Scanning Microscopy, Vol. 3, No. 1, 1989 (Pages 387-390)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/89$3.00+.00

PREPARATION OF DIPTERAN LARVAE FOR SCANNING ELECTRON MICROSCOPY WITH SPECIAL REFERENCE TO MYIASIGEN DIPTERAN SPECIES

I. Ruiz-Martinez, M.D. Soler-Cruz*, R. Benitez-Rodriguez,
M. Diaz Lopez, and J.M. Perez-Jimenez

Department of Parasitology, University of Granada,
18001 Granada, Spain

(Received for publication June 30, 1988, and in revised form January 25, 1989)

Abstract

Although controversy exists concerning the role of chemical fixatives in scanning electron microscopy (SEM) studies of Dipteran larvae, we have observed that filtered 10% formaldehyde solution gives excellent results as a preservative. After immersing in vivo in formaldehyde, the larvae material is preserved for prolonged periods (up to 8 months), before examination with SEM. As a fixative, formaldehyde preserves the structure of the larval cuticle and produces no visible artifacts. Moreover, postfixation is not necessary.

Due to pecularities of the way of life of Wohlfahrtia magnifica (principally the accumulations of necrotic tissue, purulent particles, and other types of substances that often adhere to the numerous spines of larvae), this species must be cleaned before examination by SEM. Manual cleaning with alternating bidistilled water and 0.9% saline solution proved to be a rapid, easy and inexpensive method that gave good results.

Both lyophilization drying and critical point drying were used before sputtering the material. While lyophilization drying proved to be the most effective method for instars II and III, critical point drying was the best technique for study of specimens belonging to instar I. The optimum time for drying and conditions for lyophilization and sputter-coating with gold were determined experimentally. Samples were mounted on SEM stubs with double-sided adhesive and silver conductive paint.

The method proposed is easy and effective for the SEM study of larvae myiasis-producing diptera.

Key Words: Diptera, Wohlfahrtia magnifica, larval cuticle, antennae, palpi, spines, fixation, critical point drying, lyophilization drying, scanning electron microscopy.

*Address for correspondence:

M.D. Soler-Cruz,
Department of Parasitology,
Rector Lopez-Argueta s/n.
18001 Granada, Spain

Phone No. (958) 270420

Introduction

To obtain good results from dipteran larvae by scanning electron microscopy (SEM), careful manipulation of specimens from the moment of collection is required. The use of chemical fixatives has produced no worthwhile results, because their effects on biological materials are often unknown and their effective penetration into delicate tissues is questionable (Colwell and Kokko, 1986). Several of these methods include heptane (Zalokar, 1971) or dimethylsulfoxide -DMSO- (D.D. Colwell, unpublished) based fixatives to facilitate penetration. Complex fixatives have also been used (Grodowitz et al., 1982). Some authors (e.g., Bjerke et al., 1979) have used chemical dehydration without previous fixation (2,2-dimethoxypropane). Other authors have used chemical fixatives such as trialdehyde (Mahowald and Turner, 1978), 10% formalin on ice (Sandeman et al., 1987), and 70% ethanol (Cogley et al., 1983) with satisfactory results.

Cohen (1974) proposed freeze-substitution for use with small arthropods as an alternative method to chemical fixation. This technique was modified by Wharton (1982) for parasitic nematode larvae. Recently Colwell and Kokko (1986) used this method for the preparation of dipteran larvae with notable results. The treatment of the sample in a vacuum has also been used (Wergin and Stone, 1981).

Baccetti (1975), Labuschagne and Loots (1975), Mourier (1977), Credland (1978), and Kontermann (1980) have applied several techniques to the study of insects by means of SEM. Such studies have shown themselves to be fundamental in species of Diptera with sanitary and economic interest.

In this paper, we summarize our experiences with a number of preparation techniques as applied to Dipteran larvae, with a view to arrive at the best procedure to prepare these samples for SEM.

