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Plasma etching and ashing: a technique for demonstrating internal structures of helminths using scanning electron microscopy

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Abstract

Plasma etching and ashing for demonstrating the three-dimensional ultrastructure of the internal organs of helminths is described. Adult worms of the cestode *Caryophyllaeides fennica* were dehydrated through an ethanol series, critical point dried (Polaron E3000) and sputter coated with 60% gold-palladium (Polaron E5100) and glued to a standard scanning electron microscope (SEM) stub positioned as required for ashing. After initial SEM viewing of worm surfaces for orientation, stubs were placed individually in the reactor chamber of a PT7150 plasma etching and ashing machine. Worms were exposed to a radio frequency (RF) potential in a low pressure (0.2 mbar) oxygen atmosphere at room temperature. The oxidation process was controlled by varying the times of exposure to the RF potential between 2 to 30 min, depending on the depth of surface tissue to be removed to expose target organs or tissues. After each exposure the oxidized layer was blown from the surface with compressed air, the specimen sputter-coated, and viewed by SEM. The procedure was repeated as necessary, to progressively expose successive layers. Fine details of organs, cells within, and cell contents were revealed. Ashing has the advantage of providing three dimensional images of the arrangement of organs that are impossible to visualize by any other procedure, for example facilitating testes counts in cestodes. Both freshly-fixed and long-term stored helminths can be ashed. Ashing times to obtain the desired results were determined by trial so that some duplicate material was needed.

Introduction

The internal anatomy of helminths is often difficult to visualize or interpret using conventional investigative procedures such as whole mounts, histological sections, scanning or transmission electron microscopy. Hence, the plasma etching and ashing (http://www.ebsciences.com/sem/pt7150.htm) are worth considering and may prove valuable in demonstrating three-dimensional ultrastructural details of internal anatomy or the numbers and relationships of parts. In the present study, this technique is described and illustrated using gravid, adult worms of *Caryophyllaeides fennica* (Schneider) (Cestoda: Caryophyllaeidea), although similar results have been obtained with other cestodes (*Atractolytocestus huronensis* Anthony, *Schistocephalus* spp.) and a monogenean (*Dictyocotyle coeliaca* Nybelin).

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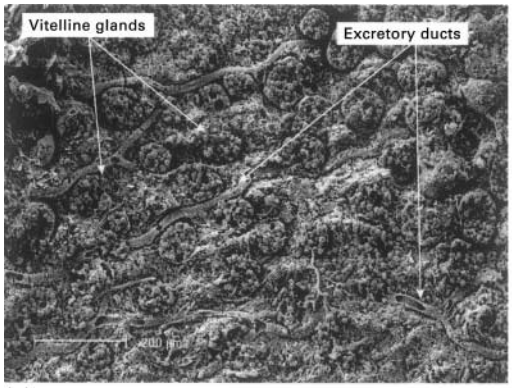
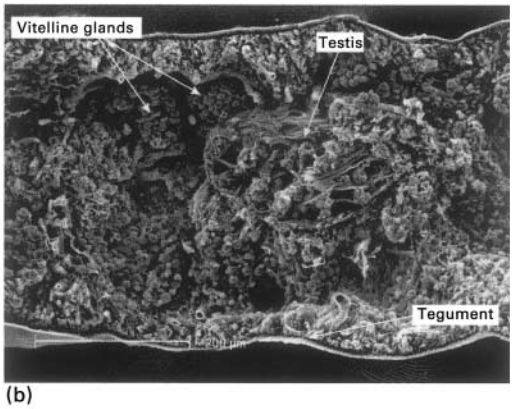
Materials and methods

Living *C. fennica* were collected from the intestines of roach, *Rutilus rutilus* (L.) from Llyn Tegid (Bala Lake) Wales, UK in October 2001, relaxed in water at room temperature and fixed in cold 5% formaldehyde. Worms



(a)



