Electron Microscopy Visualization of the Cell Surface of Trypanosomatids

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Trypanosomatids cell surfaces play an important role in the interaction process with the cells of their vertebrate hosts as well as with the epithelial intestinal cells of the invertebrate hosts. The surface of these eukaryotic organisms is considered to be made up of the glycocalix, the lipid bilayer and a layer of microtubules linked to the lipid bilayer. The surface of the infective forms exposes some macromolecules which are recognized by host cells and that trigger the invasion of these cells by the protozoan. This review describes how the surface of Trypanosoma cruzi, a member of the Trypanosomatidae family which causes Chagas’ disease, is analyzed using several electron microscopy techniques. These techniques include high resolution scanning electron microscopy and transmission electron microscopy of thin sections as well as of replicas obtained following cryotechniques such as conventional freeze-fracture, fracture-label, fracture-flip and deep-etching.

Key words: Cell surface. Trypanosomatids, scanning electron microscopy, transmission electron microscopy, freeze-fracture, fracture-flip, fracture-labeling, deep-etching, cytochemistry

Introduction

It is by means of the cell surface that a parasite first interacts with its host. This interaction takes place either by the direct participation of surface-associated components or by secretion, via portions of the cell surface, of components synthesized within the cell and released via secretory vesicles [1]. The surface of intracellular parasites, as is the case of Trypanosoma cruzi and Leishmania, interacts initially with the surface of the host cell of the vertebrate host and with the membrane of the parasitophorous vacuole. It is also through the cell surface that several protozoa, including the trypanosomatids, interact with the epithelial intestinal cells of the invertebrate host [2]. Therefore, it is important to understand the structural organization, the molecular array, the composition and the functional properties of all the cell surface components of pathogenic protozoa.

The purpose of this mini-review is to show how electron microscopy has contributed to a better understanding of the structural organization of the cell surface of pathogenic protozoa taking the trypanosomatids as examples. Flagellates of the Trypanosomatidae family include a large number of species which are responsible for diseases of great medical and veterinary importance such as Chagas’ disease (American trypanosomiasis), sleeping sickness (African trypanosomiasis), and leishmaniasis. This review will consider the cell surface from a general point of view, including the lipid bilayer and associated components that face the extracellular medium and which form the glyocalyx (also known as the cell coat or the surface coat), as well as intracellular structures, mainly formed by microtubules, which associate with the inner side of the plasma membrane.

Visualization of the actual surface

The actual cell surface here is considered as the portion of the protozoan in direct contact with the extracellular medium (the blood, the intercellular space or the lumen of the respiratory, intestinal and urogenital systems). From a biochemical point of view it is formed by the projection of integral proteins/glycoproteins, glycosylphosphatidylinositol-anchored proteins/glycoproteins, by peripheral proteins/glycoproteins and by the carbohydrate portion of glycolipids [3]. Among the trypanosomatids
some well characterized surface-associated macromolecules play a fundamental role on the biology of these important parasitic protozoa. Some of these molecules deserve to be mentioned since a considerable effort has been made to localize them using the microscopy approaches that will be described in the subsequent sections. The molecules include (a) the variant surface glycoprotein and procyclin found in *Trypanosoma brucei*, (b) gp63 protease and lipophosphoglycans found in *Leishmania*, and (c) the mucins, transialidase and Te85 family of glycoproteins found in *Trypanosoma cruzi*. Below the more common approaches to study cell structures are described.

**Scanning electron microscopy (SEM)**

SEM is the most used electron microscopy approach to analyze cell surfaces. The image is obtained either by the secondary electrons generated following the interaction of an electron beam with the sample surface or by backscattering electrons. In the first case the general shape of the cell can be seen. In the second case it is possible to obtain information on the distribution of specific surface components which have been previously labeled by incubation with gold-labeled probes (lectins, neoglycoproteins, antibodies, etc.). One important advantage of this approach is that the whole surface of the cell can be visualized allowing accurate quantitative determination of the density of labeling. SEM has been used intensely of trypanosomatids as a whole clearly showing the various developmental stages presented by the protozoan during its life cycle. It is very useful to analyze the interaction processes of the trypanosomes with cells from the vertebrate and the invertebrate hosts. For instance Figure 1 shows the disposition of promastigote forms of *Phytomonas staheli*, a pathogenic trypanosomatid which proliferates and obliterates the phloem of plants of high economical interest such as palm tree and coconut trees [4].

