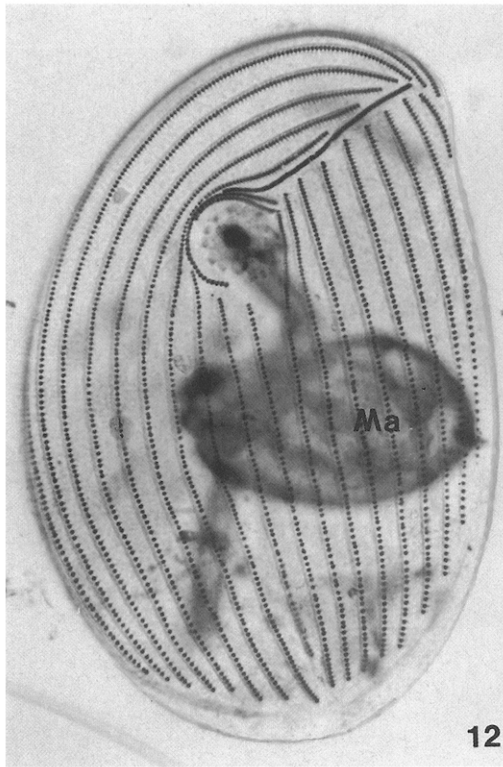
I learned this simple modification in Dr. P. Didier's laboratory (Clermont-Ferrand University). It sometimes produces excellent impregnations, especially with species having a firm pellicle (e.g., microthoracids). It also demands little material because the specimens are mounted on the slide without washing.

