The Parasites Caught In-Action: Imaging at the Host–Parasite Interface

Mohammed A. Afifi[1,2]

[1]Department of Medical Microbiology and Parasitology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia, [2]Department of Medical Parasitology, Faculty of Medicine, Beni-Suef University, Beni-Suef, Egypt

Abstract

For many decades, scientists were unable to expose the invisible existence of the parasites in their living hosts, except by scarification and then dissection of the animal model. This process just demonstrates a dead parasite in a dead host. Using this approach, very limited information can be obtained concerning the dynamics of infection and the pathways utilized by the parasite to survive within a hostile host's environment. Introduction of ultra-high-speed imaging techniques, with a time domain of barely few microseconds or even less, has revolutionized the "*in vivo* dissection" of the parasites. Such methods provide platforms for imaging host–parasite interactions at diverse scales, down to the molecular level. These have complementary advantages and relative assets in investigating host–parasite interactions. Therefore, better elucidation of such interaction may require the usage of more than one approach. Precise *in vivo* quantification, of the parasite load within the host, and better insight into the kinetics of infection are the two main advantages of the novel imaging procedures. However, imaging parasite–host interplay is still a challenging approach due to many constraints related to the parasite biology, the tissue environment within which the parasites exist, and the logistic technical limitations. This review was planned to assist better understanding of how much the new imaging techniques impacted the recent advances in parasite biology, especially the immunobiology of protozoan parasites.

Keywords: Biofluorescence, bioluminescence, imaging, parasites, toxoplasma

INTRODUCTION

A sound recognition and interpretation of the dynamics of host–parasite interplay is crucial for the development of novel therapeutics and vaccines. *In vivo* dynamics of parasitic infection have remained mysterious. Snapshot images of fixed tissues have proven to be inadequate to make such mechanisms more comprehensible. There is a compelling requirement for novel imaging methodologies to visualize living parasites within living organs and tissues. Imaging parasites in-action is a complicated task and a challenging demand. The parasite–host interplay classically takes place within highly structured, thick, and tightly packed tissue environmental conditions that have constrained the precise discern of such events. Optical microscopy has emerged as one of the most crucial research tools in biomedicine nowadays, and innovated imaging has been of imperative significance. It allowed scientists to look across diverse scales, from full model imaging down to nanoscales. Novel approaches made it possible to look at cell–cell or protein–protein interactions and to visualize molecular events.[1] The widespread availability of novel imaging systems, as well as the establishment of genetically encoded fluorescent peptides, has revolutionized the *in vivo* dissection of the parasite–host relationship. These innovations have facilitated deep tissue imaging with exceptional resolution and great target specificity. Researchers are now able to achieve real-time monitoring and measurement, of the dynamics of interactions at the host–parasite interface.

Imaging procedures either grant a two-dimensional (2D) description for a specific tissue or a 3D reconstruction model for an organ or a tissue. Novel methods use special probes or modalities to trace definite molecular processes using invasive

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Address for correspondence: Prof. Mohammed A. Afifi,
Department of Medical Microbiology and Parasitology, Faculty of Medicine,
King Abdulaziz University, P.O. Box. 80205, Jeddah 21589, Saudi Arabia.
E-mail: mohafifi36@yahoo.com

or noninvasive approaches of visualization.[2] These approaches have complementary advantages and relative merits in studying host–parasite interactions. Therefore, usage of more than one method or a simultaneous application of a combination of methods is not an uncommon requirement.[3]

Bioluminescence Imaging

A molecular imaging approach utilizes luciferase-based gene reporters to perform an *in vivo* noninvasive imaging in transgenic animals. The light emitted by the enzyme-catalyzed reactions can be applied in visualization of the specific target at cellular and molecular levels. Monitoring at such levels allows reporting the biodynamics and following the fate of cells with high sensitivity. Transgenic, luciferase-expressing cells or animals can be precisely imaged using highly sensitive charge-coupled device cameras, on applying the specific substrate (luciferin).[4] Cellular and molecular processes, such as cell proliferation, differentiation, migration, as well as gene expression and protein–protein interactions, were successfully visualized by bioluminescence.[5] A major disadvantage is the crucial necessity of adequate substrate. This is particularly challenging for long-term monitoring of *in vivo* dynamics, where continuous supply of substrate would be necessary. Moreover, homogenous substrate distribution depends on the route of administration which could prevent the precise localization of pathogens.[6]

