Cytological staining of protozoa: a case study on the impregnation of hypotrichs (Ciliophora: spirotrichea) using laboratory-synthesized protargol

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ABSTRACT

Protargol (silver proteinate) impregnation is a common method used to identify and characterize ciliated protozoa. Unfortunately, chemical companies have stopped producing the ‘strong’ protargol powder used in this method. Based on an in-house protocol for its synthesis published in 2013, more than 10 batches of protargol powder were produced and subsequently applied in taxonomic studies. During these studies, the protocol for protargol powder synthesis was slightly modified and employed a peptone not originally listed in the 2013 protocol. This modification improved the results of the impregnation protocol. Protargol preparations of hypotrichs were optimized by adjusting the pH during staining rather than during the synthesis. The pH was adjusted to 7.5–7.6, and an acetone developer was used. While the conditions used in this study are not completely comparable to those using the commercially produced protargol, access to this information could help researchers investigate the diversity of ciliates, particularly hypotrichs.

Introduction

Taxonomical and ecological studies on ciliated protozoa have been performed using a number of methods, including live observation, silver carbonate impregnation, scanning electron microscopy, and supravital staining, protargol, and dry and wet silver nitrate impregnation. These techniques have been modified and improved in several studies (Chatton and Lwoff 1930, 1936; Tuffrau 1964, 1967; Wilbert 1975, 1976; Fernandez-Galiano 1976; Foissner 1976, 1991, 2014; Dieckmann 1995; Silva-Neto 2000; Ma et al. 2003; Vdacny and Foissner 2012). Among these methods, protargol impregnation is a key approach used to identify and describe ciliate species (Corliss 1979; Lynn 2008; Foissner 2014). Unfortunately, ‘strong’ silver proteinate, which is an effective staining tool, is no longer commercially available (Pan et al. 2013). Therefore, ciliate taxonomists and ecologists may have to synthesize the protargol themselves (Pan et al. 2013).

Pan et al. (2013) published a protocol for synthesizing protargol that could be subsequently used to study ciliate diversity. To the best of our knowledge, this is the most recent publication aiding optimization and standardization of this technique. Based on this protocol, our group synthesized protargol more than 10 times. In their protocol, Pan et al. (2013) assessed five peptones for protargol synthesis; however, we were unable to locate an official supplier for some of these in Korea. Of the available peptones, three were tested: oxoid tryptone (Fisher Scientific), peptone (Beijing Aoboxing), and peptone from gelatin (Sigma-Aldrich). However, use of protargol synthesized with these peptones resulted in insufficient impregnation of ciliatures and the nuclear apparatus, compared with the commercial protargol, when an ordinary developer was used in the process of ‘Procedure A’ (Foissner 2014). Importantly, estimation of stained specimen quality is subjective and is somewhat user-dependent and depends on the taxon being studied. While we have previously described characterizing hypotrichs using laboratory-synthesized protargol (Jung et al. 2015, 2016; Kim and Jung 2016; Park et al. 2017a, 2017b), we have continued to optimize this process. As mentioned by Pan et al. (2013), an optimal peptone for protargol synthesis is still awaiting our discovery. To determine the optimal conditions for studying hypotrichs, the protocol used to synthesize protargol was modified slightly and tested with another peptone not listed by Pan et al. (2013).

In this article, we briefly report modifications to optimize preparations of hypotrichous ciliates. Pleurotricha...
oligocirrata Park et al. 2017 was used to identify the optimal conditions for protargol impregnation. Microphotographs of additional impregnated hypotrichs impregnated with this protocol are presented.

Materials and methods

Protargol powder was synthesized using a method previously published by Pan et al. (2013) with modifications. One such modification was the use of peptone derived from peas (Fluka, cat. No. 96174), which was not used in the Pan et al. (2013) study. In addition, the pH was not adjusted (eight step in Pan et al. 2013) during protargol synthesis in this study due to difficulties controlling the rapidity of the adjustment (Procedure A [Foissner, 2014]). Alternatively, the pH was adjusted using ammonium hydroxide during protargol impregnation, which was described as ‘Procedure A’ by Foissner (2014). To identify the optimal conditions for hypotrich impregnation, six different pH values (7.1, 7.3, 7.5, 7.6, 7.8, and 7.9) were tested with P. oligocirrata. Furthermore, additional hypotrichs were assessed under the condition selected as an optimal pH in this study.

