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Refinements of and commentary on the silver staining techniques of Fernández-Galiano

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Abstract

The original ammoniacal silver carbonate staining technique and subsequent modification developed by Fernández-Galiano are useful for investigating ciliate protozoan systematics and/or ciliate cortical structure and morphogenesis. The technique is complicated, however, by both uncertainties arising from the need to count drops of reagents and subjective control of the staining intensity. I have resolved these complications by defining volumes of reagents rather than using drops and by defining a range of staining times. I also comment on various steps of the techniques. My techniques are simplified and refined to produce consistent, high quality staining results.

Keywords

ciliates; cortical structures; Fernández-Galiano staining; *Paramecium*; protozoa; silver staining

Fernández-Galiano's adaptations of the of the Rio-Hortega ammoniacal silver carbonate staining protocol (Rio-Hortega 1942 produce useful images of the cortical organization of ciliophoran protozoa (Fernández-Galiano 1976, 1994). Properly done, the technique demonstrates cells with delicately stained cortical structures that enable critical analysis of cytoskeletal and membranous components and their morphogenesis for systematics analysis, and cell and developmental studies (Aufderheide 1986, 1999, Foissner 1991, Ma et al. 2003, Small et al. 2005, Pérez-Uz et al. 2010, Fokam et al. 2011). Unlike fluorescent antibody labeling, Fernández-Galiano staining can be performed quickly and requires no special reagents or microscopy equipment. Stained cells can be viewed readily with bright field microscopy optics. Some structures revealed by Fernández-Galiano staining are not evident after using other staining protocols, so the Fernández-Galiano technique is complementary to the other staining methods used for ciliophorans (Foissner 1991, 2014).

Unfortunately, the Fernández-Galiano protocols can be challenging to perform and control. The reagents for the staining mixtures are measured by counting drops. Also, in the original, first-reported technique (Fernández-Galiano 1976), the intensity of staining was controlled by assessing a rapid color change as the indicator of when the reaction should be terminated. Descriptions of the color change are subjective, such as "fine cognac," "good whiskey" or

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even “chocolate milk.” Subsequently, Fernández-Galiano (1994) published modifications of his original technique that addressed some of these issues; unfortunately the revised technique also quantified the reagents to be added to the staining mixture in drops.

I present here refinements of the published Fernández-Galiano techniques. I have converted drops of reagents to metric volumes and have established a range of durations for the staining reaction before its termination. With these modifications, inexperienced workers can be trained quickly to produce consistent, publication quality stained cells. The results reported here are specific to our work with *Paramecium tetraurelia* and *Paramecium sonneborni*; investigators using other species should adjust times and reagents for their organisms.

Methods and results

Modifications of the original, first-published technique (Fernández-Galiano 1976)

My modifications of the original procedure and my commentary are detailed below. The reagent solutions are prepared as described in the original publication.

1. Place 5 ml of the culture to be stained in a 100 ml flask. Add 160 μ l concentrated formaldehyde (37%) and fix for 4.5 min.
2. With mixing, quickly add the following reagents in the order specified; additions should be completed within about 15 sec:
 - 350 μ l pyridine (C_5H_5N)
 - 450 μ l 4% aqueous bactopectone solution
 - 2.5 ml Rio-Hortega silver carbonate solution
 - 20 ml distilled water
3. While swirling, heat the mixture in a 60° C water bath until the color turns from clear or white to light brown. The optimal staining time ranges from 1.5 to 2 min. The reaction is very rapid once it starts, so one must be careful not to let the solution get too brown. I have found that 1 min 30 sec to 1 min 40 sec staining time gives the best results.
4. Stop the reaction by adding 15 ml 5% aqueous sodium thiosulfate ($Na_2S_2O_3$)
5. Wash the stained cells twice by centrifugation ($1,000 \times g$) for 2 min and re-suspension in distilled water. Store in the dark in the refrigerator at 4° C.
6. Examine cells using temporary wet mounts.

Notes and comments—Step 1. The original description of the technique specified 3–5 drops of formaldehyde and fixation for 3–5 min. I found that 160 μ l formaldehyde and 4.5 min fixation gave consistently good results. The volume of formaldehyde used to fix the specimen is critical for staining quality; I found that 75–80 μ l produced specimens stained so

lightly that little cellular detail was visible, while 250 μ l of formaldehyde yielded excessively dark staining with poor differentiation between cortical structures and background cytoplasm.

