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Dissertation booklet

New approaches to identify and follow key pathways of the malaria parasites

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Introduction

Malaria is annually responsible for ~400.000 deaths, making it one of the most prevalent diseases of our time. The causative agents of the disease are eukaryotic parasites with a very complex life cycle including both a vertebrate and a blood-feeding insect host. A particularly important phase of parasite development occurs in the blood stream of the vertebrate host, called the intraerythrocytic cycle. It starts with the invasion of red blood cells, and its followed by an asexual development of 48 hours in case of the most prominent human malaria parasite, the *Plasmodium falciparum*. The parasites develop from rings to trophozoites and finish the cycle with the schizont stage. During their development, hemoglobin molecules are important sources of nutrients, however, they cannot digest heme compounds. In order to avoid the negative effects associated with free heme, the parasites crystallize these compounds, creating hemozoin, also known as malaria pigment. The blood stage is the infection period when the symptoms occur and, therefore, when diagnosis and treatment is possible.

Malaria parasites have been shown to have a unique genome architecture, and Plasmodia are among the organisms containing the highest level of A:T base pairs in their genome compared to G:C base pairs, often reaching 75-85%. High mutation frequencies are also observable in the parasite genome, and mutations are undoubtedly associated with the resistance characteristics of parasites against antimalarials. As of now, there are no commercially available vaccines against malaria, therefore the prevention and treatment relies on antimalarial chemotherapy. Parasites, however, are capable of developing resistance against antimalarials quickly. The first “miracle drug”, chloroquine, was widely applied all throughout the second half of the 20th century. However, the fast-spreading resistance has rendered the drug unusable worldwide by the millennium. Artemisinins provided a new hope, and are the basis of the current first-line treatment guidelines. Sadly, delayed clearance-times are already well-recorded in several regions.

The better understanding of the parasite genome, its mutations and their related repair mechanisms could facilitate the fight against drug resistance and the development of new antimalarials. During the blood stage, parasites are periodically exposed to the stressful effect of the immune system, are largely dependent on the nutrients of the host, and it is also the venue of antimalarial drug treatment. The presence of drugs imposes a selective pressure on the parasites, which manifests in the occurrence of genetic mutations. However, if we take a closer look on the parasite environment and genetic composition, the parasites are likely to be prone to certain types of mutations even without the presence of drugs. The extreme A:T richness of their genome promotes polymerase slippages, and poses a high demand for thymidines. In contrast with mammals, parasites can only synthetize thymidine molecules from uridine precursors. Under certain circumstances, polymerases are able to incorporate uridine instead of thymidine into the DNA, and the requirements of *P. falciparum* inherently involves elevated uridine levels compared to organisms with a more balanced G:C-A:T ratio. Furthermore, it must be noted that the parasite DNA synthesis proceeds in an environment of high oxidative stress related to their hemoglobin metabolism. This notable level of oxidative stress promotes the oxidative deamination of cytosine, which leaves uracil moieties in the DNA. A section of my work presented in this thesis therefore focuses on the examination of the occurrence and underlying processes of a certain mutation type, namely the genomic uracil content of *P. falciparum*.

The constant monitoring of drug resistance and screening for potential new antimalarials is another key aspect of the fight against malaria. There are many *in vitro* assay methods available for such purposes, however they are generally associated with long incubation times, complex sample preparation and measurement techniques, and often require significant expertise. Efforts to provide results about drug efficacy after short incubation times are well-known, still, for many methods, 48-72 hour incubation times are routinely applied. The reduction of incubation time is not only beneficial in terms of decreasing labor and providing faster results, but especially useful in case of fast-acting drugs aiming at the early stages of parasite development. Another aspect of fast drug assessment could be the potential of personalized treatment, which would provide a better option for the patient and could also decrease the overuse of antimalarials, contributing to slow down the spread of resistance. Incubation times are often determined by the availability of parasite maturation markers. Many methods rely on DNA-intercalating dyes or markers, and certain proteins produced by the parasites. However, the DNA synthesis is only initiated during the second half of the intraerythrocytic cycle, and the amount of proteins reaching the detection limit of these methods often requires a relatively long time.

