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Isolation, primary culture and morphological characterization of oenocytes from *Aedes aegypti* pupae

G.F. Martins[a,*,1], B.A.M. Guedes[d,e,1], L.M. Silva[b], J.E. Serrão[a], C.L. Fortes-Dias[b], J.M. Ramalho-Ortigão[c], P.F.P. Pimenta[d,**]

[a] *Laboratory of Cell and Molecular Biology, Departamento de Biologia Geral, Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais, Brazil*
[b] *Laboratory of Cell Biology, Fundação Ezequiel Dias (FUNED), Belo Horizonte, Minas Gerais, Brazil*
[c] *Department of Entomology, Kansas State University (KSU), Manhattan, KS, USA*
[d] *Laboratory of Medical Entomology, Instituto René Rachou (IRR), Fundação Oswaldo Cruz (Fiocruz-MG), Av. Augusto de Lima, 1715, Belo Horizonte, Minas Gerais, CEP 30190-002, Brazil*
[e] *Department of Morphology, Universidades Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil*

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ABSTRACT

Oenocytes are ectodermic cells that participate in a number of critical physiological roles such as detoxification and lipid storage and metabolism in insects. In light of the lack of information on oenocytes from *Aedes aegypti* and the potential role of these cells in the biology of this major yellow fever and dengue vector, we developed a protocol to purify and maintain *Ae. aegypti* pupa oenocytes in primary culture. *Ae. aegypti* oenocytes were cultured as clustered and as isolated ovoid cells with a smooth surface. Our results demonstrate that these cells remain viable in cell culture for at least two months. We also investigated their morphology *in vivo* and *in vitro* using light, confocal, scanning and transmission electron microscopes. This work is the first successful attempt in isolating and maintaining *Ae. aegypti* oenocytes in culture, and a significant step towards understanding the role of this cell type in this important disease vector. The purification and the development of primary cultures of insect oenocytes will allow future studies of their metabolism in producing and secreting compounds.

1. Introduction

Oenocytes are cells of ectodermal origin that may be associated with the epidermis or distributed amongst insect fat body cells. The distribution and association to the epidermis may depend on insect species or developmental stage. Due to their localization in the hemocele, large size and uncommon morphology, oenocytes have previously attracted the attention of insect physiologists. Typically, they have been considered analogous to the vertebrate steroidogenic cells specialized in fatty acids synthesis (Dean et al., 1985; Wigglesworth, 1988; Haunerland and Shirk, 1995; Gould et al., 2001; Rollo and Camargo-Mathias, 2006).

Primary cultures of insect oenocytes have helped unravel, at least in part, the functions of these cells in many insects. For instance, Romer et al. (1974) demonstrated that oenocytes from newly emerged larvae of the beetle *Tenebrio molitor* produce ecdysone from cholesterol, while oenocytes from fifth instar larvae of the grasshopper *Schistocerca gregaria* (Diehl, 1975) and from adult females *Blattella germanica* (Fan et al., 2003) synthesize cuticular hydrocarbons. In the last two years, other studies also have shown that oenocytes are even more complex cells, participating in neuron morphogenesis through the secretion of semaphorin, a peptide that drives axon elongation in *Drosophila melanogaster* embryos (Bates and Whitington, 2007), and involved in metabolism, store and regulation of lipid concentration in the hemolymph of fruit fly larvae (Gutierrez et al., 2007). Additionally, oenocytes in adult *Anopheles gambiae* oenocytes also act as detoxifying cells during homeostasis (Lycett et al., 2006).

Aedes aegypti is the major vector of dengue and urban yellow fever and a significant wealth of data is now available on this mosquito including the complete genome. In contrast, little is known about *Ae. aegypti* oenocytes and the role of these cells in mosquito biology and interaction with pathogens. As a first step towards developing a platform to investigate interactions between *Ae. aegypti* oenocytes and pathogens, we developed a protocol to purify and maintain *Ae. aegypti* pupa oenocytes in primary culture. The morphology of these oenocytes was analyzed *in vivo* and *in vitro* by light, confocal, scanning and transmission electron microscopy.

* Corresponding author at: Laboratory of Cell and Molecular Biology, Departamento de Biologia Geral, Universidade Federal de Viçosa (UFV). Viçosa, Minas Gerais, CEP 36570-000, Brazil.
** Corresponding author. Tel.: +55 31 38993492; fax: +55 31 38992546.
E-mail address: gmartins@ufv.br (G.F. Martins).
[1] These authors collaborated equally to this work.

