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

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8

# Summarizing discussion and future perspectives

## INTRODUCTION

In this thesis the development and application of quantitative imaging-based assays suitable to study parasite migration have been described that aimed to support the development of antiparasitic vaccines needed to reduce the global disease burden caused by parasitic infections (Figure 1). In this final chapter key concepts from this thesis will be discussed:

1. SMOOT (Sporozoite Motility Orienting and Organizing Tool) was developed and established as a quantitative software analysis tool for tracking the migration of malaria sporozoites *in vitro* and in human skin explant. This tool provided a readout with high kinematic detail, enabling the quantitative characterization of novel factors influencing the migration capability of malaria sporozoites (**chapter 1-4**).
2. A hybrid tracer labeling approach for parasites was developed which yielded viable parasites that were both fluorescently and radioactively labeled. With this approach the *in vivo* dissemination of malaria sporozoites could be revealed and human skin invasion behavior of helminths was monitored in both a qualitative and quantitative way (**chapter 5-6**).
3. By imaging the migration of parasites, insights were gained into questions that have arisen during the development of malaria vaccines, and the broader potential for imaging technology to advance the development of new diagnostic methods, therapeutic interventions and vaccines for combating parasitic infections was discussed. (**chapter 1-7**).

In this chapter, the development of new building blocks for quantitative imaging-based assays suitable to study parasite migration will be discussed, covering the first two novel concepts. The second part of this chapter will focus on the application of quantitative imaging-based assays in exploring questions related to malaria vaccine development, covering the third concept.

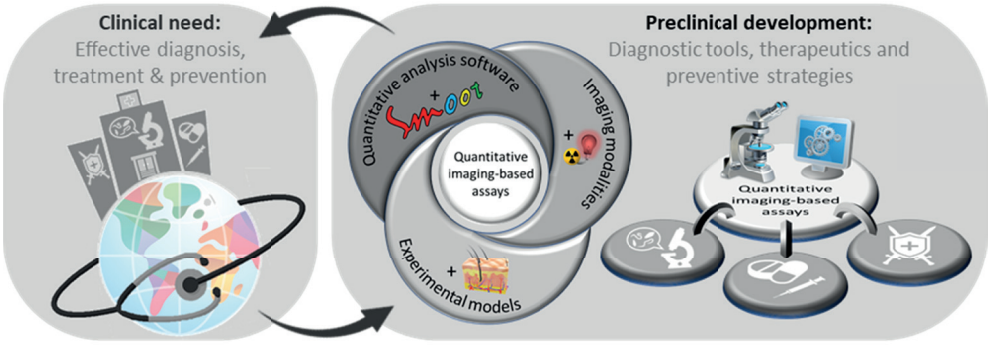


**Figure 1 Schematic representation of the interaction between clinical need and preclinical development.** The interaction is shown between the clinical need (left panel) for diagnostic methods, therapeutics and preventive measures to combat parasitic infections and the preclinical use of quantitative imaging-based assays (right panel) to help fulfil this need. The three elements of quantitative imaging-based assays are shown on the right panel: quantitative analysis software, imaging modalities and experimental models. In this thesis three building blocks were added; the software analysis tool SMOOT to quantitatively analyze parasite migration, a radiolabeling suitable for parasites and human skin explant as an experimental model.

