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CHAPTER 6

TOPOGRAPHY AND CELLULAR INTERACTION: APPLICATION OF ATOMIC FORCE MICROSCOPY IN STUDIES WITH TOXOPLASMA GONDII

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Everson Reili de Souza Teles[1]

ABSTRACT

Toxoplasma gondii is an obligate intracellular protozoan capable of infecting a wide range of warm-blooded animals and remains a global health concern. Understanding the mechanisms underlying parasite–host cell interaction is essential for elucidating the initial steps of infection. In this study, we applied atomic force microscopy (AFM) combined with fluorescence microscopy to characterize, at nanometric resolution, the early stages of interaction between *T. gondii* tachyzoites (RH strain) and mammalian host cells (LLC-MK2 and HFF). A fixation protocol optimized with 4% formaldehyde and 1% glutaraldehyde ensured morphological preservation while maintaining suitable mechanical properties for topographical analysis. Quantitative evaluation of 100 cells per experiment revealed that, after 15 minutes of incubation, the majority of tachyzoites remained adhered to host cell surfaces, with fewer in contact or fully internalized. AFM imaging revealed host cell participation during invasion, showing localized membrane invaginations, conoid projections, and numerous actin-rich extensions resembling filopodia and tunneling nanotubes directed toward the parasite. Nanomechanical mapping demonstrated distinct height and elasticity patterns at the host–parasite interface, indicating active cytoskeletal remodeling and membrane engagement during internalization. These results highlight AFM as a powerful complementary approach to fluorescence microscopy, providing unprecedented insights into the topography, elasticity, and dynamic remodeling of host cell membranes during *T. gondii* invasion. Understanding these structural and mechanical interactions contributes to elucidating the early determinants of infection and may aid in identifying novel therapeutic targets.

Keywords: *Toxoplasma gondii*; Atomic force microscopy; Host–parasite interaction; Cell invasion; Nanomechanics; Cytoskeletal remodeling.

[1] Master of Science in Biomedical Sciences
Federal University of Rio de Janeiro - UFRJ
Hertha Meyer Cellular Ultrastructure Laboratory, Precision Medicine Research Center, Carlos Chagas Filho Institute of Biophysics, National Center for Structural Biology and Bioimaging
E-mail: everson.rsteles@gmail.com

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan of worldwide distribution, being highly efficient in its invasion process, with the ability to infect a wide variety of homeothermic vertebrates, birds, and economically important animals (Dubey, 2010; Montoya & Liesenfeld, 2004). In Brazil, *T. gondii* infection shows high prevalence across all regions, affecting both humans and domestic animals (Dubey et al., 2012; Pena et al., 2008). The invasion process of *T. gondii* into host cells occurs in less than 20 seconds and is characterized by an active penetration mechanism mediated by secretory proteins from apical complexes such as micronemes, rhoptries, and dense granules (Carruthers & Boothroyd, 2007; Lorenzi et al., 2016). A moving junction is formed as a result of the strong association between the apical end of the parasite and the host cell surface, during which the parasite secretes adhesion and anchoring proteins into the host cell membrane, leading to its internalization within a parasitophorous vacuole (Dubey, 2021; de Souza & Portes in Martins-Duarte & Adesse, 2021).

Among the methodologies available for morphological and topographical studies of these interactions, atomic force microscopy (AFM) stands out as a promising tool. This technique enables high-resolution visualization of the cell surface, allowing three-dimensional and nanomechanical analysis of events such as adhesion, membrane invagination, and the formation of cytoskeleton-associated structures during the invasion process (Binnig et al., 1986; Hoh & Engel, 1993; Cappella & Dietler, 1999). AFM has been successfully applied to the study of protozoa such as *Plasmodium* and *Giardia*, and more recently, to investigations of *T. gondii* interactions with host cells (de Souza & Rocha, 2011).

In this study, we employed atomic force microscopy to characterize, with high resolution, the initial events of interaction between tachyzoites of the *T. gondii* RH strain and epithelial LLC-MK2 cells and HFF fibroblasts. The objective was to map, at topographic and nanomechanical levels, the alterations in the host cell surface and the structures involved in moving junction formation, aiming to deepen the morphological understanding of this crucial infection process.