Materials and Methods

Larvae belonging to the three developmental instars of W. magnifica were placed in vivo into filtered 10% formaldehyde. Fixation with glutaraldehyde and 2% osmium tetroxide solutions was alternatively assayed in order to evaluate and compare the results obtained after using this method. In subsequent steps we removed particles adhereing to the larval cuticle by three different methods: a) manual cleaning, using a fine brush, with alternate 10 min washes in 25 ml bidistilled water and 0.9% saline solution (9 g NaCl- 100 ml of water); b) by means of

Table 1: Effects of different preparation procedures on the quality of results

+++ : Optimum results; ++ : Good results; + : Unsatisfactory results; 0 : No results

		Instar I	Instar II	Instar III	Body surface	Soft and delicate structures	Conservation
FIXATION	Chemical: 10% Formaldehyde	+++	+++	+++	+++	+++	+++
	Chemical: Glutaraldehyde + Osmium tetroxide	+	0	++	++	0	++
	Physical: Freeze-Substitution	++	++	++	++	+	++
CLEANNESS	Manual (Bidistilled water + 0.9% ss*)	+++	+++	+++	+++	+++	+++
	Sonication (water-filled sonicator)	+	0	0	+	0	+
	Antistatic solution	0	0	0	0	0	0
DEHYDRATION	Lyophilization: Conservation	++	+++	+++	+++	+++	+++
	Lyophilization: Lyophilization time	2-2.40h	5-5.30h	7-7.30h			
	Critical Point	+++	++	+	++	+	++
MOUNTING	Double-sided adhesive	++	+	+			+
	Silver conductive paint	++	+	0			+
	Combined method	+++	+++	+++			+++
SPUTTER-COATED WITH GOLD	Times	1.5 min	2 min	3 min			+++

* saline solution

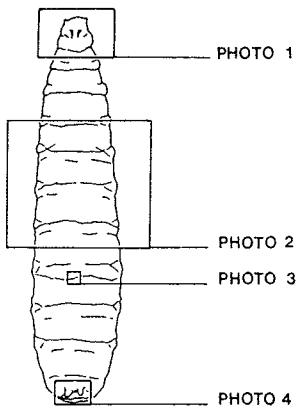


Figure 1. Position of photographs (Figs. 2 - 5 corresponding to Photos 1 - 4 respectively) on ventral surface of larva Wohlfahrtia magnifica.

a water-filled sonicator (Cogley et al., 1981), and c) with antistatic solution (96.6% glycerol, 0.05% KCl, and 3.35% bidistilled water; Brody and Wharton, 1971).

After placing the material in sterilized Eppendorf tubes, dehydration was carried out by lyophilization (without water) at -40°C and 125 bars. The optimum time of lyophilization was determined after several tests.

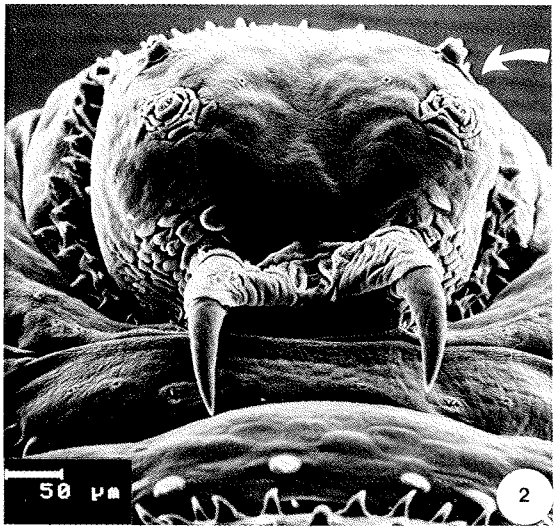
Critical point drying (CPD) with CO_2 as the transition fluid (at 31°C and 90 bars) was also used. Dehydration before CPD was performed in 50 to 100% gradient acetone for 30 min at room temperature, with amyl acetate as the intermediate fluid.