## DEVELOPMENT OF QUANTITATIVE IMAGING-BASED ASSAYS

### Towards quantitative microscopical analysis of parasite migration

Optical microscopy is the most commonly used imaging technique for visualizing parasites and also the imaging technique most used in this thesis (**chapter 1-6**). Historically, optical microscopy has been considered a qualitative observational technique. However, advances in camera technology and imaging analysis software have increasingly enabled the extraction of quantitative data from images[1]. At the interface of software engineering and biomedical imaging, optical microscopy can be transformed into a quantitative observational technique. Our interdisciplinary environment enabled us to develop SMOOT (Sporozoite Motility Orienting and Organizing Tool), a software analysis tool for automatically tracking sporozoites by fluorescence microscopy. This tool provided a quantitative assessment of malaria sporozoite migration *in vitro* and in (human) skin tissue (**chapter 1-2**). Previous studies on sporozoite motility were mainly *in vitro* studies using qualitative or semi-quantitative readouts[2-4]. Only recently, the use of projections of time-lapse videos of moving sporozoites has been used to replace the post hoc staining of sporozoite trails[5, 6]. SMOOT increased the level of kinematic detail, revealing that a complex interplay of different (macro) molecules balances strong attachment and fast detachment needed for forward locomotion (**chapter 1**). The few studies that have assessed sporozoite migration *in vivo* (in a murine model), described sporozoite migration in terms borrowed from diffusion physics (e.g. velocity and mean squared displacement)[7, 8]. However, as sporozoites purposely migrate through tissue, we included measures of directional movement such as path tortuosity measures (e.g. straightness index and angular dispersion[9]) to describe the

movement patterns of sporozoites. This increased level of detail proved to be valuable to reveal for example the impact of radiation attenuation on sporozoite directionality (**chapter 2**).

Various aspects of sporozoite migration can be assessed independently, however together they can provide a multidimensional comprehension of sporozoite migration under different conditions. In **chapter 3**, we took the first steps in presenting a multidimensional view of sporozoite migration to describe the movement exhibited by sporozoites inoculated into the skin by mosquitoes versus sporozoites intradermally injected by needle. With the growing use of quantitative imaging assays to study sporozoite behavior, the volume and complexity of the data acquired will increase, which will in turn increase the need for comprehensive multidimensional data visualization. The recent advances in other biomedical fields (e.g. genomics and immunology) in exploring and visualizing large high-dimensional data sets may offer computational methods that can be adapted for the assessment of parasite behavior[10-12]. Taken together, quantitative microscopical imaging of parasite migration appeared at the interface of software engineering and biomedical imaging and needs an interdisciplinary environment for further development.

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**Expanding the imaging toolkit to enable visualization of *in vivo* parasite dissemination**

Over the past years, a broad range of imaging modalities have become available for biomedical applications. Their characteristics determine their suitability and purpose within parasitology[13, 14]. For instance, in this thesis, fluorescent microscopy enabled real-time monitoring of individual sporozoites with a small field of view and subcellular resolution in skin tissue (**chapter 1-5**), while bioluminescence-based imaging enabled whole-body imaging of the total parasite load at a whole-organ resolution in mice two days post injection of sporozoites (**chapter 3**, **5**). In **chapter 5** and **6**, we aimed to develop the first radiotracer for parasites. It is remarkable that radiotracers suitable for parasites have not been developed yet, given the fact that they have proven to be of great value in other biomedical fields by virtue of the sensitivity, quantifiability and tissue penetration capacity of radioactivity[15]. In **chapter 5**, we developed the tracer $^{99m}$Tc-Cy5-AmineC4.MAS$_3$-Methyl, which is suitable to radiolabel sporozoites and quantitatively assessing their biodistribution. The traditional methods to study sporozoite distribution (e.g. PCR or bioluminescence-based imaging) can be used to detect the large numbers of parasites which are obtained once the malaria

parasite has replicated in the liver. However, these methods are not sensitive enough to monitor the distribution of the relative low number of malaria sporozoites towards different organs directly after infection[16, 17]. In **chapter 6**, we applied the radiotracer to radiolabel schistosomal and hookworm larvae and quantify their human skin invasion. So far, the human skin invasion rate of schistosomal larvae has only been indirectly estimated by counting the tails of the cercariae that were left behind during invasion, and for hookworm larvae it has not been assessed at all[18]. Further optimization of the radiolabeling approach is needed to obtain parasites containing larger amounts of radioactivity, which would pave the way towards whole-body longitudinal SPECT imaging of the dissemination of parasites in live animal models and potentially also in humans in the context of controlled human infection studies. Overall, with the development of a radiolabeling approach for parasites, we have expanded the imaging toolkit available for imaging parasite migration.

