RESEARCH ARTICLE

The three-dimensional structure of the cytostome-cytopharynx complex of *Trypanosoma cruzi* epimastigotes

Carolina L. Alcantara$^{1,2}$, Juliana C. Vidal$^{1,2}$, Wanderley de Souza$^{1,2,3}$ and Narcisa L. Cunha-e-Silva$^{1,2,*}$

**ABSTRACT**

The cytostome-cytopharynx complex is the main site of endocytosis of *Trypanosoma cruzi* epimastigotes. Little is known about the detailed morphology of this remarkable structure. We used serial electron tomography and focused-ion-beam scanning electron microscopy to reconstruct the entire complex, including the surrounding cytoskeleton and vesicles. Focusing on cells that had taken up gold-labeled tracers, we produced three-dimensional snapshots of the process of endocytosis. The cytostome cytoskeleton was composed of two microtubule sets – a triplet that started underneath the cytostome membrane, and a quartet that originated underneath the flagellar-pocket membrane and followed the preoral ridge before reaching the cytopharynx. The two sets accompanying the cytopharynx formed a ‘gutter’ and left a microtubule-free side, where vesicles were found to be associated. Cargo was unevenly distributed along the lumen of the cytopharynx, forming clusters. The cytopharynx was slightly longer during the G2 phase of the cell cycle, although it did not reach the postnuclear region owing to a bend in its path. Therefore, the cytopharynx is a dynamic structure, undergoing remodeling that is likely associated with endocytic activity and the preparation for cell division.

**KEY WORDS:** Cytostome, Trypanosoma cruzi, Endocytosis, Electron tomography

**INTRODUCTION**

Endocytosis comprises a complex series of events that culminates in the internalization of extracellular molecules and particles. This process is well characterized in mammals and yeast, where extracellular cargo enters the cell through invaginations of the plasma membrane that are distributed all over the cell surface and passes, in an orderly manner, through the different intracellular compartments that characterize the endocytic pathways of these organisms. In most protozoan parasites from the *Trypanosomatidae* family, such as *Trypanosoma brucei* and *Leishmania* sp., endocytosis is highly polarized and restricted to the flagellar pocket. This domain is characterized by a prominent glycocalyx and plasma membrane domain between the cytostome and the flagellar pocket. This process occurs through this structure. What is known is that there is a special plasma membrane domain between the cytostome and the flagellar pocket. This domain is characterized by a prominent glycoalyx and is known as the ‘preoral ridge’ (Martínez-Palomo et al., 1976; Pimenta et al., 1989; Vataru Nakamura et al., 2005). Also, an uncharacterized set of microtubules and vesicles are aligned with the cytopharynx (Milder and Deane, 1969), but the microtubule arrangement and the origin and function of the aligned vesicles are still unknown.

In this work, we elucidate the ultrastructure of the cytostome-cytopharynx complex by using focused ion-beam-scanning electron microscopy (FIB-SEM) and serial electron tomography, followed by three-dimensional (3D) reconstruction. This was coupled to the visualization of the endocytosis of labeled tracers, in order to define the function of the different domains of the cytostome-cytopharynx in the internalization of macromolecules. The resulting detailed model of the entire cytostome-cytopharynx complex and its associated elements provides new insights into how this structure performs the endocytic function in *T. cruzi* epimastigotes.

**RESULTS**

**Shape and length of the cytopharynx**

Reconstructing a deep and curved invagination such as the cytopharynx by serial ultrathin sections or even by serial electron tomography is very labor intensive and is inappropriate for statistical analyses, which require 3D reconstruction of a
significant number of cells. By contrast, the powerful new technique of FIB-SEM allows rapid 3D reconstruction of organelles in a larger number of cells, albeit with less resolution than electron tomography. It combines the serial removal of material from the surface of a specimen (which happens inside the microscope, using focused ion-beam milling) with imaging of the newly exposed surface by scanning electron microscopy (Soares Medeiros et al., 2012). We used FIB-SEM to reconstruct the entire cytopharynx of epimastigotes that had taken up horseradish peroxidase (HRP) for 5 minutes at 28°C. The choice of HRP as a tracer was based on our previous work (de Souza et al., 1978; Soares and de Souza, 1991) and on the assumption that, as a marker for fluid-phase endocytosis, it would distribute homogeneously through the lumen of the cytopharynx, thereby facilitating the recognition of this structure in FIB-SEM images. The tracer proved to be suitable, as it filled only the cytopharynx and made it prominent in all cells. However, we observed sites of HRP concentration along the cytopharynx and a considerable variation in the diameter of the cytopharynx along its length (Fig. 1; supplementary material Movie 1).