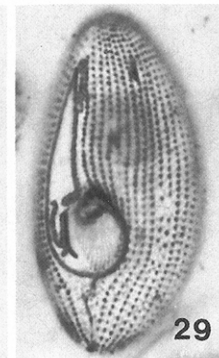
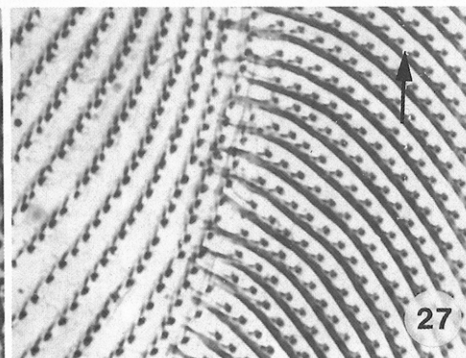
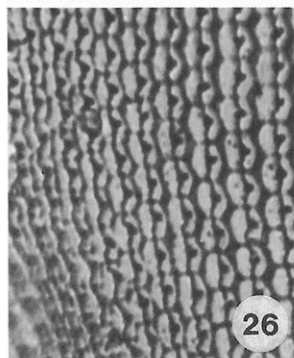
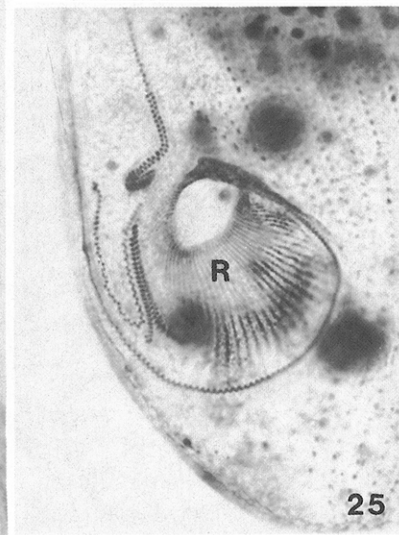
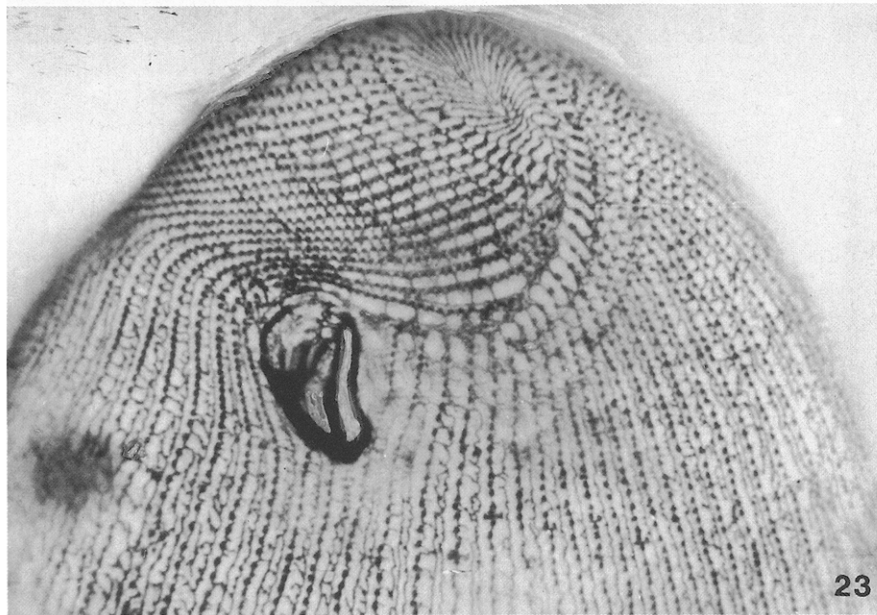
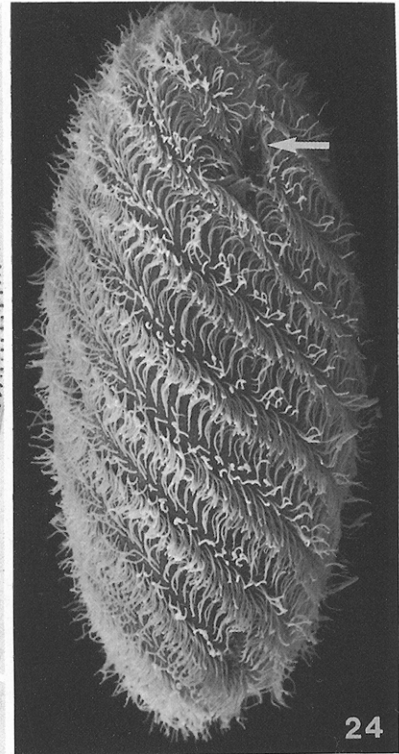
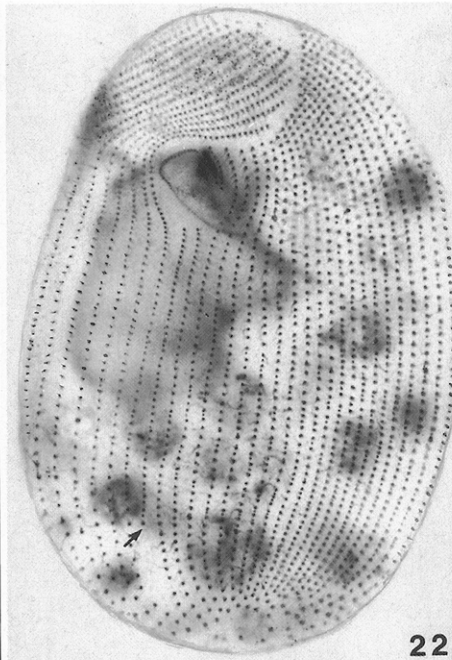
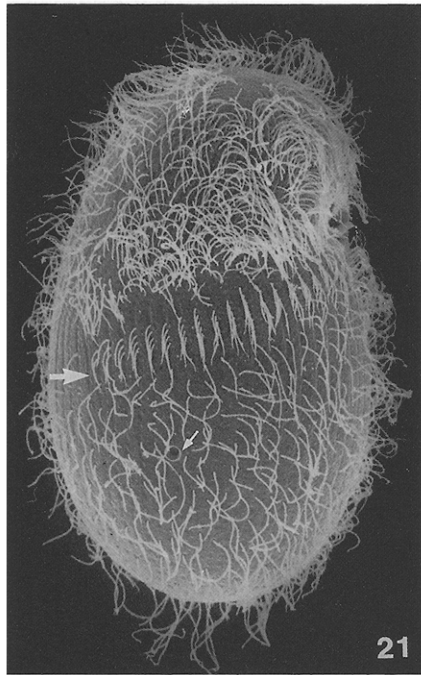
1. Collect specimens with a micropipette and place them at the centre of a grease-free slide. Remove excess fluid as far as possible.
2. Fumigate cells with 4% aqueous osmium tetroxide for about 2 minutes.
Remarks: Hold inverted slide close to the osmium tetroxide. Carry out procedure in a fume hood as osmic acid fumes are highly toxic.
3. Add an equal sized drop of albumen-glycerol, mix thoroughly but gently with a mounted needle and spread mixture in a moderately thin layer over the middle third of the slide.
Remarks: Albumen-glycerol must be prepared as described in the first protargol procedure (Foissner's modification). Cells are very fragile and frequently break or dissolve.
4. Allow to dry for about 4 hours.