Understanding the initial interaction between a host cell and an intracellular parasite is essential, as the determinants involved can be explored as potential therapeutic targets to help block infection at its earliest stages, contributing to the development of innovative treatment strategies. Considering the existence of different possible invasion processes of the parasite, we sought to describe in greater detail the events associated with invasion. For this purpose, tachyzoites of the RH strain and LLC-MK2 and HFF cell lines were used. Through synchronization of entry events, we chose to perform our analyses after 15 minutes of interaction. The analyses included fluorescence microscopy to quantify tachyzoite entry into host cells and atomic force microscopy. After synchronization, we observed the host cell's participation in parasite internalization, numerous host cell projections involved during entry, and nanotubes projected from the host cell toward the parasite.

MATERIALS AND METHODS

HOST CELLS

Two distinct host cell lines were used: rhesus monkey (Macaca mulatta) kidney epithelial cells, LLC-MK2 (ATCC – CCL7, Rockville, MD/USA), cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum; and human foreskin fibroblast cells, HFF1, cultured in complete Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA), supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 1% streptomycin and penicillin, and 2 mM L-glutamine (Sigma). Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

PARASITES

T. gondii RH strain tachyzoites were maintained by serial passages in confluent LLC-MK2 cells. Two to three days post-infection, parasites obtained from the supernatant were centrifuged at 1000 × g for 10 minutes, resuspended in RPMI medium, and counted using a Neubauer chamber.

PARASITE–HOST CELL INTERACTION

One day before the interaction experiments, 5×10^5 cells were plated on sterile round glass coverslips in 24-well plates or distributed into 25 cm^2 culture flasks. Cells were then washed three times with phosphate-buffered saline (PBS), and parasites were added at a ratio of 50:1 (parasites:cell). Subsequently, the cultures were incubated at 4 °C for 30 minutes to synchronize parasite invasion. Afterward, cells were transferred to a 37 °C incubator and incubated for approximately 19 minutes. At this stage, with the medium reaching around 20 °C, the parasite regains motility and interacts with the cell, thereby synchronizing the entry events. After 15 minutes of interaction, cells were processed for microscopy analyses.

FLUORESCENCE MICROSCOPY

After synchronization of entry events between *T. gondii* and host cells, samples were fixed in 4% formaldehyde in PBS buffer (pH 7.5) for 15 minutes at room temperature. Cells were washed three times with PBS (pH 7.5) and permeabilized with 0.1% Triton X-100 in PBS containing 3% bovine serum albumin (BSA) for 10 minutes, repeated three times. After permeabilization, samples were again washed three times with PBS (pH 7.5) and incubated overnight at 4 °C with a primary anti-SAG (surface antigen of tachyzoite) antibody diluted 1:500. The next day, samples were washed three times with PBS (pH 7.5) and incubated with Alexa Fluor 546-conjugated anti-mouse secondary antibody (1:1000) for 2 hours at room temperature, protected from light. Observations were performed in confocal mode using a Zeiss Elyra PS.1 LSM 710 microscope (Germany).

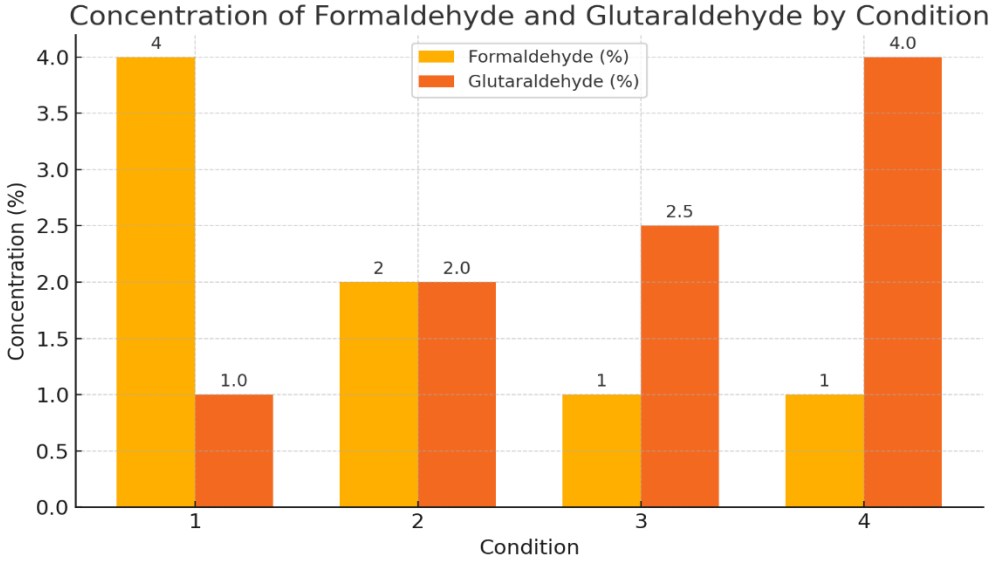
ATOMIC FORCE MICROSCOPY (AFM)

