

Review Article

Scanning Ion Microscopy and Its Application in Microbiology

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Abstract

Helium ion microscopy, a scanning microscope operating with ions rather than electrons, is one of the best equipment to analyze the surface of cells and the surface of intracellular compartments exposed by different methods. This short review aims to show the advantages of resolution and depth of focus in some microorganisms such as viruses, bacteria, fungi and parasitic protists.

Keywords: High-Resolution Microscopy; Helium Ion Microscopy; Microbiology

Introduction

The Helium Ion Microscope (HIM) uses a scanning helium (He) ion beam for surface imaging and analysis. It is based on the microscope first proposed by Muller in 1951, where a rare gas adsorbed on a pointed metal tip is ionized and accelerated straight ahead. The metal tip in a rare gas atmosphere is used as a filament of the ion source in HIM and is called a Gas Field Ion Source (GFIS) microscope. Over a decade after the Galium Focused Ion Beam (FIB) microscopy's development, the Atomic Level Ion Source (ALIS) Corporation, an American venture company, commercialized the HIM instrumentation. In this system, helium ions drawn from the tip of a sharpened metal ion source in a helium gas atmosphere are focused by a lens system and irradiated to the sample. This technique can analyze structures in the near-surface region based on the images obtained using secondary electrons and backscattered ions. Since then, there has been progress in HIM technology and new applications have been developed in areas where scanning microscopy is used. Sequentially, the microscope (designated as ORION PLUS) was commercialized by Carl Zeiss SMT (Oberkochen, Germany).

Unfortunately, Zeiss recently announced that it will no longer commercialize the equipment. Reviews on basic aspects of HIM were published previously [1-3]. This microscope provides several advantages, including (a) less charge-up; (b) reduced damage to the sample; (c) greater depth of focus and (d) higher resolution since the beam originates from an atomic scale point source. The extracted ions are accelerated down the column of the microscope as occurs in a conventional SEM. The high resolution is related to a combination of a small beam (defined by the aperture), the high brightness, the small opening angle and how the beam interacts with the sample. The latter is very different from SEM and important to reach the estimated resolution of 0.35 nm. Besides that, when the sample's surface is bombarded by, He⁺, the beam's penetration is smaller when compared to an electron beam accelerated to 30 kV, improving the resolution. The secondary electron yield is much higher than in SEM, giving the images a better signal-to-noise ratio.

Additionally, there is no need for metal coating of the samples due to the unique charge compensation mechanism in HIM. It enables direct investigation of delicate surface features that the metal coating may cover otherwise. Compared to an electron beam, the smaller beam and a narrower convergence angle allow the acquisition of images with a larger field depth. The combination of several characteristics has made HIM an instrument reaching ultra-high resolution (down to 0.35 nm) and a promising tool for the analysis of biological surfaces, as elegantly shown by Rice, et al., who characterized the surface of kidney cells using this microscope [4]. Below, we will briefly exemplify the application of HIM in microbiology.

Application to Study Virus

Recently, several authors used HIM to analyze the interaction of SARS-CoV-2 with host cells [5-7]. Visualization of the adhesion of the virus to the host cell surface, the assembly of long tunneling nanotubes that connect two or more host cells over submillimeter distances and even the presence of the particles in different compartments of the host cell could be visualized at high resolution (Fig. 1). Initial studies allowed the acquisition of excellent images of the interaction of bacteriophages with bacteria. The images obtained constitute good examples of the depth of focus of the equipment (Fig. 1) and the excellent resolution obtained, allowing the visualization of the virus particles (Fig. 1) [8].

