To IMAGE or to IMAGINE: visualization of parasite migration as a means to support (malaria) parasite vaccine development

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Imaging as a (pre)clinical tool in parasitology

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ABSTRACT

Imaging of parasites is central to diagnosis of many parasitic diseases and has thus far played an important role in the development of antiparasitic strategies. The development of novel imaging technologies has revolutionized medicine in fields other than parasitology and has also opened up new avenues for the visualization of parasites. Here we review the role imaging technology has played so far in parasitology and how it may spur further advancement. We point out possibilities to improve current microscopy-based diagnostic methods and how to extend them with radiological imaging modalities. We also highlight *in vivo* tracking of parasites as a readout for efficacy of new antiparasitic strategies and as a source of fundamental insights for rational design.

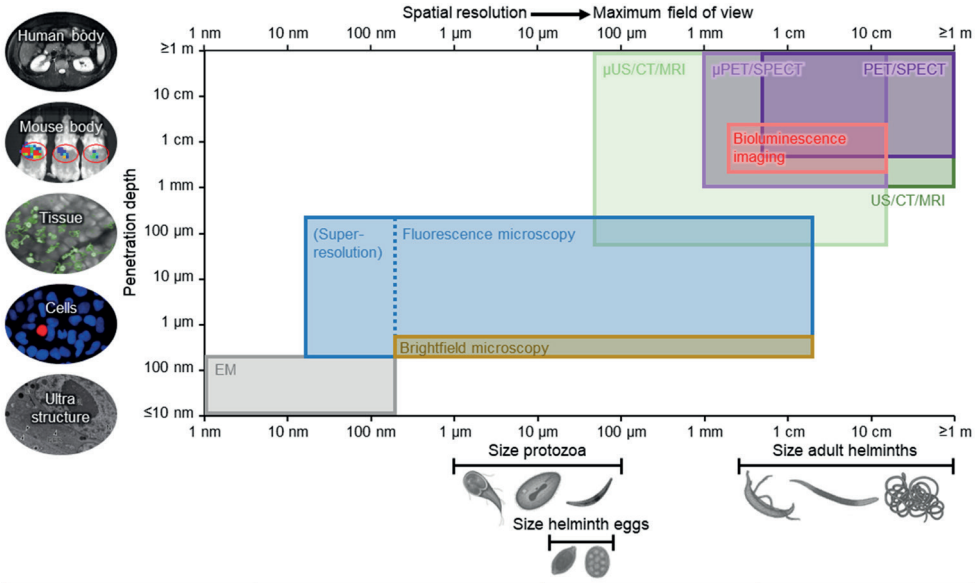
HIGHLIGHTS

- Implementation of immunohistochemistry and computer vision can improve the specificity/sensitivity of microscopy-based diagnostics for parasitic infections and decrease their labor intensity.
- For accurate diagnosis of parasitic infections, the right body sites need to be assessed. Non-invasive radiological imaging techniques provide a means to assess body sites that are not (easily) accessible for specimen collection.
- Because of the relatively high prevalence of parasitic infections in low-resource settings, innovation of diagnostics includes reducing the costs of the hardware needed.
- Quantitative imaging of parasite behavior provides a functional readout of antiparasitic strategies and has enhanced the throughput of drug screens.
- Real-time tracking of parasites in the host can provide fundamental insights needed for the rational design of antiparasitic strategies.

INTRODUCTION

Preventive measures such as clean water, sanitation and vector control have reduced morbidity due to parasitic diseases substantially, but they have not led to eradication. Because R&D investments into novel tools to help control parasite disease and transmission have suffered from neglect, only a relatively small number of drugs and no vaccines are available to confront a plethora of parasites. Also, the development of diagnostic tools for parasitic diseases has received little attention. Consequently, bright-field microscopy has been (and still is) the cornerstone for diagnosis of parasitic diseases for decades[1]. In other biomedical fields, such as bacteriology and oncology, imaging technologies have been instrumental in advancing diagnostics and spurring development of novel drugs and vaccines[2, 3]. Also in the field of parasitology, novel imaging tools have the potential to greatly enhance the sensitivity and ease with which parasites can be detected and particularly imaging-based tracking of parasites can be instrumental to identify potential targets for novel interventions.

Because of the scale of many parasites (1-100 μm diameter for protozoa and helminthic eggs to a length of millimeters or even meters for helminthic adult worms), these microorganisms are highly amenable to imaging (Figure 1). Recent advances in microscopy, clinical imaging modalities, (fluorescent/luciferase) gene tagging and computer vision have increased 1) the detectability of live parasites in host tissue (both *in vivo* and *ex vivo)* by generating contrast between the parasites and their environment using endogenous/exogenous labeling approaches, and 2) the detection capacity of imaging modalities, which have led to a higher sensitivity and spatial/temporal resolution[4]. In this review we will discuss how (novel) imaging technologies have helped and may help advance the field of parasitology and, more specifically, which role they can play in innovating diagnostic approaches (first part) and supporting the development of drugs, vaccines and other preventives (second part). The first part points out the possibilities to improve and automate the current microscopy-based diagnostic methods and how to extend them with the use of radiological imaging modalities. In the second part, we will mainly focus on imaging technologies that may enable tracking of live parasites in a 3D environment and highlight how their implementation can lead to target discovery, fundamental insights for rational design and a readout for testing the efficacy of new antiparasitic strategies.