The rotating magneto-optical diagnostic method was developed by our research group to overcome some serious hindering factors of the currently existing diagnostic practices [1]. The method utilizes the unique crystals, which are the metabolic byproduct of all known Plasmodia. Its diagnostic capabilities have been and still are studied extensively [2]. However, I believe the method is also a very promising laboratory tool candidate. Therefore, a significant section of this thesis focuses on the applicability of the RMOD method for the aforementioned purposes. Since the production of hemozoin is continuous during most of the intraerythrocytic cycle, the development of the parasites is expected to be detectable even within a single cycle *in vitro*, possibly after significantly shorter incubation times.

In vivo techniques are significant contributors to the better understanding of host-parasite interactions, immunology-related factors, infection characteristic and pathogeny; and they can also facilitate vaccine development efforts. For the study of rodent infections, however, fast, highly sensitive, simple and affordable methods are needed, and I believe the RMOD method would be an ideal alternative to the currently existing and often less optimal methods.

There are four rodent-infecting Plasmodia with variable characteristics that are widely used as models of different human malaria infections. In their natural hosts, the parasites are prone to develop synchronously during the blood stage, resulting in the release of a significant amount of hemozoin crystals after every cycle. In case of *in vitro* cultures, the free crystals are continuously accumulating in the medium unless cleared out artificially. In the body of the hosts, however, the fate of hemozoin is different, since they act as triggering factors for the immune system along with the remnants of infected red blood cells. Crystals are collected by macrophages and monocytes which can carry them for a relatively long time. Finally, hemozoin is accumulating in the liver, spleen and bone marrow. Therefore, the relation of the clearance and production of hemozoin *in vivo* may have a significant impact on the magneto-optical evaluation of malaria infections. It is important to decipher the dynamics of hemozoin removal and the production rates of the parasites, since these effects determine the amount of detectable crystals in the blood

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[1] Butykai, A. *et al.* Malaria pigment crystals as magnetic micro-rotors: Key for high sensitivity diagnosis. *Sci. Rep.* **3**, 1431 (2013).

[2] Orban, A. *et al.* Evaluation of a novel magneto-optical method for the detection of malaria parasites. *PLoS One* **9**, 1–8 (2014).

stream. Given the wide range of rodent parasite traits, huge differences in these relations may be expected based on the synchronicity, achievable parasitemia levels and multiplication rates of the parasites. Another important question is how the application of treatment changes these processes, and if the RMOD method is capable of detecting them. The last section of my thesis aims to answer all these questions and complete the evaluation of the RMOD method as a tool for *in vivo* malaria studies.

In summary, three important aspects of malaria-related fundamental research are investigated in this dissertation, in hopes of offering a promising new method for the easier, faster, and simpler analysis of malaria *in vitro* and *in vivo;* and better understanding the underlying mechanisms of high mutation rates and genome metabolism of the most lethal human malaria parasite, the *P. falciparum*.

Materials and methods

Human and rodent parasites

For *in vitro* experiments, *Plasmodium falciparum* 3D7 and *P. falciparum* W2 human pathogen parasites were used. Parasites were kept under the following conditions: Erythrocytes were used with an RMPI 1640 and Albumax I based medium. Cultures were kept at 37°C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2; at 5% hematocrit. Cultures were synchronized in a continuous manner utilizing the sorbitol and Percoll methods.

For rodent experiments, non-lethal, synchronous *P. vinckei* lentum; lethal, asynchronous *Pyp230p(-)* GFP expressing *P. yoelii* 17X-NL and lethal, asynchronous *P. berghei* ANKA strains were used. As infection methods, tail vein injection of infected red blood cells; injection of sporozoites or mosquito bites were applied. All animal-related experiments were performed according to the ethical requirements of the host institutions.