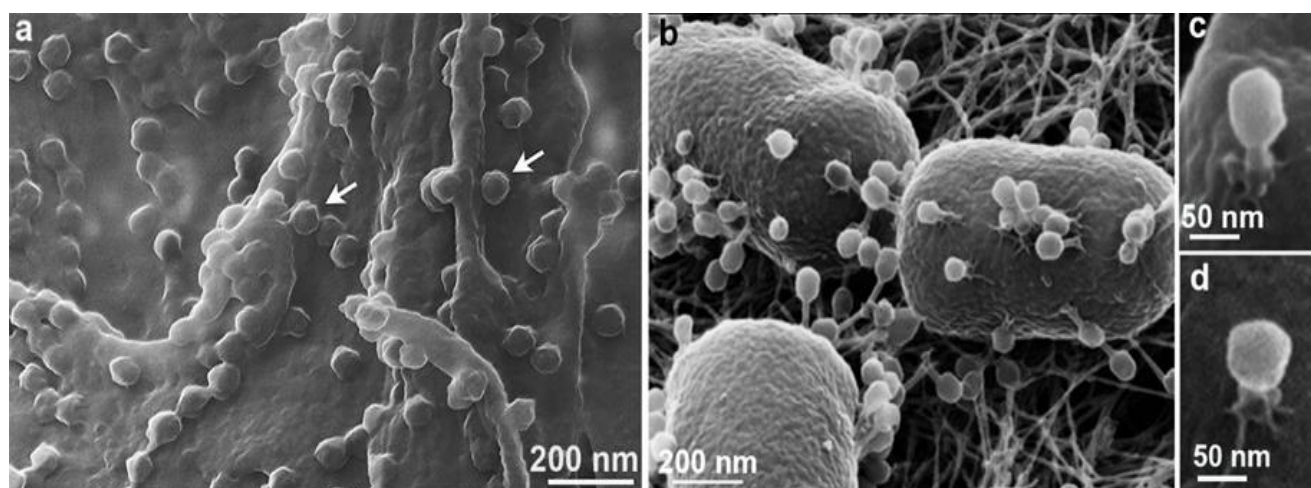


Figure 1: (a) Electron microscopy analysis of the late stages of SARS-CoV-2 infection in Vero cells. HIM image of the cellular surface showed discrete membrane projections replete with virions (arrows) at 24 hpi; bar, 200 nm (After 7); (b): HIM general view of the T4 bacteriophage infecting *Escherichia coli*; (c,d): Higher-resolution HIM image of a single T4 bacteriophage attached to the cell surface. The tail is contracted and the tail fibers are spread out, indicating a genome injection in progress. The icosahedral shape of the head is also apparent (After 8).

Application to Study Bacteria

Helium-Ion Microscopy (HIM) has rarely been employed to image microbial interactions. We will illustrate a good example of an application with the data reported by Said, et al., who used HIM to visualize the life cycle of the bacterial predator *Bdellovibrio bacteriovorus* HD100 with *Escherichia coli* and *Pseudomonas putida*, respectively, as prey is presented [9]. HIM allowed the analysis of the interaction between prey and predator by imaging the predator's flagella, the attachment and penetration of the bdellovibrios to their prey and the transformation of the membranes of prey cells during the bdelloplast formation (Fig. 2).

