# THE "WET" SILVER NITRATE METHOD

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## INTRODUCTION

The first wet ("wet" because cells are chemically fixed before being treated with silver nitrate) method was described by Chatton & Lwoff (2, 3). The technique became well known after Corliss (4) 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. Several modifications have been described (e. g., 1, 5, 6). Roberts & Causton (7) investigated the variables of this method in detail. Examples: Fig. 1 - 9.

## PROTOCOL

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 cupboard 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 suffice. 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 remains (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 minute 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 at least 10 minutes transfers 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-immerse 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 ($K_2Cr_2O_7$)
   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 ($Co(NO_3)_2 \cdot 6H_2O$)
   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 (see also remarks at step 5!)
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.

## LITERATURE CITED

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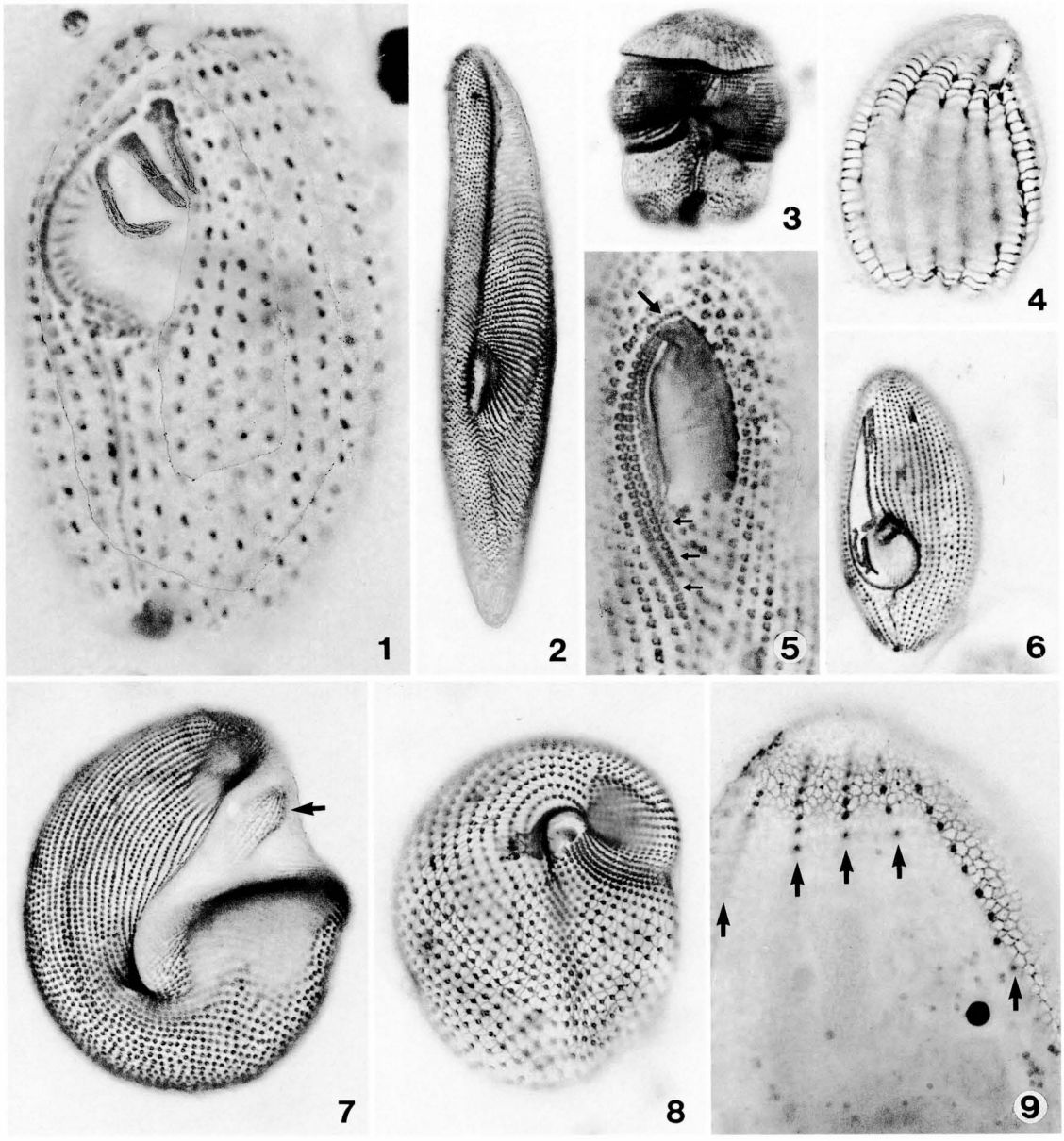


**Fig. 1 - 9.** Ciliates prepared with the wet silver nitrate protocol described. **1, 6.** *Sathrophilus muscorum* and *Pleuronema coronatum*, scuticociliatid ciliates; ventro-lateral views, length about 35 µm and 90 µm. **2, 3, 5.** *Paramecium caudatum, Urocentrum turbo*, and oral apparatus of *Frontonia depressa*, peniculinid ciliates; ventral views, length about 250 µm and 90 µm. Arrows in figure 5 mark vestibular kineties. **4, 7, 8.** *Cosmocolpoda naschbergeri, Bresslauides discoides*, and *Colpoda cavicola*, colpodid soil ciliates; ventro-lateral views, length about 50 µm, 250 µm and 100 µm. Arrow in figure 7 marks a *Tetrahymena* cell captured in the huge vestibulum. **9.** *Histriculus erethisticus*, a hypotrich ciliate; dorsal view showing the fine-meshed silverline system and the dorsal kineties (arrows).