The dynamics of eukaryotic infections by protozoan parasites such as *Plasmodium*, *Toxoplasma*, *Leishmania*, and *Trypanosoma* have all been observed using bioluminescence imaging (BLI). Sequestration patterns of the schizont stage can be analyzed within 1–2 days after infection by studying *Plasmodium berghei*, in a rodent model, using firefly luciferase (FLuc) under schizont-specific promoter ama1.[7] *In vivo* BLI has also proved superior, compared to quantitative reverse-transcription polymerase chain reaction, for real-time monitoring of the kinetics of pre-erythrocytic malaria in a murine model infected with *Plasmodium yoelii*. The same study reported a positive evaluation of the *in vivo* BLI screening of antimalarial efficacy.[8]

Real-time monitoring of the kinetics of *Toxoplasma gondii* infection in a mouse model revealed a direct relationship between emitted photon levels and the parasite burden, allowing the quantification of the parasite load *in vivo*.[9] Another noninvasive BLI study was able to demonstrate toxoplasma dissemination in a murine model. The competency of BLI for successful monitoring of toxoplasma reactivation as well as the efficacy of anti-toxoplasma chemotherapy was also displayed.[10]

The development of a double-transfected murine model of visceral leishmaniasis, with avirulent strain of *Leishmania donovani*, expressing two reporter genes – bioluminescent (FLuc) and fluorescent (E2-crimson) – enabled an *in vivo* real-time monitoring of the infection dynamics in visceral leishmaniasis. It also facilitated the quantification of the parasite load

in each organ. This bioluminescence model could allow for long-lasting follow-up studies, with easy and precise day-by-day visualization of the infection status.[11]

Biofluorescence Imaging

This is an imaging technology that depends on utilizing fluorescentlabeled peptides or dyes for precise visualization of pathogen's niches inside the host as well as the dynamics of pathogen–host interplay at cellular and molecular levels.

Typical fluorescence imaging relies on a linear interaction between light and matter where if a single photon of light reacts with a fluorescent molecule; it will emit only one fluorescent photon. However, multiphoton imaging, especially the two-photon excitation microscopy, which depends on nonlinear interactions and complex quantum mechanical effects, is the technique of choice for biofluorescence imaging.[12]

Two-Photon Microscopy and Analytical Dynamics of Parasite–Host Interplay

Little was known about the maneuvers used by parasites to combat or evade the ongoing mechanisms adopted by the host to clear such intruders. The emergence of genetically-encoded fluorescent-labeled reporters as well as the development of the two-photon microscopy has made it feasible to monitor the kinetics of infection within living tissues.[13] Multiphoton microscopy has provided the chance to accomplish a long sought-after goal, real-time imaging of the dynamic interplay between the parasite and host–cell. Multiphoton microscopy utilizes low-energy photons in short pulses to perform imaging at previously unreachable depths within tissue with reduced light scattering and minimal photobleaching.[14] It is possible to lively trace the detailed events of an immune reaction and closely monitor the kinetics of specific cells (e.g., T-cells), while interacting with parasites in a living tissue environment. This approach has been principally redefined our concepts toward the exact roles and functions of the immune cells against invading pathogens mainly through enhancing the examination of immune reactions to representative antigens in lymph nodes.[15,16]

Two-Photon Microscopy and Intracellular Parasites

Intracellular protozoans are causative agents for significantly serious parasitic diseases. Millions are infected annually with substantial morbidity, mortality, and economic loss.[17] Some of these protozoans, such as *Plasmodium*, *Leishmania*, and *Toxoplasma*, typically initiate a chronic intracellular existence justifying their abilities in manipulating host immune defenses and hijacking its metabolic machinery for survival, proliferation, and transmission. The intracellular niche also confers a shield from host's immunity.[18] While an evident heterogeneity of pathogenetic pathways is employed by such parasites, a clear consensus exists regarding the signaling

hubs orchestrated by the parasites. Therefore, dissection of the events of host–parasite interplay at all levels from system to molecule is crucial to differentiate between potentially fatal from uncomplicated infections.

Some of the unique advantages of two-photon microscopy make it a suitable approach to study the dynamics of parasitic infections, particularly those caused by intracellular protozoans. First, two-photon microscopy allows better and real-time visualization of fluorescent-labeled parasites and immune cells, as they interact. Second, two-photon microscopy has improved the *in vivo* visualization of parasite–host interplay in complex tissue environments.[19] Finally, two-photon approaches are constantly advancing and allow live-imaging parasite dynamics in thick tissues that have never accessed before. These unique advantages facilitated the study of cell signaling, real-time cytokine production, distinguishing between invasion and phagocytosis, identifying cells manipulated by intracellular parasites, and tracking the kinetics of infection and the dissemination of the parasite through photo-convert and track invaded cells.[20-22]

Considering the multiplicity of infectious behaviors of different intracellular protozoans, this section focuses only on the mechanisms utilized by *T. gondii* to manipulate and hijack host's cellular and molecular machinery. It also demonstrates how live-imaging by two-photon microscopy has advanced our understanding of such mechanisms.