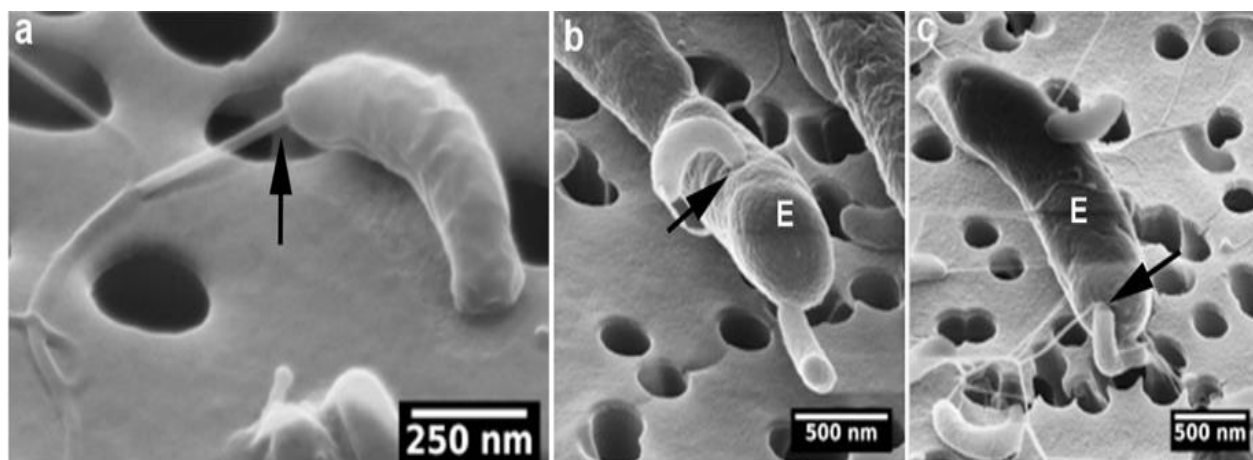


Figure 2: HIM image of the bacterium predator *Bdellovibrio bacteriovorus* HD100 interacting with *Escherichia coli*. (a): High magnification showing the vibrio-shaped; (B): Bacterivorous with its single flagellum (arrow). The sample is on a polycarbonate filter with a 0.22 μm pore size). (b,c): Images of the interaction of *B. bacteriovorus* with *E. coli* showing areas of attachment and invasion (arrows) (After 5).

Application to Study Fungi

Using *Cryptococcus neoformans*, a fungal pathogen that causes life-threatening infections in immunocompromised individuals, we will exemplify. Its main virulence factor is an extracellular polysaccharide capsule whose structure, assembly and dynamics remain poorly understood. Using HIM, Araujo, et al., used high-resolution scanning microscopy (SEM and HIM) to visualize the ultrastructure of the *C. neoformans* capsule. Although most capsule structures in nature consist of linear polymers, it was shown that the *C. neoformans* capsule is a 'microgel-like' structure composed of branched polysaccharides [10]. Moreover, it was shown that the capsule-to-cell wall link is formed by thin fibers that branch out of thicker capsule filaments and have one end firmly embedded in the cell wall structure (Fig. 3).

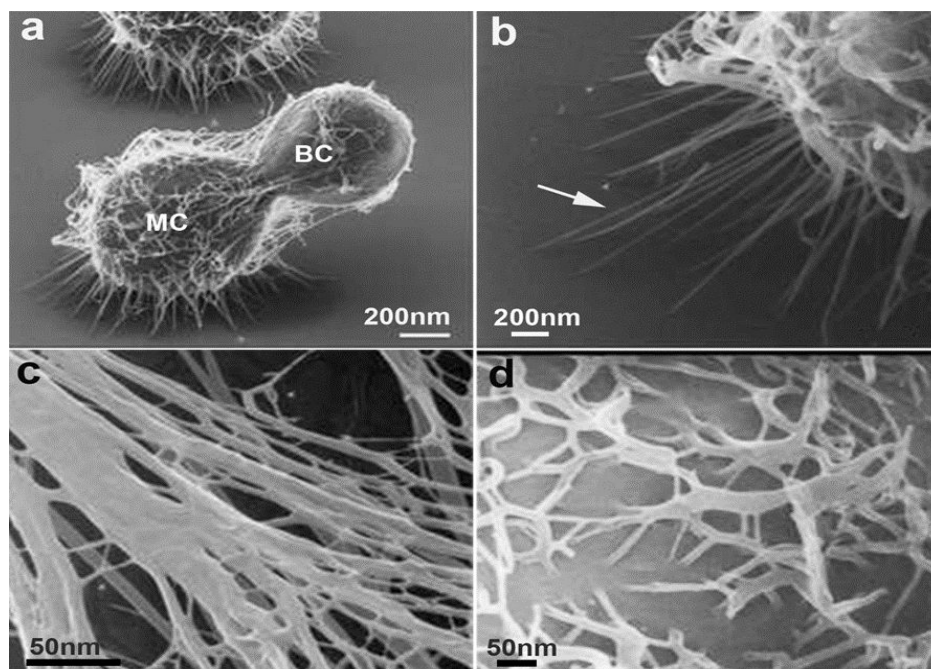


Figure 3: HIM of the *Cryptococcus neoformans* polysaccharide capsule. a: Image showing a general view of fibers surrounding yeast cells. Note that, in dividing cells, the bud (BC) capsule is less prominent than that of the Mother Cell (MC); b: Inset of the image in (a); showing polysaccharide fibers (arrows) ranging from 11.0 to 40.0 nm (mean value: 25.5 nm) linking the capsule to the adhesion surface; c: Capsule fibers interact laterally to form thicker fibers. Images of the inner region of the capsule show the edges of branched capsule filaments embedded in the cell wall, linking the capsule to the cell surface (After 10).

Application to Study Parasitic Protists

We will exemplify the results obtained with *Toxoplasma gondii*, *Giardia intestinalis* and *Tritrichomonas foetus*.

