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Optimization of a flow cytometric assay to measure antimalarial drug effects

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Abstract

Malaria is still one of the most important parasitic diseases. The emergence of parasites, resistant to most available drugs, particularly to artemisinins which are the first line treatment, has been considered as a major public health problem. Thus, monitoring parasite drug sensitivity has a high priority. Although there are different methods available for the assessment of *P. falciparum* susceptibility *in vitro*, no single method is standard because all of them have associated limitations. Moreover, the common methods fail to detect artemisinin resistance. A novel sensitivity test has been developed which is based on a flow cytometer modification that allows the detection of hemozoin (Hz). Although it has the advantages of being rapid and reagent-free, it still has some limitations. The main goal of this project was to test various conditions to further optimize this method. Our results suggest that lysing the samples before the measurement decreases the limit of detection of the method to 0.05%. Even more interesting, it allowed the detection of inhibitory drug effects as early as 10 to 14 hours after incubation. A protocol of a tight synchronization of the parasite cultures was also tested to investigate the potential of this method to detect artemisinin resistance. Along with this, the drug concentration and the time of incubation were also altered. The survival rates obtained after only 30 hours allowed to differentiate between resistance and sensitive parasites. These preliminary results show that the Hz detection assay by flow cytometry can be a promising alternative method for drug sensitivity testing including artemisinins.

Keywords: Malaria; antimalarial drug resistance; antimalarial sensitivity test; flow cytometry; hemozoin; artemisinin resistance

Resumo

A malaria é uma das doenças parasitárias mais importantes. A emergência de parasitas resistentes à maioria dos antimaláricos, nomeadamente à artemisinina, fármaco de primeira linha de tratamento, tem sido considerado um grave problema de saúde pública. Consequentemente, a monitorização da suscetibilidade do parasita tornou-se uma prioridade, existindo atualmente, diversos métodos para avaliar a sensibilidade do *P. falciparum in vitro*. No entanto, não existe nenhum considerado ideal devido às limitações inerentes a cada método. Além disso, nenhum deles consegue detetar a resistência à artemisinina. Foi então desenvolvido um novo teste baseado na deteção da hemozoina através de um citometro de fluxo modificado. Apesar das vantagens que apresenta por ser mais rápido e dispensar o uso de reagentes, existem algumas limitações. O objetivo deste projeto é otimizar o método testando diferentes condições da amostra. Os resultados obtidos sugerem que a lise das amostras diminui o limite de deteção para 0.05% e interessantemente, permite a deteção dos efeitos inibitórios dos fármacos entre as 10 e as 14 horas de incubação. Foi ainda testado um protocolo de sincronização rigorosa das culturas de forma a investigar a potencialidade do método para detetar a resistência a artemisinina. Além disso, modificou-se a concentração do fármaco e o seu período de incubação com a cultura. As taxas de sobrevivência calculadas logo após 30 horas permitiram distinguir os parasitas resistentes dos sensíveis. Estes resultados, embora preliminares, sugerem que este método pode ser uma alternativa promissora para testar *in vitro* a sensibilidade do *P. falciparum* a antimaláricos, incluído a artemisinina.

Palavras chave: malária; resistência a antimaláricos; testes de sensibilidade a antimaláricos; citometria de fluxo; hemozoina; resistência a artemisinina

