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Cytochemical localization of ZIO-stained compartments in Trypanosoma cruzi and Toxoplasma gondii

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Abstract

Electron microscopy cytochemistry has contributed to a better knowledge of the identification of some cytoplasmic components of eukaryotic cells, even when they are not highly specific, but allow the identification of cell components. Here we describe results obtained by impregnation of the cells with a Zinc-Osmium Tetroxide complex to label some organelles of *Trypanosoma cruzi* and *Toxoplasma gondii*.

Introduction

Classical cytochemical methods adapted for transmission electron microscopy have made important contributions to the identification of structures and organelles in pathogenic protozoa, allowing the localization of carbohydrates on the cell surface, characterization of organelles such as mitochondria, peroxisomes, endocytic pathways and lysosomal system, lipid inclusions, among others. In some cases, these methods allow the identification of the nature of the biochemical components analyzed, as is the case of the localization of carbohydrates or enzymes. In other cases, the methods are more general in nature, only indicating whether the structures are made of lipids (as in the case of the use of osmium tetroxide/imidazole buffered solution [1] or basic proteins (as in the case of alcoholic phosphotungstic acid (PTA) [2–3]. Another interesting method is the use of impregnation by the Zinc-Osmium Tetroxide (ZIO) complex, initially introduced by Champy [4] and Maillet [5] in light microscopy and adapted for transmission electron microscopy [6–7] initially for visualization of synaptic vesicles of cholinergic junctions, without labeling other organelles located in the same place, such as mitochondria. Labeling was also found in part of the Golgi complex, especially in the GERL, and lysosomes [8]. Later Pellegrino de Iraldi and co-workers, and other authors found labeling in the endoplasmic reticulum, part of the Golgi complex and some mitochondria [9–15]. Although the biochemical nature of the molecules that interact with ZIO remains unclear, there are indications of interaction with lipoprotein complexes, calcium affinity sites and even proteins containing sulfhydryl groups [11]. More recently, Jokhura et al. [16] used ZIO impregnation for three-dimensional reconstruction of the Golgi complex in rat hepatocytes.

To date, only two articles have described results obtained with ZIO in pathogenic protozoa. Benchimol and De Souza [17] showed labeling in the endoplasmic reticulum-Golgi complex, nuclear pore and reticulum profiles located near the region of adhesion of the recurrent flagellum to the body of the protozoan. Then Slomianny and Prensier [18] showed the labeling of the endoplasmic reticulum, Golgi complex and the lysosomal systems of *Plasmodium falciparum*.

In this article we describe the results obtained with ZIO for *Trypanosoma cruzi* and *Toxoplasma gondii*, two pathogenic protozoa that cause relevant human diseases and that present distinct cellular organization. The results obtained show the existence of variation in the labeling of the various structures and organelles, possibly indicating heterogeneity in the composition of each organelle.

Materials and Methods

Obtaining the parasites

Toxoplasma gondii tachyzoites were maintained by passages in LLC MK2 cell culture. After 3 days of infection, the parasites obtained from the supernatant were centrifuged before use. *T. cruzi* epimastigotes were maintained in LIT culture medium supplemented with 10% of fetal bovine serum. After 2 days of inoculation, the parasites were collected by centrifugation.

Zinc iodide-osmium technique

The same protocol was applied to both cells type. Fixation was carried out in 2,5% glutaraldehyde in 0,1M cacodylate buffer for 2 hours. Cells were rinsing in a solution of sucrose 8,5% in 0.1 M phosphate buffer, pH 7,2, 2 times of 10 minutes. After that cells were rinsing with trisoaminomethane buffer (1,13M NaCl; 0,011M $CaCl_2$; 0,03M $MgCl_2$; 0,01M Tris aminomethane [19]. The final pH was adjusted for 3,8–4,5 with HCl 1N) 4 times of 5 minutes and then were incubated in ZIO solution for 18 hours, at 4^{o}C in the dark. Shortly before the incubation, the ZIO solution was prepared as follows: 3 g Zn powder and 1 g iodine resublimed was suspended in 10 ml distilled water, separately. These solutions were mixed in a ratio of 1:1. After filtering 4 ml of this solution was mixed with 4 ml of the Tris aminomethane buffer. Immediately before using 4 parts of this solution were mixed with 1 part of a 2% OsO4 aqueous solution. After dehydration in graded series of acetone, cells were embedded in an epoxy resin, sectioned and observed by transmission electron microscopy.