Determination of parasite maturation

For the determination of parasitemia, the 'gold standard' of malaria diagnosis, aka. the optical microscopic evaluation of thin blood smears and/or flow cytometry were used. Hemozoin content and therefore, parasite maturation was followed by the RMOD method.

The detection of uracil moieties

Parasites of different developmental stages were collected from two independent cultures. Genomic DNA extraction was performed using the QuickDNA Miniprep Plus kit obtained from Zymo Research. A catalytically inactive UNG construct was used for the detection of uracil moieties and was visualized by a dot blot method as described by Róna *et al.*, providing a sensitivity matching mass spectrometry-based methods for similar purposes [34].

Drugs and drug assays

Three well-known antimalarials were utilized in 5 concentrations during the assays, namely: chloroquine, piperaquine and dihydroartemisinin. Assays were performed with cultures of various parasitemia levels for either 10 or 48 hours with frequent sampling. IC50 values were determined based on the RMOD data and compared to values of pLDH, HRP2 and radiolabeled-hypoxanthine assays.

[3] Róna, G. *et al.* Detection of uracil within DNA using a sensitive labeling method for in vitro and cellular applications. *Nucleic Acids Res.* **44**, 1–13 (2016).

Results

Examining the genomic uracil levels of *P. falciparum* during its intraerythrocytic cycle

For the determination of genomic uracil levels, I harvested parasites upon reaching either the ring, the trophozoite or the schizont stages for genomic DNA extraction. I determined the number of uracil moieties/million bases of genomic DNA based on a dot-blot method developed by our research group and published by Róna *et al.* [4]. I found the uracil content to be 9.6±2.8 uracil/million bases for ring stage parasites, 6.7±2.4 for trophozoite stages and 7.6±3.8 in case of schizonts. Wild type organisms and normal cell lines are known to generally possess levels of 0.1-1, or even less uracil/million bases, which are considered very low values.