Samples were fixed in a solution containing 1% glutaraldehyde and 4% formaldehyde in sodium phosphate buffer (pH 7.2) for 1 hour, washed with PBS (pH 7.2), and post-fixed for 1 hour in 1% osmium tetroxide in phosphate buffer, protected from light. Cells were dehydrated in increasing concentrations of acetone (30% to 100%) for 10 minutes at each step and dried using CO_2 critical point drying. Images were obtained at room temperature using a Bruker Dimension Icon Scanning Probe Microscope. Cantilevers with spring constants between 0.4 N/m and 2 N/m were used. For nanomechanical property analysis, full cantilever calibration was performed. To minimize damage and noise, images were acquired with a scanning frequency of approximately 1 Hz and a resolution of 512 × 512 pixels. To assist in analyses, the data matrix that generates flattened images was transformed into a three-dimensional visualization, providing better interpretation of AFM-obtained data (de Souza & Rocha, 2011).

RESULTS

The ultrastructure of *Toxoplasma gondii* invasion into host cells was observed using fluorescence microscopy and topographic images generated by AFM, in which the initial interaction events and even the invasion process of the parasite were analyzed, clearly showing host cell participation. Proper fixation is critical for preserving cellular morphology and obtaining high-quality images. In this study, different combinations of formaldehyde and glutaraldehyde were tested to identify the most effective protocol for maintaining the structural integrity of the samples, as shown in Figure 1**.**

Figure 1**.** Concentrations of formaldehyde and glutaraldehyde used under different experimental conditions. Each condition corresponds to a specific combination of fixative concentrations applied to cell samples. Bars represent the percentage of each fixative. Data are representative of standardized protocols for morphological preservation.

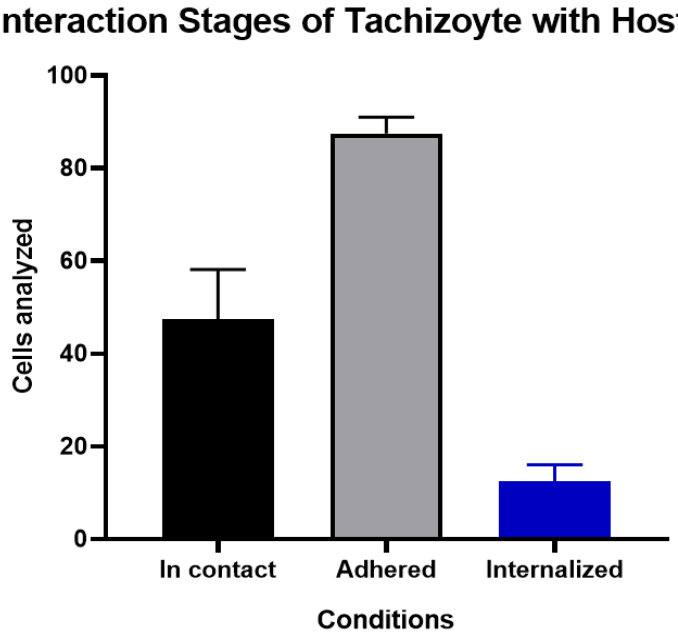


Source: Author's own work (2025).

Among the tested formulations, high concentrations of glutaraldehyde (≥2.5%) ensured excellent ultrastructural preservation but compromised topography. Balanced combinations (2% FA / 2% GA) provided good structural preservation; however, when the cantilever came into direct contact with the sample, significant topographical information was lost, reducing data fidelity. In contrast, the 4% FA / 1% GA formulation proved most suitable for this assay, as it preserved the overall morphology of both host cells and parasites while minimizing excessive stiffening. This allowed accurate visualization of the host–parasite interface and maintained suitability for morphological studies. Thus, although GA-rich fixatives are preferable for high-resolution electron microscopy, the final choice of 1% GA + 4% FA represented the best strategy to balance structural preservation with the requirements of *T. gondii*–host interaction studies by AFM.