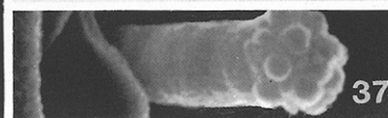
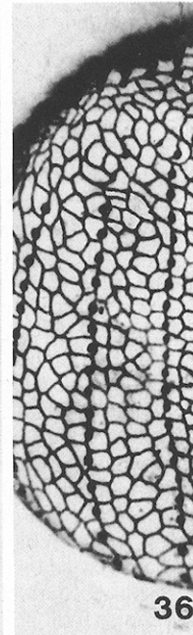
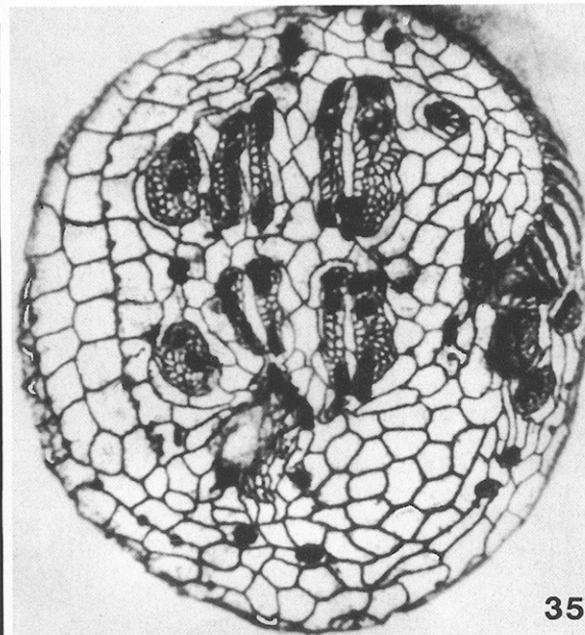
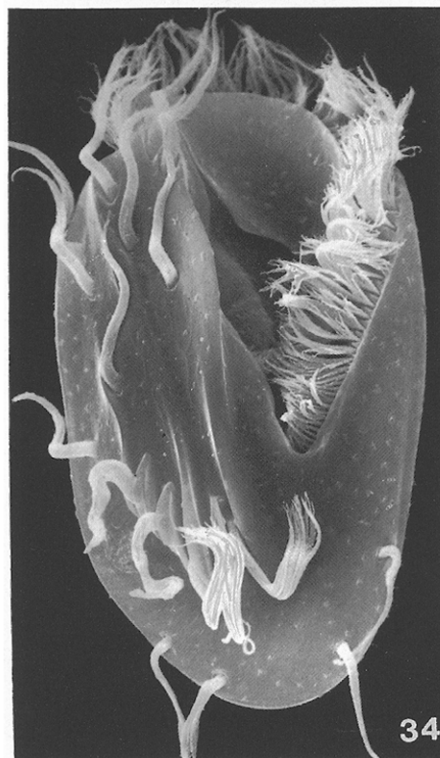
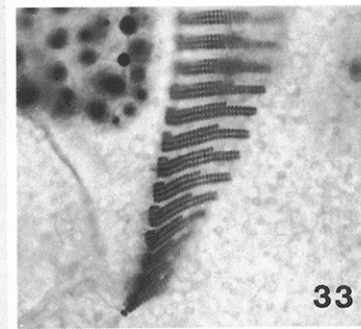
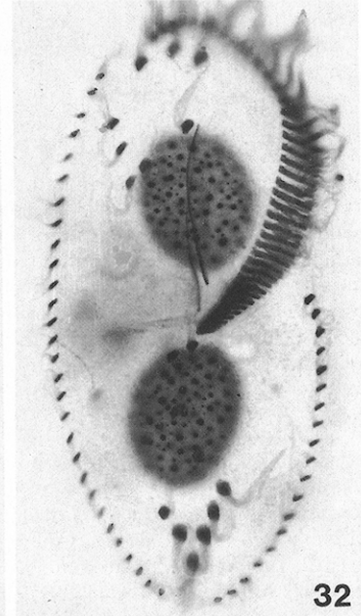
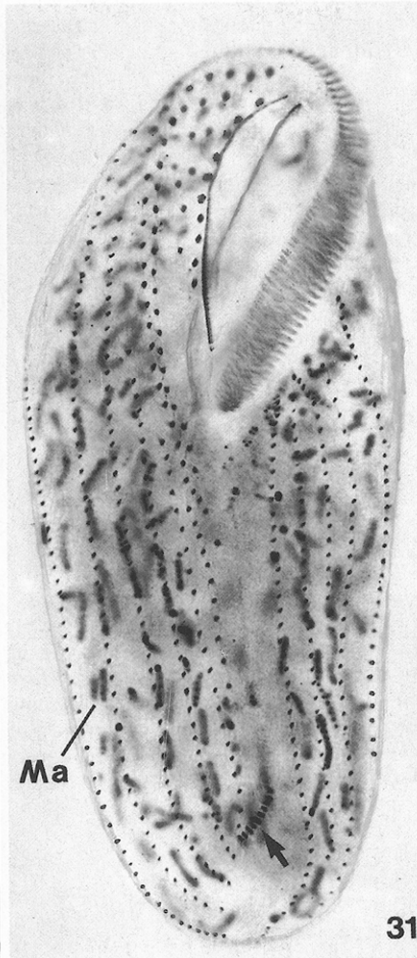
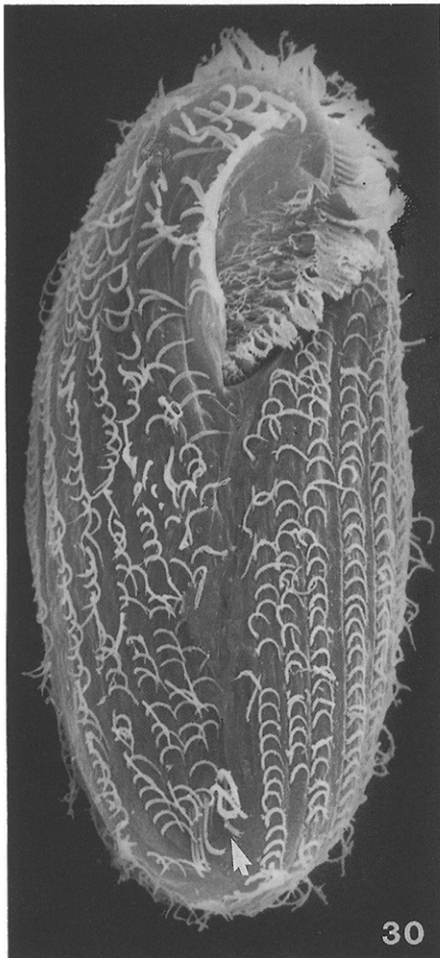
Figs. 2–38. A collection of ciliates prepared with the methods described in this article. Some micrographs have already been published (references not given to save space). ▶