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Abbreviations

ACT – Artemisinin-based combination therapy

APAD – 3-acetylpyridine-adenine-dinucleotide

ART – Artemisinin

Ato – Atovaquone

CMCM – Complete malaria culture medium

CQ – Chloroquine

DELI – Double-site enzyme-linked LDH immunodetection

DHA – Dihydroartemisinin

DHFR – Dihydrofolate reductase

DHPS – Dihydropteroate synthase

DNA – Deoxyribonucleic acid

ELISA – Enzyme-Linked Immunosorbent Assay

FCS – Forward scatter

G6PD – Glucose-6-phosphate dehydrogenase

HRP II – Histidine-rich protein 2

HPR III – Histidine-rich protein 3

Hz – Hemozoin

IC50 – 50% inhibitory concentration

iRBCs – Infected red blood cells

LDH – Lactate dehydrogenase

Mef – Mefloquine

MOT – Magneto-optical technology

NAD – Nicotinamide adenine dinucleotide

PCR – Polymerase chain reaction

pfcrt – Gene encoding *P. falciparum* chloroquine resistance transporter

Pfmdr1 – Gene encoding *P. falciparum* multidrug resistance 1 protein

PG – Proguanil

pLDH – Parasite lactate dehydrogenase

PQ – Piperaquine

QN – Quinine

RBCs – Red blood cells

RNA – Ribonucleic acid

RPMI – Roswell Park Memorial Institute

SP – Sulfadoxine-pyrimethamine

SSC – Side scatter

WHO – World Health Organization

I. Introduction

1. Malaria

Malaria is a parasitic disease with a big impact in the global human health. According to the WHO, in 2015 there was 214 million cases which were fatal for almost a half of a million individuals, mostly children [1]. This means that malaria causes approximately one death per minute. Although WHO data show a reduction in incidence and mortality in the lasts 10 years, the number of cases and deaths is concerning. It is estimated that almost half of the planet's population is at risk of developing malaria every year [1]. The impact of this disease is extended to a social-economic level once it affects mostly underdeveloped countries with limited resources and difficult access to all available means to control malaria. There are several ways to control malaria: prevention, treatment and reducing transmissibility. To achieve that, there are a number of available antimalarial drugs which act in different phases of the parasite life cycle.

1.1 Biology of the parasite

The parasite responsible for causing malaria belongs to the genus *Plasmodium* and is frequently characterized by having a complex life cycle (Figure 1) [2]. It is complex because it involves different stages within two hosts, the human and the mosquito. Humans can be infected by five species of *Plasmodium* (*P. falciparum, P. vivax, P. ovale, P. malariae and P. knowlesi*) though, their life cycles have minor differences [3]. There are among 30 species of Anopheline mosquito that are important vectors for transmitting parasites [1]. Parasites are transmitted from human to mosquito by the ingestion of sexual stages, called gametocytes. These stages circulate in the bloodstream of the infected individual even after the treatment, period which they can be ingested by the mosquito. Thus, these forms are responsible for maintaining the cycle and an ideal target to avoid parasite transmission.

Although one good gametocytocide exists, a drug that acts against gametocytes, it cannot be used easily in patients with glucose 6-phosphate dehydrogenase deficiency and for that reason it is not an universal solution [4]. Within the mosquito, gametocytes develop in a process designated sporogony resulting in sporozoites that invade human organism after having been injected during the next mosquito feed. Injected sporozoites migrate to the liver starting the hepatic phase. This phase is important because it is the phase immediately before the emergence of the disease. On the other hand, in *P. vivax* and *P. ovale* cases, it is the phase which is involved in the relapses. These two species produce dormant stages, called hypnozoites, which persist in the liver. After a period of time they may reactivate and complete the cycle leading to bloodstream infections weeks or months after the treatment [5]. These stages are the target of hypnozoiticides which are used after the treatment of the acute malaria episode to prevent future relapses.

During the hepatic phase parasite maturation occurs, resulting in mature hepatic schizonts which release merozoites into the bloodstream. This starts the erythrocytic stage, when these parasites invade red blood cells (RBCs). By acting against hepatic schizonts the tissue schizonticides avoid the disease and are important in primary prophylaxis.