Results and Discussion

Epimastigotes of Trypanosoma cruzi

In general, procedures for preparing biological specimens using metal impregnations require long incubation times. Therefore, the quality of fixation is slightly lower than that obtained by conventional methods. Figure 1A shows an image of the epimastigote form of *T. cruzi* processed in a similar way to the cells impregnated with ZIO, but without passing through this reagent. The image clearly shows the nucleus, the kinetoplast and the Golgi complex. Figures 1B and 1C and Fig. 2A-E show equivalent areas of epimastigotes impregnated with ZIO, where a clearly marked region of the Golgi complex can be seen, as well as a set of vacuoles located in the posterior region and corresponding to the endolysosomal system, consisting of the reservosomes, as well as other structures described below.

Figure 1. (A) control image of cells that were not incubated in the presence of the Zio-OsO_4 solution. No labeling is seen in the various structures of the epimastigote form of *T.* cruzi. N, nucleus; K, kinetoplast. G, Golgi complex; (B_C) low magnification of epimastigotes incubated in the presence of Zio-OsO_4. The following structures are intensely labeled: Golgi complex region (G), profiles of the endoplasmic reticulum (ER). The nucleus and kinetoplast are not labeled.

Certainly, one of the most marked structures is the Golgi complex. However, not all the cisternae and vesicles that form this organelle were marked in the same way. In some cells, all cisternae of the cis and trans faces were marked, with the mark being more pronounced in more expanded lateral regions (Fig. 2. B, E). In others, the label was more evident in the first cisternae (Cis face) although not in their entire extension, while in other cells labeling was evident in the last cisternae of the trans face. Images such as Fig. 2C clearly show a non-uniform mark of the Golgi but with a densely marked cisternae associated with dense vesicles that detach from the central cisterna). Some cells showed the electron-dense product throughout the Golgi as well as in the cisternae of the endoplasmic reticulum distributed throughout the anterior region, close to the Golgi, and in the posterior region, surrounding the reservosomes (Fig. 3A and E). These data clearly show that the system of cisternae and vacuoles associated with the Golgi complex of *T. cruzi* is highly heterogeneous, indicating a dynamic activity of the glycoprotein synthesis and glycosylation system. This information is relevant because there are no detailed studies on the Golgi complex of this protozoan, although this organelle has been isolated using cell fractionation [20].

The labeling on other sites deserves to be highlighted. First, a reticular system located in the lateral region of the flagellar pocket, possibly formed by cisternae and vesicles that form the contractile vacuole of *T. cruzi* (Fig. 1B, 3B). This compartment concentrates several ions and may also act as a transport route for components of the Golgi complex that are secreted into the interior of the flagellar pocket [Review in 21). Second, a reticular structure located just below the portion of the plasma membrane of the protozoan in the area close to the flagellum (Fig. 3D). It is possible that this component is associated with the FAZ (Flagellar Attachment Zone) complex, which contains a set of important proteins such as GP 72 [22]. Third, structures located within the endocytic system, especially in the reservosome. Based on what we know about the reservosome, it is possible that these structures correspond to the lipid component, which tends to form crystalline lipid structures [23]. Finally, we highlight the fact that some epimastigotes present strong staining of the nuclear membrane (Fig. 3C).

Tachyzoites of Toxoplasma gondii

In the case of *T. gondii*, we used cells infected with tachyzoites. Several structures of the host cell were labeled, especially the Golgi complex, lipid inclusions, lysosomes and mitochondria (Fig. 4A). This labeling pattern is like that previously described in several cell types. In the case of the parasitized cell (Fig. 4B-C), the labeling of the intravacuolar tachyzoites, as well as the parasitophorous vacuole, was very discreet, except for some intravacuolar structures (Fig. 4C). In the case of the tachyzoite, the labeling was restricted to a set of structures located in the anterior portion facing the nucleus (Fig. 4B). They are spherical or elongated structures that must correspond to secretory structures in formation as the rhoptries and micronemes [Review in 24].

Declarations

Author Contribution

The study was designed by WS. Sample collection and electron microscopy processing were performed by NA; Electron microscopy observation was carried out by WS and NA. Both authors selected the images, wrote, and reviewed the final version of the manuscript.