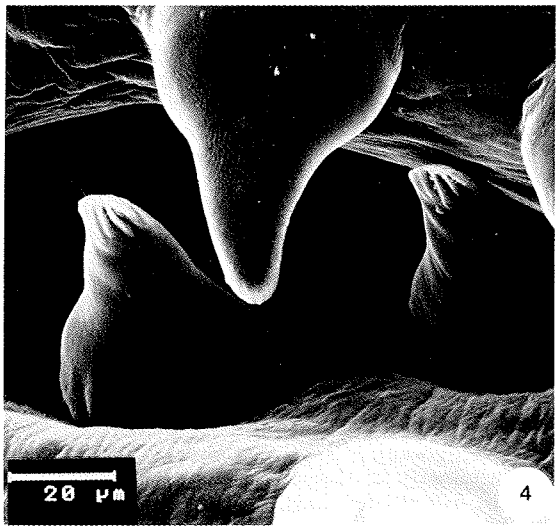
Samples were mounted on SEM stubs with double sided adhesive tape and silver conductive paint. Sputtering for different time periods (20 sec - 4 min) was also performed in order to determine the optimum time for sputter-coating with gold in the presence of argon gas. The material was examined in a Zeiss DSM 950 SEM at 15 to 25 kV. Photographs were taken with a polaroid system using 52 Polapan snapshots.

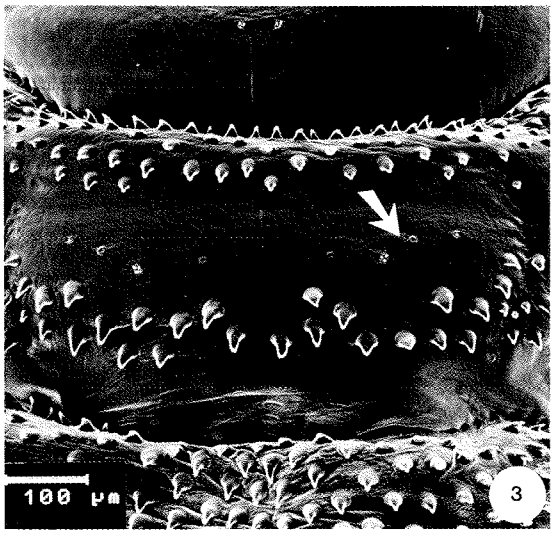
Results and Discussion

The results are shown in Table 1 and Figures 1 through 5.

Obtaining good preparations of these dipteran larvae for SEM is difficult because Wohlfahrtia magnifica Schin. is a Palearctic obligate parasite.







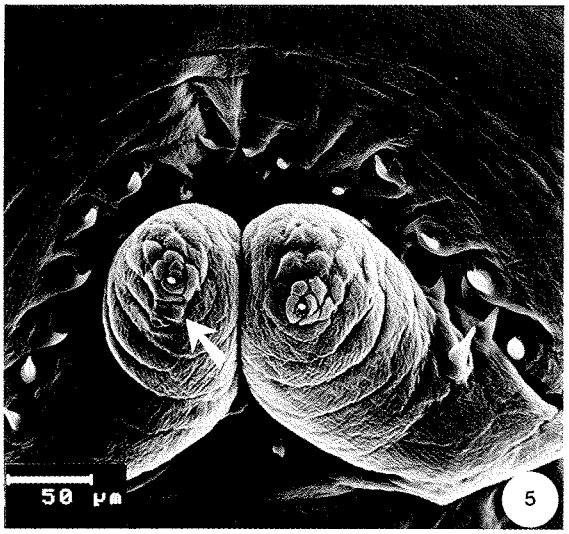


Figure 2. First segments of instar II of Wohlfahrtia magnifica; palpi and antennae: arrow.