Toxoplasma gondii

Previous studies showed that gently scraping the surface of a cell monolayer infected with *T. gondii* exposes the inner components of the host cell, including the parasitophorous vacuoles, revealing new aspects of the intravacuolar network assembly. High-resolution SEM of dry fractured cells showed that the membranous tubules within the parasitophorous vacuole exhibited a rather uniform diameter of 30-35 nm [11]. It was suggested that this network would reinforce the attachment of the parasites to the vacuolar membrane, the residual body and each other. Using HIM, several previously unknown structures within the PV were revealed (Fig. 4) [12]. The first and most prevalent structure is the Intravacuolar Network (IVN). The amount of tubules increases with the successive division of the tachyzoite by endodyogeny. Fig. 4 show three straight tubules anchored to a short tubule close to the vacuolar membrane. The opposite ends layover but are not continuous with the parasite surface. These three tubules have about the same length and are equidistant from each other, forming a tripod. This symmetry suggests that this structure is highly stable and maintains the parasite in an appropriate position. In other instances, the IVN tubules are more curved, suggesting the application of a lower tension. It was suggested that the contrast between the straight appearances of some tubules could result from tension forces generated during the intravacuolar establishment of the parasite. In the most abundant zone of tubules, such tension may not occur, probably because more structures are supporting the junction between the parasite and the inner face of the PV. The tubules are not smooth at high magnification (250,000 times) (Fig. 4). Small "bumps" measuring 31.3 ± 6 nm is randomly scattered along the tubules (Fig. 4).

These bumps may correspond to areas of IVN branching. The IVN tubules establish a bridge between the parasite surface and the intravacuolar side of the PV membrane. HIM observations confirmed that many tubules were continuous with the parasite surface (Fig. 4), but many others only touched the surface. A new feature of the intravacuolar space, as detected with HIM, was the presence of a large amount of filamentous material interconnecting the IVN tubules to each other, the parasite and the inner surface of the vacuolar membrane (Fig. 4). These filaments were uneven, varying in thickness from 2 to 10 nm within the same filament). Some filaments may correspond to actin as described using other approaches [13].

Aside from these two filamentous structures, membranous sac-like structures, previously observed by TEM, were also seen. The dry cleave technique often exposes large areas of the intravacuolar face of the PV membrane. The inner surface in the early vacuole (containing a single parasite) appeared smooth (Fig. 4), with tiny structures 100 nm long and 60 nm wide homogeneously scattered. These structures may correspond to previously described invaginations of the PVM [14]. Openings in this membrane, irregular in size and distribution, were also present. The smallest openings were pore-like, circular and measured less than 10 nm in diameter, but other openings, which were 26 and 100-200 nm wide, were also observed.

These openings could reach 300 nm in diameter. An interpretation for these observations is that the newly formed PV would have many protrusions and very few and very small (10 nm or less) pores. However, the pores would increase progressively in number and diameter, giving rise to the observed openings and reducing the number of protrusions. These pores may be involved in the passage of nutrients from the host cell into the PV. Indeed, previous studies have shown that molecules with molecular weights in the range of 1,300-1,900 Daltons can be transferred from the host cell cytoplasm into the PV [15]. The host cell cytoplasmic side of the surface of the PV membrane In a few situations, images were obtained that displayed the surface of the PV membrane in contact with the host cell cytoplasm (Fig. 4). Organelles, probably mitochondria, are seen around the PV, as are cytoskeletal filaments. As magnification is enhanced, more details, such as endoplasmic reticulum elements networking around the PV (Fig. 4), become visible.