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References

1. Soares MJ, de Souza MF, de Souza W (1987) Ultrastructural visualization of lipids in trypanosomatids. J Protozool. 10.1111/J.1550-7408.1987.TB03160.X
2. Bloom FE, Aghajanian GK (1968) Fine structural and cytochemical analysis of the staining of synaptic junctions with phosphotungstic acid. J Ultrastruct Res 22:361
3. Souto-Padrón T, De Souza W (1979) Cytochemical analysis at the fine-structural level of trypanosomatids stained with phosphotungstic acid. J Protozool 26:551–557. 10.1111/j.1550-7408.1979.tb04194.x.PMID
4. Champy C (1913) Granules et substances réduisant liodure d'osmium. J Anat 49:323–343
5. Maillet M (1962) La technique de Champy à l'osmium ioduré de potassium et la modificacion de Maillet à l'osmium-ioduré de Zinc. Trab Inst Cajal Invest Biol 54:1–36
6. Akert K, Moor H, Pfenninger K, Sandri C (1969) Contributions of new impregnation and freeze-etching to the problems of synaptic fine structure. In: Akert K, Waser FG (eds) Progress in Brains Research, vol 31. Elsevier Publishing Company, Amsterdm, pp 223–240
7. Akert K, Sandri C (1968) An electron microscopic study of zinc iodide-osmium impreganation of neurons. I. Staining of synaptic vesicles at cholinergic junctions. Brains Res 7:286–295
8. Marty F (1980) High voltage electron microscopy of membrane interactions in wheat. J Histochem Cytochem 28:1129–1132 Doi.0022-1554/80/10112904$02.25
9. de Pellegrino A (1975) Electron cytochemical demonstration of -SH groups in the synaptic vesicles of photoreceptor cells with the mixture of zinc iodide-osmium tetroxide. Experientia 7:842–844. 10.1007/BF01938497
10. Pellegrino de Iraldi A, Cardoni R (1979) ZIO staining in synaptic vesicles of the rat pineal nerves after inhibition of serotonin and noradrenaline synthesizing enzymes. Cell Tissue Res 200:91–100. 10.1007/BF00236890
11. Pellegrino de Iraldi AP (1975) Localizing -SH groups in monoaminergic synaptic vesicles with the mixture of zinc iodide-osmium tetroxide (ZIO). Brain Res 94:363–367. 10.1016/0006-

8993(75)90222-x

12. Reinecke M (1981) The Zinc Iodide-Osmium Tetroxide (ZIO) method. In: Heym et al (eds) Techniques in Neuroanatomical Research. Springer-, Berlin Heidelberg, pp 292–300
13. Chau Y-P, Lu K-S (1994) ZIO impregnation and cytochemical localization of thiamine pyrophosphatase and acid phosphatase activities in small granule-containing (SGC) cells of rat superior cervical ganglia. Histo Histopathol 9:649–656
14. Schimmig B, Vicenti CA (2005) Zinc Iodide-osmium tetroxide (ZIO) reactive Golgi apparatus in the principal cells of dog epididymal epithelium. Int J Morphol 23:337–344. Doi.org/10.4067/S0717-95022005000400009
15. Reinecke M, Wlather C (1978) Zinc iodide-osmium tetroxide reaction: Its effect on the synaptic vesicles in locust neuromuscular junctions and its chemical basis. In: Sturgess JM (ed) Electron microscopy Vol II. The imperial press Ltd. Ontario Canada, pp 614–615
16. Johkura K, Usuda N, Tanaka Y, Fukasawa M, Murata K, Noda T, Ohno N (2022) Whole-cell observation of ZIO-stained Golgi apparatus in rat hepatocytes with serial block-face scanning electron microscope, SBF-SEM. Microscopy 71:262–270. Doi.org/10.1093/jmicro/dfac024/80/129
17. Benchimol M, De Souza W (1985) *Tritrichomonas foetus*: Cytochemical visualization of the endoplasmic reticulum-Golgi complex and lipids. Exp Parasitol 59:51–58. https://doi.org/10.1016/0014-4894(85)90056-6
18. Slomianny C, Prensier G (1990) A cytochemical ultrastructural study of the lysosomal system of different species of malarial parasites. J Protozool 37:465–470
19. Urensen G, de Groot D (1974) Osmium-zinc iodide staining and the quantitative study of central synapses. Brain Res 74:131–142. 10.1016/0006-8993(74)90116-4
20. Morgado-Diaz JA, Agrellos OA, Dias WB, Previato JO, Mendonça-Previato L, De Souza W (2001) Isolation and characterization of the Golgi complex of the protozoan *Trypanosoma cruzi*. Parasitology 123:33–43. 10.1017/s0031182001007946
21. Docampo R (2024) Advances in the cellular biology, biochemistry, and molecular biology of acidocalcisomes. Microbiol Mol Biol Rev 88:e0004223. 10.1128/mmbr.00042-23
22. Souza-Melo N, de Souza GH, de Souza W (2025) Effects of TcFLA-1BP and TcGP72 Deletion on the Infectivity and Survival of *Trypanosoma cruzi* in Cell Culture. Int J Cell Biol. 10.1002/cbin.70076
23. Estevam H, Carvalho RT, Salgado LT, Alcantara CL, Aguar-Seabra J, de Souza W, Cunha e Silva NL, Pereira MG (2025) Cholesterol crystals in reservosomes of *Trypanosoma cruzi*. J Struct Biol 217(4):108259. 10.1016/j.jsb.2025.108259
24. Tachibana Y, Yamamoto M (2025) Recent advances in identifying and characterizing secretory proteins of *Toxoplasma gondii* by CRISPR-based screening. Parasitol Int 105. https://doi.org/10.1016/j.parint.2024.102997