Imaging modality	Parasite visibility	Sensitivity* (M)	Temporal resolution
Brightfield microscopy	Label-free (& exogenous labels)	N/A	ms – s
Electron microscopy	Label-free (& exogenous labels)	N/A	N/A
Fluorescence microscopy	Endogenous & exogenous labels	10^{-15} – 10^{-17}	ms – s
Bioluminescence imaging	Endogenous labels	10^{-15} – 10^{-17}	s – min
(micro-) US/CT/MRI	Label-free (& exogenous labels)	N/A (MRI: 10^{-3} – 10^{-5})	min (US: s – min)
(micro-) PET/SPECT	Exogenous labels	10^{-10} – 10^{-12}	min

*Minimum concentration of imaging agent that can be detected

Figure 1 Characteristics of molecular imaging modalities. A broad range of imaging techniques are available for biomedical applications; this graph and table summarize the most important characteristics of the different (widely applied) imaging modalities. The spatial resolution combined with the maximum field of view (x-axis) of different imaging modalities (depicted as differently colored boxes) are plotted against their penetration depth (y-axis). The spatial resolution describes how detailed the parasites can be represented in the image (ranging from ultrastructural to whole-organ resolution), the maximum field of view determines the area of the sample that can be seen (ranging from squared micrometers to a whole-body field of view) and the penetration depth defines how deeply radiation can penetrate tissue (ranging from thin tissue slides to the whole human body). For the same imaging modalities, the temporal resolution is listed in the table, which defines the amount of time needed to reacquire data for the exact same location (ranging from milliseconds to minutes). In the table, it is also annotated if labeling (endogenous or exogenous) is a prerequisite for the visualization of parasites. This determines the sensitivity of the imaging modality (detection limits range from millimolar to femtomolar concentrations of the imaging agent). Some images used were adapted from: [5, 6]. Abbreviations: CT, computed tomography; EM, electron microscopy; MRI, magnetic resonance imaging; PET, positron emission tomography; SPECT, single-photon emission computed tomography; US, ultrasound.

THE ROLE OF IMAGING TECHNOLOGY IN INNOVATING DIAGNOSTIC APPROACHES FOR PARASITIC INFECTIONS

Correct diagnosis of parasitic infections (detection of parasites and identification of their species) is crucial to provide effective treatment. Particularly in low-income settings, the use of brightfield microscopy to examine patient specimens has remained the most important diagnostic tool for parasitic disease. For this purpose, non- or minimal-invasively obtained patient specimens are mostly assessed using light microscopy to detect the presence of parasites. For example, blood is examined to detect blood protozoa and microfilariae released by parasitic worms and feces is examined to detect cysts, eggs or larvae shed by intestinal protozoa and helminths[7]. Two of the limitations of the use of bright field microscopy for diagnostics that can be (partly) addressed by (novel) imaging technologies are 1) the low sensitivity and labor-intensity of the diagnostic procedures that require highly trained microscopists and 2) the restricted number of bodily locations at which parasites can be detected due to the need for patient specimens. In this section, we discuss the potential of immunohistochemistry (IHC), automated imaging and computer-aided image analysis to improve the sensitivity and laboriousness of microscopy-based methods (first part) and the potential of affordable medical imaging modalities to assess hard-to-reach body sites (second part). Together, these approaches can revolutionize the diagnostic workflow that is schematically depicted in Figure 2.

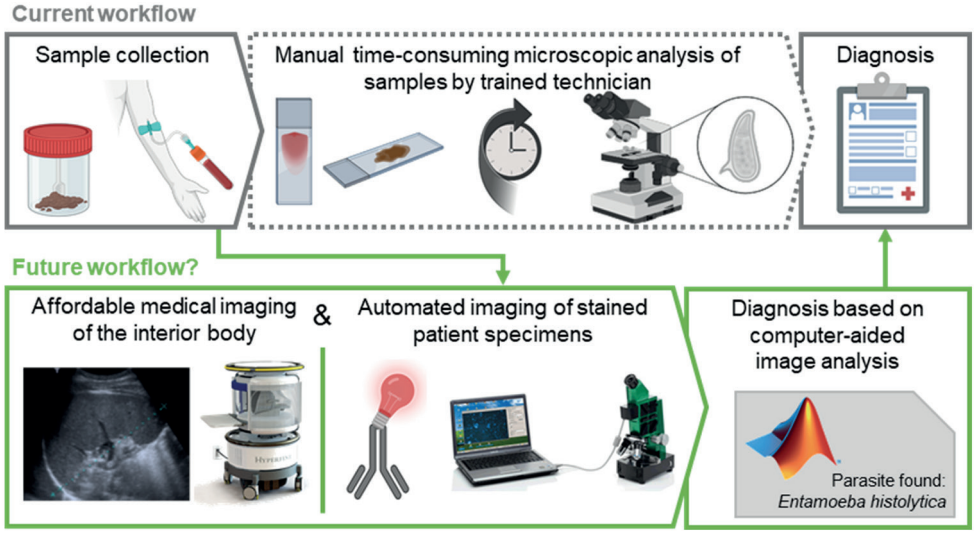


Figure 2 Innovating diagnostic approaches for parasitic infections. The traditional workflow for parasitic diagnostic methods (grey) and the potential for imaging-based technologies to accelerate this workflow (green). For the creation of this figure content form BioRender.com has been used and the ultrasound scan and Cyscope set-up have been reprinted from: [8, 9].