With the fixation protocol for AFM established, 100 cells per experiment were analyzed in triplicate, and *T. gondii* tachyzoites were classified according to interaction stage: adhesion, contact, and internalization. After 15 minutes of incubation, parasites were predominantly adhered to the host cell surface, followed by those in contact and a smaller number of internalized parasites. Statistical analysis (ANOVA with Tukey's test) revealed significant differences between groups ($p < 0.05$), indicating that the experimental time was sufficient to capture distinct phases of parasite entry, with emphasis on early adhesion events (figure 2).

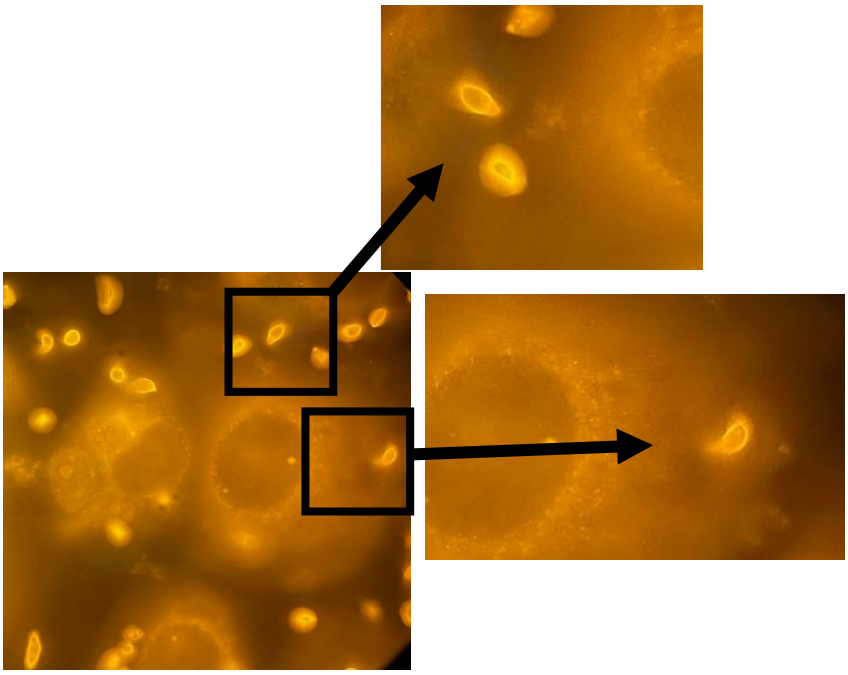
Figure 2: Quantification of *Toxoplasma gondii* tachyzoites classified according to their interaction with host cells after 15 minutes of incubation. Parasites were grouped into three categories: adhered (attached to the cell surface), in contact (in the process of entry), and internalized (completely inside the cell). Data represent the mean ± standard deviation of three independent experiments, with n = 100 cells analyzed per condition. Statistical analysis was performed by ANOVA followed by Tukey's test, with $p < 0.05$ considered significant.



Source: Author's own work (2025).

Fluorescence microscopy was used to visualize the invasion process. Labeling of the surface antigen (SAG) allowed visualization of the initial interaction between the tachyzoite and the host cell. In the obtained images, the host cell membrane showed a localized invagination at the point of contact with the parasite, indicative of the formation of the moving junction—an essential structure for the active internalization process promoted by *T. gondii*.(figure 3)