Figure 3. Ventral view of instar III; cuticular sensilla: arrow.

Figure 4. Detail of spines in instar III.

Figure 5. Anal lobes in instar II; anal sensilla: arrow.

Accumulations of necrotic tissues, purulent particles, and other types of substances often adhere to the numerous spines of larvae (Teskey, 1981; Sandeman et al., 1987). Moreover, the cuticle seems to be impermeable to fixatives and solvents.

Placing the larvae in vivo in 10% formaldehyde favours penetration of the fixative into the tissues and adequately preserves the fine structures such as palpi and antennae (Fig. 2). Although several techniques avoid the use of chemical fixatives (Cohen, 1974; Colwell and Kokko, 1986), 10% formaldehyde solution produces good results, provided it is previously filtered to prevent the formation of precipitating plaques on the larval cuticle. Moreover, fixation in 10% formaldehyde solution allows long term storage of the material (6-8 months) and firmly maintains larval tissue texture (Figs. 3 and 4). Fixation and post-fixation of this material by means of glutaraldehyde and osmium tetroxide (Mahowald and Turner, 1978; Sandeman et al., 1987) (Table 1) failed to yield satisfactory results; numerous artifacts were produced on the cuticle surface.

Neither water-filled sonicator (Cogley et al., 1981) nor antistatic solution (Brody and Wharton, 1971) removed the larger particles adhereing to the cuticle. In addition, with the latter method alcohol solutions contracted the cuticle and left glycerol residues between the body spines. In our study, manual cleaning combined with alternate washing with bidistilled water and 0.9% saline solutions is shown to be a very simple, inexpensive, rapid and effective method (Table 1).

Lyophilization gave good results in instars II and III (including small structures at high magnification); CPD was suitable for instar I, which contains very delicate structures, although treatment with a graded acetone series resulted in damage to some of

these structures (Baccetti, 1975; Principato and Tosti, 1988) (Table 1).

Mounting the sample on stubs with double-sided adhesive tape combined with the use of silver conductive paint (Colwell and Kokko, 1986; Colwell 1986) gave good results. Not only was conductivity increased, but the sample remained stable at high magnifications (Table 1).

A cooling system was used when sputter-coating to prevent artifacts from increased temperature.

Accelerating voltages at 15 to 25 kV were found to be the most effective (Cogley et al., 1981). Minimum working distance (10-15 mm) further optimized visualization of the samples (Table 1).

Acknowledgements

The authors express their gratitude to the Electron Microscopy Unit of the Technical Services of the University of Granada, for their assistance in preparing, examining, and photographing of samples, and to Ms. Karen Shashok for revising the English style of the manuscript.

This study has been supported by a grant from the General Direction of Investigation and Agrary Extension of Junta de Andalucia (Project No. PA. A3. 85. 1).

References

Baccetti B. (1975) Critical point for preservation of larvae and adult insects with soft teguments. Bull. Soc. Entomol. Ital., 107, 42-47.

Bjerke JM, Freeman TP, Anderson AW. (1979) A new method of preparing insects for scanning electron microscopy. Stain Technol., 34, 29-31.

Brody AR, Wharton GW. (1971) The use of glycerol-KCl in scanning electron microscopy of Acari. Ann. Ent. Soc. Amer., 64(2), 528-530.

Cogley TP, Anderson JR, Weintraub J. (1981) Ultrastructure and function of the attachment organ of warble fly eggs (Diptera: Hypodermatidae). Int. J. Insect Morphol. & Embryol., 10(1), 7-18.

Cogley TP, Anderson JR. (1983) Ultrastructure and function of the attachment organ of Gasterophillus eggs (Diptera: Gasterophillidae). Int. J. Insect Morphol. & Embryol., 12(1), 13-23.

Cohen AL. (1974) Critical Point Drying. In: Principles and Techniques of Scanning Electron Microscopy. Biological Applications. Vol.1. M.A. Hayat (ed.). Van Nostrand Reinhold Co., N.Y., 44-112.