Low hanging fruits: innovation of microscopical methods for assessment of patient specimens

Low levels of parasites can easily be missed when microscopically analyzing patient specimens, either by overlooking parasites that are present in the sample or by accidentally examining a part of the sample that does not contain parasites. For example, for malaria diagnosis, the WHO recommends to screen 100 oil immersion visual fields before calling a thick blood smear negative, which in practice will yield a detection limit of 10–50 parasites/µl[10]. To assess the prevalence and infection intensity of intestinal helminths (e.g. the genera *Schistosoma, Ascaris, Necator*) the WHO recommends the use of Kato-Katz. This method is based on the microscopical analysis of the presence of eggs in a standard quantity of feces (most commonly used: 41 mg), which limits sensitivity of this tool to >20 eggs per gram feces[11]. To increase this sensitivity, different concentration methods have been developed and implemented. For example, the FLOTAC apparatus has been designed, based on the concept of centrifugal flotation of a fecal sample suspension and allows for quantitative copromicroscopic analysis[12]. To enable faster and more sensitive examination of patient specimens, and food samples in the case of screening for foodborne parasites[13], histological stains and to a lesser extent fluorescent dyes that enhance contrast between the parasites and the rest of the specimen can be used (Box 1). Enhancing contrast between parasites and the rest of the specimen aids the detection of parasites, since it improves the sensitivity by enabling the faster screening of higher volumes of sample. The advancement of enhancing contrast can be further exploited by the implementation of parasite-specific stains.

In addition, specific staining such as IHC can be used to identify parasites. IHC relies on antibodies that bind to a specific antigen, which results in highly specific staining methods that allow for identification and speciation of microorganisms beyond morphology seen with simple light microscopy. For example, IHC on patient specimens is routinely used in certain laboratories to diagnose *Toxoplasma gondii*. However, in general, remarkably, IHC barely plays a role in parasite diagnostics, whereas it has dramatically transformed the approach to histopathologic diagnosis in oncology[17]. Recently, several reports were published using IHC in parasitology. For example, Saïdi *et al*., amongst others, have reported the development of an immunofluorescent method suitable to stain *Leishmania* parasites in dermal scrapings, which improved the sensitivity of microscopy-based assessment of patient specimens[18]. In addition, Reinehr *et al.* re-examined metacestode material, which was archived as formalin-fixed, paraffin-embedded specimens in a biobank, for echinococcosis using IHC; their results show that IHC can contribute to diagnosing echinococcosis with greater certainty and can robustly discriminate between cystic and alveolar echinococcosis[19]. Beside its potential, IHC has also limitations since, despite the use of specific antibodies, nonspecific staining or cross-reactivity can occur which limit its specificity. Overall, development of

immunohistochemical methods for parasites provides a relatively easy and cheap way to increase the sensitivity and specificity of microscopy-based diagnostics of parasitic infections and can also aid the identification of parasite species needed to decide the right treatment.

Box 1. Current staining methods for patient specimens

The most commonly used histological stains for parasites[7]:

- Mixtures of polychromed methylene blue and eosin (e.g. Giemsa staining) for microfilariae and protozoa in blood.
- Iodine which makes several parasitic worms appear brown while the rest of the stool specimen remains clear.
- Red acid-fast stains whereby coccidian species stand out clearly against a blue or green background.

Examples of successful implementation of fluorescent stains:

- Fluorescent acid-fast stains (e.g. auramine-rhodamine) have improved the diagnostic procedures for coccidian species by further increasing contrast between the parasites and their surroundings, which led to a higher sensitivity and a shorter observation time needed[14].
- The fluorescent nucleic acid binding dye acridine orange enables rapid identification of the nucleated blood parasites within a blood sample. Based on this fluorescent staining, the quantitative buffy coat test has been developed, which allows for more sensitive and rapid diagnosis compared with the traditional fix-and-stain techniques[15, 16].

Another simple method to improve the diagnostic workflow is to bypass the laborious process of preparing microscope slides of patient specimens to manually screening and interpreting these slides, which relies on the experience of trained technicians. Automated microscopy imaging and image analysis have the potential to reduce hands-on time, can standardize image interpretation (omitting the need for highly trained personnel) and can reduce the number of false negatives by screening larger samples. Current advances in the field of oncology exemplify what can be achieved when it comes to automation of histopathologic diagnosis; automated whole-slide imaging combined with computer-aided diagnosis are becoming the core of modern histopathology[20]. These concepts also have potential to innovate parasite diagnostics. For example, recent studies have shown that images of feces can automatically be generated and subsequently analyzed with learning software good enough for accurate diagnosis of multiple intestinal helminths with high accuracy[21, 22]. Other examples are the automated analyses of trichrome stained slides

for the presence of protozoa in feces (both cyst and trophozoites) and the automated classification of stained malaria parasites in blood smears[23, 24]. These examples also suggest that implementation of more parasite-specific staining methods, as described in the previous paragraph, would increase the applicability of automated image interpretation. In the future, automated detection and classification can potentially be extended with artificial intelligence-based reasoning to provide a treatment proposal based on the findings.

To enable implementation of new diagnostic methods in low-income countries, tools need to be affordable. Thus, innovation of imaging-based diagnostic methods for parasitic infections also means reducing the costs of equipment such as microscopes. In this respect, two promising lines of research are the development of LED microscopy that offers the benefits of fluorescence microscopy without the associated costs and the development of smartphone-based imaging devices. For example, the portable LED microscope CyScope has achieved a sensitivity and specificity of up to 90% regarding the detection of malaria parasites in blood[25, 26]. Moreover, although presently moderate, the accuracy of smartphone-based imaging devices is constantly improving and has, for example, been used to quantify *Loa* microfilariae in blood and *Schistosoma haematobium* eggs in urine[27, 28]. Such technical innovations at the hardware site may boost the accessibility of imaging devices in low-income countries.

