Improving TEM Fixation with Additives

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Introduction

Attempts to fix the gregarine *Pterospora floridiensis* (a protozoan parasite found in marine bristle worms) for TEM using a conventional glutaraldehyde and osmium protocol produced poor preservation of the outer pellicular membranes and internal membranous structures. However, fixation success was achieved using the well-known fixative additives tannic acid and potassium ferrocyanide, which improved membrane preservation, cytoplasmic integrity, and contrast in both the unencysted feeding stage of *P. floridiensis* as well as the difficult-to-fix cyst stage. The longer fixation times (24 hours) needed for the cysts were compatible with the modified fixatives. Interestingly, *both* additives were needed to improve fixation.

Materials and Methods

General. The polychaete worm *Axiothella mucosa* was collected from the sandy sediment in St. Andrew Bay, Florida. The feeding gamont stages (Figure 1a) and the transmissive oocysts (Figure 1b) were collected from the coelom of the host and fixed for TEM with a conventional glutaraldehyde/osmium protocol as well as with experimental protocols that included the fixative additives tannic acid and/or potassium ferrocyanide. The tannic acid and potassium ferrocyanide methods were modified from earlier studies [1–3]. All fixatives were used at room temperature. Suppliers for reagents were as follows: tannic acid, sodium cacodylate, glutaraldehyde (Electron Microscopy Sciences), osmium tetroxide (Polysciences), and potassium ferrocyanide (Aldrich).

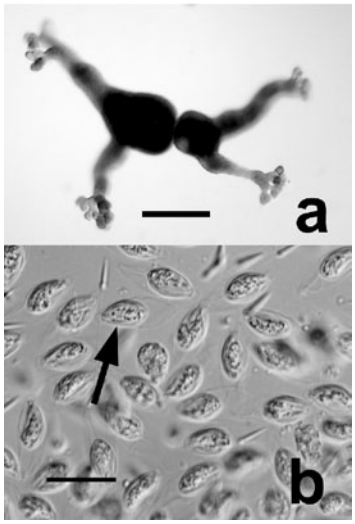

Figure 1: *Pterospora* light microscopy. (a) The live gamont stage showing the bizarre shape of the two cells joined prior to gamete formation. Each cell has a large soma and two trunks through which the cytoplasm moves back and forth. (b) The transmissive oocyst stage. The inner capsule containing the sporozoites is indicated at the arrow. Bar lengths: Figure 1a = 150 µm, Figure 1b = 20 µm.

Fixation of the gamont (unencysted feeding stage) [4]:

(1) Conventional fixation: 3% glutaraldehyde buffered with 0.05M Na cacodylate pH 7.5 for 1–6 hours. Post-fixation in 2% OsO4 for 1–2 hours in cacodylate buffer.

(2) Modified fixation with additives: *1.5% glutaraldehyde with 0.2% tannic acid*, buffered with 0.05M Na cacodylate pH 7.5 for 30–60 minutes. Post-fixation in *1% OsO4 with 1% potassium ferrocyanide* for 30–60 minutes in cacodylate buffer.

Fixation of the oocyst [5]:

(1) Modified fixation with additives: *2% glutaraldehyde with 0.2% tannic acid*, buffered with 0.05M Na cacodylate pH 7.5 for 24 hours. Post-fixation in *0.5% OsO4 with 1% potassium ferrocyanide* for 24 hours in cacodylate buffer.

Tissue processing. Gamonts and oocysts were dehydrated in isopropanol and then placed in a 1:1 solution of isopropanol:Spurr epoxy, followed by two changes of Spurr prior to embedment in TEM molds. As opposed to the gamont stage, the oocysts were much more difficult to dehydrate and infiltrate with plastic and were processed over a period of several days. The cells were then incubated at 68° C (154° F) overnight to polymerize the resin. Thin sections were cut on a Sorvall MT2B ultramicrotome, collected on uncoated copper grids, and stained with uranyl acetate and lead citrate (~7 minutes each).

TEM stains. Uranyl acetate was used as a saturated solution in 25% ethanol (ethanol has been added to this same bottle, which has been kept at room temperature and used for over 12 years). Lead citrate was made using a method similar to that of Venable and Coggeshall [6]. Lead citrate batches have been used for years. Both the uranyl and lead stains were pipetted from below the meniscus of the fluid, upon which precipitate floated, and were centrifuged at over 5000 RPM for 5 minutes before using. Small droplets of stain were taken from below the meniscus after centrifugation. Grids were stained individually on a sheet of dental wax.

Photography. Thin sections were photographed with a Hitachi H-7000 (The University of Alabama at Birmingham) or a Zeiss EM-10CR TEM (Auburn University) operating at 75KV or 60KV, respectively. All TEM images were scanned from negatives and adjusted for brightness, contrast, and gamma using Adobe Photoshop® Elements 6.0. Images of live material were taken on a Nikon E600 light microscope using either a digital or film camera and were adjusted similar to the TEM images.