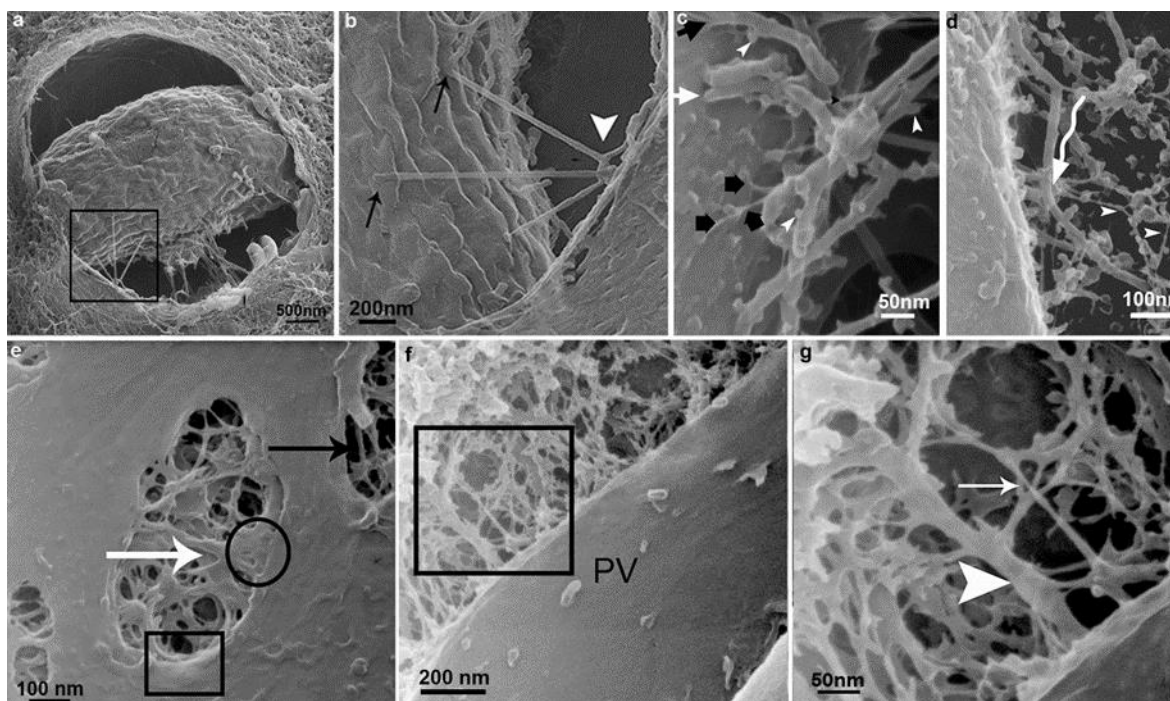


Figure 4: HIM of *Toxoplasma gondii* tachyzoites within the Parasitophorous Vacuole (PV). a parasite inside a vacuole. A: Soon after the invasion, the secretion of dense granules leads to the formation of the intravacuolar network (squares); b: Higher magnification shows three straight tubules anchored to a shorter one in the PV wall. These three tubules are set from each other, forming a tripod and the extremities are opposed but not fused with the parasite surface; c: The intravacuolar network is seen at high magnification. White arrowheads point to “bumps” that may correspond to the point of origin of a new tubule branch. The thick white arrow points to a closed tubule touching the surface of the PVM and the thick black arrow points to a tubule that appears continuous with the PVM. Small black arrowheads point to tiny filamentous structures that link the IVN tubule to the PVM inner surface; d: A sinuous arrow runs parallel to a longer IVN tubule that also curves. Filamentous extensions (arrowheads) are seen linking IVN tubules; e,f: Views by HIM of the cytoplasm around the parasitophorous vacuole. Filaments of variable caliber associated to other filamentous structures are seen around the PV. A higher magnification of the area marked by square elements that probably belong to the endoplasmic reticulum is seen close to the PV (white arrowhead). The white arrow points to what probably is a filament of the host cell cytoskeleton (After 12).

Giardia

Giardia intestinalis is a zoonotic enteroparasite that inhabits extracellularly in the small intestine of its hosts. This protozoan holds significant medical importance as the causative agent of giardiasis, one of the most prevalent intestinal parasitic diseases [16]. Its biological life cycle involves trophozoite and cystic forms, where these phases are respectively responsible for the clinical disease and its transmission. *G. intestinalis* trophozoite is binucleate and measures approximately 12-15µm long, presenting a wider anterior portion and a conical caudal region. The protozoan cytoskeleton comprises microtubules and filaments that play a significant structural and functional role within the cell. In the flagellated parasite *G. intestinalis* context, the cytoskeleton organizes into complex and elaborated structures crucial for maintaining the biological cycle. Among these structures are the ventral disc, the four pairs of flagella, the funis and the median body [17]. These elements are directly associated with important processes such as trophozoite motility and host intestine attachment, which are fundamental for the parasite's infection mechanisms. The ventral disc is the characteristic structure of the trophozoite and occupies nearly the entire ventral side of the cell. This structure is not observed in other eukaryotes and is responsible, at least in part, for the parasite's adhesion to host cells [17,18]. The cytoskeleton of the ventral disc is formed by a spiral layer of microtubules arranged in a clockwise direction. The microribbons, fibrous structures with a trilaminar arrangement, interact with the microtubules perpendicularly and along their entire length. Small elements known as cross-bridges connect the microribbons [17]. The parasite has eight flagella organized into four pairs and named according to their position: anterior, postero-lateral, ventral and caudal. All the flagella consist of microtubules arranged in the typical pattern of eukaryotic axonemes and originate from basal bodies between two nuclei in the middle region of the cell. The axonemes retain a significant cytosolic region before emerging as active flagella. Some structures