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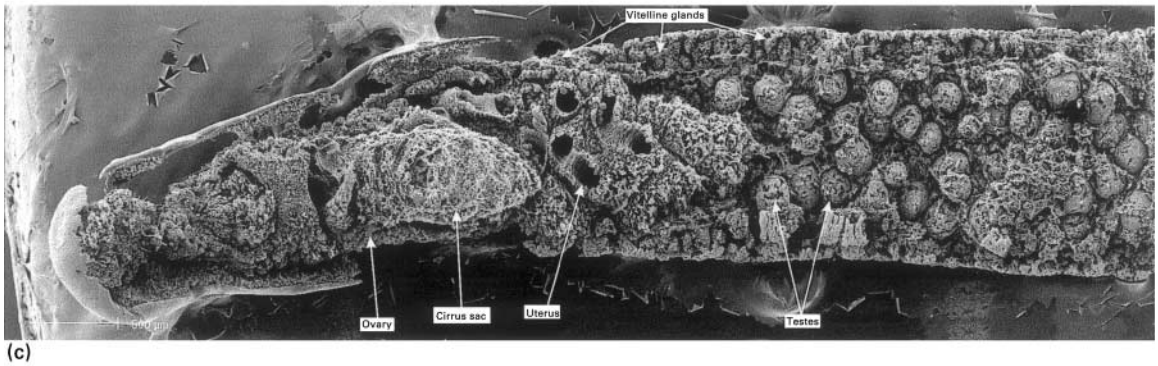
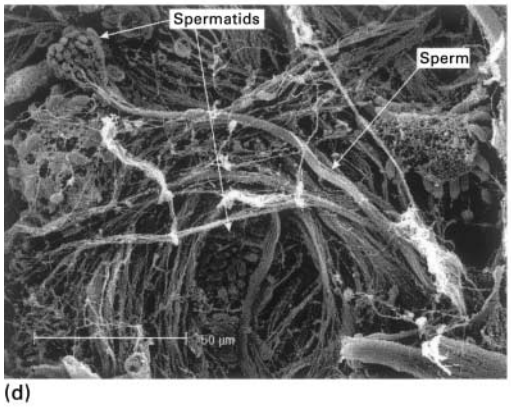
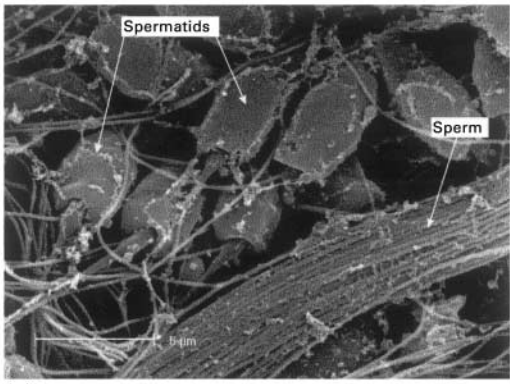


(c)



(d)



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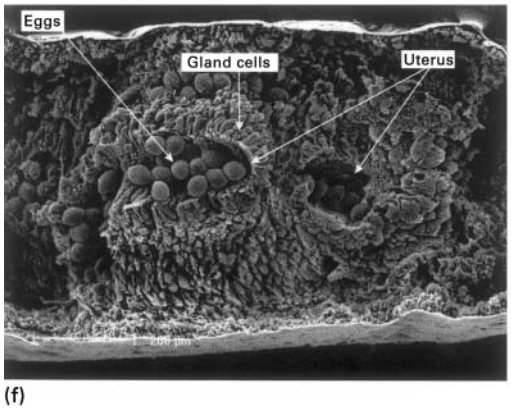
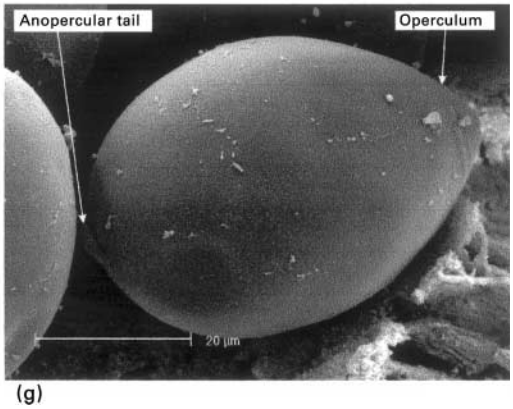


(f)



(g)

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were dehydrated through an ethanol series, with two changes of absolute ethanol, critical point dried (Polaron E3000), sputter coated with 60% gold-palladium (Polaron E5100) and glued to a standard scanning electron microscope (SEM) stub using Bostik clear adhesive (Costa *et al.*, 2003). Specimens of *C. fennica* were orientated on stubs as required for viewing after ashing and mounted with the dorsal surface uppermost to facilitate comparison with whole mounts. Prior to ashing the external features were viewed with a Philips XL30 SEM and recorded as TIF files.

Specimens mounted on SEM stubs were placed individually in the reactor chamber of a PT7150 plasma etching and ashing machine and exposed to a radio frequency (RF) potential in a low pressure (0.2 mbar) oxygen atmosphere at room temperature. The exposed upper surfaces of *C. fennica* were progressively oxidized whilst the underlying parts remained undamaged. The oxidation process was controlled by varying the time of exposure to the RF potential. Exact times were found by trial and error using duplicate specimens and these times varied between 2 and 30 min or more, depending on the depth of surface tissue to be removed to expose the target organs or tissues. After each exposure, the oxidized layer was carefully blown from the surface with compressed air, the specimen sputter-coated, and viewed under the SEM. The procedure was repeated as many times as necessary, to progressively expose and view successive layers, until the specimen was ashed away.