Two-Photon Microscopy and Space/Time Characterization of *Toxoplasma gondii* Infection

A complex relationship does exist between *T. gondii* and its human host. Infection with *T. gondii* has two distinct phases, regarding the developmental parasitic stage as well as the niches of parasite existence. In the acute phase, infection starts in the intestine; however, *Toxoplasma* tachyzoites soon use the host immune cells as migratory vehicles to spread through the body and cross several unique anatomical and cellular barriers such as the blood–brain barrier, blood–retina barrier, and mother–fetus placental interface. The replication of parasites, at these sites, results in the most serious clinical presentations which are encephalitis, chorioretinitis, and congenital toxoplasmosis, respectively.[23,24] Because of a robust immune response, tachyzoites transform into the slow-replicating bradyzoites that are packed in tissue cysts that characterize the chronic phase and predominantly exist in the nervous tissues as well as muscles. Throughout such stage, T-cells and interferon-γ are crucial to maintain chronicity and inhibit recrudescence, leading to persistence of infection for the lifetime of the host.[25]

No therapeutics are readily available to kill the cyst, the hallmark of chronic toxoplasmosis. This is mainly because of the deficient monitoring of the host–parasite interplay and the progression events of toxoplasma infection. Tracing the host–parasite interactions throughout acute as well as chronic phases of the disease may result in developing sound approaches for future therapeutics.[26] The events that take place throughout such dynamic host–toxoplasma interactions are complicated and difficult to monitor *in vivo*. A comprehensive recognition of these events requires an efficient tracking, within a milieu of multiplex tissue environments, of toxoplasma, its secretory and excretory products, as well as incriminated immune cells. This can be significantly achieved by direct imaging of toxoplasma and immune cells within an *in vivo* model.[27]

Two-Photon Imaging and Toxoplasma Dynamics in the Intestine

Apart from congenital transmission, toxoplasmosis in humans is mostly acquired through the oral route. Ingestion of the sporulated stage of the oocyst passing in cat's feces or the tissue cyst in undercooked meat of the many animal intermediate hosts is the main route of infection.[24] Therefore, intestine is usually the first station in the long journey of *T. gondii* in the human body. After oral infection, toxoplasma starts to replicate within intestinal epithelium and then initiates a rapid dissemination throughout the body. The exact kinetics of this intestinal phase and the triggering of an immune reaction were poorly understood. Visualization of the events, during acute toxoplasma infection, has achieved better understanding of the essence of a protective immune response against toxoplasma during the intestinal phase. Two-photon imaging was able to elucidate the kinetics of spread and colonization of toxoplasma tachyzoites, in the intestines of orally infected murine model.[28] Very early during infection, *T. gondii* parasites scatter among the villi with a frequent pattern of distribution of one villous containing one parasite. Few days later, foci of infection have developed showing clusters of villi packed with parasites that tend to settle toward the tips of the villi.[28,29] Another interesting point cleared by live-imaging is the phenomenon of hasty relocation of toxoplasma-infected dendritic cells (DCs) far from the intestine. *T. gondii* infects intestinal CD11c+ DCs within 3 days after infection and is thought to take these cells as vehicles to translocate from the intestine to the lymph nodes.[30] Live-imaging 6–8 days after oral toxoplasma infection of CD11c-YFP reporter mice has demonstrated that heavily infected villi became dramatically devoid of CD11c-YFP+ cells, in contrast to the neighboring uninfected villi that retained a dense existence of CD11c-YFP+ cells.[31]

Two-Photon Imaging and *Toxoplasma gondii* in Lymph Nodes: A Captured Sequence of Events