For protargol preparations, cells were fixed using concentrated Bouin’s fluid and then centrifuged at 3000 rpm for 1 min (Beckman GS-15R, CA). These fixed cells were washed with tap water and then harvested by centrifugation. Laboratory-synthesized protargol solution (0.8% w/v in distilled water) was adjusted to the desired pH adjusted by ammonium hydroxide during protargol impregnation, which was described as ‘Procedure A’ by Foissner (2014). To identify the optimal conditions for hypotrich impregnation, six different pH levels were compared to the ordinary developer (95 ml distilled water, 5 g sodium sulfite [Na2SO3], 15 ml acetone) was used and compared to the ordinary developer (95 ml distilled water, 5 g sodium sulfite [Na2SO3], 1 g hydroquinone [C6H6O2]; Dieckmann 1995; Foissner 2014) diluted 1:20. The duration of development was adjusted using a stereomicroscope. General terminology is according to Berger (1999).

Results and discussion

This section has been subdivided based on key diagnostic characteristics used to identify ciliates. Hypotrichs, which are one type of ciliate, are commonly found in a range of habitats, including terrestrial, freshwater, and saline environments (Lynn 2008). Diagnostics of hypotrichs based on morphology, especially for stained specimens, involve the evaluation of (1) the nuclear apparatus, (2) configuration and the number of adoral membranelles (AMs), (3) the arrangement of undulating membranes (UMs), (4) cirral patterns, and (5) the number and arrangement of dorsal kineties (DKs) (Berger 1999). For the terminology and morphology used in this study, see Figure 1(C).

Nuclear apparatus and cytoplasmic inclusions

The nuclear apparatus of ciliates comprises one or more macronuclear nodules with one or more micronuclei (Figure 1; Lynn 2008). One of the species tested in this study, P. oligocirrata, has two macronuclear nodules with several micronuclei (Park et al. 2017a). During experiments in this study, the unadjusted pH of the protargol solution was typically <6.0, which resulted in transparency of the nuclear apparatus due to insufficient silver deposition (data not shown). With an increase in pH, the nuclei and cytoplasmic inclusions turned brownish (Figure 1). When the pH ranged from 7.3 to 7.9, the apparatus could be clearly visualized following an incubation of less than 1 min in the acetone developer. However, when the pH was 7.8 and 7.9, development took more than 2 min and resulted in the inclusions being too dark. In addition, the longer duration of development caused deposition of silver in the albumin layer that obstructed the view of the specimens.

AMs and cirral patterns

The adoral zone of membranelles of P. oligocirrata is question mark-shaped without a gap (Park et al. 2017a). The impregnation of the AMs was not affected by pH (Figure 1). When the pH ranged from 7.3 to 7.6, each basal body forming the AM could be clearly seen. The somatic ciliature of P. oligocirrata consists of 23–27 frontal–ventral–transverse cirri with two rows of marginal cirri (Park et al. 2017a). The cirral patterns were distinct at all pH levels with the exception of a pH of 7.9. In addition, development at the pH levels of 7.8 and 7.9 took longer (often at least three times) than at other pH levels. In addition to the longer time required for the deposition of silver, a pH of 7.9 failed to display the cirri clearly (Figure 1F).

UMs and DKs

The undulating membranes of P. oligocirrata consist of two rows of kinetids in an Oxytricha pattern (Figure 1; Kumar and Foissner 2015). The structure of the UMs could be visualized clearly for all pH levels tested (Figure 1). However, as mentioned above, when the pH 7.9 solution was used, there was less deposition of silver on the UMs. The DKs of P. oligocirrata consist of six or seven rows with widely spaced dikinetids (Figure 2; Park et al. 2017a). A single caudal cirrus is located at the posterior
end of DK 1, 2 and 4. The quality of DK impregnation ended up being the critical criterion by which the optimal pH was selected because there was a relatively narrow pH range that resulted in the deposition of silver on DKs in this study (Figure 2). In contrast to the AMs, UM, and cirri, DKs consist of sparsely distributed basal bodies (Lynn 2008) that decrease the amount of silver deposition on these structures. Based on the amount of the deposition, the contrast between the non-targeted structures and the infraciliation with the nuclear apparatus creates distinct diagnostic morphology. The DKs were clearly defined only at a pH of 7.5 and 7.6.