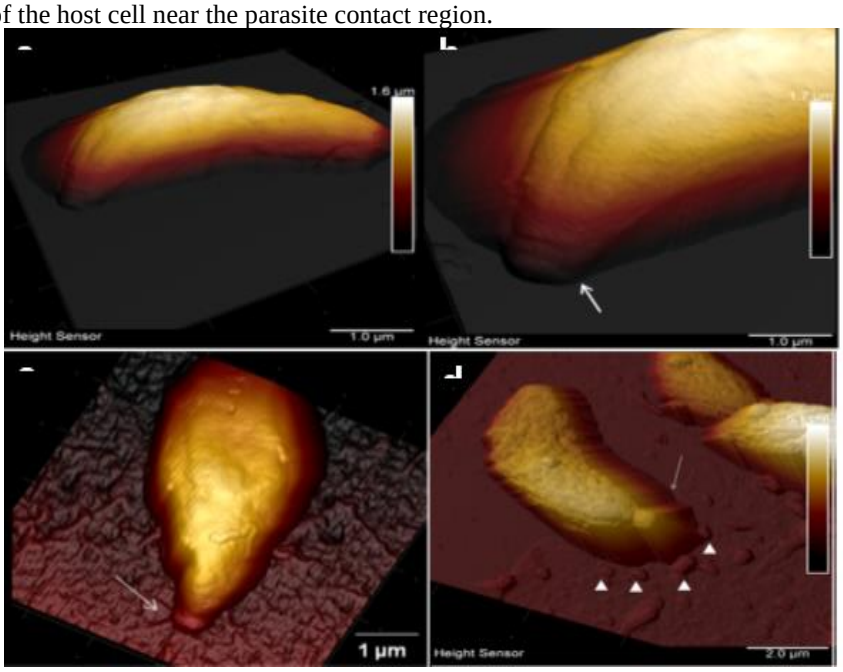
Figure 3. Fluorescence microscopy showing labeling of *Toxoplasma gondii* surface antigen (SAG) during the initial invasion events. Membrane invagination of the host cell is observed at the point of contact with the tachyzoite, suggesting. The site of formation of the structure known as the moving junction.



Source: Author's own work (2025).

In the early stages of the invasion process, which consists of cell recognition (**Figure 4a**) followed by parasite adhesion (**Figure 4b**), conoid projection occurs (**Figure 4c**) together with secretion of proteins required for moving junction formation. During these initial events, membrane projections from the host cell surrounding the parasite were also observed (**Figures 4c and 4d**).

Figure 4. Ultrastructure of *T. gondii* by AFM interacting with LLC-MK2 cells. a. Fixed with formaldehyde after 15 minutes of interaction, showing the entire body of the tachyzoite with conoid (arrow) interacting with the host cell. b. Higher magnification of the conoid projection (arrow).c. Arrow indicates the conoid on the host cell surface. d. Arrowhead shows membrane projections of the host cell near the parasite contact region.



Source: Author's own work (2025).

In **Figure 5**, filopodia-like projections emitted by the host cell are observed in regions of contact with *T. gondii*. AFM image analysis revealed distinct height and elasticity signals in these areas, indicating the presence of possible filaments directed toward the parasite. These structures are consistent with tunneling nanotubes—actin-rich extensions commonly associated with adhesion and cellular recognition events.

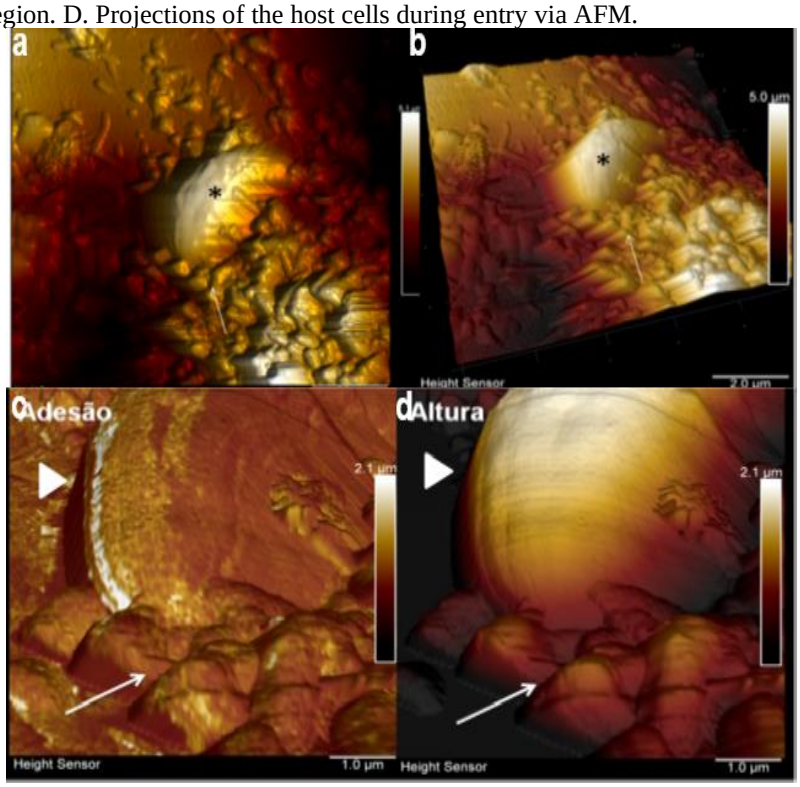
Figure 5. Filopodia-like projections emitted by the host cell in contact regions with the parasite. Height and elasticity signals near these regions suggest possible filaments emitted from the host cell (arrow). Scale bar: 1 µm.