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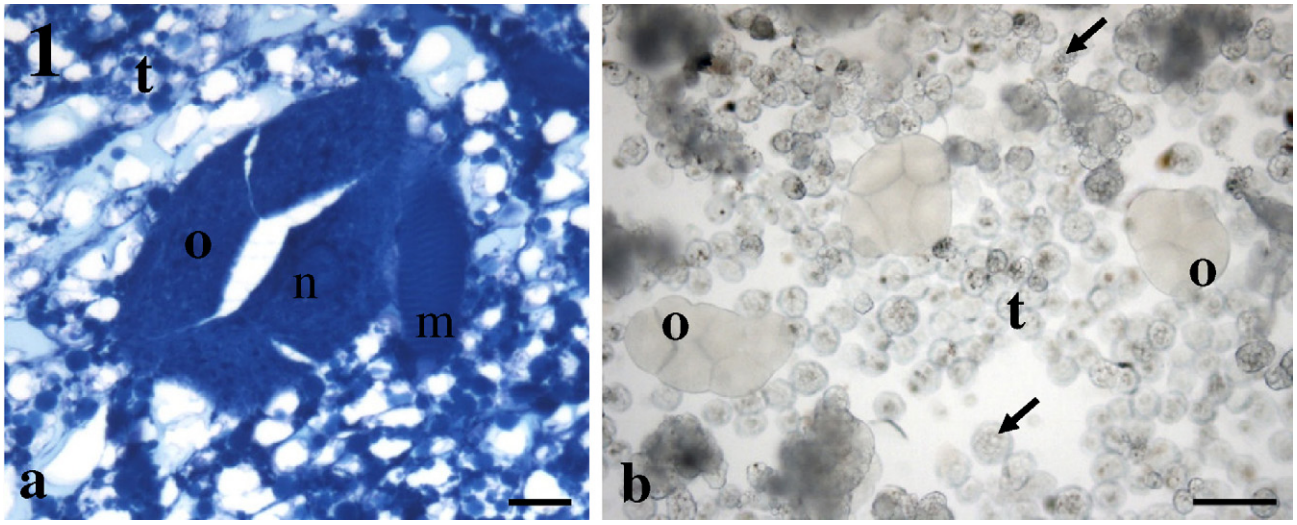

Fig. 1. General aspect of oenocytes and trophocytes from *Ae. aegypti* pupae. (a) Histological section showing a cluster of oenocytes (o) surrounded by trophocytes (t) in the abdominal fat body. Oenocytes are bigger, while trophocytes have cytoplasm rich in lipid droplets (L). Section was stained with toluidine blue; bar = 10 μm. (b) Three clusters of oenocytes (o) excised from the abdomen of a single pupa. Note that they are not attached to trophocytes (t). Trophocytes are smaller than oenocytes and have lipid droplets (arrows) into their cytoplasm; bar = 100 μm.

To our knowledge, this work represents the first successful isolation and primary culture of *Ae. aegypti* oenocytes. It also represents the first step in understanding the role of this cell type on vector-pathogen interaction regarding this important vector of human diseases.

2. Material and methods

2.1. Mosquito collection

Ae. aegypti strain PP-Campos (Campos dos Goytacazes, RJ, Brazil) were obtained from a colony maintained at the Laboratory of Medical Entomology of the Instituto René Rachou (IRR-FIOCRUZ, MG, Brazil). Mosquitoes were kept in an acclimated insectary at 28 °C and 70–80% relative humidity in a cycle of 12 h (dark and light); adult mosquitoes were maintained on 10% glucose solution and water *ad libitum*. Mouse blood also was provided to females for egg laying.

2.2. Histology

Female pupae were dissected under stereoscope microscope using 0.1 M Phosphate Buffered Saline (PBS) at pH 7.2. The abdomen was separated from the thorax, cut at the last abdominal segment and transferred to 4% formaldehyde fixative in PBS. Whole fixed abdomens were processed for histological *in situ* examination of the oenocytes in pupae. Samples were rinsed in PBS, dehydrated in a crescent series of ethanol (30–100%) and embedded in Historesin (Leica). Four-μm thin serial sections were stained with 1% toluidine blue-borax.

2.3. Dissection and oenocyte culture

Female pupae were rinsed in 0.001% ordinary dish detergent, surface sterilized in 0.1% sodium hypochlorite followed by 70% ethanol, 5 min each, and washed three times (2 min each) in ultrapure water. Clean insects were transferred to plates containing PBS and dissected under sterile conditions inside a hood. The last abdominal segment from each pupa was cut and the abdomen removed. Fat bodies from fifty pupae were excised and the oenocytes mechanically separated from other cells by gentle agitation. Cell types were continuously monitored under the phase microscope. Unlike fat body trophocytes, oenocytes are larger and do not display a cytoplasm filled with lipid droplets (Fig. 1b). They were recognized as large isolated cells or in clusters, and harvested using a 10 μL micropipette. Harvested oenocytes were transferred to siliconized microcentrifuge tubes containing 10 μL of supplemented IPL41 culture medium (Sigma) (0.1% lipid concentrate, 4% yeastolate, 1% pluronic acid, 1% tryptose, 0.025% gentamicin, 0.025% tetracycline, 0.05% fungizon and 0.025% streptomycin/penicillin). Following a brief spin, cells were re-suspended in culture medium, placed onto glass cover slips, and into 6-well plates. Oenocyte cultures were maintained at 27–28° C with 3 μL of fresh medium added every 3-to-5 days until the completion of the experiments.

2.4. Scanning electron microscopy (SEM)

Coverslips containing adhered cultured oenocytes were pulled out from the well plates and submersed in a fixative solution (2.5% glutaraldehyde in 0.1 M sodium caccodylate buffer, pH 7.2) followed by a post-fixation in 1% osmium tetroxide containing 0.8% of potassium ferricyanide in 0.1 M sodium caccodylate buffer, pH 7.2 (Pimenta and De Souza, 1983). Then, the samples were dehydrated in a graded acetone series (30–100%) and dried at the critical point device using liquid CO_2. The dried samples were mounted in stubs and coated with gold particles with a sputtering to be analyzed and photographed in the JEOL JSM-5600.