The next level: molecular imaging technology for the detection of parasites inside the body

With brightfield microscopy as the most important diagnostic tool, parasites are commonly detected in specimens that can be easily and non- or minimal-invasively obtained, such as feces, blood and sputum. However, parasites migrate through the human body (Figure 3), which means that diagnosis of parasitic disease at any organ level can be time-sensitive. Thus, for accurate diagnosis of parasitic infections, it is important to assess both enough and the right body sites. This is exemplified by the fact that only recently two other reservoirs in the host for trypanosomes besides the blood were discovered, namely the interstitial fluid of adipose tissue and skin[29, 30]. These reservoirs were even identified in individuals without trypanosomes in their blood, highlighting the limitations of only blood slides to diagnose trypanosomiasis[31]. A step in the right direction, is the recent translation of confocal laser scanning microscopy in combination with standard cystoscopy to the clinic. This technique permits non-invasive cell imaging *in vivo* in order to obtain images with a microscopic resolution. As an example, this technique enabled the non-invasive visualization of *Schistosoma* eggs in the urothelium of patients with urinary schistosomiasis without the need of a patient specimen[32].

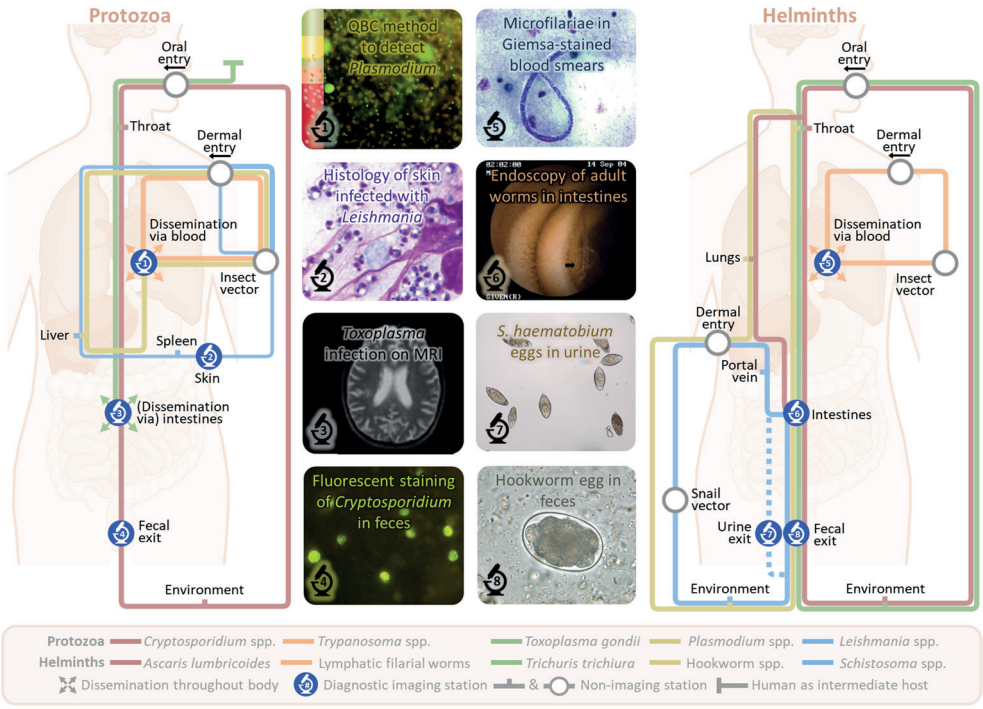


Figure 3 The role of imaging technology in diagnostics. The life cycles of the ten most clinically relevant protozoa and helminths are schematically depicted (five protozoa species in the left figure panel and five helminth species in the right figure panel), showing the different 'stations' these parasites pass while migrating through the human body. The 'stations' at which parasites can be detected for diagnosing an infection are annotated. Examples of images that can be obtained at the different diagnostic 'stations' are shown, which were kindly provided by Eric Brienen (1, 2, 4, 5, 7, 8) or adapted from: [33, 34]. Abbreviations: MRI, magnetic resonance imaging; QBC, quantitative buffy coat.

For detection of parasites at different body sites (e.g. brain, lungs, liver and intestines) without the need to obtain diagnostic material through puncture or biopsy, non-invasive radiological imaging techniques can provide a solution. Radiological imaging modalities (ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI)) are macroscopic, noninvasive imaging modalities that allow the visualization of tissue morphology deep inside the body. Over the past years, the soft tissue contrast of US, CT and MRI has improved enough to allow for visualization of large adult worms. However, in the field of parasitology, these medical imaging modalities are mainly used to visualize how a parasitic infection has altered pathology. Especially in cystic diseases, such as neurocysticercosis caused by the *Taenia* tapeworm and cerebral toxoplasmosis caused by the protozoa *Toxoplasma gondii*, radiological imaging techniques are already applied to aid identification of disease stage (e.g. active/inactive disease) and thus guide the choice of treatment[9]. More recent advances are morphological MRI imaging to visualize and quantify liver tissue alterations

resulting from an infection with *Schistosoma* parasites in a rodent model[35]. It is, however, important to note that high costs are associated with most of these radiological imaging procedures which results in low availability of equipment in low-income countries with a high prevalence of parasitic infections (Uganda: <0.5 MRI and CT units per million people versus the Netherlands: >10 MRI and CT units per million people[36]). Nevertheless, US especially has become more and more accessible and affordable (price drop >10x, hand-held scanners available) and different studies have shown its potential to diagnose a broad range of parasitic infections by detecting the parasites or the tissue alterations they induce[37].