Toxoplasma parasites reach the mesenteric lymph nodes either transported via DCs coming directly from the intestine or reach freely through lymph. Therefore, they infect CD169+ macrophages in the subcapsular sinus and initiate a strong immune response few days after oral infection.[32] Other immune cells incriminated in such reactions are the neutrophils. One of the early live-imaging reports regarding experimental toxoplasmosis demonstrated a fast neutrophil

recruitment to the lymph node in reaction to toxoplasma infection.[33] The *in vivo* multiphoton microscopy studies[33,34] revealed characteristic transient and persistent dynamic swarms of neutrophils, coinciding with macrophage clearance from the subcapsular spaces. The "swarms" are signaled by toxoplasma exit from cells and/or invited migration of some "pioneer" neutrophils.[33] As mentioned earlier, DC another innate immunity cell type is essential to develop resistance to acute toxoplasma infection.[28,35] DCs are well-recognized providers of interleukin-12 in early toxoplasma infection, initiating defensive T-cell responses very early during infection.[36] Multiphoton imaging demonstrates that toxoplasma-infected DCs are hypermotile which facilitate dissemination of the parasite.[37] Early after infection (first 48 h), the antigen-primed T-cells and DCs were involved in an intimate cross-talk,[32,34] within the lymph node. An active handover of toxoplasma was observed, from parasitized DCs to primed T-cells.

The characteristic "neutrophil swarms" caught by multiphoton microscopy coincide with lymph node tissue remodeling, especially the CD169+ macrophage layer. This results in "gaps" that permitted other immune cells such as natural killer (NK) cells to have access.[33] The lymph node remodeling predominantly took place in the subcapsular region and results in toxoplasmatriggered accumulation of NK cells in the remodeled regions.[38] Twophoton imaging showed a striking proliferation of collagen fibers in toxoplasmainfected lymph nodes.[34] This proliferation allows better interaction between collagen and NK cells and results in better activation of such immune cells with subsequent control of infection.[31]

Two-Photon Imaging and *Toxoplasma gondii* in the Brain: A Partially Revealed Layout

On reaching the brain, *T. gondii* invades microglia and astrocytes, before transforming into cysts mainly within neurons. A continuing adaptive immune response critically orchestrated by CD8 T-cells.[39,40] Immune responses to toxoplasma are tailored to control the chronic phase of toxoplasma infection in the brain as well as to limit the damage to host tissues. This balance is exceptionally crucial in the brain, which is a vital, delicate tissue that exhibits a limited power of regeneration.[41] Therefore, immune cell migration to the brain is largely controlled, and the actions of the recruited ones are strictly checked. Multiphoton technology revealed unprecedented details of toxoplasma and brain cell behaviors during chronic infection. It has offered significant insights into the kinetics of the interplay between toxoplasma and brain immune cells, explaining how this balance is sustained.

Two-photon microscopy-based study showed that antigen-primed CD8+ T-cells migrated to the brain tissues of chronically-infected murine models and remained there as long as antigen existed.[42] Another study revealed clustering and arrest of T-cell populations near infected brain cells as well as remodeling and upregulation of the brain fibrous network, like what is seen in the lymph nodes. It was also noted that CD8 T-cells migrated to areas of toxoplasma replication along this fibrous network. The same study, further, demonstrated the already recognized phenomenon of astrocyte activation during chronic toxoplasmosis,[43] including astrocytic swelling.[44] Two-photon technology has demonstrated a novel viewpoint of CD8 reactions to chronic toxoplasma brain infection. It has revealed an exceptional mechanism of Antigen (Ag) recognition where T-cells overlook Ag-bearing brain cysts, while adopting transient contacts with antigen-presenting cells (APCs) and granuloma-like foci may close to areas of toxoplasma dissemination. This manner of interplay most probably indicates immune evasion by toxoplasma. It could also reflect the ability of the local brain environment to restrict Ag presentation and modulate immune reaction. This mode is close to that done by CD8 T-cells where they act as source of cytokines to APCs rather than commit direct killing of target cells.[42]

Thus, we can deduce that dynamic multiphoton imaging has allowed unprecedented insight into the dynamics of toxoplasma infection, explaining (i) how specific immune responses, against the parasite, are dynamically orchestrated in different local tissue environments, (ii) how defensive reactions to toxoplasma are elicited and controlled, and (iii) how the parasite evades such immune mechanisms to survive inside the host.[31]

The fundamental advantages of two-photon microscopy are better 3D and deep tissue imaging with reduced phototoxicity and photobleaching. However, it does not achieve an enhancement of spatial resolution with a high possibility of photodamage, especially for visible flurophores. Therefore, weighing the advantages and limitations, in each situation, is crucial to determine when the two-photon microscope is appropriate to use.[45]