2.5. Transmission electron microscopy (TEM)

TEM was applied to cells obtained by separate procedures. For all TEM, samples were kept in 500 μL microcentrifuge tubes submitted to a fast spin on each step of procedures to keep cells pellet on the tube bottom.

Freshly dissected cells were fixed as indicated above for SEM. Samples were dehydrated using a graded series of acetone (30–100%) and embedded in Epon resin. Semi-thin sections (1 μm) were obtained and stained with 1% toluidine blue-borax to be observed in a light microscope. Ultra-thin sections (0.6 μm) were stained with uranyl acetate and lead citrate to be analyzed by Zeiss TEM 109.

After fixation, two-month old cultured oenocytes were carefully scrapped off the coverslips with a cell scrapper, collected at

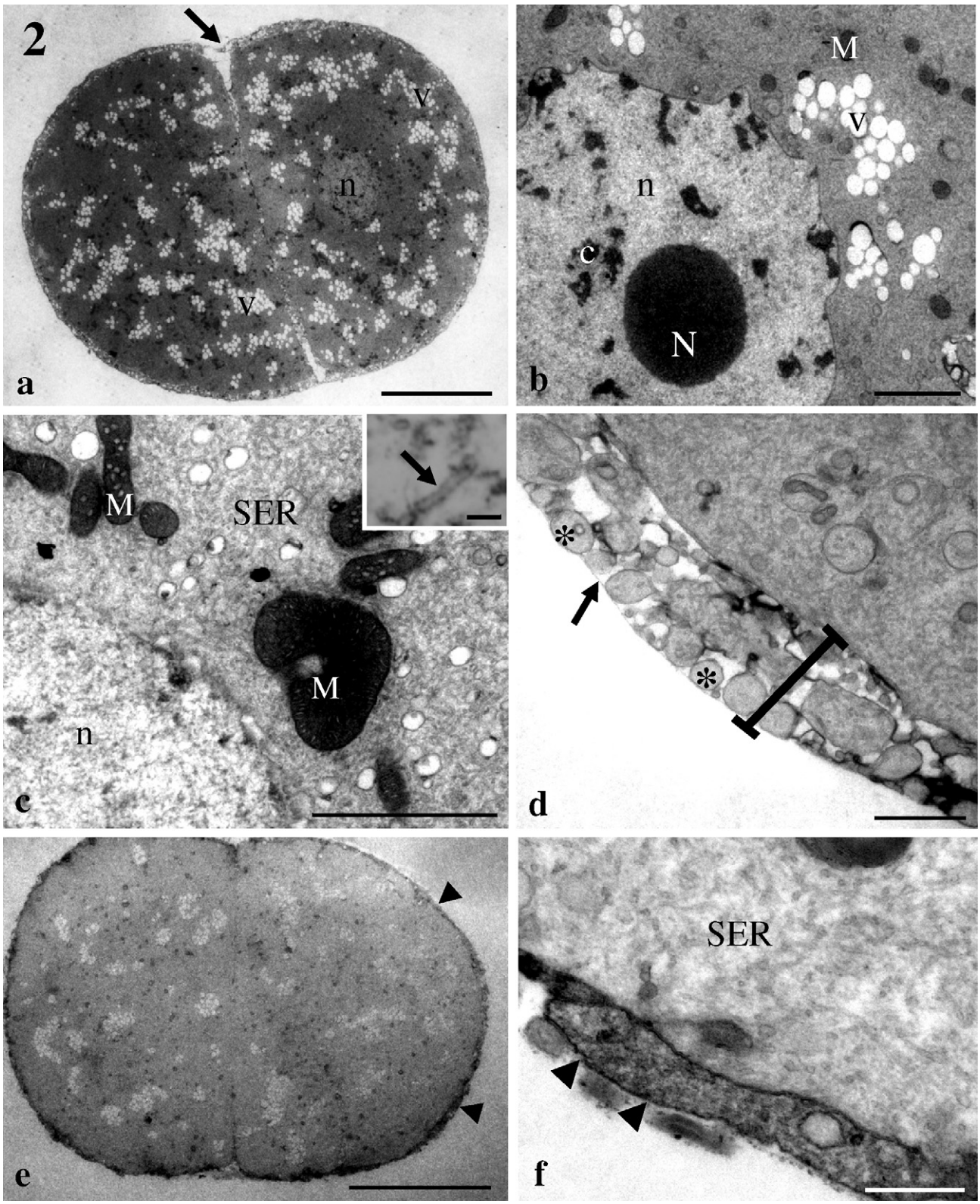

Fig. 2. Low and high magnifications TEM pictures of freshly dissected oenocytes of *Ae. aegypti*. (a) General view of two oenocytes enclosed by a basal lamina (arrow). Note the central nucleus (n) and several clustered translucent vesicles (v) in the cell cytoplasm; bar = 20 μm. (b) Oenocyte detail with a prominent nucleus (n) and clumps of condensed chromatin (c). M – mitochondrion; bar = 2 μm. (c) Detail of oenocyte cytoplasm showing several electron-dense mitochondria (M) with different profiles, and the well-developed smooth endoplasmic reticulum (SER); bar = 1 μm. Inset – tubular SER (arrow); bar = 0.3 μm. (d) Details of the oenocyte periphery with protrusions on the cell membrane (*) which form the lymph space (diagonal bar) at cytoplasm cortex (bar = 20 μm). (e) General view of two oenocytes with the cell membrane stained by ruthenium red (arrowheads); bar = 1 μm. (f) Detail of an oenocyte protrusion positively stained for ruthenium red (arrowheads). Note the SER network extending towards the cell periphery; bar = 1 μm.