A second category of imaging modalities that allows the detection of cells inside the body are nuclear imaging modalities (positron emission tomography (PET) and single-photon emission computed tomography (SPECT)) which are based on the principle of detecting gamma rays emitted from exogenous radiotracers present in target tissue. The advantage of using gamma rays is that they easily penetrate tissue allowing the non-invasive visualization of signal coming from deep within a patient, which have proven to be of great value in diagnosing cancer and subsequently monitoring the effect of treatment[38]. The success of these approaches fully relies on the ability to target cells with an exogenous radiotracer. While radiotracers are increasingly developed and used for specific types of cancer, there are no radiotracers available that target parasites yet. As a proof of concept, the nonspecific metabolic tracer 2-deoxy-2[^{18}F]fluoro-D-glucose (FDG) has once been successfully used to visualize the helminth burden in mice[39]. Although these techniques have shown great potential for diagnostic innovation in other fields, the benefits of increased sensitivity might not outweigh the high costs (development of an imaging agent is estimated to cost 50 to 100 million dollars and also their use within diagnostics is expensive[40]), especially not in the context of low-income countries.

THE ROLE OF IMAGING TECHNOLOGY IN DEVELOPING DRUGS, VACCINES AND PREVENTIVE MEASURES

A characteristic of parasites is that they migrate throughout their host and transition from one life cycle stage to the other. Recent advances in imaging technology have enabled real-time tracking of parasites, both *in vitro* and *in vivo*; individual parasites can be tracked using microscopy and the total parasite load can be monitored using bioluminescence-based imaging. These technologies are the result of developments in 1) the generation of transgenic parasite lines that express reporter proteins which has largely increased the detectability of parasites in tissue[41], 2) advanced microscopes with increased imaging resolution (e.g. development of laser scanning confocal), imaging speed (e.g. development of spinning disk confocal) and signal penetration depth (e.g. development of multiphoton laser excitation) which have improved their capacity to detect parasites[4] and 3) the availability of software tools for spatio-temporal cell tracking which are capable of automatically identifying

parasites in images and following them over time[42, 43]. In this section the application of these technical advances is discussed. Tracking of parasites during their journey through the body helps to unravel mechanisms that can serve as potential targets for drugs, vaccines and preventive measures, as the feature of migration is essential to the survival of the parasite (first part). In addition, during development of new drugs, vaccines and preventive measures, monitoring parasite migratory behavior can serve as a readout for testing their efficacy (second part).

Imaging of parasite migration to generate fundamental insights needed for rational design of antiparasitic strategies

Historically, it was through imaging technology (bright field microscopy) that parasite transmission routes were discovered, which provided the basis for many of the preventive measures that are available nowadays (Box 2). Whilst these initial studies were instrumental to uncovering fundamental preventive measures, more advanced imaging tools were subsequently applied to understand the fundamental mechanisms that play a role when parasites enter the human host. These imaging studies can provide insights needed for the rational design of new antiparasitic strategies (such as preventive measures, vaccines, drugs).

Box 2. From microscopical findings to preventive measures

Bright field microscopy has played a pivotal role in the discovery of parasite transmission from one host to the other, shifting the initial paradigm that parasites were capable of 'spontaneous generation'.

- The oral route of parasite transmission was unraveled by amongst other things feeding humans with larvae of the *Taenia* tapeworm or eggs of the *Enterobius* roundworm whereafter the adult worms could be visualized respectively in the intestines and the feces. Friedrich Küchenmeister published in 1855 his findings that *Taenia* larvae fed to a man under death sentence (currently considered unethical) had transformed into *Taenia* worms within the human intestinal tract and in 1865, Rudolf Leuckart and some of his students swallowed *Enterobius* eggs and found *Enterobius* worms in their feces around 2 weeks later[44].
- Microscopical analysis of skin previously exposed to hookworm larvae, revealed that hookworm larvae were able to penetrate intact skin and uncovered the transdermal route of parasite transmission. In 1898 Arthur Looss noted reddening of his skin and a burning sensation after a drop of water containing a high number of *Ancylostoma* larvae had fallen on his hand. He scraped of the last moisture residue from the epidermis and found numerous empty worm skins and just a few larvae. He was the first to report dermal penetration of any

worm[44].

- The role of vectors in parasite transmission was discovered using microscopy, starting with the discovery, made by Patrick Manson in 1877, that filarial parasites metamorphosed in the mosquito' abdomen. Subsequently the uptake and development of several parasite species in arthropod vectors was visualized (e.g. *Babesia* parasites in ticks, *Plasmodium* parasites in mosquitoes, *Trypanosoma* parasites in tsetse flies and blood-sucking bugs and *Leishmania* parasites in sand flies)[45].

The knowledge gained by detecting parasite transmission has formed the basis for many of the preventive measures that are available nowadays (Figure I). For example:

- Safe water, sanitation and hygiene (WASH) activities to prevent the release of parasitic eggs into the environment[46].
- Promoting awareness of how to handle potentially infected food to prevent food-borne parasite transmission[47].
- Increasing access to footwear to prevent soil-borne parasite transmission[48].
- Using insect repellants and nets to reduce vector exposure[49].

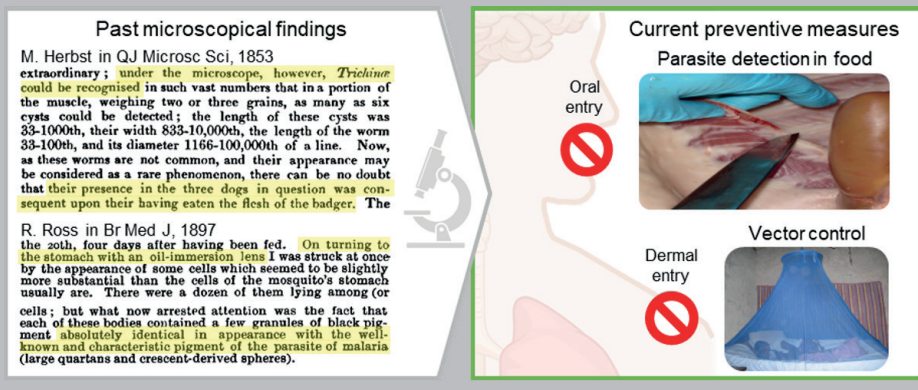


Figure I Past microscopical findings have formed the basis for the current preventive measures.