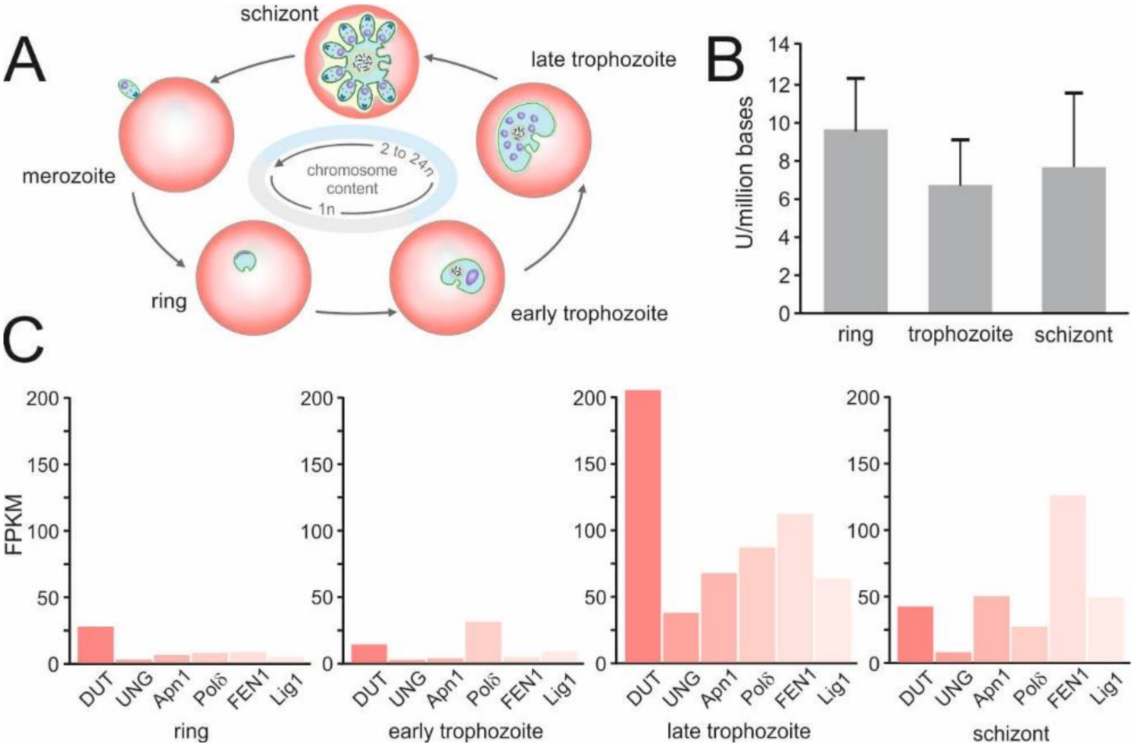

Figure 1. – Uracil-DNA and its metabolism in *P. falciparum*. (A) Changes in chromosome content in the different stages. **(B)** Genomic uracil levels of ring, trophozoite and schizont *P. falciparum* developmental stages based on our dot blot measurements. **(C)** Expression levels of the enzymes in the proposed long-patch BER pathway of *P. falciparum* and the dUTPase (DUT). Abbreviations: uracil-DNA-glycosylase (UNG); AP-endonuclease 1 (Apn1); DNA polymerase δ (Polδ); Flap endonuclease 1 (FEN1); DNA ligase 1 (Lig1). FPKM means the Transcript levels of fragments per kilobase of exon model per million mapped reads.

Besides the conditions favoring uracil incorporation presented in the Introduction section, the unsuccessful DNA repair mechanisms may also facilitate the high levels of this non-DNA-building base. I methodically examined and compared the participating proteins of the uracil-related DNA repair pathways, the long- and the short-patch base excision repair (BER) in case of mammals and *P. falciparum,* looking for a possible lack of key enzymes. I found that many enzymes associated with the short-patch pathway were not identified in the parasites, corroborated by other reports. By contrast, mammals almost exclusively rely on the short-patch BER for the removal of uracil from the DNA. Furthermore, only one of the uracil-cleaving enzymes, the uracil-DNA glycosylases was found in *P. falciparum*, while it has been shown that in a diverse set of organisms, uracil-DNA glycosylases of four enzyme families are generally present, all fulfilling specific roles.

I checked the expression levels of the identified long-patch BER enzyme orthologues during the ring, early trophozoite, late trophozoite and schizont stages. The transcriptome levels indicate that the onset of the BER pathway can probably be placed during the late trophozoite stage (Figure 1). The parasitic DNA metabolism corroborates the aforementioned theory, since the DNA replication is initiated during the late trophozoite stage starting approximately 30 hours after invasion and stretches into the early schizont period, followed by the packaging of the newly produced genetic material into new daughter merozoites by the end of the cycle.

Utilization of the RMOD as a drug efficacy testing tool *in vitro*

By the monitoring of hemozoin production of the laboratory-adapted *P. falciparum* 3D7 parasite strain in several independent experiments, we created a general hemozoin production profile. Based on my analysis, I concluded that the onset of hemozoin production occurs already during the early ring stage of the intraerythrocytic cycle, corresponding to parasite ages of 6-10 hours.

Building on the aforementioned findings, I designed and performed several assays using three antimalarials and two parasite strains. All drugs were tested utilizing the laboratory-adapted *P. falciparum* 3D7, while chloroquine was also utilized for the study of the chloroquine resistant *P. falciparum* W2 strain. The effect of piperaquine was analyzed during short-term studies with cultures of three parasitemia levels. Chloroquine was used to monitor the drug effect throughout the complete intraerythrocytic cycle at two parasitemia levels. The new line treatment, dihydroartemisinin was also applied over a 48-hour period. IC50 values of the drugs were determined at every sampling point, covering a wide range of parasite development.