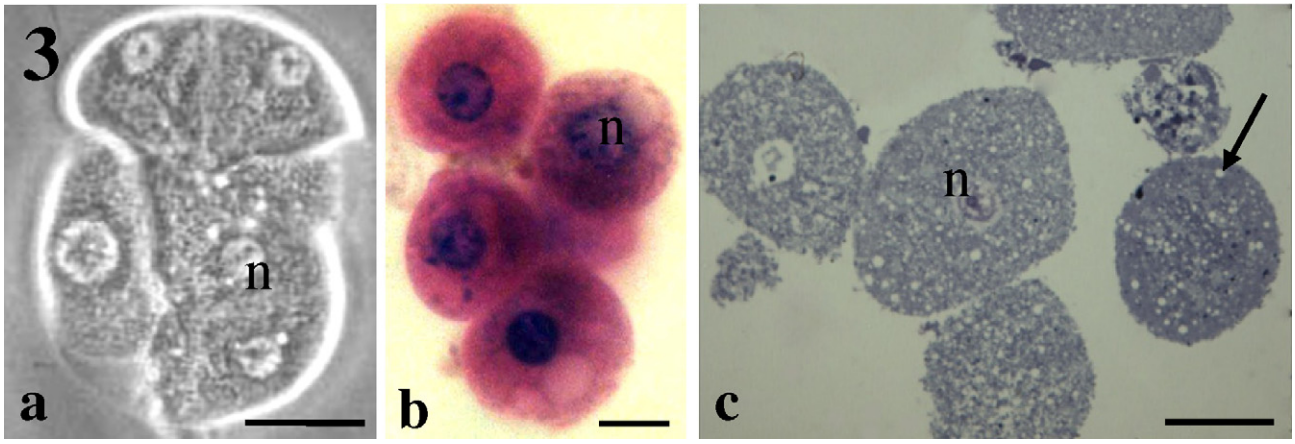

Fig. 3. Light microscopies (LM) of two-month old cultured *Ae. aegypti* oenocytes. Cell clusters display rounded nuclei (n). (a) Cluster of oenocytes under phase microscope; bar = 50 μm. (b) Similar cluster of oenocytes after staining by Giemsa. Cells display acidophilic cytoplasms (red) contrasting the basophilic nuclei (n, blue); bar = 30 μm. (c) Semi-thin section of oenocytes stained with toluidine blue-borax. Arrow – vesicle-like structures; n – nucleus; bar = 40 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the bottom of well plates, transferred to microcentrifuge tubes and processed as described above for fresh oenocytes.

For cell surface staining, following fixation, freshly dissected oenocytes were washed twice in 0.1 M sodium caccodylate buffer with 0.5 mg/mL ruthenium red for 10 min, and post-fixed in 1% osmium tetroxide with 0.5 mg/mL ruthenium red for 2 h (Wight and Ross, 1975) and processed for TEM.

2.6. Laser confocal microscopy (LCM)

Coverslips with the adhered oenocytes were fixed by fixative solution (4% formaldehyde solution in PBS, pH 7.2) for a period of 30 min. The samples were incubated in PBS/BSA (PBS with 2% of bovine serum albumin) for 1 h at room temperature. After a triple-washing in PBT (PBS with 0.1% Triton X-100) the samples were incubated overnight with fluorescent Phalloidin-FITC (1:100) (Molecular Probes) diluted in the PBT inside a humid chamber. The following day, the coverslips with the labeled cells were washed four times in PBT for 5 min and mounted with Mowiol (anti-fading medium). Images were obtained by using either fluorescence microscopy and a digital camera or multiple confocal sections by Zeiss LCM 5100.

2.7. Viability of cultured oenocytes

Two-month old cultures were incubated with 0.001% acridine orange diluted in IPL41 for 1 h. After washing cells three times in 1 mL PBS, cells were observed using an epifluorescence microscope to check for viability. A total of 300 cultured cells from three wells were analyzed for fluorescent nuclei. For comparative morphological analysis, cultured cells were also stained with 0.01% Giemsa solution and observed under a light microscope.

3. Results

Through the use of a simple series of dissecting methods we were able to establish primary oenocyte cultures isolated from *Ae. aegypti* pupa. Oenocytes were free of other cells as demonstrated by our microscopy analyses, and a number of cellular characters were assessed. Oenocytes were analyzed both *in vivo* and *in vitro* via light microscopy, SEM, TEM and LCM.

Serial sections obtained from the abdomen of *Ae. aegypti* pupa revealed that oenocytes were detected as clusters of large cells within the fat body or in close proximity to the integument (Fig. 1a). In fresh preparations the oenocytes were completely detached from other tissues and could be easily distinguished and sorted from trophocytes (Fig. 1b).

Under TEM, pupa oenocytes were clustered and enclosed by a basal lamina (Fig. 2a). These cells had a central nucleus with a well-developed nucleolus and the condensed chromatin appeared in irregular granular clumps, especially around the edge of the nucleus (Fig. 2b). The cytoplasm is replete with mitochondria and translucent rounded shape vesicle-like structures with different sizes (referred simply as vesicles) (Fig. 2a and b). The mitochondria were strongly electron-dense with distinct profiles (Fig. 2c), while these vesicles were closely associated in bundles and not dispersed through the cytoplasm (Fig. 2b). In addition, the cytoplasm was almost filled with numerous narrow, coiled and tubular structures of the smooth endoplasmic reticulum (SER) (Fig. 2c, inset).

