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Short Communication

An Improved Silver Carbonate Impregnation for Marine Ciliated Protozoa

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Summary. An improved silver carbonate impregnation method for marine ciliates is described. Compared to conventional methods, the new method of fixing and staining is easy to learn and yields easily reproducible results. Structures that are impregnated with silver carbonate such as nuclear apparatus, infraciliature as well as cortical and cytoplasmic microtubules and even contractile vacuole pore are stained evenly and with high contrast in all cells on the slides, therefore yielding perfect photographs. This method has the characteristic of simplicity, quickness and good reproducibility, so it is especially suitable for ecologists and ontogenetists. The slides with mounted cells can also be stored for months without loss in quality or staining capability.

Key words: marine ciliate, silver carbonate impregnation, staining method.

INTRODUCTION

In order to identify ciliates for taxonomic evaluation, silver impregnation techniques are indispensable. Even though there are many methods for ciliated protozoa (Klein 1926, Chatton and Lwoff 1930, Dragesco 1962, Deroux and Tuffrau 1965, Wilbert 1975, Fernandez-Galiano 1976, Montagnes and Lynn 1987, Foissner 1992), marine protozoologists and ecologists are still perplexed about the staining of the marine ciliated protozoa. Along with the development of marine ecological research, many ciliated protozoa are being more closely examined, so a simpler kind of and more user-friendly staining method for ciliates is necessary for marine ecologists and biologists. The method of silver carbonate impregnation described in this paper was improved so it is easy to learn and consistently yields easily reproducible results for marine ciliated protozoa.

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DESCRIPTION OF THE METOD

Some of the ciliate species that were used in this work were either laboratory cultures or freshly collected and cultivated in Incheon, Korea or Qingdao, China. The ciliates were cultivated either in normal or in deep Petri dishes and kept at 20-25°C according to their special requirements, using either rice or organisms as food source. The following ciliate species were tested:

Chaenea teres, *Coleps hirtus*, *Dexiotricha granulose*, *Dysteria ovalis*, *Euplotes vanus*, *Fabrea salina*, *Glauconema trihymene*, *Halteria grandinella*, *Metanophrys sinensis*, *Paramecium caudatum*, *Paranophrys magna*, *Parauronema virginianum*, *Pelagostrobilidium simile*, *Pleuronema coronatum*, *Pseudocohnilembus hargisi*, *P. persalinus*, *Rimostrombidium orientale*, *Rimostrombidium venilie*, *Strombidinopsis elegans*, *Uronema marinum*.

Staining Procedure (reagents according to Fernandez-Galiano 1976)

(1) Place 2-3 drops of a rich ciliate culture or even a single specimen in an embryonic dish or in a concave slide.

(2) Add 2-3 drops of 10% formalin quickly to the embryonic dish if the organisms are freshwater species (final concentration is 5%), or add 2-3 drops of 10% (or even 50% depending on the kind of species) seawater formalin if the organisms are seawater species and fix for 1-3 min. Shake and try to concentrate the cells to the middle of the dish or concave slide.

(3) Carefully wash out the water with micropipette and add distilled water three times. Leave about two or three drops of water and organisms.

(4) Add 2 or 3 drops of formalin (10%) and refix for about 1 to 2 min (the final concentration is 5%).

(5) Add 2-3 drops of Fernadez-Galiana's fluid to the refixed ciliates, and mix one minute.

(6) Place the embryonic dish on a pre-heated (60°C) hot-plate for staining. Shake the dish in the process of heating. Immediately add 2-3 ml of 5% sodium thiosulfate solution as soon as the color turns golden brown. The process usually needs 5-7 min.

(7) Check the impregnation under microscope, take pictures with digital camera or draw figure if the impregnation is perfect.

(8) After stabilization with 5% sodium thiosulfate solution for about 8-15 min, wash the organisms very thoroughly in distilled water at least 3 times. For morphogenesis or genus identification, steps 1-8 are enough. For mounting slide, according to the following steps.

(9) Add a small drop of well impregnated ciliate to a clean slide and add an equal sized drop of albumen-glycerol, mix thoroughly but gently with a mounted needle and spread mixture in a moderately thin layer in the middle third of the slide and remove the redundant fluid with a thin micropipette.

(10) Allow to dry in the oven at 55-60°C for 10-30 min.

(11) Dehydrate in alcohol series (70-80-90-95-100-100%) for 5 min in each.

(12) Transfer the slide through 1:1 alcohol and xylene, then through xylene 2 times for 5 min in each.

(13) Mount the slide with Permount and cover with a coverslip. Ensure that no air is trapped under the coverslip and put the slide into the oven at 55-60°C for 24 h.