Transverse sectional views were obtained by cutting the body of worms with a razor blade at the desired positions along their length after critical point drying. A smooth surface is left that is difficult to interpret, but mounting the specimens on SEM stubs with the transverse cut uppermost, ashing this surface for 3 min, then sputter coating, gave excellent results.

Results

Entire specimens of *C. fennica* were ashed from the dorsal surface (after preliminary viewing under SEM). Successive 5-min periods of ashing established the depth for each set of observations. After 10 min a surface view of the cortical layer of vitelline glands with excretory ducts interwoven between the glands was obtained (fig. 1a). Where surface membranes of vitelline cells were ashed away, the central nucleus and peripheral vitelline droplets contained within were seen at higher SEM magnifications. A transverse view (fig. 1b) in the anterior region of a worm demonstrates cortical vitelline glands and medullary testes (family Lytocestidae).

Figure 1c, at a depth of about 35% through the worm, displays the characteristic A-shaped ovary of *C. fennica*, the cirrus sac, uterus, vitelline glands and testes in their respective natural positions. The round cells of the vitelline glands (fig. 1b) and spermatids and sperm in the testes (fig. 1d and e) readily differentiate these organs. Viewed at this magnification (fig. 1c) testes were readily counted.

A transverse view of the uterus (fig. 1f) shows eggs, with gland cells surrounding the uterine wall. At a higher magnification, eggs (fig. 1g) were clearly shown to be operculate.

Discussion

Plasma etching and ashing are techniques used in a range of industrial processes, but in biology are typically applied to visualize mineral elements in tissues (Hohman & Schraer, 1972; Dinsdale *et al.*, 1979; Chapman, 1985; Arnold *et al.*, 1990; Bogren *et al.*, 1995). A higher SEM resolution of etch-resistant cell components of fixed and embedded tissues is often claimed (Humphreys & Henk, 1979; Yang *et al.*, 1988). Initially, the procedure was used to obtain accurate counts of the number of testes in the cestode *Atractolytocestus huronensis* that were needed for taxonomic purposes. *Atractolytocestus huronensis* has vitelline glands and testes that are about the same size, thus counting was impractical in stained whole mounts and inaccurate with reconstructions from serial sections. In ashed specimens, however, a good resolution of the testes was obtained, although care was necessary not to ash too deeply otherwise testes were lost. This latter constraint applies particularly in helminths where testes are arranged in more than one layer, a condition easily checked with ashed transverse views. *Caryophyllaeides fennica*, illustrated here, has testes in a single layer, and much larger than the vitelline glands (fig. 1b), and although the two organ systems were distinguishable in a well-stained whole mount, counting was greatly facilitated by ashing.

Fine details were revealed at all levels, i.e. in the organs, cells within, and even cell contents. For example, when cell membranes of vitelline cells were ashed to expose the contents, the central nucleus surrounded by peripheral vitelline droplets was clearly distinguishable. Ashing also revealed the relationships of spermatids to sperm in the testes (fig. 1d) (compare Swiderski & Mackiewicz, 2002).

Fig. 1. (a) The dorsal surface of *Caryophyllaeides fennica* about 15% through the depth of worm: to show vitelline glands in the parenchyma, with excretory ducts. Ashing 10 min. (b) Transverse view of the vitelline glands and testes of *C. fennica*; vitelline glands are cortical, the testes are medullary; excretory ducts are visible in section (see fig. 1a). Ashing 3 min. (c) The dorsal surface of *C. fennica* about 35% through the depth of the worm to show cirrus sac, ovary, uterus with eggs, vitelline glands and testes. In *C. fennica* the testes are much larger than the vitelline glands. Ashing 45 min. (d) Groups of spermatids and sperm of *C. fennica*. Ashing 45 min. (e) Spermatids and sperm of *C. fennica* at a higher magnification; the spermatid-sperm relationship is shown clearly. Compare with Swiderski & Mackiewicz (2002). Ashing 45 min. (f) Transverse view of the uterus of *C. fennica* to show eggs with gland cells that surround the uterine wall and open into the uterus. Ashing 3 min. (g) Transverse view of eggs in the uterus of *C. fennica*. Note operculum (right) and anopercular tail (left). Eggs hatch after ingestion by an oligochaete intermediate host. Ashing 3 min.

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Ashing has a particular advantage in that it provides three-dimensional images of the internal organs (fig. 1c) which can be interpreted easily and confirmed readily in contrast with the same information gained by light microscopy and histology. The procedure has a further advantage of providing information that was impossible to visualize by any other procedure, as noted above, the testes counts for *A. huronensis*. A further merit was that helminths that have been fixed and stored for very long periods were equally amenable to ashing. Such specimens were processed as described earlier.

However, there were some disadvantages with the procedure. A reasonable amount of duplicate material is desirable to trial the times needed, and to relate with the particular requirements of an investigation. Experience with a range of specimens soon provided a reasonably accurate guide to times that guaranteed consistent and repeatable results.

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