Step 2. The original description listed drops of reagents to be added. I have converted all drop counts to microliter volumes. The order of addition of the reagents is critical; several investigators have specified the precise order for adding components to the staining solution (Fernández-Galiano 1976, Foissner 1991, 1992) and I concur. I also found that the timing of addition is crucial. All chemicals should be added within 15 sec for best staining results; longer times for adding all components of the staining solution produced lower quality stains, especially for differentiating the cortex and background cytoplasm.

Different batches of the Rio-Hortega stock solution can produce different intensities of staining. Up to 3.5 ml of a particular batch of Rio-Hortega stock solution can be added to the staining mixture, depending on the desired results.

Step 3. This step “develops,” i.e., reduces the silver of the stain. The amount of time the mixture is kept at 60° C is critical to the amount of silver that is deposited on cellular structures. Too little time produces understained cells with little contrast, while too much time turns the entire cell too dark to distinguish structures; the interval between these extremes is short and difficult to control. Fernández-Galiano (1976) specified stopping the reaction by adding thiosulfate when the mixture reached a particular color, described as “fine cognac” or “quality whisky.” Assessment of this color change is subjective and does not ensure consistent staining quality. For example, I found that a sample with higher concentrations of bacteria darkened quickly during the development step and could prompt early termination of the stain, but the ciliates then were stained only lightly, although the bacteria were extremely dark. Therefore I sought to establish a staining period that consistently produced good differentiation of cortical structures and low background regardless of the overall color change of the reaction mixture. In general, this period is between 1.5 and 2 min depending on the particular batch of Rio-Hortega solution. I found that approximately 1 min 30 sec to 1 min 40 sec is optimal for paramecia in my laboratory. The user should develop times appropriate for the particular organism under study.

Step 4. Prompt use of thiosulfate is essential to stop the staining reaction before the cells become too dark. I found it essential to have the thiosulfate solution premeasured and in one’s hand, ready to add at the correct time.

Step 5. This step is essential if one wishes to store the stained cells for more than a few days. If the cells are left in their original staining solution, they darken within a day and become opaque. If stained cells are washed and stored in the refrigerator, the quality of their staining remains good for several days. Washing the cells also reduces the odor of the pyridine.

Step 6. There are published procedures for dehydrating and mounting the cells for permanent preparations (Augustin et al 1984, Foissner 1991), but I have been unable to obtain good results with these approaches.

Modifications of the second-published technique (Fernández-Galiano 1994)

My modifications of the procedure and my commentary are detailed below. The reagent solutions are prepared as described in the original publication.

1. To a 100 ml flask, quickly mix the following in the order listed:
 - 75 μ l concentrated formalin
 - 2 ml culture to be stained
 - 2 ml 5% Tween 80 in water
 - 900 μ l 5% aqueous bactopectone solution
 - 350 μ l pyridine (C_5H_5N)
 - 2 ml modified Rio-Hortega solution
 - 30 ml distilled water
2. Shake to mix the contents. Heat the mixture in a 70° C water bath for 5–10 min until the color of the solution turns from clear or white to dark brown.
3. Wash the stained cells a few times by centrifugation ($1,000 \times g$ for 2 min) and re-suspension in distilled water. After the first centrifugation, decant the supernatant from the pelleted stained cells, add about 2.5 ml of 5% sodium thiosulfate solution, re-suspend the pellet, then fill the tube with distilled water. Subsequent centrifugations involve washing with distilled water only. Store stained samples in the dark in the refrigerator at 4° C. Treatment with sodium thiosulfate at the beginning of the first wash appears to preserve the quality of stained cells during subsequent storage.
4. Examine the cells using temporary wet mounts.

Notes and comments—Reagent solutions were prepared as described by Fernández-Galiano (1994). Fernández-Galiano significantly modified his own protocol in 1994. Although he continued to count drops of reagents, he changed the formula of the Rio-Hortega stock solution by adding excess sodium carbonate, then modified the staining protocol. I adjusted this protocol by converting drops of reagents into metric volumes.

Step 1. The cells and the formaldehyde are mixed and allowed to stand for a few seconds before the remaining ingredients are added. Mixing all the ingredients in less than approximately 1 min produces good staining.

Step 2. Fernández-Galiano (1994) claimed that his revised staining reaction was self-limiting, so overstaining samples would be unlikely. I found that there is no need for precise timing or constant agitation. The solution begins to turn color after a few minutes and eventually becomes very dark brown. Nevertheless, I found that a total staining time of approximately 5 min produced a more delicate stain with little background; extended staining times produced coarser staining and much darker cytoplasmic background.