Remarks concerning the steps of the staining procedure

(1) This method is especially suitable for species with a firm pellicle. It is necessary to adjust the concentration of formalin and the solvent, if the organisms are freshwater species, use 10% distilled water formalin; seawater species, add 10% filtered seawater formalin. Some species cannot be fixed well with formalin and cells may even burst.

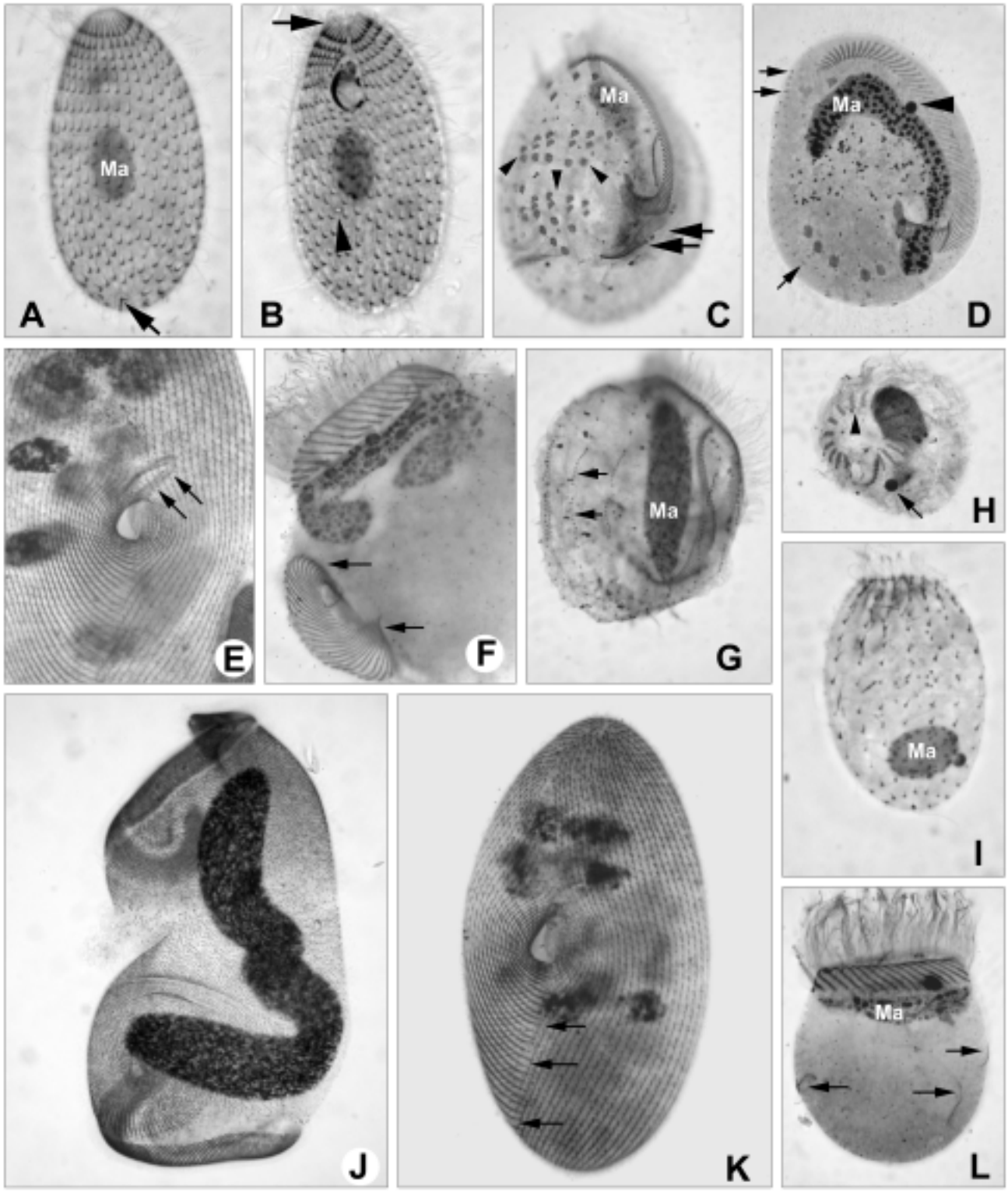
(2) Washing out the redundant liquid is also a most important step. Since the chemical component of the original liquid in which the organisms live are different, these chemicals can affect the staining result, so the original liquid should be washed out thoroughly with distilled water at least 3 times.

(3) The ratio of Fernandez-Galiano's fluid usually need not change, but the amount needed depends on many unpredictable factors, e.g., amount and concentration of fixative, kind of organisms. The trial and error method must be frequently used to obtain best results.