Three slice-and-view series of FIB-SEM were used to measure the length of the cytopharynx in 32 cells. The mean cytopharynx length was ~8 μm (Fig. 2A). In these samples, there was a mixture of cells in different stages of cell cycle; thus, we classified these cells based on their stage of cell cycle, assuming that epimastigotes with one nucleus, one kinetoplast and one flagellum (1N1K1F) were in G1, whereas epimastigotes with one nucleus, one kinetoplast and two flagella (1N1K2F) were in an early stage of G2 (Elias et al., 2007). We noticed a small, albeit significant, increase in the length of the cytopharynx in parasites in G2, compared with those in G1. (Fig. 2A). The mean cytopharynx length of cells in G1 was 6.8 μm±1.9 μm (n=16; ±s.d.), whereas cells in G2 had a mean cytopharynx length of 8.9 μm±1.4 μm (n=16). Moreover, during G1 the cytopharynx had a helical shape, bypassing the nucleus and reaching the posterior of the cell (Fig. 2B–D), whereas in G2 there was a bend in the path of the cytopharynx, at a region close to the anterior portion of the nucleus, that turned the cytopharynx towards the anterior region of the cell (Fig. 2E–G). As a result of this bend, the cytopharynx of G2 parasites did not reach the posterior of the cell.

**The ultrastructural organization of the cytostome-cytopharynx complex**

To understand the ultrastructural organization of the cytostome-cytopharynx complex we used high resolution electron tomography. Owing to the long length of the complex, we analyzed several tomograms of different parts of the complex, taken from different cells. To preserve the architecture of the complex, after cells had taken up the endocytic tracer [transferrin coupled to colloidal gold (TF–Au)] for 10 minutes, we added isothermal glutaraldehyde directly to the incubation medium (Gadelha et al., 2009), to increase the speed of fixation and minimize cytoskeleton and membrane destabilization.

The first tomogram (Tomo 1, Fig. 3; supplementary material Movie 2) was produced by joining tomograms of seven adjacent 200-nm sections, resulting in 597 virtual slices, each 1.5-nm thick. The reconstructed volume comprised part of the flagellar pocket and the region of the cytostome opening. In this transversely positioned epimastigote it was possible to see the entire preoral-ridge membrane domain, characterized by an electron-dense surface coat running from inside the flagellar...
pocket (Fig. 3A–D, purple brackets) to a position close to the cytostome (Fig. 3C,D, purple brackets; Fig. 3E,F, purple surface). In the cytoplasm, a set of four microtubules was observed underlying this membrane domain (Fig. 3A–D), then lining the cytopharynx and going deep inside the cell body (Fig. 3G–I). A different set of three microtubules initiated just below the cytostome membrane and also accompanied the cytopharynx (Fig. 3B,G,I). These two sets of microtubules were arranged in a 'gutter' shape around the cytopharynx, leaving a side of the cytopharynx membrane devoid of underlying microtubules (Fig. 3I).

We confirmed these findings by analyzing a second tomogram of the same region in a different cell (Tomo 2; 321 virtual slices, each 3.14-nm thick). In this tomogram (Fig. 4), we could clearly observe the existence of the four microtubules that have one end underneath the flagellar-pocket membrane and follow the path of the preoral ridge (Fig. 4A). The first to disappear was microtubule Q3 (Fig. 4E), followed by T1 (Fig. 4F), leaving five microtubules around the cytopharynx on one end of Tomo 2 (Fig. 4F,I). In this tomogram, an endocytic vesicle containing Tf–Au particles was observed very close, although not fused, to the 'naked' side of the cytopharynx (i.e. the side where the cytopharynx membrane is devoid of associated microtubules) (Fig. 4D,G,H). In the cytoplasm, we could find other membranous elements in close proximity to the cytopharynx (Fig. 4E,F), although we could not unambiguously identify them, because they were on the edge of the tomogram.