Plasma membrane protrusions touching the delicate basal lamina also were detected (Fig. 2d), and labeling with ruthenium red indicated that such protrusions surround the cell cortex, forming the lymph space, except in the intercellular space (Fig. 2d–f).

Once in culture, oenocytes could be kept viable for at least two months. The two-old month cultured oenocytes observed under phase contrast microscope (Fig. 3a) and stained by Giemsa (Fig. 3b) confirmed the presence of a single type of adhered cells, isolated or in clusters. Cell clusters were consistently greater in number than isolated cells.

The SEM confirmed the presence of clusters and of isolated oenocytes (Fig. 4a–d). Interestingly, clusters containing up to six oenocytes also were observed covered by the original basal lamina. The outlines of clustered cells were easily detectable as they were marked by the tightly covering basal lamina (Fig. 4b). The basal lamina appeared smooth with few small depressions on the surface of clustered or isolated cells (Fig. 4d). The shape and the surface of the attached oenocytes were well preserved as seen by SEM analysis of isolated oenocytes or cell clusters with broken basal lamina or without it (Fig. 4c and d). Oenocytes were large oval shaped cells with a smooth surface with adhered cell debris detected on occasion. Their contact with the coverslip typically triggered the spreading of the cell over the substrate through small surface projections around the entire basal region (Fig. 4c and d).

The cytoskeleton of *Ae. aegypti* oenocytes was analyzed under LCM using Phalloidin-FITC, a fluorescent stain for actin filaments. Sequential confocal images from the top (Fig. 5a) to the base (Fig. 5b) of the same oenocyte revealed the entire