Step 3. Cells stained by the second-published protocol generally are lightly and delicately stained with little cytosolic background. Although the reaction does not require addition of thiosulfate to stop it, I found that treating the stained cells with thiosulfate inhibited darkening of stained cells during storage at 4° C. Washing the cells with distilled water a few times after staining also reduced the objectionable odor of pyridine.

Discussion

Several techniques have been described for obtaining high quality, high resolution views of the fine structure of living immobilized protists and other cells (Aufderheide and Janetopoulos 2012, Yan et al. 2014, Zinskie et al. 2015). Many staining techniques using fixed cells, however, can provide valuable structural information not easily visible in living cells, even with the most advanced light microscopy techniques.

The Fernández-Galiano protocols (Fernández-Galiano 1976, 1994) are relatively simple, quick to prepare and perform, and they produce outstanding staining. The results can be assessed readily using bright field optics. Undergraduate students with little experience can be trained quickly to produce publication quality stained cells.

Consistent with other descriptions of Fernández-Galiano staining (Fernández-Galiano 1976, 1994, Foissner 1991, 2014), my protocol reliably stains the basal bodies, parasomal sacs and kinetodesmal fibers of the cell cortex (Allen 1971, Aufderheide 1986, Cohen and Beisson 1988) (Fig. 1). Also, in some stained cells, the boundaries of the alveolar membranes and even inserted trichocysts can be seen. The macro- and micronuclei are stained darkly as are undigested bacteria in food vacuoles. Despite differences in details and reagents, the two protocols described here produce similar staining results.

Although silver staining techniques are widely used to visualize ciliate surface structures, the chemical basis of silver staining of the cell cortex is not well understood. Using *Paramecium*, Tellez et al. (1982) combined light, scanning and transmission electron microscopy to determine where silver granules were deposited by the Fernández-Galiano (1976) staining protocol. These investigators found that silver is deposited in and near basal bodies and their ancillary structures, especially in the kinetodesmal fibers. The reasons for this staining pattern or why silver deposition patterns differ among the different silver staining techniques, however, is obscure (Foissner 1981, 1991, 2014).

I found two elements of Fernández-Galiano's published protocols to be significant. One is the uncertain amounts of reagents specified. Fernández-Galiano (1976 Fernández-Galiano (1994) specified drops of liquid for many of the reagents used in the protocols. Depending on the size of the pipette used and how the pipette was held when the drops were counted, significant differences in the volumes can result; I found that these differences change the quality of the staining. For consistency, it preferable to specify the amounts of reagents added in objective volumes using digital micropipettes rather than drops.

The second issue is the period required for the stain to develop. The color change identified in the original paper ("fine cognac" or "quality whisky") (Fernández-Galiano 1976) might not be interpreted consistently. I also found that a sample with a large bacterial population

caused the solution to turn dark quickly, because the bacteria also are stained by the silver, but the ciliates in the sample were only lightly stained; therefore, monitoring the color change would not produce good results. The color change also is rapid, so an inattentive or inexperienced individual could easily miss the desired light brown end point before adding thiosulfate; consequently, the cells would be overstained. Therefore, a clear definition was necessary for the staining period that produces optimal results for the ciliate species being studied. Although the precise period might vary slightly depending on the species examined, the periods specified here are optimal for my species in my laboratory.

For all aspects of the protocols discussed here, the user is advised to adapt the times of staining and amounts of the various reagents added to optimize the results for the species of organisms studied. Consistent with the advice of Foissner (1992), if the staining is too light, the addition of more formaldehyde and more pyridine to the staining mixture of both versions of the protocol should produce darker staining of cortical structures. In addition, special modifications to the protocol likely would be required if one were staining marine specimens (Foissner 1991, Martín-Cereceda et al. 2001, Ma et al. 2003, Small et al. 2005) and investigators should consult the literature appropriate to this application.

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Fig. 1. Anterior suture region of *Paramecium tetraurelia* stained using the revised Fernández-Galiano protocol. Cortical units containing basal bodies and parasomal sacs (bb), and kinetodesmal fibers (kf) are clearly visible. In some regions, outlines of the alveolar membranes (av) that define the edges of cortical units also are stained. Planapochromatic objective. 100 × oil immersion. Image processed and labeled using ImageJ software.