**Internalization of the endocytic tracer Tf–Au occurred by vesicle budding along the 'naked' side of the cytopharynx**

The arrangement of the microtubules along the cytopharynx has an important consequence for the architecture of the endocytic process that takes place in the cytopharynx. This could be best illustrated in Tomo 3 (Fig. 5), which covers 3.62 μm in length of the cytopharynx, with a slice thickness of 3.14 nm. Fig. 5A gives an overall view of the model generated from the reconstructed

---

**Fig. 2. Analysis of cytopharynx length and shape.** By using FIB-SEM microscopy we were able to reconstruct and measure the length of the cytostome-cytopharynx complex in 32 cells that had endocytosed HRP for 5 minutes. (A) Morphometric analysis of the length of the cytopharynx of T. cruzi epimastigotes in all cells analyzed (Total) and in cells from G1 and early G2 phase of the cell cycle. The mean cytopharynx length when all cells were analyzed was 8.0 μm ± 2.0 μm (± s.d.). The mean cytopharynx length of cells in G1 was 6.8 μm ± 1.9 μm, whereas cells in G2 had a mean cytopharynx length of 8.9 μm ± 1.4 μm. The data was analyzed using an unpaired t-test and post-analyzed by two-tailed test. *P < 0.01.

(B–D) 3D models of the cytopharynx (Cy, pink) in three representative G1-phase cells. The cytopharynx followed a helical path around the nucleus (N, blue), reaching the posterior of the cell. White arrowhead, the cytostome. (E–G) 3D models of the cytopharynx in G2-phase cells showing a bend in this structure (* towards the anterior of the cell. K, kinetoplast (green). Scale bars: 0.5 μm.

---

**RESEARCH ARTICLE**


---

2229
Many uncoated vesicles, loaded (light blue vesicles) or not (silver vesicles) with Tf–Au particles, could be seen to be aligned with the naked side of the cytopharynx, along the length of this structure (Fig. 5A,B). We noticed that the Golgi complex was in close proximity to the cytopharynx (Fig. 5B). Importantly, vesicles containing Tf–Au (Fig. 5E,F) or without this tracer (Fig. 5C,D) were observed clearly budding from or fusing with the cytopharynx membrane. All vesicles aligned with the cytopharynx had the approximate diameter of 90 nm, and the gold-containing vesicles seemed to be more frequently found at the distal portion of the cytopharynx.

Tf–Au particles were not evenly distributed inside the cytopharynx

The distribution of Tf–Au particles inside the cytopharynx was not homogeneous, forming expansions where the tracer concentrated. In Fig. 5A,G, a diluted portion of the cytopharynx was sectioned at the edge of the tomogram. Close to this diluted portion, endocytic compartments with a tubule-vesicular morphology and containing a large number of gold particles could be observed and should correspond to the early endosomes of this organism (Fig. 5G).

Fig. 6 shows a serial tomogram that covers the final portion of the cytopharynx (Tomo 4; 239 virtual slices, each 1.9 nm thick). We could find Tf–Au particles gathered in clusters inside enlarged portions of the cytopharynx, separated by regions devoid of the tracer (Fig. 6A,B), as we observed in cells that had taken up HRP. Therefore, we have shown that, when it is engaged in endocytosis, the cytopharynx varies in diameter along its length. Although the variations in diameter and length of the cytopharynx have been observed using different techniques, different tracers and in different cells, we cannot rule out some contribution of osmotic variation inherent to chemical fixation.

The cytopharynx ended in a large bag-shaped structure, positioned very close to the reservosomes (a lysosome-related organelle of T. cruzi epimastigotes) at the posterior of the cell (Fig. 6C). At this point, only four microtubules (two from each set) were observed around the cytopharynx, and they seemed to continue even after the cytopharynx ended (Fig. 6B,C). In all cells we have examined so far, after 2–10 minutes of incubation.
with endocytic tracers, the tracers were contained in the cytopharynx, which ended in a bag-shaped dilation.

**The flagellar pocket of *T. cruzi* epimastigotes has two microtubule quartets**

The existence of a microtubule quartet that is common to both the flagellar pocket and the cytostome-cytopharynx complex led us to question whether these microtubules correspond to a conserved quartet of microtubules that surrounds the flagellar pocket in other trypanosomes (Preston, 1969; Taylor and Godfrey, 1969; Lacomble et al., 2009; Girard-Dias et al., 2012). In *T. brucei*, this conserved quartet is often referred to as the MtQ (microtubule quartet), and it comprises the only microtubules associated with the flagellar-pocket membrane.

The MtQ underlies a membrane domain in the flagellar pocket that forms a channel through which extracellular components can gain access to the lumen of the flagellar pocket (Gadelha et al., 2009).