Because multiple parasites enter the human body via the skin, which can be easily imaged, thus far many *in vivo* imaging studies that involved real-time tracking of parasites have focused on the skin stage of parasitic infections (Figure 3, 4). These studies have uncovered important invasion mechanisms. For example, it was initially assumed that most *Plasmodium* parasites were directly inoculated into the capillaries present in the skin, while imaging studies revealed that significant numbers of them reside in avascular tissue[50-52]. Also, *Leishmania* parasites were found to be phagocytosed by immune cells infiltrating the bite site because of the bite of the vector[53-55]. Microscopic tracking of helminths that use the

transdermal route to enter their host, revealed that infective larvae can respond to stimuli like chemical cues, water turbulence and temperature to increase their chances for successful host skin penetration[56-58]. These studies have also enabled assessment of time taken to invade and investigation of invasion which uncovered the process by which schistosomes lose their tail and surface glycocalyx and release excretory/secretory molecules (which are potent stimulants of innate immune cells) as they enter the skin [59, 60]. Together, these imaging studies focusing on the host entrance of parasites have revealed that the skin is an important anatomical reservoir and immune sentinel site for these parasitic infections, which are valuable insights for vaccine design.

Next to the skin, tracking of individual parasites in other organs has also led to an increased understanding of organ-specific parasitism and development. For example, intravital imaging of the liver revealed that *Plasmodium* sporozoites use several pathways to reach hepatocytes, rather than only passing through the Kupffer cells as the earlier accepted model proposed [61]. Also, *Trypanosoma* parasites have been found inside the brain parenchyma within hours after entering the bloodstream, which was assumed only to happen at the final stages of the disease[62, 63]. For schistosomes, imaging revealed that schistosomula migration in the lungs and liver is restricted to the vascular system, solving the question of whether or not extravascular routes exist and that eggs lodge in the venule part of the intestinal vasculature (and dependent on the species, also the urogenital vasculature) and can subsequently pass through the vessel wall[64]. The mechanisms behind the processes described in this paragraph serve as a potential target for drugs and vaccines[65], because blocking these essential mechanisms may prevent the establishment of a full-blown infection.

Despite successes in imaging of parasites in specific organs, real-time imaging of parasites during their dissemination throughout the human body has not been performed yet, due to technical challenges (except recently in the transparent zebrafish model[66]). However, bioluminescence imaging of the total parasite load in animal models such as mice has aided our understanding of parasite dissemination (Figure 4AB). For example, bioluminescence-based imaging of the total parasite load has played a key role in understanding the differential spread of parasites in cutaneous leishmaniasis versus visceral leishmaniasis[67, 68]. Another example of insight into parasite dissemination is the testis tropism of *Trypanosoma brucei* parasites, which might be of importance considering the role of the blood-testis barrier in determining drug efficacy[69]. More importantly, the presence of parasitized red blood cells in the brain of mice was identified as crucial for the onset of experimental cerebral malaria, the most severe complication of infection with *Plasmodium* parasites[70]. Together, these imaging studies have brought parasite migration into the limelight as a potential target for new drugs and vaccines. For *Plasmodium falciparum* malaria, these findings were

translated to potential novel therapeutics with the visualization of a direct inhibitory effect of circulating antibodies targeting parasite motility[71].