Fig. 2. *Epispathidium amphoriforme*, left lateral view in the scanning electron microscope. Arrow marks a long row of short cilia belonging to the dorsal brush. – Figs. 3, 4. *Dileptus mucronatus* and *Litonotus lamella*, left lateral views of distal portion of proboscis. The specialized cilia of the brush (arrows), the perioral kinety (pK) and several other details can be revealed with such clarity only by the scanning electron microscope. The figures base on raw material cleaned with the method described. – Figs. 5–7. *Furgasonia blochmanni*, infraciliature of ventral side after silver carbonate (5, 6) and silverline system after wet silver nitrate impregnation (7). The oral basket (B), the paroral membrane (pM), the adoral organelles (aO), the macronucleus (Ma), the cytophyge (large arrow) and the inclined kinetodesmal fibres (small arrows) are revealed with high clarity. The silverline system consists of small, polygonal meshes. – Fig. 8. *Obertruria gracilis* after supravital staining with methyl green-pyronin. The mucocysts form a distinct envelope. – Fig. 9. *Lepidotrachelophyllum* sp., epipellicular scales in the scanning electron microscope. – Fig. 10. *Paramecium caudatum*, infraciliature of ventral side after wet silver nitrate impregnation. The shape of the cell is perfectly preserved. – Fig. 11. *Prorodon viridis*, argyrophilic lattice after silver carbonate impregnation. – Fig. 12. *Trithigmostoma cucullulus*, infraciliature of ventral side after protargol (Wilbert's procedure) impregnation. Ma = macronucleus. – Figs. 13, 14, 17. *Bursaria truncatella*, ventral view (13) and dorsal vestibular wall with emerging mucocysts (14; arrows) in the scanning electron microscope. Extruded mucocysts stain distinctly with methyl green-pyronin (17). Arrow in Fig. 13 marks distal portion of large zone of adoral organelles. – Figs. 15, 18. *Colpoda steinii*, silverline system after dry silver nitrate impregnation (15). Arrow marks oral polykinetids whose details are clearly revealed by silver carbonate impregnation (18); their location is, however, slightly distorted since the cell has been squashed to reveal these fine details. – Fig. 16. *Bresslauides australis* after wet silver nitrate impregnation. The shape of the cell and of the huge vestibulum are perfectly preserved. – Fig. 19. *Vorticella extensa*, silverline system after dry silver nitrate impregnation. Arrow marks anlage of aboral ciliary wreath. – Fig. 20. *Trichodina pediculus*. The fine details of the holdfast organelle can be revealed with such high clarity only by the dry silver nitrate method. – Figs. 21–23. *Colpidium colpoda* in the scanning electron microscope (21), after protargol (22; Wilbert's procedure) and dry silver nitrate (23) impregnation. Figure 21 is a right lateral view showing a ribbon of short cilia (large arrow) and slightly elongated caudal cilia. The small arrow marks the excretory pore of the contractile vacuole. Figure 22 shows the infraciliature of the ventral side. Arrow marks cytophyge at end of right postoral kinety. Figure 23 shows the silverline system of the anterior ventral portion. – Fig. 24. *Ophryoglena* sp., ventral view. The metachronal ciliary waves can be revealed with such clarity only by the scanning electron microscope. Arrow marks buccal opening. – Figs. 25, 29. *Pleuronema coronatum* after protargol (25; Wilbert's procedure) and wet silver nitrate (29) impregnation. Figure 25 shows the proximal portion of the oral apparatus where even single basal bodies within the adoral membranelles and the oral ribs (R) are recognizable. – Figs. 26, 27. *Paramecium caudatum* (cf. Fig. 10), silverline system after dry silver nitrate impregnation (26) and kinetodesmal system (arrow) of ventral side after silver carbonate impregnation (27). – Fig. 28. *Urocentrum turbo*, infraciliature of ventral side after wet silver nitrate impregnation. – Figs. 30, 31. *Urostyla grandis*, infraciliature of ventral side in the scanning electron microscope and after protargol impregnation (Foissner's procedure). Ma = macronuclear segments. Arrows mark transversal cirri. – Figs. 32, 33. *Stylonychia pustulata*, infraciliature of ventral side and nuclear apparatus after protargol impregnation (Wilbert's procedure). The details of the adoral membranelles are clearly recognizable in Fig. 33. – Fig. 34. *Euplotes patella*, ventral view in the scanning electron microscope. – Fig. 35. *Euplotes moebiusi*, silverline system of ventral side in an early divider after dry silver nitrate impregnation. – Fig. 36. *Euplotes muscicola*, silverline system of dorsal side after dry silver nitrate impregnation. – Fig. 37. *Prodiscophrya collini*, distal end of tentacle with haptocysts in the scanning electron microscope. – Fig. 38. *Australothrix australis*, subpellicular granules (extrusomes) stained with methyl green-pyronin.