![Figure 1](image_url)

**Figure 1.** Scanning electron microscopy showing the presence of a large number of promastigote forms of *Phytomonas staheli* (arrows) within an opened phloem. The star indicates the phloem wall. Bar, 0.5 µm (After Attias et al., 1987).
Recently more detailed information has been obtained with the use of a high resolution field emission scanning electron microscope [5]. Using such an instrument it is possible to visualize specialized areas of the cell surface such as the cytostome of epimastigotes, a structure involved in the uptake of macromolecules from the medium through a typical endocytic process. SEM has also been used to show surface domains of the protozoan, especially with the use of gold-labeled lectins and their localization by imaging using backscattering electron. For instance, Figure 2 shows that gold-labeled concanavalin A is preferentially localized in the cytostome region of the epimastigote forms of *T. cruzi* [5].

![Figure 2](image)

**Figure 2.** High resolution scanning electron microscopy of an epimastigote form of *Trypanosoma cruzi* showing the cytostome (C), the flagellum (F) and the flagellar pocket (FP). The arrow points to the cytostome opening. Bar, 0.1 µm. (After Nakamura et al., 2005).

Transmission electron microscopy (TEM) of thin sections

Conventional transmission electron microscopy of thin sections is certainly the most usual approach to analyze the structural organization of cells. However, it only gives a general idea on the thickness and density of the glyocalyx. In the case of trypanosomatids it was shown that the bloodstream trypomastigote forms, as well as the metacyclic forms found in the salivary gland of the insect vector, of members of the genus *Trypanosoma*, sub-genus *Trypanozoon*, which includes the *T. brucei* complex, present a 15 nm thick and dense surface coat [6], essentially made of about $10^7$ VSG molecules per protozoan. In contrast, other developmental stages of the same group, as well other trypanosomes, do not present such a thick surface coat, although a thin fibrilar layer can be seen. This surface coat can be better visualized using cationic dyes which bind to the cell surface and are intrinsically dense, as in the case of cationized ferritin particles or colloidal iron hydroxide particles, or others which are osmiophilic, as is the case of ruthenium red. TEM of thin sections is also informative if associated to cytochemistry and immunocytochemistry, allowing information on the distribution of specific surface exposed molecules, as is the case of Tc85 glycoprotein on the surface of *Trypanosoma cruzi* trypomastigotes [7].
Transmission electron microscopy of Freeze-fracture, Deep-etched Replicas

The conventional freeze-fracture technique, where the cells are initially fixed in glutaraldehyde, cryoprotected with glycerol, frozen in Freon-liquid nitrogen, fractured at -115 C and then replicated with platinum (45°) and carbon (90°) has been intensely used to analyze the inner structure of the plasma membrane, as will be discussed in section 3a. However, if we avoid the use of glycerol it is also possible to sublimate part of the extracellular water (etching process) allowing simultaneous observation of the protoplasmic face of the plasma membrane and a small area of the actual cell surface. The extension of the surface area varies according to the etching extent, which is basically dependent on the temperature used for the etching process (usually at –100°C, immediately following the fracture made at –115°C) and the time of etching (variable from seconds to minutes). One important advance in this area was the introduction of quick freezing techniques of living cells, especially by impact of the sample onto a liquid nitrogen or a liquid helium cooled metal surface, followed by freeze-fracture, deep-etching and rotary and low angle metal replication [8]. Figure 3 shows an extensive area of the surface of *T. cruzi* as visualized using this approach [9].

![Figure 3](image_url)

**Figure 3.** General view of the etched surface of the epimastigote form of *Trypanosoma cruzi*. Bar, 0.25 μm. (After Souto-Padron et al., 1984).
Transmission electron microscopy of Fracture-flip replicas

Another possibility to visualize large areas of the actual cell surface is the use of the fracture-flip technique. It was developed in 1988 [10] and corresponds to an adaptation of the conventional freeze-fracture technique. In this case the cell suspension is processed as described for the conventional freeze-fracture. Following fracture the exposed surface is initially coated with carbon, rather than with platinum, in order to obtain a stable replica. It is then removed from the freeze-fracture machine and the carbon replica is released in water and then rinsed several times in water. After which the replica is inverted and returned to the freeze-fracture machine where a thin layer of platinum is deposited at 45°C. Subsequently, the replica is released in water, cleaned with sulfuric acid and/or bleach, collected on a grid and examined in a transmission electron microscope. Figures 4 and 5 show examples of images obtained from the surface of epimastigote and trypomastigote forms of *Trypanosoma cruzi*, respectively [11]. It is clear that the surface of trypomastigotes is much more rugose than that of epimastigotes. The rugosities probably correspond to proteins exposed on the protozoan surface. In the case of epimastigotes, the surface lining the cytostome region is more rugose than that covering the cell body and the flagellum. Studies carried out with *Leishmania major* showed significant differences between the surface of the non infective promastigotes and that of infective metacyclic forms [12].