Comparison of the acetone and ordinary developers

The protargol preparations using the acetone and ordinary sodium sulfite/hydroquinone developers were shown to denote the difference of staining quality on each specific pH levels (Figure 1; Supplementary figure 1). The disadvantages of using ordinary developer were as follows: (1) the mismatch of impregnation timing between nucleus apparatus and ciliature, (2) less contrasted between infraciliatures and cilia, and (3) indistinct nucleoli (Supplementary figure 1). However, the number and arrangement of DKs were rather conspicuous because cilia usually

Figure 1. Ventral views of Pleurotricha oligocirrata impregnated with protargol solutions at different pH levels. (A) pH 7.1, (B) pH 7.3, (C) pH 7.5, (D) pH 7.6, (E) pH 7.8, and (F) pH 7.9. AZM: adoral zone of membranelles; BC: buccal cirrus; FC: frontal cirri; FVC: frontoventral cirri; LMR: row of left marginal cirri; Ma: macronuclear nodules; Mi: micronuclei; PTVC: pretransverse ventral cirri; PVC: postoral ventral cirri; RMR: row of right marginal cirri; TC: transverse cirri; UM: undulating membranes.
impregnated strongly in most of pH levels (7.1–8.1), but the impregnation quality conspicuously decreased under high pH levels (8.6–9.0) when we used the ordinary developer (data not shown for pH 9.0). The cytoplasm rather strongly stained under pH 7.1–7.6 and became faintly stained from pH 7.6 to 8.6 gradually (Supplementary figure 1). This result revealed that the optimal pH conditions are not the same and depend on which developer we use. The optimal pH of ordinary developer was in between pH 7.8 and 8.1 in our experiments. According to the previous studies, Pan et al. (2013) provided a protocol for protargol synthesis that was originally modified from that published by Davenport et al. (1952), which suggested adjusting the pH to between 8 and 9. While the pH was measured and adjusted during protargol synthesis, pH rapidly increased, resulting in the solution becoming too dark and not working for staining. The protargol solution

**Optimal protargol impregnation conditions**

Conditions were optimized to characterize hypotrichs using laboratory-synthesized protargol powder. Because the pH was not constantly adjusted to between 8 and 9 during each attempt at synthesizing protargol, the pH of the protargol solution itself was adjusted immediately prior to the impregnation. Pan et al. (2013) provided a protocol for protargol synthesis that was originally modified from that published by Davenport et al. (1952), which suggested adjusting the pH to between 8 and 9. While the pH was measured and adjusted during protargol synthesis, pH rapidly increased, resulting in the solution becoming too dark and not working for staining. The protargol solution

**Figure 2.** Dorsal views of *Pleurotricha oligocirrata* impregnated with protargol solutions at different pH levels. Each specimen is identical to those in Figure 1. (A) pH 7.1, (B) pH 7.3, (C) pH 7.5, (D) pH 7.6, (E) pH 7.8, and (F) pH 7.9. CC: caudal cirri; DK: dorsal kineties.
generated in this study (0.8% w/v in distilled water) had an average pH <6 without any adjustment, resulting in an insufficient protargol deposition on key morphological structures, particularly nuclear apparatus. A range of pH levels was tested and the acetone developer, first reported by Dieckmann (1995), was used according to the protocol published by Foissner (2014). Microphotographs of stained specimens were created using protargol solutions that had not yet been optimized from previous studies (Jung et al. 2015, 2016; Kim and Jung 2016; Park et al. 2017a, 2017b). Based on assessments of the five morphological attributes (see above, for the diagnostics of hypotrichs), a pH of 7.5–7.6 was considered optimal for hypotrichs when using ‘Procedure A’ published by Foissner (2014). Other hypotrichs were assessed under the pH 7.5–7.6 presented in Figure 3. However, each time the protargol is synthesized, the optimal condition may vary slightly because few batches had the condition at level of pH 7.9 with acetone developer. Further studies are still necessary to

Figure 3. Ventral views of hypotrichs impregnated with a protargol solution at a pH of 7.5–7.6. (A) Allotricha mollis, (B) Gastrostyla steinii, (C) Gonostomum affine, (D) Hemiurosoma similis, (E) Notohymena australis, (F) Pseudokeronopsis flava, (G) Hemicycliostyla franzi, and (H) Pseudourostyla subtropica.
find more optimal laboratory-synthesized protargol and an optimal condition for the Quantitative Protargol Staining (QPS; Montagnes and Lynn 1987). In particular, no tests for the QPS using the laboratory-synthesized protargol have yet been attempted.

**Conclusion**

This study was performed to optimize the preparation of high-quality hypotrich specimens using laboratory-synthesized protargol. Based on our case study, protargol synthesis was modified as follows: (1) protargol powder was created without adjusting the pH during synthesis, (2) the pH of the protargol solution was adjusted to a pH of 7.5–7.6 during staining, (3) the acetone developer was more effective, and (4) a previously untreated peptone derived from peas was used successfully for protargol synthesis. While the conditions used in this study to synthesize protargol are not completely comparable to commercial protargol, this information could help taxonomists/ecologists investigate the diversity of ciliates, particularly in hypotrichs. In addition, we recommend testing the pH levels to find the optimal condition for each batch of the laboratory-synthesized protargol.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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