Source: Author's own work (2025).

Topographical analysis revealed lateral internalization of the parasite, with the tachyzoite assuming a “half-moon” shape (**Figure 6a**). Multiple host cell membrane projections were also observed

in this region (**Figure 6b**), appearing in all parasite-contact areas, forming a pattern supported by adhesion and height data (**Figures 6c and 6d**).

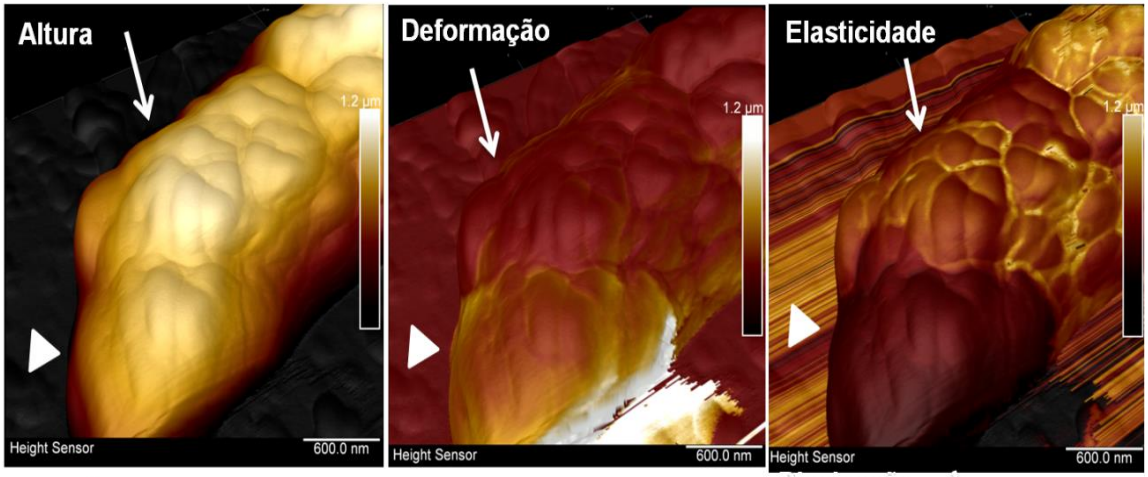
Figure 6. Three-dimensional representation of height images during tachyzoite invasion, as observed using a single-tip probe. A. The arrows indicate projections of the host cell, and the lateral orientation of the parasite is marked with an asterisk during entry. C. At the arrowhead, the tachyzoite is surrounded by the host cell membrane, indicated by an arrow, with numerous projections in this entry region. D. Projections of the host cells during entry via AFM.



Source: Author's own work (2025).

Surface density in the tachyzoite entry region was higher compared to adhesion-only regions. In **Figure 7**, elasticity signal readings allowed differentiation between host cell membrane regions—those with higher elasticity signals corresponding to membrane enveloping the parasite, while the non-internalized portion emitted lower elasticity signals.

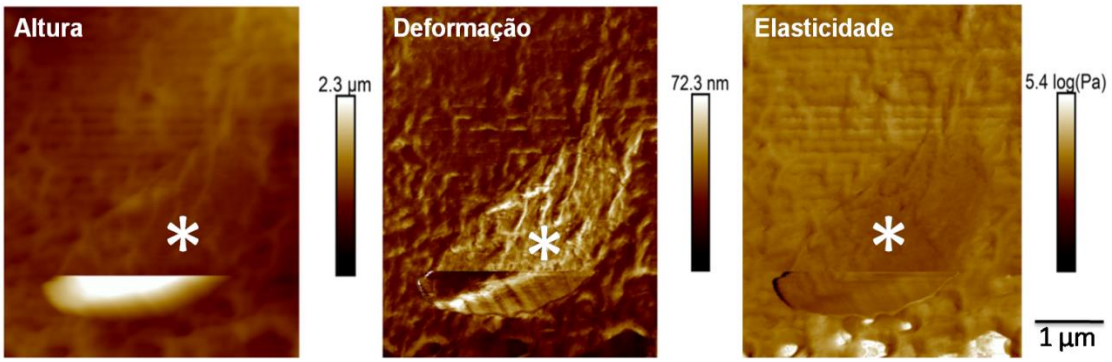
Figure 7**.** Host cell membrane surrounding *T. gondii* tachyzoites observed by AFM with height, deformation, and elasticity data. Arrow indicates the host cell membrane enveloping the parasite, with the arrowhead showing the partially unengulfed parasite body.