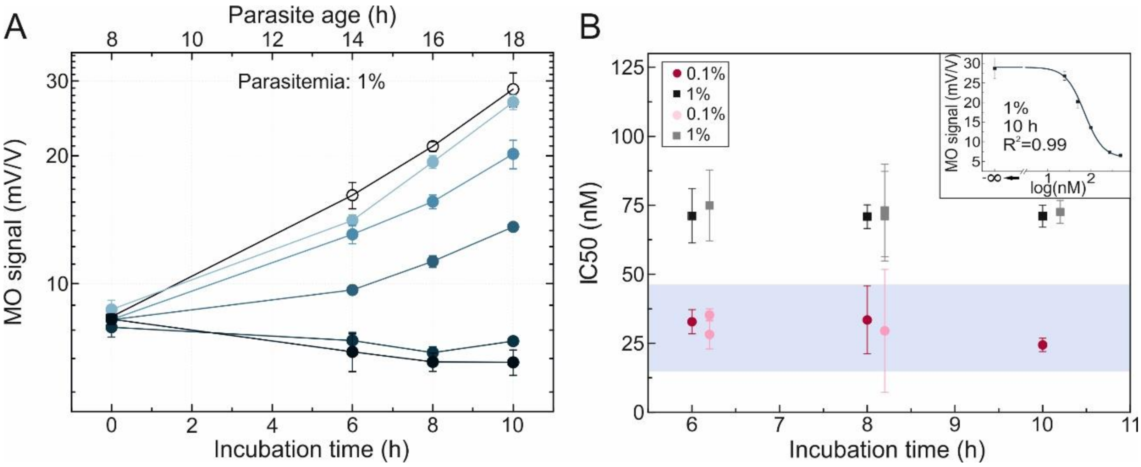

Figure 2. – Inhibitory effect of piperaqune on *P. falciparum* 3D7 cultures. Panel A: Growth curves representing MO values as a function of incubation time for the cultures with 1% parasitemia. The top axis determines the age of parasites based on optical microscopy. Light to dark shades of the curves indicate increasing drug concentrations. Empty, black circles correspond to the drug-free control samples. Each circle is the average of data obtained from three biological replicates. **Panel B: IC50 values determined after 6, 8 and 10 hours of incubation for 0.1 and 1% parasitemia samples.** Red and pink circles show the IC50 values originating from independent 0.1% parasitemia assays (not shown). Similarly, black and grey squares show IC50 values of the 1% parasitemia assay on panel A, and another independent assay, respectively. The blue background shading marks the range of IC50 values presented in literature. A dose-response curve corresponding to the 10 h time point is included in the top right corner of panel B to serve as example.

All drugs were effective against the sensitive strain, and the drug effect was already detectable and IC50 values were obtained after 6-10 hours of incubation (Figure 2.). The thorough comparison of IC50 data obtained by the RMOD method revealed that our results are in an overall good agreement with the IC50 values reported based on other drug susceptibility assay methods after much longer incubation times. The relevance of these results is further corroborated by the observation that IC50 values covering the whole intraerythrocytic cycle showed no significant time-dependence. In case of the chloroquine assays, the values obtained throughout the complete erythrocytic cycle showed a very little variance, corroborating that the assessment of drug efficacy at the earliest time points is indeed relevant. The method was tested at a range of parasitemia values, ranging from 0.01 to 1%. In case of the lowest parasitemia experiment, the growth of the drug-free sample was detected by the RMOD method even after 6 hours of incubation, however, IC50 values were not possible to determine. In case of 0.1, 0.5 and 1% parasitemia cultures, the RMOD showed an excellent sensitivity, and relevant IC50 values were obtained at all aforementioned levels. The sensitivity of the device allows the sub-cycle resolution analysis of the intraerythrocytic development of parasites, making it an ideal candidate for drug susceptibility assays.