Confocal Imaging

This is an optical technology that is particularly useful for 3D imaging. It provides an image sectioning potential to obtain 3D imaging data from specimens with diverse depths by differentiating between in-focus signals from the disturbing background flaw.[46] Confocal microscopy assembles light from a diffraction-limited focused spot (<1 μm in thickness) in the specimen. A pinhole is assigned to track the path and cast-off the out-of-focus light to enhance resolution and contrast. The light source, the detection pinholes, and the in-focus specimen spot are collectively called "con-focal."[2]

Numerous models of confocal microscopy are accessible. The one that is commonly used is laser-scanning confocal microscope, which seizes images through specimen scanning with a compact laser beam, then assembling the emitted signals using a photodetector.[47]

Confocal microscopy is usually applied in imaging the interaction of microorganisms within their hosts,[48] including microbial adhesion and invasion,[49] intracellular motility,[50]

and immune response to infection.[51] It was used for direct imaging the motility of living malaria parasites within the salivary glands of infected mosquito vectors.[52] A high-speed laser spinning disk confocal imaging system allowed an *in vivo* 4D imaging of malaria parasite vector–host transmission.[53,54] Imaging showed motile sporozoites gliding away from the bite site and targeting capillary blood vessels. It then showed sporozoite penetration of capillary endothelium as well as its entrance to the capillary lumen to flow in the blood stream, presumably heading toward the liver.[55]

Confocal microscopy was also used to measure glutathione (GSH), a key player in the redox mechanisms in malaria parasites. Parasite's GSH combats the oxidative stress that faces the parasite once internalized within the host red blood cells. Reactive oxygen and nitrogen species are also produced by the host's immune system, as a product of hemoglobin digestion, and by antimalarial drugs.[56] GSH is usually measured with conventional methods, such as reverse-phase high-performance liquid chromatography or glutathione reductase-dependent reactions, which cannot achieve a dynamic thiol measurement.[57] Real-time imaging of the potential of glutathione redox in the obligatory intracellular *Plasmodium falciparum* was performed using a glutathione biosensor consisting of human glutaredoxin-1 linked to a green fluorescent peptide. Confocal microscopy was able to demonstrate the rapid changes in GSH levels as a reaction to the host oxidative and nitrosative stress. This technique was also able to detect thiol changes induced by oxidative actions of some antimalarial drugs.[58]

Confocal microscopy, however, has its limitations. While it allows the capture of high-resolution 3D images for cells in cultures or thin specimens, its capability to image thick tissues is limited. Confocal technology necessitates a point-by-point scan in the lateral and axial directions to facilitate 3D reconstructive images of the assigned tissue. In addition, axial scanning in confocal is slower than scanning in lateral directions.[46] There is also a poor confocal imaging on focusing deeper in tissues due to rapid decay of intensity and contrast as the number of emitted photons, arising from the scanned sample, decays exponentially with the increasing depth. Confocal technique is particularly vulnerable to photon scattering. The dispersed photons not only unable to gather in an assigned focus, but they also contribute in diffuse background and consequently photobleaching. Thus, confocal microscopy has a major flaw that renders it impractical in samples of <100-μm thickness where only 1% of the emitted fluorescence contributes to confocal imaging.[2] Therefore, good resolution is practically confined to depths of 60–80 μm.[59] One of the inherent drawbacks of confocal microscopy is that it needs ultraviolet (UV) excitation. Whenever UV emission is needed to capture specific signals from live specimens, we must trade out imaging resolution with sample integrity. Photons emitted from UV excitation exhibit excessive energy, and therefore, cell damage and/or photobleaching are expected on using high intensity.[12]

Conclusion

Innovative microscopic approaches, together with the emergence of smart genetically-encoded bioluminescent and/or biofluorescent probes, have consistently improved our tools for real-time monitoring of pathogens and immune cells. Our understanding of the host–parasite interaction has transformed from just descriptive 2D information to precise 3D and 4D *in vivo* checking of such interactions down to the cellular as well as the molecular levels. This has significantly improved our understanding of how the host–parasite dialog proceeds in complex tissue environments. We described herein examples of advances in microscopy that contributed to upgrade the concepts related to host–parasite interactions of some intracellular parasites. We are confident that with the widespread usage of such modern techniques as well as the introduction of the next-generation imaging approaches, such as light sheet fluorescence microscopy, label-free 3D imaging by holotomography microscopy, and superresolution microscopy, specifically single-molecule localization microscopy will lead to update the fundamental knowledge on the immune responses as well as the pathophysiology of parasitic infections. This might create new platforms for drug discovery and vaccine development.

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Conflicts of interest
There are no conflicts of interest.

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