considered unique to *Giardia* are associated with the axonemes of each pair of flagella. Among them, the marginal plate and the dense rods, both related to the axonemes of the anterior flagella; the electron-dense material associated with the axonemes of the postero-lateral flagella; and the microtubules of the funis that wrap around and extend from the axonemes of the caudal flagella [19,20]. Another cytoskeleton element is the median body, which consists of a set of microtubules organized in layers [21]. Its function is still unknown, but it is believed to act as a pre-polymerized tubulin reserve for disc assembly or as a microtubule organizing center [21].

Ion microscopy has potential applications for studying these cytoskeletal elements and other cellular structures. Nevertheless, it is crucial to note that conventional sample preparation techniques designed for surface cell observation cannot reveal insights into the internal organization of cells. An alternative approach to enhance the applicability of ion microscopy in studying cytoskeletal elements involves using detergents to remove the plasma membrane. This procedure exposes the internal structures, allowing the characterization of filaments, intracellular elements and their connections, as previously demonstrated (Fig. 5) [22]. Ion microscopy's distinctive features render it a fascinating tool for cytoskeleton observation. This quality is particularly advantageous for studying the cytoskeleton, which extends to practically all cell regions. These characteristics make ion microscopy a high- resolution technique and a promising tool for analyzing biological surfaces and filamentous structures, as demonstrated in previous applications [23,24]. Using a previously established protocol for plasma membrane removal and employing Helium Ion Microscopy (HIM), Gadelha et al., obtained new insights into the organization of the ventral disc of *G. intestinalis* [24]. The authors showed that the elements of the disc (microtubules, microribbons and cross-bridges) could have a different arrangement depending on the region of the disc analyzed. These distinctive configurations highlighted the disc as a non-uniformly organized structure, delineating specific regions as the margin and the ventral groove.

Additionally, it was observed that the microtubules of the disc originated from a region delimited by the banded collar and in the perinuclear region contrasting with early observations [25]. Using HIM, the authors provided detailed images of the banded collars and their associations with basal bodies (Fig. 5). They reported the presence of two banded collars in the parasite, repeated on both sides of the cell. Each banded collar was associated with a set of microtubules: the disc microtubules emerged from the region surrounded by the left banded collar. In contrast, supernumerary microtubules originated from the region surrounding the right banded collar. Integrating these two microtubules sets with the banded collar resulted in a goblet-shaped structure [24]. HIM revealed cytoplasmic filaments interacting with cytoskeletal structures, such as the median body. These filaments had 9.4 nm in diameter and were identified as actin filaments. Ring-like configurations could also be seen. This network was observed continuously, with filaments extending towards the periphery of the trophozoite. The three-dimensional distribution of these elements had yet to be visualized previously. Unraveling *Giardia*'s cytoskeletal organization enhances our comprehension of the parasite's pathogenesis and provides potential opportunities for targeted interventions.