Utilization of the RMOD for investigating *in vivo* hemozoin production and clearance characteristics in mice

I examined the relation of hemozoin production and parasitemia values and found that there is a good correlation between the two traits, however, the MO signals corresponding to a given parasitemia value have an order of magnitude ambiguity. That is well explained by the biological traits of the parasites, namely that the hemozoin production is continuous within a single intraerythrocytic cycle, while parasitemia is constant.

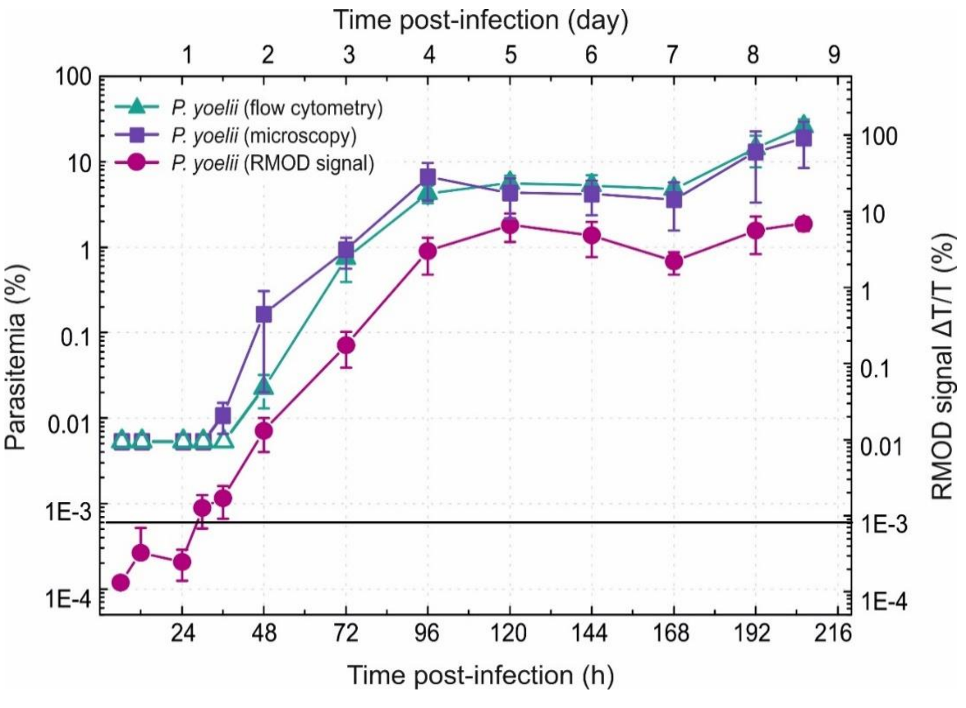

Figure 3. Comparison of parasitemia and hemozoin production of *P. yoelii* 17X-NL infection in mice. Parasitemia values are indicated as green triangles for data obtained by flow cytometry and purple squares for microscopy results. The MO signals are shown as magenta circles. Empty symbols of flow cytometry and microscopy results show the data points tested negative by the aforementioned methods. The black line represents the detection limit of the RMOD method.

The course of infection in case of the self-resolving *P. yoelii* 17X-NL strain (Figure 3.) and the *P. vinckei* lentum strain was followed by different diagnostic methods to compare the relations of parasitemia and hemozoin content over longer periods of times. The MO value reflected changes in parasitemia very well, including ranges of intense multiplication and the following decline. Moreover, multiplication rate variances of the two species were also detected over the course of days. Different methods of infections were applied, the *P. vinckei* and *P. yoelii* parasites were injected into the tail vein of mice. The detection of the *P. yoelii* infection was possible during the 2nd intraerythrocytic cycle post-infection by the RMOD method, similar to microscopy and flow cytometry. The *P. vinckei* infection was first detected during the 3rd intraerythrocytic cycle both by microscopy and the RMOD method. In a different experimental setup, mice were infected with the lethal *P. berghei* ANKA parasite strain by mosquito bites, modeling the natural course of infection. Detection of the infection in this case was possible during the 1st or 2nd cycle, similar to microscopy, and sooner compared to results achieved by two settings of flow cytometry.