Figures

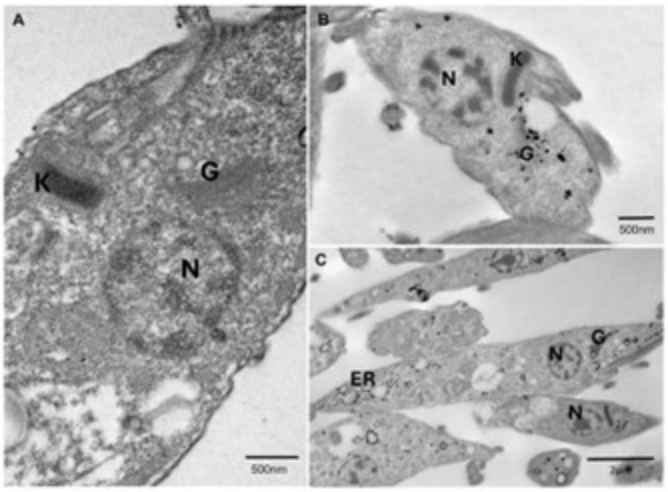


Figure 1

(A) control image of cells that were not incubated in the presence of the Zio-OsO_4 solution. No labeling is seen in the various structures of the epimastigote form of *T.* cruzi. N, nucleus; K, kinetoplast. G, Golgi complex; (B_C) low magnification of epimastigotes incubated in the presence of Zio-OsO_4. The following structures are intensely labeled: Golgi complex region (G), profiles of the endoplasmic reticulum (ER). The nucleus and kinetoplast are not labeled.

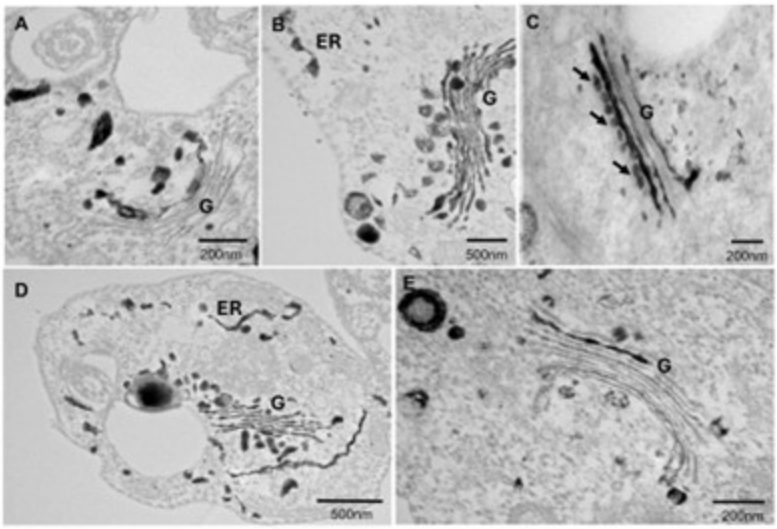


Figure 2

Different views of labeled Golgi complex (G) and cisternae of the endoplasmic reticulum (ER) in different cells. Some Golgi complex cisternae show a variable labeling intensity. Fig. C shows intensely stained

vesicles aligned to trans region of the Golgi (arrows).