We performed a serial tomogram of a cell that covered the entire volume of the flagellar pocket (Tomo 5, Fig. 7). The reconstructed volume, consisting of 771 virtual slices, each 1.58-nm thick (Fig. 7E), demonstrated the presence of two different quartets of microtubules underlying the flagellar-pocket membrane of *T. cruzi* epimastigotes. One of the quartets originated close to the basal body, surrounded the flagellar pocket in a helical pattern and inserted into the subpellicular microtubule cortex, near the opening of the flagellar pocket (Fig. 7A–E). This clearly corresponded to the MtQ.
The microtubules of the other quartet represented the ones shown in Tomos 1 and 2. One end of these microtubules was positioned below the flagellar-pocket membrane, but anterior to the basal body area. Also, the end of each microtubule was found at a different position relative to the anteroposterior axis of the cell (Fig. 7A–C,E). Their path accompanied that of the preoral ridge, and then these microtubules extended into the parasite body, along with the cytopharynx. To investigate these microtubules in the context of the entire cytoskeleton, we detergent-extracted the membranes of epimastigotes and negatively stained the resulting whole-mount cytoskeletons for observation by transmission electron microscopy (TEM). In these preparations, we could identify the two different microtubule quartets and follow the cytostome quartet from the flagellar pocket to the posterior region of the cell (Fig. 8A–C). Interestingly, the arched shape of the cytostome microtubules as they pass underneath the predicted region of the preoral ridge was always preserved in the cytoskeletons, even after total membrane extraction (Fig. 8A,B). This region appeared to be structurally complex relative to other areas of the cytostome-cytopharynx cytoskeleton, where microtubules appeared comparatively ‘bare’ (in Fig. 8, compare the structures of the cytostome-cytopharynx cytoskeleton in B and C). The cytopharynx followed a helical path around the nucleus from anterior to posterior. Also, our negative-staining images confirmed that the anterior portion of the cytopharynx microtubules always reached the plane of the nucleus from the opposite side of the cell relative to the one where the cytostome is found. These microtubules remained closely associated with each other along the entire path to the posterior of the cell, even without the cytopharynx membrane. We could count five cytopharynx microtubules immediately posterior the nucleus,
and these were reduced to four towards the end of the cytopharynx (Fig. 8A–C).

In T. brucei, the subpellicular microtubule corset is known to depolymerize after treatment with CaCl$_2$ or high concentrations of salt, whereas the flagellar microtubules and the MtQ remain (Gull, 1999). We confirmed that this was also the case in T. cruzi epimastigotes by treating cytoskeletons with 1 M NaCl (Fig. 8D) or 60 mM CaCl$_2$ (Fig. 8E). Importantly, the cytostome cytoskeleton was also resistant to these salt treatments. Distal to the cytostome region, sections of varying lengths of the cytopharynx microtubules remained in salt-treated cytoskeletons (not shown). As expected, the subpellicular microtubules disappeared upon salt treatment.

In our reconstruction of the flagellar pocket (Tomo 5), we have also found other recently described cytoplasmic microtubules (Girard-Dias et al., 2012). These were not included in the model shown in Fig. 7, because they are not related to the cytostome-cytopharynx microtubules. All of the microtubules we could observe near the flagellar pocket in Tomo 5 are shown in supplementary material Fig. S1.

In Tomo 5, two rare invaginations were observed at the flagellar-pocket membrane; one of them appeared to represent a small budding vesicle of 32 nm in diameter (supplementary material Fig. S2A,B; Box 1), and the other resembled a precursor of a tubule-vesicular structure (supplementary material Fig. S2A,C; Box 2). A large vesicle, measuring 274 nm in diameter (supplementary material Fig. S2D,E; Box 3), was also seen in direct contact with the flagellar-pocket membrane. Note that, although the endocytic tracer was present in the extracellular milieu, no tracer was found inside the flagellar-pocket vesicles.

**DISCUSSION**

Since the initial description of the cytostome-cytopharynx complex of T. cruzi epimastigotes (Milder and Deane, 1969), very little further knowledge about this structure was gained, despite its great importance as the main site of endocytosis at this stage of the life cycle. This was due to the difficulty in following this structure in its entirety when using only analyses of two-dimensional (2D) micrographs. In the present work, we used high resolution electron tomography combined with FIB-SEM microscopy to generate the first integrated view of the 3D architecture of the cytostome-cytopharynx complex in T. cruzi epimastigotes.