Although a broad range of imaging modalities is available, parasite samples are typically examined using only one imaging modality. Nevertheless, combining different imaging modalities and correlating their results would provide a more comprehensive (both structural and functional information) and multi-scale view (with high spatial/temporal resolution as well as a large field/depth of view) on parasite behavior. The tracer that we developed and used in **chapter 5** and **6** was a radiolabeled analogue of a fluorescent tracer. It contained both a radioisotope and a fluorophore, making it suitable for bimodal imaging. Especially in **chapter 6** the bimodal imaging approach was applied to its full advantage and allowed for detailed real-time monitoring of human skin invasion behavior of individual larvae as well as quantitative assessment of the total amount of invasion. So far, different multimodal imaging approaches have been developed for biomedical applications[14, 19, 20]. For example, correlative light and electron microscopy (CLEM), which combines optical and electron microscopy, has been developed. This approach can put the functional results obtained by optical microscopy in an ultrastructural context[21]. Adapting this approach to the imaging of sporozoite migration in skin could reveal the cellular context of the migrating sporozoites, which so far has remained a 'black box' in our studies. For *in vivo* studies, the most widely used multimodal approaches are PET/CT and SPECT/CT which offer integrated functional and anatomic whole-body imaging[22]. Taken together, both adding imaging modalities to the toolkit for parasite imaging and using them in a correlated multimodal imaging approach are means to support comprehensive assessment of parasite migration *in vivo*.

Combining different imaging modalities can provide a more holistic and multiscale view of parasite behavior

**Diversifying experimental models to better approach the human situation**

We studied parasite migration in different experimental models, each of which has its own possibilities and limitations. In **chapter 2** and **4**, we have implemented the use of human skin explant to investigate the migration of malaria sporozoites in their natural human host environment. Until now, *in vivo* studies of sporozoite migration have been performed in animal, mainly mouse, models. Because of the differences between animal and human skin regarding physical and chemical properties, a human skin model is a valuable contribution to the available experimental models[23]. Comparing our data regarding the tortuosity of malaria sporozoite tracks obtained in mouse abdominal skin (**chapter 3**) and human abdominal skin (**chapter 2**), suggested that the differences between mouse and human skin induced differential movement patterns; sporozoites in human skin travelled along more straight lines compared to their counterparts in mouse skin. A human skin explant model represents part of the human environment, but since the skin is no longer connected to a living organism, sporozoites cannot leave the skin via the bloodstream as they normally would. Therefore, in **chapter 3** and **5**, we used a mouse model to be able to correlate findings from the skin and liver stage of infection and to track malaria sporozoites beyond the skin to other organs. To dissect the role of different factors which influence parasite migration inside their host separately, more simplified imaging-based *in vitro* models can be of value. In **chapter 1** and **4**, we have used an *in vitro* assay to specifically assess the effects of chemical stimuli and anti-CSP antibodies on sporozoite motility. Together, these examples illustrate how a variety of experimental models can complement each other since each of them is suited to answer different research questions.

Experimental models can complement each other since each of them is suited to answer different research questions

The lack of coherence between the results of our *in vitro* and human skin explant models in **chapter 4** raises the question to which extent the results obtained with our different experimental models are representative for the human *in vivo* situation. While anti-CSP antibodies were able to completely block the motility of malaria sporozoite *in vitro*, they could not completely stop their migration in human skin. This question about coherence is linked to a broader challenge, generally acknowledged within the biomedical field; how to develop and validate experimental pre-clinical models with a high clinical predictive value[24, 25]. There are different criteria proposed according to which an experimental model can be validated[25]. An example is predictive validity; the criterium that the effects of an intervention in the human situation and in the experimental model should be comparable. We used that criterium in **chapter 3** where we first validated that the difference in infectivity between mosquito-inoculated and intradermal syringe-injected malaria sporozoites, which

had been observed in humans[26, 27], also occurred in our mouse model. A second criterium is face validity; the closer the species used as a model is to humans, the better[28]. Besides the *ex vivo* use of human tissue as we describe in this thesis (**chapter 2**, **4**, **6**), recently, humanized mouse models have been developed for *in vivo* assessment of human malaria species[29, 30]. These models, although costly, combine the advantages of the use of an animal model and human tissue. Ideally, a preclinical assay can be validated using data from clinical studies. For example, when in addition to CIS43LS[31], more anti-CSP antibodies will be tested in future controlled human infection studies, their protective efficacy against a malaria infection can be compared to their $IC_{50}$ value obtained with our assay, to assess the predictive value of the latter. Nonetheless, despite shortcomings, experimental models have been invaluable for parasitological research. A diverse range of experimental models can help to investigate different aspects of the human situation and together approach the clinical reality as good as possible.