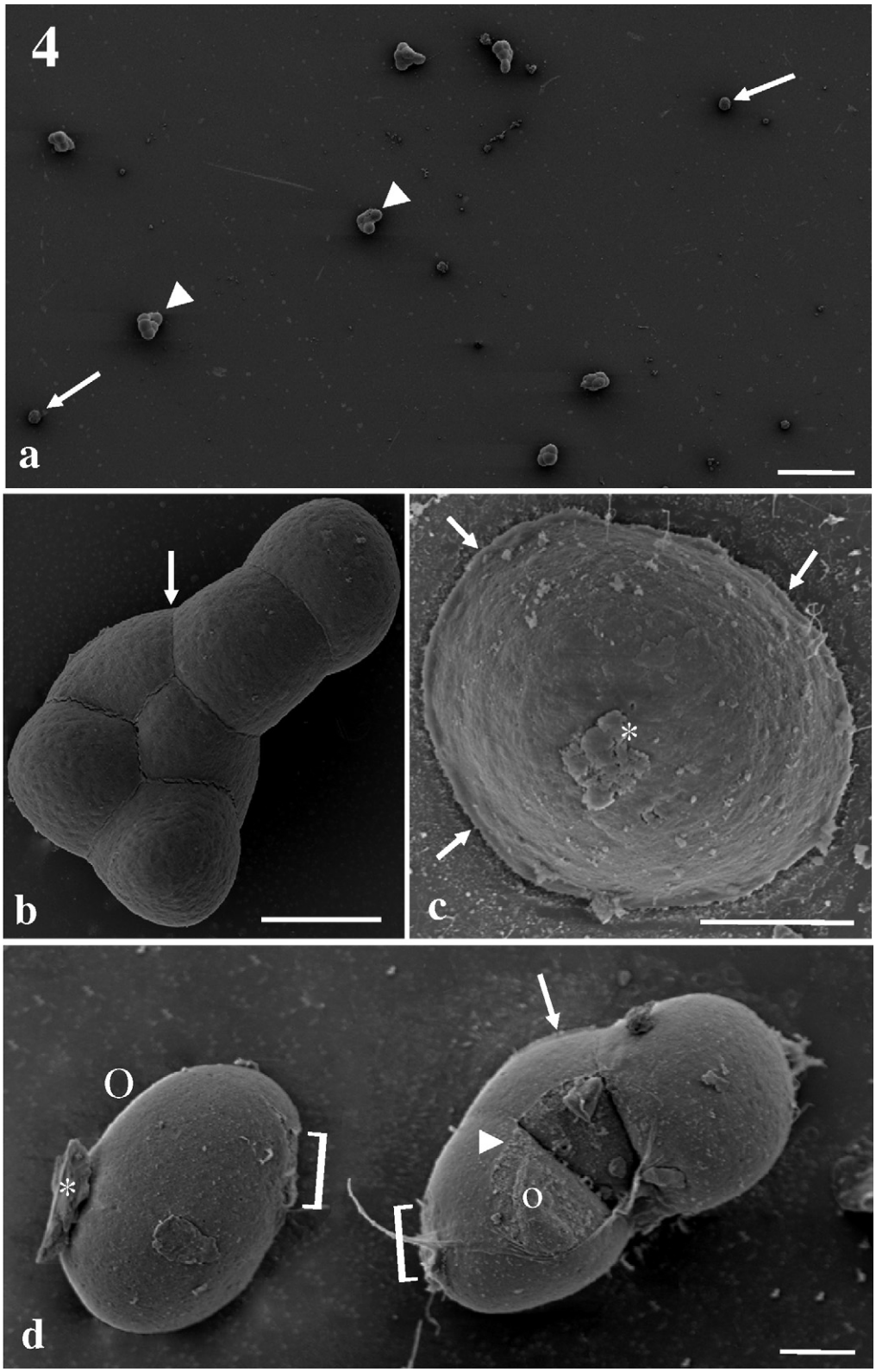

Fig. 4. SEM pictures of two-month old cultured oenocytes of *Ae. aegypti*. (a) Low magnification showing isolated (arrows) and clustered oenocytes (arrowheads); bar = 400 μm. (b) Cluster of oenocytes covered by the basal lamina. The basal lamina is smooth with few small depressions and granules on the surface. Cellular profiles of the clustered oenocytes due the tightness of the basal lamina are shown (arrow); bar = 50 μm. (c) Large oval-shaped oenocyte with an occasional cell debris adhered (asterisk). Note cell contact with the coverslip, with the cell spreading over the substrate through small surface projections around the entire basal region (arrows); bar = 20 μm. (d) Clustered (o) and isolated (O) oenocytes with open, broken basal lamina (arrowhead) revealing the cell membrane. Brackets indicate the point of contact prior to separation between the two oenocytes depicted. Arrow – cell projection; asterisk – cell debris; bar = 20 μm.

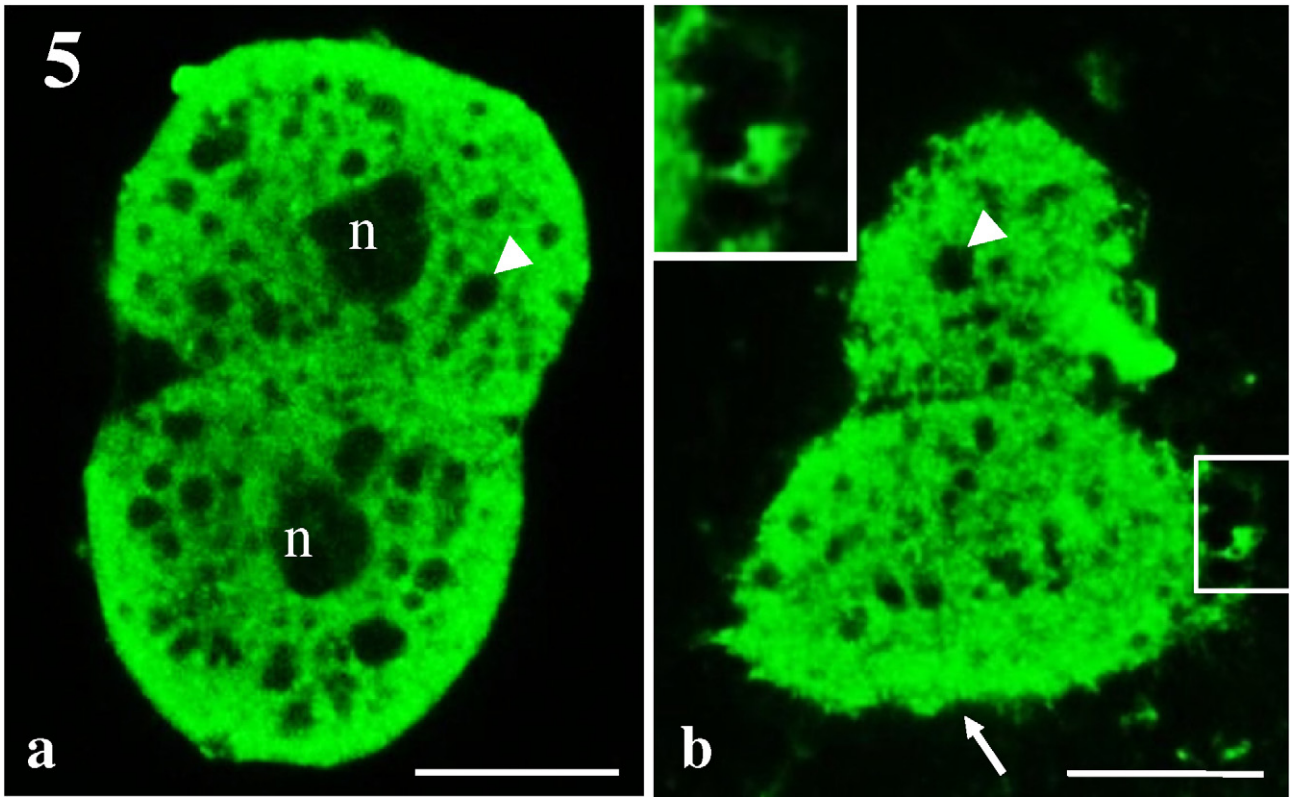

Fig. 5. Laser confocal microscopy (LCM) analyses of two-month cultured oenocytes of *Ae. aegypti*. LCM images of oenocyte close to the apex (a) and the base (b) of the cell. The cytoskeleton is distinctly labeled by the fluorescent stain of the Phalloidin-FITC, a marker for actin filaments. Note several cell membrane expansions-like-filopodia (inset) and lamellipodia (arrow) on oenocyte surface (b); and non-fluorescent nuclei (n) (a), and dark vesicles of different sizes and shapes (arrowheads); bars = 20 μm.

cytoskeleton and the organelle profiles. The oenocyte was distinctly fluorescent in the entire cell cytoplasm (Fig. 5a and b) unveiling the notably non-fluorescent nuclei, as well as, dark vesicle structures of different sizes and shapes. These vesicles were distributed throughout the cytoplasm. It was also possible to observe several plasma membrane expansions (collectively known as filopodia and lamellipodia) on the oenocyte surface (Fig. 5b).