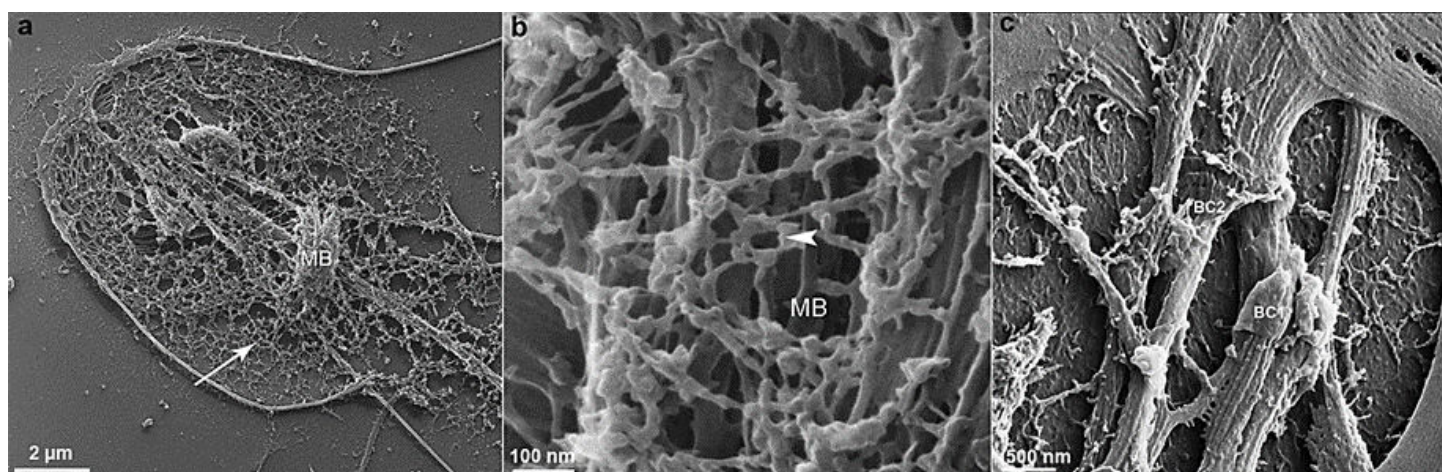


Figure 5: HIM images of the *G. intestinalis* trophozoite. (a): Actin-like filaments (arrow) spread out along the parasite dorsal region; (b): High magnification of filaments contacting other cytoskeletal elements as the Median Body (MB). The ring-like conformation is seen (arrowhead); (c): The “bare area” of the parasite is observed. Some basal bodies are associated with Banded Collars 1 (BC1) and 2 (BC2). (After 24).

Trichomonads

Trichomonas foetus is a cattle parasite that provokes trichomonosis, an important sexually transmitted disease. It has a worldwide distribution and causes veterinary and economic losses. The infection occurs in cows, cats, pigs and other domestic animals. In cows, trichomonosis can induce vaginitis, infertility, early embryonic death, or abortion. The parasite presents three anterior flagella and one recurrent flagellum. It adheres to the vaginal epithelial host cell inducing cell death. The parasite cell surface is smooth when seen in routine SEM. The mode of infection and cell contact are important elements for better understanding the parasite's behavior. Therefore, it is important to know the structural organization of this microorganism surface in more detail. Thus, Scanning Electron Microscopy (SEM) provides good information on the cell surface before and during the infection process. Using Helium Ion Microscopy (HIM), which is based on ions rather than electron beams, allowed the obtainment of high-quality parasite images in a sub-nanometer resolution. It provided important details that could not be detected before with other types of equipment. It was the first time using a scanning microscope that rounded depressions on the anterior flagella surface were seen, but not of the recurrent flagella (Fig. 6). When the size and localization were compared with structures named rosettes visualized by freeze- fracture, we consider that they may be the same [25]. Afterward, using quick freezing and deep-etching, it was reported that the rosettes protruded to the flagellar surface [26]. This publication showed the high resolution provided by the HIM. Another important observation was the region where the recurrent flagellum attaches to the parasite cell body.

By HIM, it was possible to notice that the emergence of nanotubes occurs in this region (Fig. 6). It exhibits several pits and ribbon-like arrays of particles along the length of the recurrent flagellum. The images confirmed previous freeze-fracture images where ribbon-arrays of particles were reported. It highlights the special protein arrangements, thus indicating that HIM can produce images that surpass what is obtained with transmission electron microscopy. In addition, HIM revealed thin membrane protrusions measuring between 60 and 80 nm in thickness. In addition, thin nanotubes measuring about 27 nm were seen with this equipment. Several surface projections, bulbous and tubular thin protrusions measuring 73 and 100 nm, respectively, appeared [27]. Thus, high-resolution scanning helium ion microscopy provides new morphology, allowing important advances in the knowledge of the structure of the parasite *T. foetus*.