Colwell DD, Kokko EG. (1986) Preparation of Dipteran larvae for scanning electron microscopy using a freeze-substitution technique. Can. J. Zool., 64, 797-799.

Colwell DD. (1986) Cuticular sensilla on newly hatched larvae of Cuterebra fontinella Clark (Diptera: Cuterebridae) and Hypoderma spp. (Diptera: Oestridae). Int. J. Insect Morphol. & Embryol., 15(5-6), 385-392.

Credland PF. (1978) The application of scanning electron microscopy to the study of chironomal larvae. Acta Univ. Carol. (Biol.), 1978(1-2), 35-41.

Grodowitz MJ, Krchma J, Broce AB. (1982) A method for preparing soft bodied larval diptera for scanning electron microscopy. J. Kans. Entomol. Soc., 55, 751-753.

Kontermann R. (1980) Haftorgane von Insekten. Mikrokosmos, 69(4), 362-365.

Labuschagne MC, Loots GC. (1975) Techniques for the preparation of ticks for scanning electron microscopy. Reeks B. Natuurwet., 67, 1-5.

Mahowald AD, Turner FR. (1978) Scanning electron microscopy of Drosophila embryos. Scanning Electron Microsc., 1978;II; 11-20.

Mourier H. (1977) House flies. Naturens Verd., 1977(1), 2-13.

Principato M, Tosti M. (1988) Scanning electron microscope observations on the anterior thoracic and postabdominal spiracles of Gasterophillus larvae (Diptera: Gasterophillidae). Int. J. Parasitol., 18(2), 191-196.

Sandeman RM, Collins BJ, Carnegie PR. (1987) A scanning electron microscopy study of Lucilia cuprina larvae and the development of blowfly strike in sheep. Int. J. Parasitol., 17(3), 759-765.t

Teskey HJ. (1981) Morphology and terminology-larvae. In: Manual of Nearctic Diptera. (Ed.) McAlpine JF, Peterson BV, Shewell GE, Teskey HJ, Vockeroth JR, Wood DM. Vol. 1, 65-88. Department of Agriculture. Ottawa, Canada. Monograph no. 27.

Wergin WP, Stone AR. (1981) Techniques for preparing and examination of plant parasitic Nematodes in the scanning electron microscope. Scanning Electron Microsc., 1981;III: 169-176.

Wharton DA. (1982) Observations on the coiled pasture of trichostrongyle infective larvae using freeze-substitution method and scanning electron microscopy. Int. J. Parasitol., 12(4), 335-343.

Zalokar M. (1971) Fixation of Drosophila eggs without pricking. Drosophila Inf. Ser., 47, 128-129.

Discussion with Reviewers

J. Krchma: How were the larvae frozen? In our experiences with freeze drying insects, freezing method strongly influenced results. Also, were the larvae frozen in water or removed from water before freezing?

Authors: The larvae were frozen in the lyophilization drying process which was carried out at -40°C and 125 bars or by means of critical point drying (with the following conditions: -30°C and 90 bars).

Certainly, freezing process influences the results remarkably, principally when it occurs suddenly, influencing the thin apendages such as palpi, antennae, wings, etc. on soft tissues (very common in larvae).

The dried larvae were frozen (taking advantage of the no deforming effect of the formaldehyde). If larvae are frozen in water, the rapid formation of ice crystals produces alterations and destroys the larval surface.

J. Krchma: What tests were performed to check on the results of sputtering experiments?

Authors: In table 1 the results of 10 previous sputtering experiments, performed in order to determine an optimum sputtering time, are included. The coating process can cause a rise in temperature at specimen, which can influence larvae greatly. Moreover, an excessive sputtering time (and resultant thick coatings) can hide and deform the smaller structures of the cephalic capsule and peritremal cavity (visualized at magnifications of 2000-7000 X).