Semi-thin sections and TEM revealed that *Ae. aegypti* cultured oenocytes display a central, rounded nucleus with evident nucleolus, as described for freshly processed oenocytes. Chromatin was detected as irregular granular clumps especially around the edge of the nucleus (Figs. 3c and 6a). These techniques also revealed unstained vesicles detected as non-fluorescent structures under the LCM (Figs. 3c and 6b) and these vesicles displayed different sizes and fairly uniform rounded shapes (Fig. 6b). The cytoplasms of cultured oenocytes were also almost filled by coiled and tubular structures of the SER. On the other hand, the cultured cells displayed fewer and smaller ovoid mitochondria than the freshly processed cells (Fig. 6d). Cultured oenocytes also displayed plasma membrane evaginations (corresponding to filopodia) and infoldings (Fig. 6c and d).

We routinely assessed the long term primary culture (up to two months) for viability using acridine orange. Acridine orange is known as a vital stain and induces an intensely photo-active staining of nuclei of dead or dying cells. We examined nearly 300 cells obtained from three separate cultures and the average percent of viable cells was 85% (not shown). Comparatively, when these oenocytes were stained with Giemsa or observed using contrast phase microscopy, they appeared morphologically well preserved (Fig. 3a and b). We did not observe any cellular division indicative of cell proliferation when the oenocytes were maintained in culture.

4. Discussion

Here we described a method for isolation and establishment of oenocytes from mosquito pupae in culture. Mosquito oenocytes can be maintained in primary cultures for up to 2 months. Cultured oenocytes tend to form clusters similarly to previously described for oenocytes in *Drosophila* (Hartenstein et al., 1992; Elstob et al., 2001; Gould et al., 2001) and in *Ae. aegypti* larvae (Wigglesworth, 1942). Oenocyte clusters are formed during *Ae. aegypti* metamorphosis and are thought to spread throughout the interior and the periphery of the mosquito fat body during the imago development (Christophers, 1960).

We investigated the morphology of cultured pupae oenocytes via TEM, SEM and light microscopies. Overall, cultured oenocytes maintained main cytoplasmic characteristics found in freshly isolated cells, such as the general chromatin organization in the nucleus, and the ovoid shape of the cells with the cytoplasm filled with SER and vesicles. However, we noticed a decrease in the mitochondria number and size in the cultured cells.

Interestingly, fresh and cultured oenocytes from pupae were quite different from adult mosquito oenocytes. For instance, in pupae, the SER almost completely filled the cytoplasm, while in adults the SER was restricted to some areas of the cytoplasm. Also in adults, the plasma membrane displayed deeply invaginated canaliculi (supplementary data) which were not detected in either fresh or cultured oenocytes. Moreover, adult oenocytes were polymorphic, clearly distinct from the rounded pupae cells (supplementary data), also reported by Tadkowski et al. (1977).

Pupal oenocytes had prominent SER and numerous bundles of vesicles. It can be inferred that these vesicles corresponded to lipid droplets that were abundantly found in the *D. melanogaster*