Several characteristics of this structure could be addressed. The cytopharynx is a deep and spiral-shaped invagination, with considerable variation in length from cell to cell. Parasites with a shorter cytopharynx would present a decrease in endocytic activity, as suggested by previous work that reconstructed one epimastigote in the early G2 phase of the cell cycle using conventional ultrathin serial sections (Ramos et al., 2011). In the present work, using FIB-SEM to obtain 3D models and quantitative information about the cytopharynx in several cells, we confirmed the apparent retraction of the cytopharynx in G2, but demonstrated that this was not due to shortening in length. Rather, the cytopharynx become slightly longer in G2, but appeared retracted owing to a change in its shape. Moreover, by using the endocytic tracer, we could guarantee that all of the cells in which the cytopharynx was measured presented endocytic activity. Thus, cytopharynx remodeling from G1 to G2 was not associated with an obvious decrease in the endocytic capacity of the cells. Based on this, we strongly suggest that the changes in cytopharynx shape and length in G2 reflect the remodeling of the cytopharynx in preparation for cell division. The events involved in the duplication of the cytostome-cytopharynx complex during cell division are now under detailed investigation.

The participation of microtubules in the structuring of the cytostome-cytopharynx complex is a hallmark of trypanosomatids (Steinert and Novikoff, 1960; Preston, 1969) and bodonids (Attias et al., 1996). However, the origin of these microtubules and their exact positioning and arrangement around the cytopharynx remained unclear. The existence of a physical cytoskeleton-mediated link between the flagellar pocket and the cytostome had been suggested by Okuda and co-workers (Okuda et al., 1999). Nevertheless, a clear description of the structure of this physical link was lacking, requiring the analysis presented here using serial electron tomography of epimastigotes, where
microtubules could be individually followed from the flagellar pocket all the way to the end of the cytopharynx by high-resolution 3D reconstruction.

Our results describe the existence of seven microtubules following the path of the cytopharynx of *T. cruzi* epimastigotes. The new microtubule quartet described here follows the path of the preoral ridge and of the cytopharynx. Therefore, this new quartet is likely to be responsible for the arched shape of the preoral ridge, and for the formation and/or maintenance of the preoral-ridge membrane domain. Indeed, even after the membranes were extracted for examination of whole-mount cytoskeletons, the cytopharynx microtubule quartet remained together and described a helical path toward the posterior of the cell, a strong indication that these microtubules are responsible for the curved shape of the cytopharynx. The reconstruction of the cytopharynx of several parasites from FIB-SEM images confirmed this shape. It is possible that this is a consequence of the positioning of the kinetoplast between the cytostome and the nucleus, which might force the cytostome to adopt a helical path towards the opposite side of the cell (as opposed to running straight towards the posterior of the cell). We could not identify clearly the cytostome portion of the
microtubule triplet in whole-mount cytoskeletons. However, in the perinuclear region, at least five microtubules could be counted in the cytopharynx cytoskeleton. Therefore, at least one of these microtubules must be part of the cytopharynx microtubule triplet seen in tomographic reconstructions. It is possible that, in negatively stained cytoskeletons, the cytostome end of the triplet microtubules is obscured by the complexity of insoluble (i.e. detergent-resistant) structures found in the region of the cytostome. We speculate that the structural complexity of this region is due to the presence of detergent-resistant components of the preoral-ridge membrane domain that are stably linked to the cytostome-cytopharynx microtubules.

The presence of a cortex of closely spaced subpellicular microtubules beneath the epimastigote plasma membrane impairs the events of budding or fusion of vesicles. Although the cytostome-cytopharynx complex lacks microtubules of the subpellicular cortex, it is nevertheless associated with two sets of microtubules. Given that this complex is the main site of endocytosis in epimastigotes, understanding the positioning of microtubules around the cytostome-cytopharynx allowed us to make important conclusions regarding the architecture of the endocytic processes in this parasite. If the cytostome-cytopharynx microtubules formed a closed cage around all sides of the cytopharynx, it is likely that the endocytosed cargo would have to travel all the way to the bottom of the cytopharynx in order to be endocytosed in budding vesicles. Instead, our tomograms showed that these microtubules are arranged around the cytopharynx in a ‘gutter’ shape, leaving a side of the cytopharynx membrane devoid of underlying microtubules. Close to this ‘naked’ side of the cytopharynx, many uncoated vesicles could be observed. In fact, some of these vesicles were connected with the cytopharynx membrane and contained the endocytic tracer Tf–Au. Although the presence of vesicles near the side of the cytopharynx is documented in the literature (Milder and Deane, 1969; de Souza et al., 1978), the occurrence of endocytosis in this region had never been demonstrated. Therefore, by using a labeled endocytic tracer, we have demonstrated that there is active membrane traffic at the naked side of the cytopharynx. With the techniques used here, we could not be certain whether the vesicles devoid of Tf–Au that were observed in contact with the cytopharynx membrane were budding from or fusing with this structure. We have shown that the Golgi apparatus is in close proximity to the cytopharynx. Thus, it is possible that the vesicles devoid of the gold tracer near the cytopharynx originated from the Golgi complex. In this case, the endocytosed cargo must travel to the bottom of the cytopharynx to be endocytosed in budding vesicles.