Results

Overview. Tannic acid and postassium ferrocyanide improved the fixation of membranes for two unique life cycle stages, the gamont and the sporozoites within the oocyst (Figures 2–3). The gamont stage fixed quickly, as it had no

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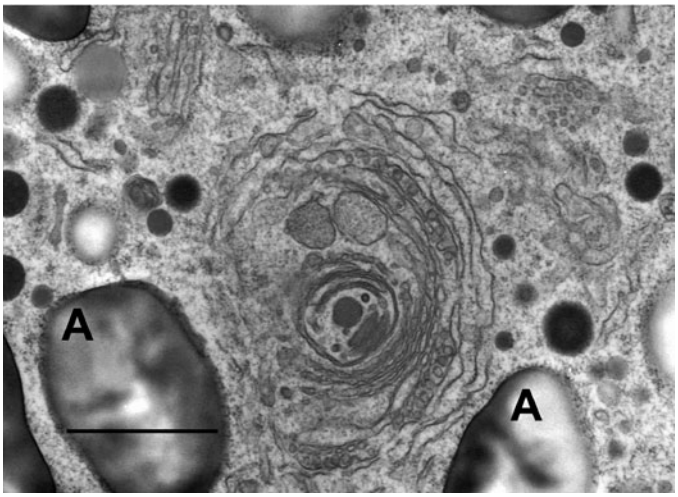

Figure 2: TEM of the endomembrane system. The golgi apparatus and transport vesicles of the gamont are well preserved. "A" indicates amylopectin granules. Bar = 1 μm.

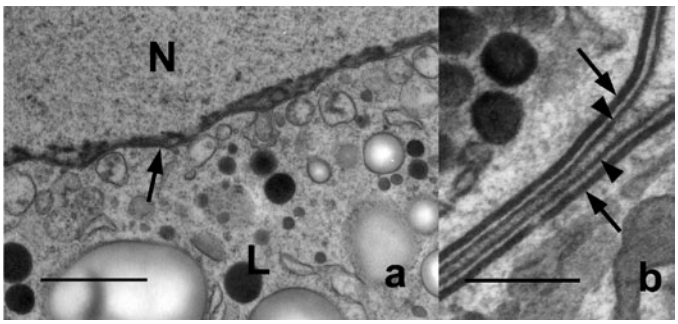

Figure 3: TEM of the nuclear membrane and external membranes. (a) The inner cytoplasm and nuclear membrane (arrow) of the gamont. Lipid droplets (L), amylopectin granules, and cytoplasmic vesicles are well preserved. "N" indicates the nucleus. (b) High magnification of two sporozoites within the oocyst capsule. Each sporozoite has a three-membraned pellicle: one outer membrane (arrowheads) and two closely appressed inner membranes (arrows). Bar lengths: Figure 3a = 1 μm, Figure 3b = 0.2 μm.

external covering. The sporozoites were surrounded by 2 oocyst walls and a gametocyst wall, making long fixations (24 hours) necessary.

Gamonts. Conventional glutaraldehyde and osmium fixation provided a uniform cytoplasmic matrix and adequately fixed the lipid droplets, amylopectin granules, and outer pellicle. However, the three-membraned nature of the pellicle was poorly preserved, as were other membranous structures such as the golgi apparatus and ER. In other preparations, fixation with only one additive (tannic acid or potassium ferrocyanide) did not improve the results, even though each additive has been reported to improve membrane structure. Tannic acid used without the other additive did not improve the fixation of internal membranes. Potassium ferrocyanide used without tannic acid did improve internal membrane structure, but it poorly preserved the cytoplasm. The use of both additives, however, gave superior fixation of all cytoplasmic components as well as the membranous organelles (Figures 2 and 3a). The success of using tannic acid and potassium ferrocyanide indicates a complementary effect of the additives. Particularly well preserved were the golgi apparatus (unrecognizable with standard fixation), the ER, and small transport vesicles.

Oocysts. Both additives were used in a later study of the oocyst, based on the success of the gamont study. As with the gamont results, the sporozoites revealed well-preserved golgi, ER, and secretory vesicles. This was more pronounced in early stage sporozoites in which membranous organelles are well developed. Additionally, the three membranes of the pellicle of developing sporozoites were well preserved (Figure 3b).

Discussion

Standard fixation provided poor preservation of the outer pellicular membranes and internal membranes of *Pterospora* [7]. Later studies of the gamont [4, 8] and the developing oocyst [5] demonstrated the usefulness of tannic acid and potassium ferrocyanide as additives in combination. Tannic acid is a well-known additive to the glutaraldehyde fixation step [1–2, 9–10]. This may act as a mordant, binding to membranes to enhance their staining with osmium and standard EM stains [2, 9, 10]. Hayat [2, 10] discusses the literature and proposed mechanisms by which tannic acid may work, especially in combination with osmium. Potassium ferrocyanide is likewise a known additive to osmium tetroxide [2–3, 9–11], which reduces the fixative. In studies with *Pterospora*, both additives were mixed with the fixative the same day they were used. Separately, these additives did not improve the protozoan's fine structure, but they were successful in combination with each other. This combination was compatible with both long (24 hours for each fixative) and short (<1 hour for each fixative) fixation times.

Conclusions

Tannic acid and potassium ferrocyanide should be considered as useful additives, even if they have been tried separately and were not successful. When used in combination they may dramatically improved membrane structure as they did for the marine protozoan *Pterospora*.

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