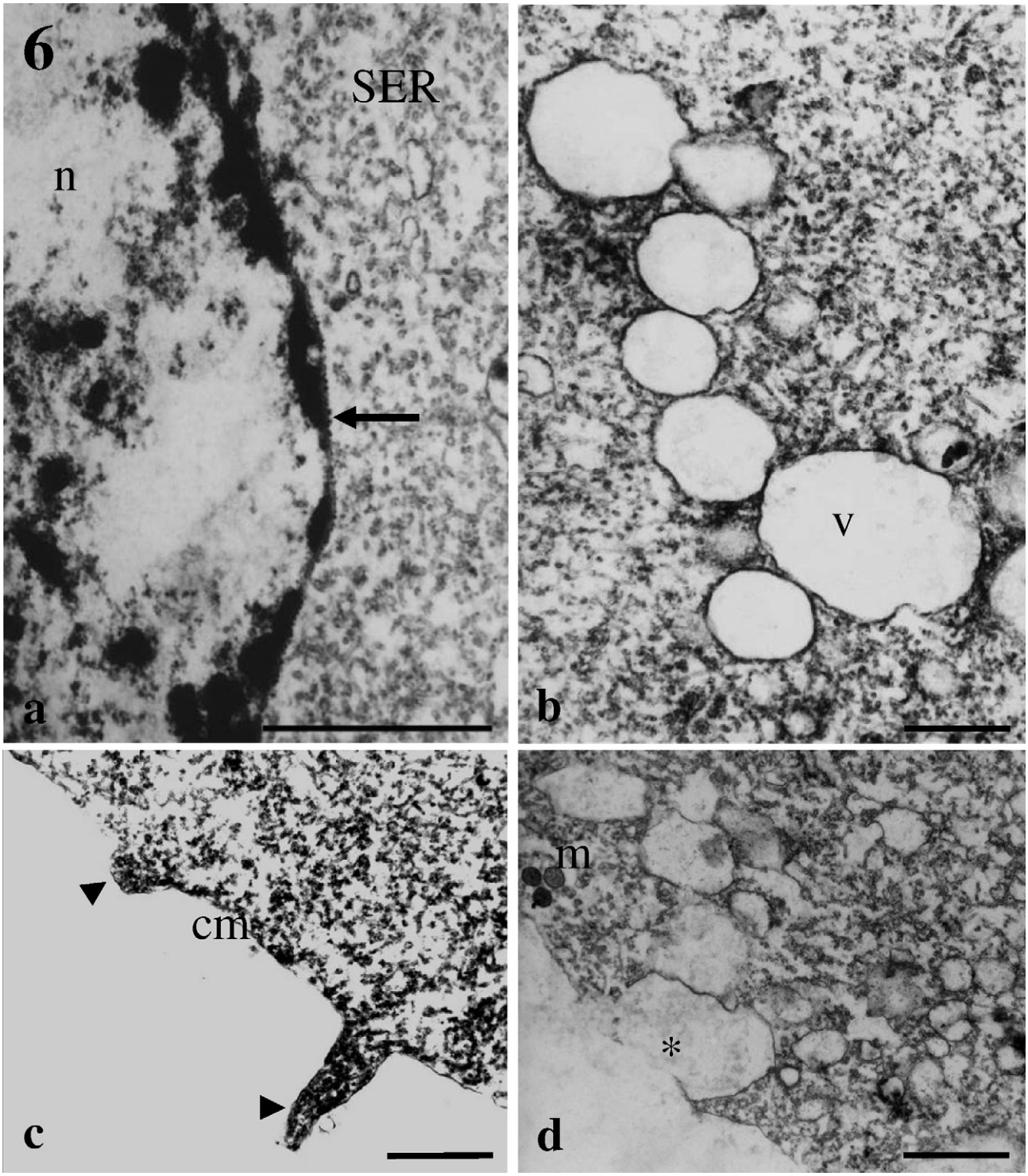

Fig. 6. TEM pictures showing details of two-month old cultured oenocytes of *Ae. aegypti*. (a) Cell nucleus (N) with closed cisterns of the smooth endoplasmic reticulum (SER). Arrow point ribosomes adhered to the nucleus membrane; bar = 1 μm. (b) Translucent rounded vesicles (v) of different sizes in the oenocyte cytoplasm; bar = 60 nm. (c) Cell periphery with the cell membrane (cm) with lamellipodia (arrowheads); bar = 60 nm. (d) Cell periphery with cell membrane invaginations (*) and mitochondria (M); bar = 60 nm.

larval oenocytes (Gutierrez et al., 2007) and in adult ant oenocytes (Camargo-Mathias and Caetano, 1996; Roma et al., 2008). These two organelles have been associated with the oenocyte lipid metabolism and storage in the caterpillar *Calpodes ethlius* (Lepidoptera) (Locke, 1969) and in adults of *T. molitor* (Coleoptera) (Romer et al., 1974), *S. gregaria* (Orthoptera) (Diehl, 1973, 1975) and *B. germanica* (Blattaria) (Fan et al., 2003).

The ruthenium red is specific for cell surface staining and indicated the presence of a lymph space on the external surface of fresh oenocytes. This is also known as reticular system and was reported in oenocytes and trophocytes of *C. ethlius* pupae (Locke, 1969, 1986). Lymph spaces are formed through plasma membrane protrusions that increase the cell surface area (reviewed by Locke, 2003). However, lymph spaces were no longer observed after cell

culturing. Modifications of the surface of cells also included the formation of pseudopodia (filopodia and lamellipodia), which were due to cultured settling on the glass substrate.

In semi-thin sections and under TEM, the basal membrane was not observed in cultured oenocytes even though they were evident under SEM in the majority of cells. Possibly during the scraping of the adhered cells and processing for TEM, the basal lamina was mechanically disrupted, releasing isolated oenocytes. Furthermore, clustered oenocytes were enclosed by a basal lamina and this structure had fractures under SEM, suggesting possible mechanical disruption of this structure.

Under SEM oenocytes are large ovoid cells with a smooth surface and occasional adherence of cell debris. Generally similar SEM aspects also were detected *in vivo* in oenocytes from the caterpillar *C. ethlius* (Jackson and Locke, 1989), and the ants *Atta sexdens rubropilosa* and *Pachycondyla striata* (Thiele and Camargo-Mathias, 2003; Rollo and Camargo-Mathias, 2006). The contact of the oenocytes with the coverslip typically triggered the spreading of the cell over the substrate through small surface projections around the entire basal region.

The results obtained using acridine orange indicated that oenocytes can be viably maintained *in vitro* for a relatively long period of time (at least two months). We did not observe any cellular division indicative of cell proliferation when the oenocytes were maintained in culture. This result was expected since oenocytes are highly differentiated and specialized cells and supported by data suggesting that oenocytes are non-dividing cells (Gould et al., 2001).

The development of a successful method to isolate and maintain *Ae. aegypti* oenocytes *in vitro* will significantly contribute towards studies aimed at understanding the metabolism of such an important cell type. Moreover, the long-term survival of viable oenocytes in primary culture also provides a useful tool for investigating their interactions with pathogens (e.g. dengue virus) naturally transmitted by the *Ae. aegypti*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tice.2010.12.003.

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