## APPLICATION OF QUANTITATIVE IMAGING OF PARASITE MIGRATION

### Investigating questions raised during malaria vaccine development

In **chapter 1-3**, we gained insights into three aspects of live attenuated malaria sporozoite vaccination strategies that influence their efficacy: 1) the vaccine formulation (**chapter 1**), 2) radiation-attenuation of the sporozoites (**chapter 2**) and 3) the route of administration (**chapter 3**). We observed that after intradermal administration the injected liquid spreads through the skin and fills the interstitial space (**chapter 3**), impacting the chemical properties of the environment in which the sporozoites need to migrate. Combined with the *in vitro* findings described in **chapter 1** that different (macro) molecules regulate sporozoite motility, these findings suggest that it would be worthwhile to further investigate the *in vivo* effects of formulation on sporozoite migration. Adjusting the attenuated whole sporozoite vaccine formulation, which is currently only enriched with the motility-regulatory protein albumin[32], may provide a means to support attenuated sporozoites in reaching the liver after intradermal administration. In **chapter 2**, we showed that radiation-attenuation impairs the sporozoite's capability to migrate through human skin. Ideally, an optimal balance should be struck between sufficient attenuation to ensure safety and minimal impairment to support continuation of the first part of the life cycle needed to induce protective immunity. Besides radiation-attenuated, genetically attenuated sporozoites are also under clinical investigation[33]. This type of attenuation is likely to be better suited to generate sporozoites that can safely be used while still capable to completely accomplishing the intended part

> Quantitative imaging-based assays to study parasite migration can be used to investigate questions raised during clinical trials

of their life cycle in the host. In **chapter 3**, we examined the dermal site after sporozoite administration by syringe compared to mosquito inoculation. The differences found suggest that investigating engineering solutions that better mimic mosquito inoculation with regard to the volume and distribution of the sporozoite sample (e.g. a micro needle patch[34]) may provide a means to increase the efficacy of intradermally delivered attenuated whole sporozoite vaccines. Together, these examples reveal how quantitative imaging-based assays to study parasite migration can be used to investigate questions raised during clinical testing of vaccine candidates and can offer possibilities for further optimization of vaccine strategies.

Since migration is crucial for sporozoites to continue their life cycle within the host, it not only influences the efficacy of live attenuated sporozoite vaccines, but also provides a potential target for malaria vaccines. In **chapter 4**, we showed that anti-CSP antibodies directly impact sporozoite motility both *in vitro* and in human skin. In the future, our quantitative imaging-based assay can be used as an additional tool to comprehensively assess the parasite inhibitory capacity of anti-CSP antibodies and potentially other antibodies targeting sporozoite motility. These antibodies become available in the context of clinical trials testing malaria vaccine candidates[35-37], and selecting the most efficacious ones can support optimization of subunit and passive immunization strategies.

Sporozoite migration plays a major role in the performance of the current malaria vaccine candidates

In **chapter 1-4**, the migration capacity of sporozoites during the skin stage of the infection has been used as a readout to investigate factors that (may) impact the efficacy of attenuated whole sporozoite vaccine strategies, such as the vaccine formulation and the route of administration. In the future, this readout can be expanded with the assessment of the dissemination of sporozoites through the body, an approach developed in **chapter 5**. This would provide more information on how different vaccine strategies impact the spatial and temporal dynamics of sporozoite dissemination, which in turn would give insights into the parasite-host interactions that may take place and contribute to the overall immune response. Also, in this thesis and in most other imaging-based studies on *in vivo* parasite behavior, parasites are monitored without simultaneously visualizing their host environment (in high detail). This makes it challenging to explain observed parasite migration behavior in terms of parasite-host interactions. As a step in the right direction, brightfield imaging was used in **chapter 3** to visualize the cellular environment, revealing structural differences that correlated with differences in sporozoite migration patterns. However, interactions of sporozoites with host cells, such as dermal antigen-presenting cells like macrophages and