- Proceed with steps 6 (coagulation of albumen in concentrated alcohol) to 17 of the first protargol procedure (Foissner's modification). Bleaching times are usually about 50% shorter than with my modification.

Preparation for Scanning Electron Microscopy (SEM)

Ciliate species cannot usually be identified solely by scanning electron microscopy because only a limited number of characters is revealed. However, SEM is useful for the beginner by allowing a three-dimensional view of the object and for the specialist in documenting details which are difficult to reveal with other methods. Only the method used by ourselves is described here. See text-books for general SEM-techniques. Examples: Figs. 2–4, 9, 13, 14, 21, 24, 30, 34, 37.

Procedure

- Pour ciliates into Parducz' fixative and leave for about 30 minutes.

Remarks: Concentrate and clean material as thoroughly as possible (see step 2). Ratio of sample: fixative should be at least 1 : 1, better 1 : 2. Add some drops of 5N HCl if fixative becomes milky when the material is added. Parducz' fluid fixes most ciliates very well. However, the cirri of the hypotrichous ciliates usually disintegrate into their component cilia. Hypotrichs should thus be fixed either in concentrated sublimate (dissolve 60 g HgCl₂ in 1 litre hot distilled water and allow to cool) or in a mixture composed of 4 parts concentrated sublimate and 1 part 2% osmium tetroxide.

- Wash ciliates at least 5 times with 0.05 M Na-cacodylate buffer.

Remarks: Ciliates in the buffer may be refrigerated for years. Washing must be done in a watch-glass (Fig. 1F) and a micropipette should be used to remove bacteria and detritus. This cleaning of the material is essential but rather difficult and laborious, especially with small species (< 100 µm) and field material; thus cultures and/or pre-cleaned material (see below) should be used. The cleaning is performed as follows: Ciliates settle at the bottom of the fixation tube after 30 minutes (cp. step 1). Remove as much supernatant as possible with a pipette (do not centrifuge!). Then transfer the material to a watch-glass and allow to settle for about 5 minutes (use fume hood). Quickly remove most of the fixative with a micropipette under the dissecting microscope. Now wash the ciliates with the buffer by several passages through a large-bore (diameter about 1 mm) pipette. Bacteria and detritus adhering to the ciliates are hereby mechanically removed. Again allow to settle, but control sedimentation with the dissecting microscope; remove supernatant containing bacteria and detritus with a micropipette as soon as ciliates settle. This procedure must be repeated until the material is clean and all of the detritus is removed. Use fractionated sedimentation if the sample contains several species differing in size and/or mass.

Field material: Larger species (> 100 µm) are picked out with a micropipette and sprinkled into the fixative. Several hundred specimens must be collected because loss of material may be considerable during the following steps. Small species can be prepared by this method only if abundant material is available. Accumulation can often be achieved by the following simple method: leave a freshly collected sample containing ample mud to stand for some hours at room temperature. Due to oxygen depletion the ciliates usually move to the surface where they can be skimmed off with a teaspoon.

- Transfer cleaned ciliates with a small drop of buffer to the preparation chamber (Fig. 39).

Remarks: Place the drop on the bottom plankton net of the chamber which is weighted with washer 1. The net must be dry to avoid spreading of the drop to the chamber margin and the washer. Place the top plankton net carefully on the drop, i.e. on washer 1, using forceps. Weight top net with washer 2, close chamber with lid and immediately transfer into 30% ethanol. The plankton net must have a mesh-size < 12 µm and should be used only once. It should fit exactly into the chamber, which is best achieved using an appropriate punch.