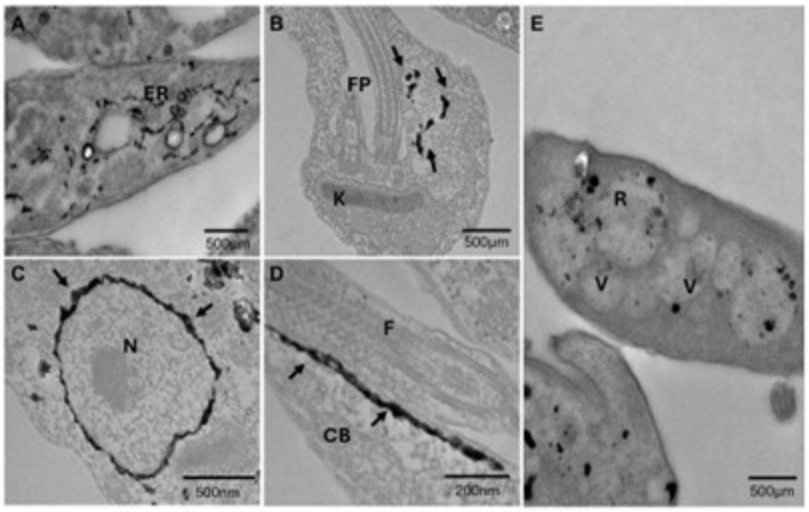


Figure 3

Cells incubated in the presence of the Zio-OsO_4 solution. (A) a reticular system, part of the ER, is labeled leaving in between unlabeled vacuoles; (B) Tangential section of the region of emergence of the flagellum. Only some cisternae, localized around the flagellar pocket (FP) is labeled (arrows). They may correspond to part of the contractile vacuole. (C) in a few cells the nuclear membrane was intensely labeled.; (D) Labeling in a cisterna that lines the region of the adhesion of the cell body (CB) to the flagellum (F). (E), labeling of several vacuoles (V) and the reservosome R localized at the posterior region of the cell.

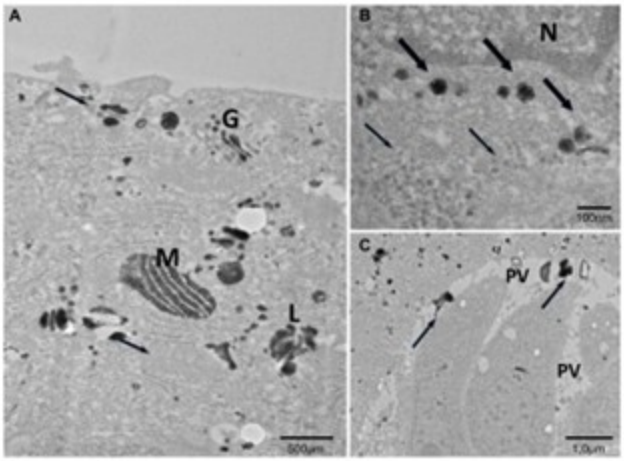


Figure 4

(A) LLC MK2 cells incubated in the presence of Zio-OsO_4 solution. Labeling of some cisternae of the Golgi complex (G), Lysosome-like structures (L) and some profiles of the smooth ER are labeled (arrow). Profiles of the rough endoplasmic reticulum were not labeled. One single mitochondrion (M) was intensely labeled. (B-C) Images of a host cell with intracellular tachyzoites within the parasitophorous vacuole (PV). Some components of the PV are labeled (arrow in C). In contrast, only the anterior pole of the parasite shows some labeled structures (arrows in B). Other structures localized in the anterior region of the tachyzoite are also lightly labeled (thin arrows). They may represent components of the secretory system (rhoptries and micronemes). _