(4) The correct impregnation time depends on many factors that are difficult to control. The amount of pyridine and silver carbonate in the Fernandez-Galiano's fluid is especially important. Add another drop of pyridine and/or silver carbonate solution to the Fernandez-Galiano's fluid if impregnation is repeatedly too faint. Some species can be impregnated well before the fluid turn brown, while others even very faint after the fluid becomes dark brown, so try several times to get good results.

(5) Immediately add 2-3 ml 5% sodium thiosulfate solution as soon as the color turns golden brown. Because impregnation can develop well within 1-2 seconds, it is not advisable to check the impregnation results under the microscope. The quickly changing golden brown color is the best opportunity to stop the staining with the 5% sodium thiosulfate solution. Stabilization should occur in no less than 10 min, and only in this way can the mounting slide stay perfect in quality.

(6) Before preparing a permanent slide, the sodium thiosulfate solution must be washed out thoroughly with distilled water at least 3 times, or it will affect the staining quality.



Figs 1A-L. Overview of slides stained by the silver carbonate impregnation method. **A, B** - *Dexiotricha granulose*, a scuticociliatid, arrow in **A** shows the caudal cilium complex, arrow in **B** indicates the cytoplasmic microtubules, arrowhead in **B** means the contractile vacuole pore; **C, D** and **G** - *Euplotes vanus*, a hypotrichid, arrows in **C** point to the new adoral membrane anlagen, arrowheads in **C** indicate the anlage of ventral cirri, arrows in **D** mean the dorsal kineties, arrowhead in **D** points to micronucleus, arrows in **G** display the dorsal kineties anlagen; **E, K** - *Paramecium caudatum*, a hymenostomatid, arrows in **E** indicates the buccal membranelles, arrows in **K** show the post suture; **F, L** - *Pelagostrobilidium simile*, an oligotrichid, arrows in **F** point to the new built buccal field, arrows in **L** show the kineties; **H** - *Halteria grandinella*, an oligotrichid, arrow shows the micronucleus, arrowhead indicates the external adoral membrane; **I** - *Coleps hirus*, a prostomatid; **J** - *Fabrea salina*, a heterotrichid. Ma - macronucleus.

(7) Pay extra attention to avoid foaming when adding the albumen-glycerol to the slide. The key is to use a very thin micropipette and suck a small amount of distilled water before removing the albumen glycerol. Only leaving the least possible amount of albumen glycerol behind results in good staining quality.

In the ciliates species we tested, the infraciliature, the nuclear apparatus, and part of the cortical and cytoplasmic fibrilla systems were stained excellently with the method (Figs 1A-J). Many of the published modifications of the protargol impregnation method, do not yield desired results. The main reason for this situation is that these modifications need more steps and much experience (Dieckmann 1995). Compared to the protargol impregnation, silver carbonate method can save much time, the whole staining procedure can finish within 20 min. The whole procedure is easy to grasp, even a newcomer can also stain slides well, so this method is welcome to ecologists.

Now, the digital camera is widely used to take photos under the microscope. The staining quality can be checked under a microscope directly, and when it is perfect, the photos can be taken right away. If not good, another staining procedure can start within a few minutes. Compared to mounting samples, taking photos with a digital camera has more advantages. Mounting the cells with albumen also has a decisive influence on the staining quality (Foissner 1991). A special advantage of the method is that it is very useful for ontogenetic specialists. A large amount of ciliate can be stained within a very limited time and the morphogenetic stages can be scouted under microscope with a wet slide directly. It is clearer and easier than mounting samples because the cells can be stressed heavily, and the color and structure of the organisms will not change within one or two hours.

Compared to Fernandez-Galiano's original method, a rinsing and post-fixing step was added in our method. This ensures the high staining quality and reproducibility, and also avoids the chemical interference of the solution. As for the seawater species, we do not use the centrifugal method as Fernandez-Galiano used, because in that way cells can easily burst and immingle with other sundries, and affect the mounting result. Compared to Foissner's (1992) modification, the content of Fernandez-Galiano's fluid is different, while two modifications can all produce perfect staining quality. We preferred embryonic dish or concave slide to slide because it is easy to rinse organisms and to avoid the loss of cells during washing, this also keeps a steady condition for staining.

The main problem of Fernandez-Galiano's is not fit for species of thin pellicle. So we fixed seawater sample with filtered seawater formalin and changed the salinity according to species. This is very useful, because most of species even that of thin pellicle can also get perfect staining quality with high concentration of Formalin. For some hypersalinitic species such as *Fabrea salina*, which grows better at the salinity of 90 psu, hypersalinitic seawater formalin can remedy the deficiency of distilled water formalin.

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