Fig. 8. Whole-mount negatively stained cytoskeleton after detergent extraction. (A) Detergent-extracted whole-mount cytoskeletons of T.cruzi epimastigotes. (B) High magnification of the area in rectangle B in A, showing the flagellar-pocket region. The flagellar-pocket quartet (MtQ, orange bracket) and the cytostome quartet (blue asterisks) are indicated, as well as the predicted region for the cytostome (Cy, white arrowhead). (C) High magnification of the area in rectangle C in A, showing the distal portion of the microtubules that accompany the cytopharynx. In a more anterior region, five microtubules (white asterisks) were seen, whereas at a more posterior region only four were found. (D,E) Resistant microtubule preparations were obtained after treatment of the cytoskeletons with 1 M NaCl (D) or 60 mM CaCl2 (E). In both cases, the axoneme, the MtQ and the cytostome microtubule quartet were preserved. N, nucleus; Axo, axoneme; BB, basal body; PBB, probasal body. Scale bars: 500 nm (A–C,E), 200 nm (D).
context, vesicles coming from the Golgi would deliver their contents to the cytopharynx, maintaining and replacing its differentiated membrane domain and acidifying its lumen. Indeed, we have demonstrated that the lumen of the cytopharynx is acidified by the action of a P-type H¹-ATPase (Porto-Carreiro et al., 2000; Vieira et al., 2005).

Some of the microtubules around the cytopharynx terminate as this structure becomes thinner and goes deeper inside the cell. Therefore, fewer microtubules are found at the end of the cytopharynx, which is likely to contribute to the fact that this is the preferred site of exchange of content with other compartments. We have noticed that other endocytic compartments are often in close proximity to regions of the cytopharynx where only four microtubules are found. At these locations, the cytopharynx was often enlarged and contained the endocytic tracer. Thus, the diameter of the cytopharynx varies along its length. Until now, the cytopharynx was described as having a funnel-shaped opening – the cytosome – followed by a tube-like element with a roughly homogeneous diameter along its length – the cytopharynx proper – which terminates in a bag-shaped structure (Milder and Deane, 1969; de Souza et al., 1978). Based on this description, it was assumed that endocytosis occurred exclusively at the enlarged end of the cytopharynx. By contrast, our analyses of different serial tomograms and FIB-SEM series showed that the cytopharynx varies in diameter along its length. Also, the regions of enlarged diameter along the cytopharynx contained clusters of endocytic tracers, strongly suggesting that they are engaged in endocytosis. This might have been missed in previous publications where only 2D images of the cytopharynx, from ultrathin TEM sections, were analyzed. Depending on the plane of observation, an enlargement of the cytopharynx along its length might have been mistaken for the distal end of this structure, thus leading to the assumption that endocytosis occurs exclusively at the end of the cytopharynx.

Interestingly, the aggregation of cargo in clusters along the cytopharynx was observed when using Tf–Au, an insoluble particulate tracer, as well as with peroxidase, a fluid-phase tracer, showing that this feature is independent of the nature of the cargo. The region where these clusters form along the cytopharynx might characterize preferential ‘exit points’ where vesicles bud into the cytoplasm. At the final portion of the cytopharynx, four microtubules were still present and continued towards the posterior of the cell, running past the end of the cytopharynx. Although we did not observe any internal cell compartments directly connected with these microtubules, one could speculate that they play a role in directing organelles or vesicles towards the posterior of the cell.

In conclusion, the 3D reconstruction work presented here has proven to be more adequate for the analysis of the spatial dynamics of endocytosis in epimastigotes, compared with conventional methods of observation that have been used so far. The high-resolution 3D analyses revealed new features of the long and complex structure of the cytosome-cytopharynx and opened the possibility of understanding the highly efficient endocytic process in this interesting and relevant eukaryotic model.

**MATERIALS AND METHODS**

**Parasites**

*T. cruzi* epimastigotes from clone Dm28c were cultivated in liver infusion tryptose (LIT) medium (Camargo, 1964) supplemented with 10% (v/v) fetal calf serum (FCS) at 28°C. Exponential-phase parasites from 3–5-day-old cultures were used for the experiments.