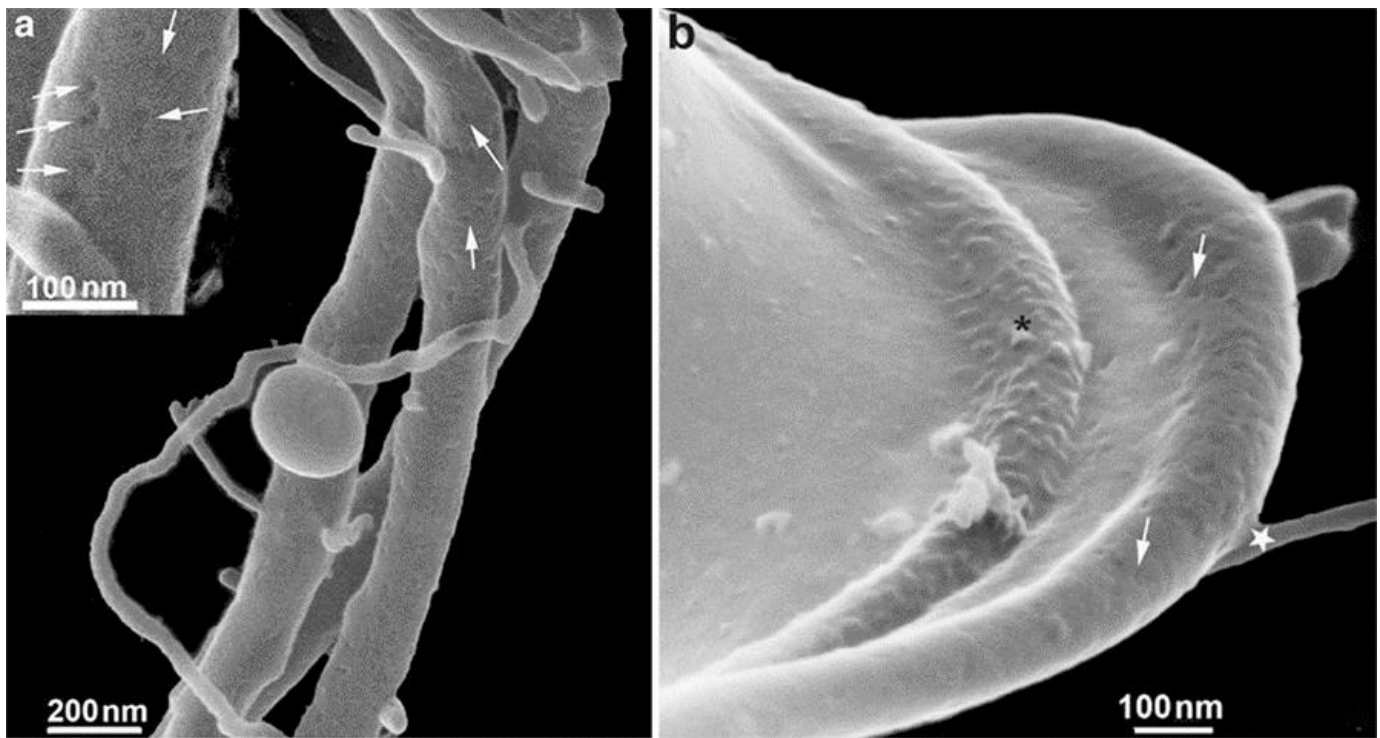


Figure 6: HIM of *T. foetus* with filamentous structures (a): of different sizes in the length and thickness of about 60 nm. Several pits are seen (arrows), which may correspond to the rosettes, better seen in the inset; (b): Region of adhesion of the recurrent flagellum forming the undulating membrane with a decoration (asterisk). Note that several pits are also observed (arrows). The star indicates a thin nanotube projecting from the undulating membrane (After 27).

Conclusion and Perspectives

It is clear from the few examples selected in this brief review that HIM is very important for analyzing the cell surface of intact cells and the inner portions of cells whose cytoplasm are exposed. One great advance would be to incorporate a cryo attachment to the equipment to allow the observation of fracture samples. Unfortunately, with the decision to suspend the production of the microscope, new advances will require the work of a few laboratories harboring the equipment and the capacity to maintain it and add new attachments.

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Conflict of Interest

The authors have no conflict of interest to declare.

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