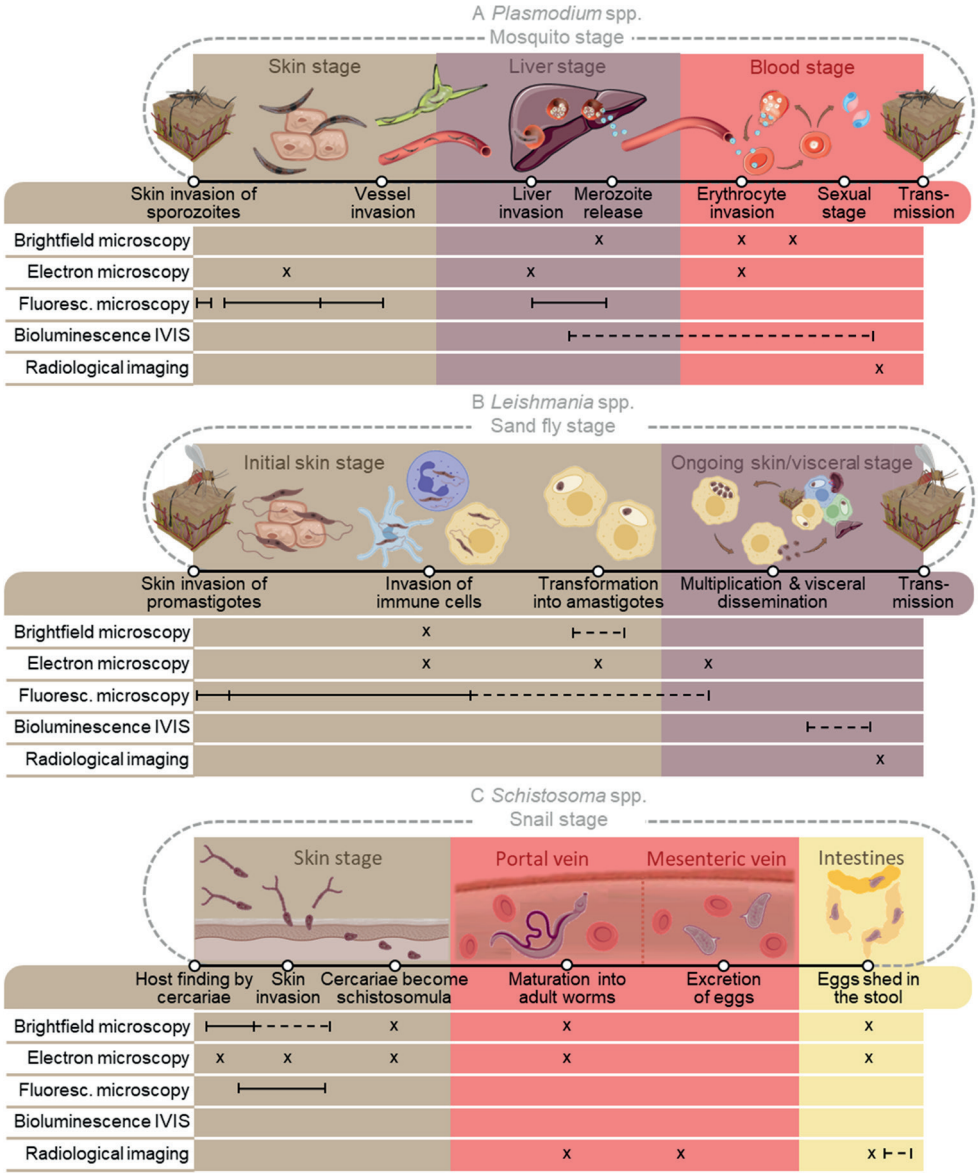


Figure 4 Imaging of parasitic life cycles. The life cycles of *Plasmodium* spp. (A), *Leishmania* spp. (B) and *Schistosoma* spp. (C) are schematically depicted (top). Imaging modalities used to visualize parasites are indicated (left) and their particular role within visualizing the parasite life cycle is highlighted for a single timepoint (x), for recording a time-lapse (- -) or for real-time tracking (—). For the creation of this figure content form BioRender.com has been used.

The majority of *in vivo* imaging studies regarding parasites make use of transgenic protozoa lines that express fluorophores or luciferase[72]. Unfortunately, the difficulties with transfecting multicellular organisms to obtain stable reporter lines have slowed down the progress made in intravital imaging of helminths. The first transgenic helminths expressing reporter proteins have been documented; however, stable transgenic lines suitable for *in vivo* tracking are not available yet[73, 74]). Thus, most of the recent *in vivo* imaging studies regarding helminths make use of larvae that are labeled *ex vivo* with fluorescent dyes and subsequently detected in specific organs of their (intermediate) host[60, 75, 76]. To study their dissemination through the host the development of radiotracers could provide a solution. Although not applied in the field of parasitology yet, tracking of low numbers of cells migrating throughout the body is achieved in other research fields using the sensitivity, quantifiability and tissue penetration capacity of radioactivity[77, 78]. A suitable radiolabeling method would facilitate *in vivo* study of migration and development of helminths (and protozoa) without the need for transgenic lines.

Collectively, different imaging modalities have been applied to track protozoa such as *Plasmodium* and *Leishmania* species (Figure 4AB) and a few helminths such as schistosomes (Figure 4C) during their journey through the body. These studies can provide insights needed for the development of new drugs, vaccines and preventive measures. Unfortunately, there are only a few imaging studies investigating other parasite species beyond those listed. The progress made so far demonstrates the potential of what can be achieved for other parasite species in the future.

Imaging of parasite migration to test the efficacy of potential antiparasitic strategies

Over the past years, imaging-based approaches have played an increasingly important role in the screening of novel antiparasitic compounds. For example, imaging-based models that enable monitoring of schistosome skin invasion have been used to test compounds potentially having schistosome cercariae anti-penetrant properties (repellent, barrier or cercaricide); for example DEET formulations, dimethicone-based barrier creams and topical applied chemicals such as niclosamide[79]. Several of these compounds have now shown to prevent schistosomiasis in clinical (field) trials[80]. Similarly, transmission of vector-borne protozoa such as *Plasmodium* and *Leishmania* parasites has been visualized in rodent models using confocal imaging of transgenic parasites expressing a fluorophore. *Plasmodium* sporozoites have been followed from the mosquito salivary cavities, through the salivary ducts, into the skin, pointing out that motility is crucial within the vector and thereby a potential target for transmission-blocking compounds[81]. To assess the transmission reducing activity of new antimalarial drug candidates, a standard membrane feeding assay is used; mosquitoes are fed with infected blood including the compound of interest and subsequently imaged

by brightfield/fluorescence microscopy or by a bioluminescence-based imaging system to check for transmission events[82]. Similar assays for other vector-borne parasites do not exist yet, but may have applications for parasites such as trypanosomes. Together, these imaging-based setups to study parasite transmission offer a platform that supports the discovery of transmission blocking compounds needed as part of a strategy to prevent parasitic infections (Figure 5A).

To screen compound libraries for the identification of compounds with antiparasitic properties *in vitro* imaging-based screening platforms have been developed (Figure 5B). Screening of already approved drugs, aiming at repurposing them (while avoiding expensive approval procedures) seems an especially promising strategy given the low commercial attractiveness of developing new drugs for most parasitic diseases. The effect of compounds has commonly been assessed by manual scoring of viability using brightfield microscopy[83, 84]. This has recently been replaced by automated image analysis based on video recordings of parasite motility and the uptake of fluorescent viability dyes to generate more high-throughput screening assays[85-87]. For example, using an imaging-based screening assay, the compound tolfenpyrad, which nearly completely reduced the motility of third-stage *Haemonchus contortus* larvae (ruminant helminth), was identified[88]. Several reviews have provided an overview of the compounds with anthelmintic or antiprotozoal properties identified by imaging-based screening of compound libraries[89-91].