**Endocytic tracers**

Colloidal gold particles (Au, 8–10 nm) were prepared as described previously (Slot and Geuze, 1985). Bovine holotransferrin (Tf, Sigma, St Louis, MO) was coupled to gold particles as described previously (Roth, 1983). Horseradish peroxidase (HRP, Sigma) was used as fluid-phase tracer at a final concentration of 1 mg/ml in culture medium.

**Endocytosis experiments**

Parasites were collected by centrifugation at 1500 g for 10 minutes and were washed twice in phosphate-buffered saline [10 mM sodium phosphate buffer with 0.9% (w/v) NaCl, pH 7.2]. Cells were incubated in RPMI medium (without FCS) that contained transferrin coupled to gold particles (Tf–Au) for 10 or 15 minutes; or HRP for 5 minutes, both at 28°C. To stop endocytosis, cells were fixed rapidly by the addition of isothermal glutaraldehyde to a final concentration of 2.5% directly to the incubation medium. After fixation, the cells were washed twice in PBS to remove the endocytic tracer and processed for transmission electron microscopy (TEM).

**3-3-diaminobenzidine (DAB)-cytochemistry**

Before processing for TEM, cells that had taken up HRP were incubated in a solution containing 0.5 mg/ml DAB (Sigma) in Tris-HCl buffer, pH 7.6, for 15 minutes in the dark and at room temperature. After this incubation, H₂O₂ was added to a final concentration of 0.03% (v/v), and the cells were kept in the dark for a further 15 minutes at room temperature (Graham and Karnovsky, 1966). Cells were then washed and processed for TEM.

**Transmission electron microscopy**

Cells were fixed by using 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 hour at room temperature, or fixed in isothermal glutaraldehyde (after endocytosis, as described above). Following a wash in cacodylate buffer, the cells were post-fixed using an osmium-tetroxydride-osmium (OTO) protocol (Willingham and Rutherford, 1984). Briefly, the cells were incubated in a post-fixative osmium solution [1% (v/v) osmium tetroxide, 0.8% (v/v) potassium ferrocyanide and 5 mM calcium chloride in 0.1 M cacodylate buffer, pH 7.2] for 40 minutes, washed twice in water and then incubated in a solution of 1% (w/v) thio-carbohydrazide (TCH, Sigma) in water for 5 minutes. After three washes in water, the cells were incubated again in the post-fixative osmium solution for 3 minutes. Following post-fixation, the samples were washed in water, dehydrated in an acetone series and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and were observed by using a Tecnai-G² electron microscope operating at 200 kV.

**Preparation of whole-mount cytoskeletons**

Epimastigotes were mounted onto glow-discharged formvar-coated grids, extracted with 1% (v/v) NP-40 in PEME buffer (100 mM PIPES, 1 mM MgSO₄, 0.1 mM EDTA and 2 mM EGTA, pH 6.9), followed by fixation with 2.5% (v/v) glutaraldehyde in PEME (Parussini et al., 2003). For the preparation of salt-treated cytoskeletons, grids were incubated in 1 M NaCl or 60 mM CaCl₂ immediately after detergent extraction and prior to fixation. Grids were negatively stained with 0.7% (v/v) uranyl acetate and lead citrate, and were observed by a Tecnai-G² electron microscope operating at 200 kV.

**Electron tomography**

For electron tomography, ribbons of 200-nm thick serial sections were produced from TEM blocks prepared as described above. These ribbons were collected onto formvar-coated copper slot grids and stained post-embedding as described above. Colloidal gold particles (10 nm) were deposited on both surfaces of the sections, to be used as fiducial markers for alignment of the tilted views. Single-axis tilt series (±60° with 1° increments) were produced from samples using XploRE3D software and a Tecnai-G² (FEI Company, Eindhoven, Netherlands) electron microscope operating at 200 kV and coupled to a 4k×4k CCD camera. All 3D reconstructions and subsequent 3D data analyses were performed using the IMOD software package (Kremer et al., 1996). Tomogram generation
by R-weighted back-projection was performed using ETOMO, followed by joining of adjacent tomograms (of sections in a series) using the JOIN window in IMOD. Virtual slices were manually segmented using 3DMOD, which was also used to produce 3D models.