dendritic cells, have only been studied *in vitro*[38]. Nevertheless, real-time *in vivo* imaging of the immune system is a rapidly emerging field and the first findings at the interface of *in vivo* immuno-imaging and parasitology have been reported[39, 40]. Combining real-time *in vivo* monitoring of parasites and immune cells would provide a means to deepen our understanding of where, when and how migrating (vaccine) parasites can interact with specific parts of the immune system. In conclusion, sporozoite migration throughout the human host is a key characteristic that plays a major role in the performance of current malaria vaccine candidates; both whole sporozoite vaccine candidates and subunit vaccine candidates. This underlines the importance of visualizing sporozoite migration in the context of understanding and optimizing malaria vaccine performance.

**Applying quantitative imaging of parasite migration beyond malaria vaccine development**

In most chapters of this thesis (**chapter 1-5**), quantitative imaging assays have been used to study the migratory behavior of malaria sporozoites in the context of vaccine development. However, **chapter 6** investigates the migratory behavior of schistosomal and hookworm larvae, while **chapter 7** explores the applications of imaging of parasite migration beyond vaccine development. First of all, the distribution of chapters in this thesis between malaria and other parasitic diseases reflects the imbalance in research efforts; most imaging studies have focused on malaria parasites and a few other protozoa, while other harmful parasites such as schistosomes and hookworms remain neglected[41]. The gained insights so far because of the imaging of malaria parasites should inspire for more research regarding other parasite species.

Vaccines are considered a promising strategy to combat parasitic infections, but there are other important strategies. For example, accurate diagnosis of parasitic infections followed by effective treatment is crucial to avoid morbidity and mortality. In **chapter 7** the role preclinical development of imaging technology can play in fulfilling this clinical need has been extensively reviewed. **Chapter 6** provides an example of this potential role; a readout for helminthic skin invasion was established, which is needed to assess the efficacy of potential parasite invasion blocking strategies aiming at selecting an efficacious one. Another example is the screening of already approved drugs and aiming at repurposing them (while avoiding expensive approval procedures) which is highlighted in **chapter 7** as a promising strategy for antiparasitic drug discovery. Imaging-based whole-organism screening tools have been developed to enable screening of large compound libraries to identify compounds with

> The translational value of the quantitative imaging of parasite migration goes beyond supporting parasite vaccine development

antiparasitic effects[42, 43]. The imaging-based *in vitro* assay developed and used in **chapter 1** and **4** to investigate the effects of formulation components and anti-CSP antibodies on sporozoite migration is an example of such a screening tool and can potentially be applied to identify a new malaria prophylaxis that targets sporozoite motility. Taken together, the translational value of the quantitative imaging of parasite migration can go beyond supporting parasite vaccine development.

## CONCLUDING REMARKS

Parasitic infections have a major impact on the global disease burden and molecular imaging technology can play a role in the development of strategies to reduce this burden. This thesis highlights the role that quantitative imaging of parasite migration can play in supporting the development of parasite vaccines. To fully realize the potential of imaging technology in parasitology, investment is needed 1) to translate the use of imaging technology already successfully applied in biomedical fields such as oncology and immunology to the field of parasitology and 2) to foster interdisciplinary research teams which form the ideal environment for the development of quantitative imaging-based assays with translational potential. Quantitative imaging-based research is most effective when there is room for exploration; first and foremost, imaging of parasite migration is an invitation to explore and observe uncharted territory, only thereafter quantitative data should be collected to transform observations into interpretable charts. This will help us uncover the behavior of parasites and how to combat them driven by, but not limited to, our imagination.

First and foremost, imaging of parasite migration is an invitation to explore and observe uncharted territory

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