Following *in vitro* identification of antiparasitic drug and vaccine candidates, imaging also plays a role in the *in vivo* characterization of their effect (Figure 5B). Regarding helminths, testing new drug or vaccine candidates involves microscopical assessment of the worm burden after sacrificing the animal. For instance, this approach was used to validate the *in vitro* anti-schistosomal activity of hydroxyquinoline derivatives in a mouse model and to evaluate recombinant hookworm antigens as vaccine candidates in a hamster model[95, 96]. In contrast, the availability of transgenic reporter lines of protozoa has facilitated the *in vivo* monitoring of the temporal dynamics of the effect of, for example anti-trypanosomal compounds and anti-leishmanial compounds[92, 97-99]. Moreover, bioluminescence-based monitoring in mice has been applied to assess the protective efficacy of immunization with genetically attenuated *Plasmodium* parasites with complete late liver stage arrest, which are currently in clinical trial[100, 101]. Taken together, imaging technology can play an indispensable role in the preclinical phase of the development of drugs, vaccines and preventive measures.

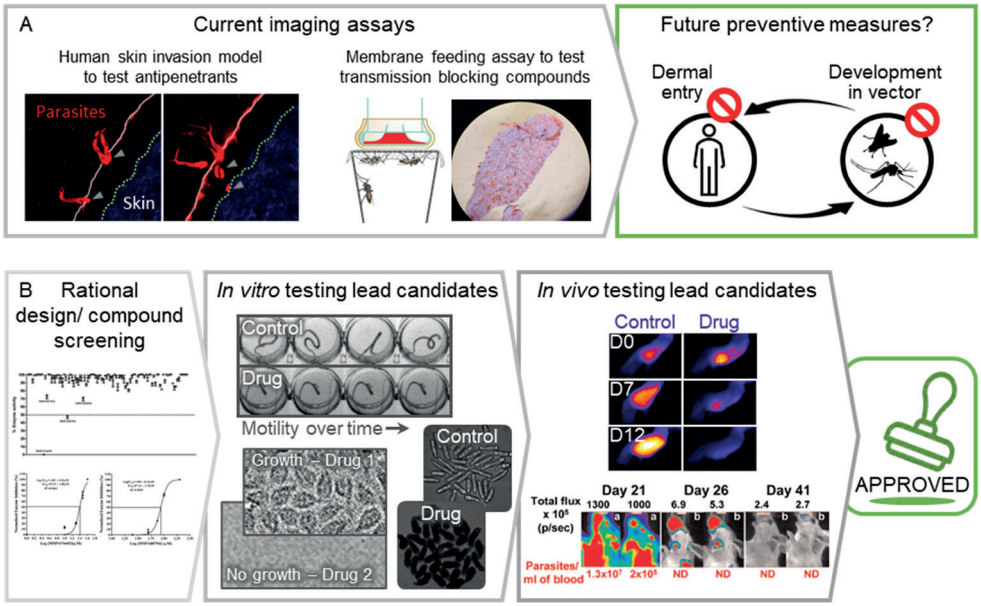


Figure 5 Preclinical testing the efficacy of antiparasitic strategies. A) The currently available imaging assays (grey panel) to study parasite transmission and the effect of potential transmission blockers aimed at the development of new preventive measures (green panel). B) A workflow diagram summarizing the drug/vaccine discovery process. After rational design or compound screening, the second and third panel give examples of how imaging technologies can be used for the *in vitro* and *in vivo* testing of lead compounds, respectively. The images used as examples were adapted from: [39, 85, 92-94].

CONCLUDING REMARKS

Implementation of IHC and automated image acquisition/interpretation are potential approaches to improve microscopy-based parasitological diagnostics. We envisage that further implementation can be boosted by leveraging the experience available in other biomedical fields, while keeping in mind the importance of cost reduction. When innovation in divergent areas (imaging hardware, staining methods, computer vision etc.) occurs in alignment, it may be mutually reinforcing and lead towards the widespread availability of improved affordable diagnostic approaches.

A growing number of *in vitro* and *in vivo* screening approaches relying on imaging of parasites has become available, suitable for identifying antiparasitic compounds and validating the efficacy of potential drug/vaccine candidates. We expect that *in vivo* tracking of parasites can also point towards targets for rational design of antiparasitic strategies by unraveling mechanisms essential for parasite survival that can be translated into suitable targets. The development of radiotracers for *in vivo* tracking of parasites might even pave the way

towards obtaining such insights in controlled human infection studies (see Outstanding Questions).

OUTSTANDING QUESTIONS

- The low-cost widespread availability of brightfield microscopy often outweighs the application of novel molecular (imaging) tools in low- and middle-income countries. Can technical innovations at the hardware site aimed at improved accessibility tip the scale?
- How can the experience available in the field of oncology regarding the use of immunohistochemistry and computer vision be leveraged for application in diagnostics for parasitic infections?
- How can innovation in divergent areas (imaging hardware, staining methods, computer vision etc.) be coordinated to assure a better linkage that makes advances in different areas mutually reinforcing?
- Which so far unknown parasite reservoirs would we encounter in the human body if we could follow individual parasites on all potential paths through the host?
- How can we ensure that fundamental insights in parasite migration obtained by imaging translate into the identification of a suitable target for antiparasitic strategies?
- Can the development of radiotracers suitable to label helminths provide a means to study their dissemination bypassing the difficulties with the generation of transgenic lines that are used so far to study the dissemination of protozoa?

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