**Focused-ion-beam scanning electron microscopy**

For observation by FIB-SEM, cells that had taken up HRP for 5 minutes were processed for TEM as described above. The top of the resin block containing the sample was trimmed to a pyramidal shape, and its surface was smoothed by sectioning using a conventional diamond knife. The block was then glued to an SEM stub using silver paint, with the surface of the resin block pointing upwards, perpendicular to the microscope column. Samples were imaged by using a Nova Nanolab 600 dual-beam microscope (FEI Company) equipped with a gallium-ion source for focused-ion-beam milling, and a field-emission gun and an in-lens secondary electron detector for SEM imaging. Prior to milling and SEM imaging, the entire sample surface was coated with a 3-nm-thick platinum layer using a gas injector system located inside the main specimen chamber. The specimen stage was tilted to 52˚ and exposed to the focused ion beam so that the plane of the stage was parallel to the beam. A cross-sectional cut was introduced in two stages. First, a coarse cut was made at high beam currents (typically 7–20 nA) and at an accelerating voltage of 30 kV to create a trench that enabled viewing of the cross-section. Usually, 50–150-μm-wide trenches were cut into the specimen. In the second step, the ion beam was scanned using a current of 3–7 nA to polish and smooth the surface. Back-scatter electron images were recorded at accelerating voltages of 1–5 kV and a beam current of 4000 pA in the immersion lens mode. To create a slice-and-view image series, a step size of 25 nm was chosen for the removal of material from the specimen surface with the focused ion beam. After image capturing, the back-scattered electron images had their contrast inverted so that they looked like conventional TEM images. Slice-and-view series were automatically aligned using the XFAign algorithm of IMOD, and then a fine alignment was performed using MIDAS. All 3D models and automatically aligned using the XfAlign algorithm of IMOD, and then a

---

**References**


Fig. S1. Cytoplasmatic microtubules at the vicinity of the flagellar pocket. (A-E) Virtual slices of Tomo 3 showing sections of 6 different cytoplasmatic microtubules (numbered 1 to 6, brown arrows) in the vicinity of the flagellar pocket (FP). These microtubules are clearly distinct from those of the flagellar pocket microtubule quartets, namely: the MtQ (orange brackets) and the preoral ridge quartet (blue arrows). (F-H) Different views of a model of Tomo 3 showing the cytoplasmic microtubules and their distribution relative to other cellular structures, such as the Golgi complex (G, red), the kinetoplast (K, dark green), the basal body (BB, beige) and the pro-basal body (PBB, yellow), and the flagellar pocket (FP, white) and its associated microtubule quartets (blue and orange tubes). Microtubules number 1 and 2 initiated close to the probasal body and appeared intimately associated with the base of the flagellar pocket. Microtubule number 3 originated close to the kinetoplast, and extended towards the Golgi complex. Microtubule number 4 appeared close to cytopharinx microtubule quartet (blue arrows/blue tubes) and extended until a position close to the Golgi complex. Microtubules number 5 and 6 were short and originated close to the cytopharinx microtubule quartet. F (flagellum). Scale bars: 200 nm.
Fig. S2. Rare events of fusion or fission of vesicles with the flagellar pocket membrane. The model in panel A shows two of these events (rectangles), depicted in the tomogram slices shown in B and C (corresponding to the regions highlighted by rectangles 1 and 2, respectively). (B) A transversal image of the flagellar pocket (FP) region showing the point where a small vesicle (yellow arrow in rectangle 1) is fusing with or budding from the flagellar pocket. (C) A flagellar pocket invagination (white arrow in rectangle 2) was clearly visible. (D) Another view of the model showing the area indicated by rectangle 3 in E, where a large vesicle (yellow arrow) was observed fusing to or budding from the flagellar pocket. The orange brackets in B, C and E (and orange tubes in A and D) indicate the position of the MtQ. The blue tubes in A and D represent two of the four microtubules of the cytostome/cytopharinx. F (flagellum). Scale bars: 200 nm
Movie 1. FIB-SEM series of the cell showed in Figure 1. The FIB-SEM series was followed by the 3D model of the entire cell. Cytopharinx (pink), Kinetoplast (green), Nucleus (blue), Flagellar pocket (white), Flagellum (orange), Reservosomes (red) and plasma membrane (white transparent).

Movie 2. Virtual slices from Tomo 1. At the movie beginning, two of four microtubules that accompany the cytopharinx can be identified (blue arrows). They are followed by the appearance of the third and fourth microtubules, respectively. The beginning of the preoral ridge membrane domain at the flagellar pocket is delimitated by the purple bracket until it reaches the cytostome (Cy). The microtubule triplet is also pointed (green arrows).