- Dehydrate chamber with ciliates in ethanol series (30–50–70–90–100–100%) for 5 minutes each.
- Transfer chamber with ciliates to an ethanol: amylacetate series (2 : 1, 1 : 1, 1 : 2) for 5 minutes each.
- Transfer chamber with ciliates to pure amylacetate (C₇H₁₄O₂) for about 12 hours.
- Dry chamber with ciliates in a critical-point drying apparatus.

Remarks: We use CO₂ and change the amylacetate at least 10 times. Sometimes good results are achieved by transferring the ciliates directly from the last ethanol step to CO₂.

- Open chamber and place ciliates on the SEM-stub. Use a mounted eyelash if ciliates do not fall from the net.

Remarks: The dried ciliates usually form a lump at the bottom plankton net. This lump is carefully transferred (by holding the net over the stub and stripping off the ciliates with the eyelash) to the SEM-stub, where it is dispersed under the dissecting microscope with the mounted eyelash. The ciliates spread easily if drying was sufficient.

Preparation of the SEM-stub: We use commercial aluminium SEM-stubs. To get a black, homogenous background the

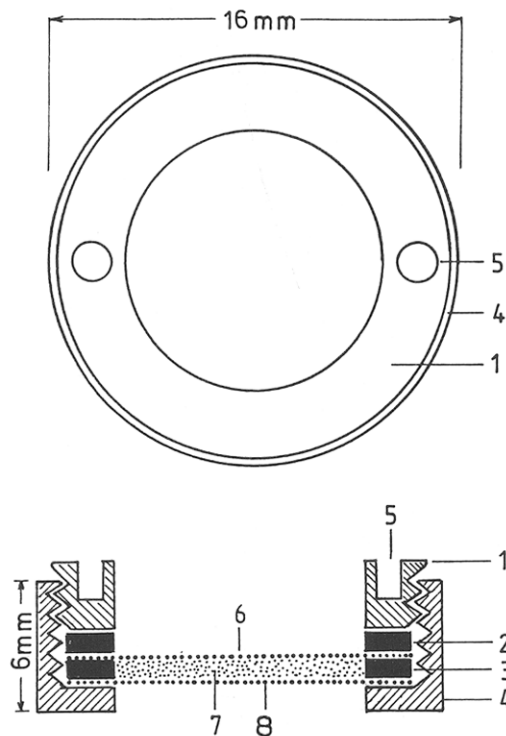


Fig. 39. Brass chamber for critical-point drying of protists (after P. Simonsberger, unpubl.). 1 = threaded chamber lid, 2 = washer 2, 3 = washer 1, 4 = threaded chamber jacket, 5 = holes for forceps tips, used to screw lid into jacket, 6 = top plankton net, 7 = sample, 8 = bottom plankton net.

slide is covered with a *very thin layer* of Mixtion à Dorer Clarifiée (Lefranc and Bourgeois, Le Mans) 1.5–3 hours before use. This fluid is used by artists to attach goldfoil and is available in ordinary stores. It dries slowly (whereby its surface becomes smooth) and attaches the ciliates to the slide. The mixture may be diluted with turpentine. Note that small species sink into the lacquer more easily than large ones and the mixture should thus be allowed a longer drying time (2–3 hours).

9. Harden the lacquer for at least 48 hours at room temperature.

Remarks: This drying is not essential but prevents the development of cracks when the preparation is placed in the vacuum of the scanning electron microscope.

10. Sputter with gold.

Reagents

- a) Parducz' fixative (prepare immediately before use)
 - 4 ml aqueous 2% osmium tetroxide (OsO_4)
 - 1 ml saturated, aqueous mercuric chloride (HgCl_2 ; preparation see protargol impregnation; Foissner's method)
- b) 0.05 M Na-cacodylate buffer (can be stored for several months in the refrigerator; adjust to pH 7 with HCl)
 - 10.7 g dimethylarsinacid-sodium salt ($\text{C}_2\text{H}_6\text{AsNaO}_2 \cdot 3\text{H}_2\text{O}$)
 - ad 1000 ml distilled water
- c) Amylacetate ($\text{C}_7\text{H}_{14}\text{O}_2$). Use commercial product.

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Key words: Ciliophora – Live observation – Methyl green-pyronin staining – Silver impregnation techniques – Scanning electron microscopy

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