Monitoring the effect of treatment was also successful, moreover, the decline of hemozoin content was observed one cycle sooner by the RMOD method than the decline of parasitemia by optical microscopy. The rapidly changing MO signal suggests that a significant amount of hemozoin was released and removed from the blood stream even within the first cycle after drug administration.

The RMOD method was capable of detecting hemozoin production of *P. vinckei* and *P. berghei* at a sub-cycle resolution, revealing dynamic, declining and increasing MO signals over multiple cycles. The declining periods are attributable to the periods of cycle changes, where hemozoin is released into the blood stream, and the young parasites of the next cycle do not produce enough crystals to balance the fast clearance of free hemozoin.

Based on the results summarized here I conclude that the RMOD method can be an ideal candidate for the observation of *in vivo* rodent malaria infections including fast detection, treatment efficacy, and disease monitoring regardless of the lethal or self-resolving, synchronous or asynchronous nature of the parasites.

Thesis points

1. I concluded that all three intraerythrocytic developmental stages of the human pathogen *Plasmodium falciparum* contain ~7-10 uracil/million bases in their genomic DNA. Uracil is not a DNA-building base in case of the vast majority of organisms. Compared to the general genomic uracil levels of a variety of wild type organisms (0.1-1 uracil/million bases), the *P. falciparum* genomic uracil levels are significantly higher. **Publication #1**
2. I concluded that the RMOD method is a promising new technique for the assessment of *in vitro* antimalarial drug efficacy using *P. falciparum* laboratory cultures. The method provides relevant half-maximal inhibitory concentration (IC50) values even after 6-8 hours of incubation times in case of treatment with chloroquine, piperaquine and dihydroartemisinin; with parasitemia values as low as 0.1%. Considering that most other, routinely used drug assay techniques require 48-72 hours of incubation and often rely on additional markers and expertise for the specific instruments, the RMOD offers a significantly shorter incubation time, marker-free and fast detection combined in an easy-to-use platform. **Publication #2**
3. I concluded that the RMOD method is capable of the *in vivo* detection and monitoring rodent malaria infection in mice over several cycles in case of infections with both the non-lethal, synchronous *P. vinckei* lentum and the self-resolving, asynchronous *P. yoelii* 17X-NL parasite strains with similar sensitivity as optical microscopy and flow cytometry. **Publication #3**
4. I concluded that the RMOD method indicated the effect of chloroquine treatment in case of the rodent parasite, *P. vinckei* lentum infections 24 hours *in vivo*, aka. one intraerythrocytic cycle earlier than the gold standard optical microscopy, and I determined the hemozoin kinetics in case of the same strain on a sub-cycle level with a precision and resolution that is, to the best of my knowledge, has not been done before. **Publication #3**
5. I concluded that in the examined *in vivo* rodent models the hemozoin production of the parasites and the hemozoin clearance by the host immune system is in a state of a dynamic equilibrium. The measured hemozoin amount at every given detection point reflects the amount of hemozoin produced by the parasites, making it an ideal indicator of parasite development *in vivo*. **Publication #3 and #4**

Possible applications of my findings

Given the significantly elevated uracil levels of *P. falciparum* compared to a variety of other organisms, this phenomenon probably contributes to the mutation-related traits and mechanisms of the malaria parasites. Monitoring uracil level changes under drug pressure could reveal resistance-related mutations. It would be especially interesting to see the effect of drugs that interfere with uracil-related pathways, such as the folate pathway inhibitors, sulfadoxine and pyrimethamine. By monitoring mutation rates and the occurrence of high-frequency SNPs in case of resistant cultures, possible resistance-related mechanisms or genes could be identified, linking the unusually high prevalence of uracils in the genome of *P. falciparum* to its remarkable adaptation capabilities towards antimalarials.