![Figures 4 and 5. Fracture-flip views of the surface of epimastigote and trypomastigote forms of *Trypanosoma cruzi*, respectively. The surface of epimastigotes is smooth, except the region containing the cytostome (C). The surface of trypomastigotes shows many surface projections. CB, cell body; F, flagellum. Bar, 0.1 µm. (After Pimenta et al., 1989).](image)

**Visualization of the plasma membrane**

Since plasma membrane plays a fundamental role in several biological processes several groups have made concentrated efforts to identify its molecules. However, there is still very little information on the architecture of the plasma membrane, especially the topographical relationship between structure and composition. Below I will discuss the most common approaches to study the structural organization of the plasma membrane.

**Conventional freeze-fracture**

Conventional freeze-fracture, briefly described above, is the only technique which opens up the possibility of examining the inner portion of the membranes. During freeze-fracture the hydrophobic portion of the membrane bilayer is cleaved exposing the inner portions of both the cytoplasmic and the extracellular leaflet, which can be replicated and the replicas obtained examined in a transmission electron microscope [13]. Generally there is a smooth area, which corresponds to the tail of the
phospholipids, with globular structures, designated as intramembranous particles, which correspond mainly to proteins inserted into the lipid bilayer. Figure 6 shows an example of the image obtained in an epimastigote form of *Trypanosoma cruzi* [14]. The use of this technique showed that the plasma membrane is not homogeneous in terms of density and distribution of intramembranous particles. Indeed it was possible to identify at least three macrodomains of the membrane [15]: the cell body, the flagellum and the flagellar pocket. Each of these macrodomains present specific microdomains such as (a) the flagellar necklace, localized at the basal portion of the flagellum; (b) the zone of attachment of the flagellum to the cell body; and (c) the cytostome region, observed in epimastigote and amastigote forms of *Trypanosoma cruzi* as well as in other members of the *Schizotrypanum* sub-genus, such as *Trypanosoma vespertilionis* and *Trypanosoma dionisi*. The cytostome is an invagination of the plasma membrane followed by a few special microtubules that penetrate so deep into the cell that they may even reach the nuclear region. The opening of this complex, which is known as the cytostome, may reach a diameter of 0.3 µm but it is significantly smaller in the deeper portion, the cytopharynx, resembling a funnel. There is a specialized region of the membrane lining the parasite that starts in the opening of the cytostome and projects towards the flagellar pocket. Freeze-fracture studies have shown that this area is delimited by a palisade-like array of closely associated particles, corresponding to transmembrane proteins that remain unidentified thirty years after. The delimited area is almost devoid of transmembrane proteins, appearing smooth in freeze fracture replicas. However, if the replicas were flipped, exposing the actual surface, the membrane lining the cytostome appeared very rugous. As described above this area contains a fibrillar material exposed on the surface. Association of freeze fracture and cytochemical data indicates that the membrane lining the cytostome is rich in glycoconjugates that are not inserted in the membrane. The morphologic and functional characteristics of the membrane lining the cytostome entry, therefore, point to a specialized membrane domain.

**Figure 6.** Freeze-fracture image of an epimastigote form of *Trypanosoma cruzi* showing the variation in the distribution of intramembranous particles in the membrane lining the cell body (CB), the flagellum (F) and the cytostome (C). The cytostome opening is indicated by the arrow. A linear array of particles (small arrows) separates the membrane lining the cytostome. Bar, 0.25 µm. (After Martinez-Palomo et al., 1976).
Freeze-fracture followed by fracture-labeling

In the conventional freeze-fracture technique the replicas obtained are treated with sulfuric acid and/or bleach in order to remove any organic material from the sample completely, leaving only the platinum/carbon replica. In the case of freeze-fracture of isolated cells it has been shown that when the replica is released into water in those areas exposing the P fracture face of the plasma membrane the actual cell surface is maintained. Therefore, if the replica is incubated in the presence of gold-labeled probes labeling of the cell surface can be visualized [16]. This technique is known as freeze-fracture labeling. Figure 7 is an example of such an approach, showing the concentration of concanavalin A-binding sites, revealed using gold-labeled concanavalin A on the surface lining the cytostome of epimastigotes of *Trypanosoma cruzi* [11]. It is also possible to incubate living cells with gold-labeled probes before freeze-fracture and then release the replicas into water, as described above. With such approach the results are basically the same and the technique is known as label-freeze fracture [17].