Source: Author's own work (2025).

During scanning, the cantilever probe swept across the parasite; upon its removal, deformation signals were detected in the area previously occupied by the parasite, indicating possible rearrangement of the host cell membrane surface at the adhesion and contact site **(**Figure 8**).**

Figure 8. Host cell surface after scanning of the parasite by the probe. The asterisk marks the area that, before scanning, corresponded to the tachyzoite anchoring site on the host cell, which was unexpectedly removed during the cantilever process.



Source: Author's own work (2025).

DISCUSSION

The study of interactions between pathogenic protozoa and host cells—particularly those capable of penetrating and replicating intracellularly—has been the focus of extensive research. In the case of *Toxoplasma gondii*, evidence indicates that it can be internalized by various cell types; however, mere entry does not guarantee successful infection and intracellular multiplication. Successful infection requires an active invasion mechanism involving the parasite's apical organelles. In this work, we analyzed observable data from the early interaction process using atomic force microscopy, focusing on

the first fifteen minutes of interaction, a period previously shown to involve diverse host cell morphologies (Teles *et al*., 2023).

The choice of fixative concentration is critical for morphological preservation, antigenicity, and cellular rigidity, particularly in studies of interactions between mammalian cells and *T. gondii*. In search of an efficient and standardized fixation protocol, different combinations of formaldehyde (FA) and glutaraldehyde (GA) were evaluated to optimize morphological preservation without compromising structural integrity. Comparative analysis of four formulations—(1) 4% FA + 1% GA, (2) 2% FA + 2% GA, (3) 1% FA + 2.5% GA, and (4) 1% FA + 4% GA—revealed that high GA concentrations, although promoting excellent ultrastructural preservation, caused excessive stiffening that limited elasticity analyses.

Conversely, the 4% FA + 1% GA formulation provided the best overall performance: it preserved cellular morphology, prevented material loss during cantilever contact, and minimized structural artifacts. This balance between fixatives and sample response made the combination ideal for AFM-based topographical analysis and was thus adopted as the standard protocol for subsequent analyses. Traditional microscopic techniques have long been employed to study *T. gondii*–host cell interactions (Dubey, 2021; de Souza & Portes in Martins-Duarte & Adesse, 2021; Teles, 2023). However, most focus on parasite development within the parasitophorous vacuole.

Our AFM observations support the involvement of the host cell during interaction with the tachyzoite. This methodology was initially applied by Aikawa (1997) to examine *Plasmodium*–erythrocyte interactions and later extended to other protozoa (de Souza & Rocha, 2011). Signals captured by the probe indicated increased elasticity in regions where the tachyzoite was partially covered by the host cell membrane. Considering that *T. gondii* possesses a trilaminar pellicle (Cintra & de Souza, 1985), this overlap explains the observed elasticity increase at probe–sample contact, while reduced height and elasticity signals in the lower region suggest areas of the parasite body not yet fully internalized.

Numerous alterations on the host cell surface were detected during scanning. Depending on morphology and cellular context, these protrusions may be classified as microvilli, lamellipodia, filopodia, or growth cones (Condeelis, 1993). The biogenesis of these structures depends on cytoskeletal organization and plays essential roles in cell motility and endocytosis (Pantaloni *et al*., 2001), processes also associated with passive entry of *T. gondii* (Portes *et al*., 2019).

Protrusive processes require a specialized actin filament system driving membrane movement. Actin filaments are polarized and, upon myosin binding, elongate rapidly, pushing the membrane forward (Pantaloni et al., 2001). Although both are cytoskeleton-related membrane projections, filopodia have been described in numerous biological contexts.

Using atomic force microscopy, we observed that, following scanning of an adhered tachyzoite, the host cell membrane surface underwent reorganization, indicating parasite anchoring. During *T. gondii*–host cell interaction, intense remodeling occurs at the contact area, involving the aforementioned structures and others, underscoring the need for new approaches and techniques to fully understand host–parasite interactions.

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