I concluded that the RMOD method can be an ideal candidate for the assessment of antimalarial drug activity by significantly improving the necessary incubation times and decreasing the complexity of the assay procedure, which have been long-standing goals of the field. The method's applicability should be further evaluated for new drug candidates; *ex vivo* assays and for the assessment of the stage-specific nature of different drugs. Resistance against artemisinins is a serious threat and results in a dormant state of parasites capable of re-emerging after the drug is no longer present. During this state, no DNA or protein synthesis can be detected, and dormancy is very hard to recognize under the microscope. Therefore, artemisinin resistance can only be detected ~60-70 hours after drug removal. Given the sensitivity of the RMOD in the detection of hemozoin even in relatively young parasites, it is possible that the recovery of parasites via hemozoin production could be detected earlier than in case of methods relying on other markers.

I concluded that the RMOD method is an ideal candidate for the observation of *in vivo* rodent malaria infections including fast detection, treatment efficacy, and disease monitoring regardless of the lethal or self-resolving, synchronous or asynchronous nature of parasites. By proving that the hemozoin is an ideal marker of parasitemia changes even under *in vivo* conditions, an important future prospect is the evaluation of the method during human clinical and field trials. Results of our group obtained from mice experiments indeed formed the basis of our first extensive field study. The test series were performed in Papua New-Guinea, and the results are under publication at the moment.

List of publications

Publications utilized for thesis points:

1. **Molnár, P.**, Marton, L., Izrael, R., Pálinkás, H. L. & Vértessy, B. G. Uracil moieties in *Plasmodium falciparum* genomic DNA. *FEBS Open Bio* **8**, 1763–1772 (2018). doi: 10.1002/2211-5463.12458. **IF: 2.23 I:6 FI:4.**

2. **Molnár, P.** *et al.* Rapid and quantitative antimalarial drug efficacy testing via the magneto-optical detection of hemozoin. *Sci. Rep.* **10**, 14025 (2020). doi: 10.1038/s41598-020-70860-y. **IF: 3.99**

3. Pukáncsik, M., **Molnár P.**, *et al.* Highly Sensitive and Rapid Characterization of the Development of Synchronized Blood Stage Malaria Parasites Via Magneto-Optical Hemozoin Quantification. *Biomolecules* **9**, 579 (2019). doi: 10.3390/biom9100579. **IF: 4.08 I:2**

4. Orban, A., Rebelo, M., **Molnar, P**., et al. Efficient monitoring of the blood-stage infection in a malaria rodent model by the rotating-crystal magneto-optical method. Sci. Rep. 6, 23218 (2016). doi: 10.1038/srep23218
IF: 3.99 I:12 FI:9

Publication under revision:

Arndt, L., Koleala, T., Orbán, A., Ibam, C., Timinao, L., Lorry, L., Butykai, A., Karman, P., **Molnár, A. P**. et al. n.d. “Magneto-Optical Diagnosis of Symptomatic Malaria in Papua New Guinea.” Under revision. doi:10.1101/2020.05.14.20101543 **IF: 12.1**

Conference attendances:

2017 - Deutsche Physikalische Gesellschaft Spring Meeting; Dresden, Germany: **oral presentation**

2018 - Grandmaster Early Carreer Workshop, Vienna, Austria: **oral presentation**

2018 - Oláh György Doctoral School PhD conference, Budapest, Hungary: **oral presentation**

2018 - BioMalPar - Biology and Pathology of the Malaria Parasite, Heidelberg, Germany: **poster presentation**

2018 - McDonnell Academy 7th International Workshop, Beijing, China: **poster presentation**

2019 - Grandmaster Early Carreer Workshop, Split, Croatia: **oral presentation**

2019 - Malaria in Melbourne, Melbourne, Victoria, Australia: **oral presentation**