Figure 7. Freeze-fracture labeling showing the distribution of gold-labeled concanavalin A on the protoplasmic face of an epimastigote form of *Trypanosoma cruzi*. Gold particles are more concentrated in the region lining the cytostome (arrows). Bar, 0.25 µm. (After Pimenta et al., 1989).

Freeze-fracture cytochemistry

Freeze-fracture cytochemistry opens up the possibility of identifying some components of the plasma membrane. The most successful example in this area is with the use of digitonine and filipin to localize cholesterol and other β-hydroxy sterols. These compounds establish complexes with the sterol inducing the appearance of cylindrical or protrusion structures, in the case of digitonine and filipin, respectively, which can be easily recognized in freeze-fracture replicas. Figure 8 shows the distribution of filipin-sterol complexes in epimastigote forms of *T. cruzi* [18].
Figure 8. Freeze-fracture view of the protoplasmic face of the plasma membrane of an epimastigote form of *Trypanosoma cruzi* previously incubated in the presence of filipin. Filipin-sterol complexes (asterisk) and intramembranous particles (star) aggregate at some points. The arrow indicates the presence of few intramembranous particles within the filipin-sterol aggregate. Bar, 0.1 µm. (After Souto-Padron & De Souza, 1983).

Visualization of the membrane-associated cytoskeleton

One characteristic feature of the trypanosomatids is the presence of a layer of microtubules localized immediately below the plasma membrane, forming the so called sub-pellicular microtubules. This structural organization makes the cell resistant to lysis. Below I will comment on the most common techniques to study the cytoskeleton.

Transmission electron microscopy of thin sections

The observation of thin sections of trypanosomatids by TEM revealed the presence of the sub-pellicular microtubules distributed throughout the protozoan body, except the flagellar pocket region. Using conventional fixation techniques it is possible to see that the microtubules maintain a constant distance from each other (about 44 nm) and to the plasma membrane (about 12 nm). In favorable sections it is possible to see some connections between these two structures (Fig. 9). Profiles of the endoplasmic reticulum can be seen in between and below the sub-pellicular microtubules [19]. When tannic acid is added to the glutaraldehyde solution the protofilaments, which make up the microtubules, can be visualized [20].
Transmission electron microscopy of a thin section of a promastigote form of *Leishmania amazonensis*. Profiles of the endoplasmic reticulum (ER) are seen in between the sub-pellicular microtubules (M) and touching the plasma membrane (arrow). Bar, 0.1 µm. (After Pimenta & De Souza, 1985).

**Figure 9.** Transmission electron microscopy of a thin section of a promastigote form of *Leishmania amazonensis*. Profiles of the endoplasmic reticulum (ER) are seen in between the sub-pellicular microtubules (M) and touching the plasma membrane (arrow). Bar, 0.1 µm. (After Pimenta & De Souza, 1985).

Transmission electron microscopy of whole extracted cells

One approach to observe the whole cytoskeleton of trypanosomatids is the lysis of the protozoan on a water surface followed by critical point drying (21) or during drying following adsorption of the cells to a formvar-carbon coated grid and negative staining. With both techniques it is possible to obtain images showing the helicoidal array of the microtubules.

Scanning electron microscopy

More recently, informative images of the organization of the sub-pellicular microtubules have been obtained using high resolution scanning electron microscopy. The use of stable cold field emission scanning electron microscopes produces images of excellent resolution such as that shown in figure 10 where the sub-pellicular microtubules of *Herpetomonas megaseliae* can be seen [22].
Freeze-fracture followed by deep-etching

Certainly the quick-freeze, freeze-fracture, deep-etching and rotary replication technique is the best way to reveal details of the cytoskeleton organization [8]. Using this approach new details of the organization of the sub-pellicular microtubules and the flagellum of trypanosomatids have been obtained. In relation to the sub-pellicular microtubules it was possible to see the filaments which connect the microtubules to each other, to the inner portion of the plasma membrane and to profiles of the endoplasmic reticulum clearly [9] (Figure 11).

Figure 10. High resolution scanning electron microscopy of detergent-extracted promastigote form of *Herpetomonas megaseliae* where the sub-pellicular microtubules (arrows) are seen. Bars, 3.5 and 1.0 µm, respectively (After Sant’ana et al., 2005).

Figure 11. Deep-etching view of the sub-pellicular microtubules of an epimastigote form of *Trypanosoma cruzi* where the filaments which connect them (arrows) are clearly seen. Bar, 0.1 µm. (After Souto-Padron et al., 1984).
References