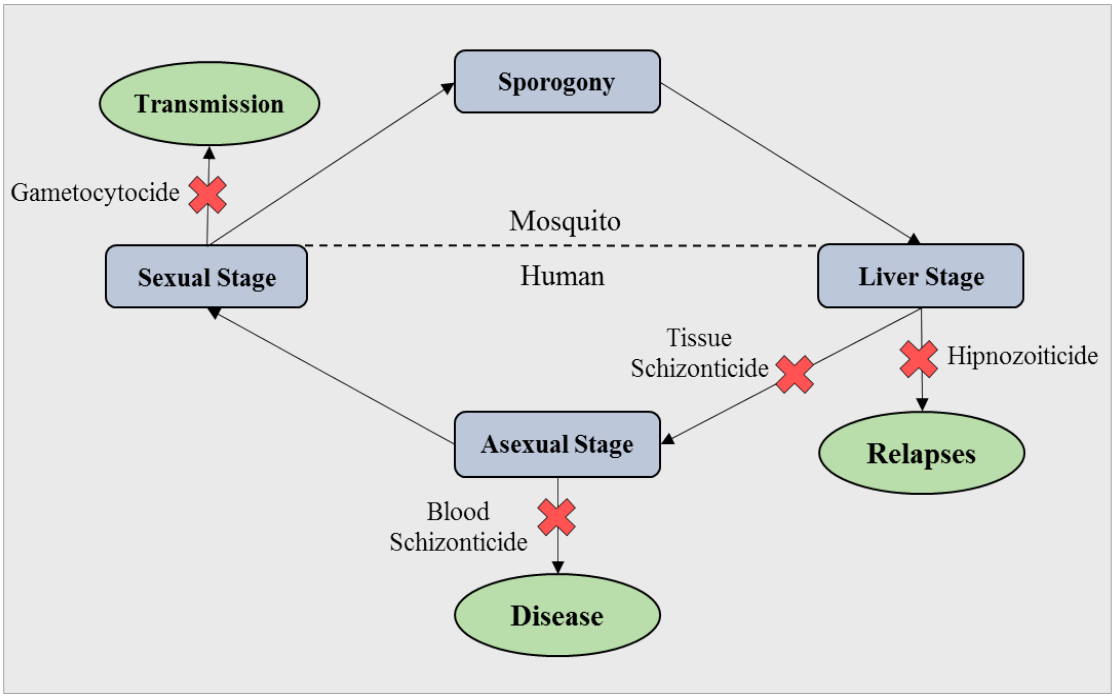


Figure 1. Life cycle of *Plasmodium* spp.
Plasmodium life cycle is constituted by four stages and involves two hosts. Within the mosquito occurs the sporogony and the rest of the cycle takes place in humans where the parasite development continues with a liver stage, an asexual stage and a sexual stage. Each human stage has a consequence associated that can be avoid with an adequate treatment. Depending on the site of action of drugs (red crosses) they are classified as: hipnozoiticide, tissue schizonticide, blood schizonticide and gametocytocide. Hipnozoiticides prevent relapses in *P. vivax* and *P. ovale* infections. Tissue and blood schizonticides act to avoid clinical symptoms and signs (disease). Gametocytocides are the drugs that prevent the transmissibility of the parasite [6].

The disease, malaria, with all its symptoms, derives from the development of asexual forms during the erythrocytic phase (Figure 2). This is the phase where drugs act which treat the disease, they are considered blood schizonticides. During this stage, *P. falciparum* matures inside the RBCs which makes it very useful for certain *in vitro* applications; for example, to test drug effects. These assays tend to measure the maturation of the parasite when exposed to drugs instead of measuring the replication and thus, the quantity of parasites. This is different to drug sensitivity tests in bacteria, which are based on replication and number of bacteria. A possible explanation for this is the fact that there is not an exact number of merozoites produced by one schizont but a number which can varies [3,7]. There are several ways to measure parasite growth.

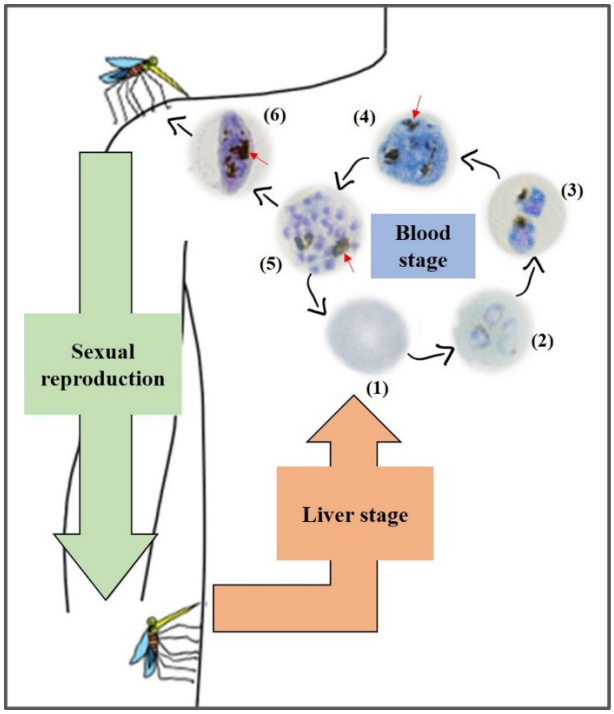


Figure 2. Blood stage of *P. falciparum*.
Merozoites from liver stage enter the bloodstream and invade RBCs (1) by surface interactions (ligand-receptor). Within the RBC, the merozoite develops forming a structure with the appearance of a ring (2). Parasites continue to replicate and are designated trophozoites (3) until they occupy the entire RBC. After that they are called schizonts (4) and they look spherical and segmented. Subsequently, schizonts will rupture and release new merozoites (5) into the bloodstream. The merozoites reinvade RBCs and the erythrocytic developmental cycle starts again. Once inside the erythrocyte, some merozoites will differentiate into a sexual form, male or female gametocytes (6). The red arrows indicate hemozoin crystals that can be found in mature trophozoites, schizonts and gametocytes. Modified image from [8].

During the blood phase, the parasite follows a maturation process where it degrades components of the cell, namely hemoglobin. When digested, hemoglobin releases free heme which is toxic to the parasite. To overcome this toxicity, the parasite converts free heme into a crystal called malaria pigment or hemozoin [9]. The amount of hemozoin increases during parasite maturation and therefore is considered a good indicator of growth. By monitoring the amount of hemozoin it is possible to assess parasite maturation [10]. Additionally, for being a crucial process to parasite survival it is the target of many antimalarial drugs.

2. Antimalarial Drugs

Currently, a number of drugs exists which can be used to treat or prevent malaria. It is possible to categorize them by chemical structure and mode of action (Figure 3) [11].

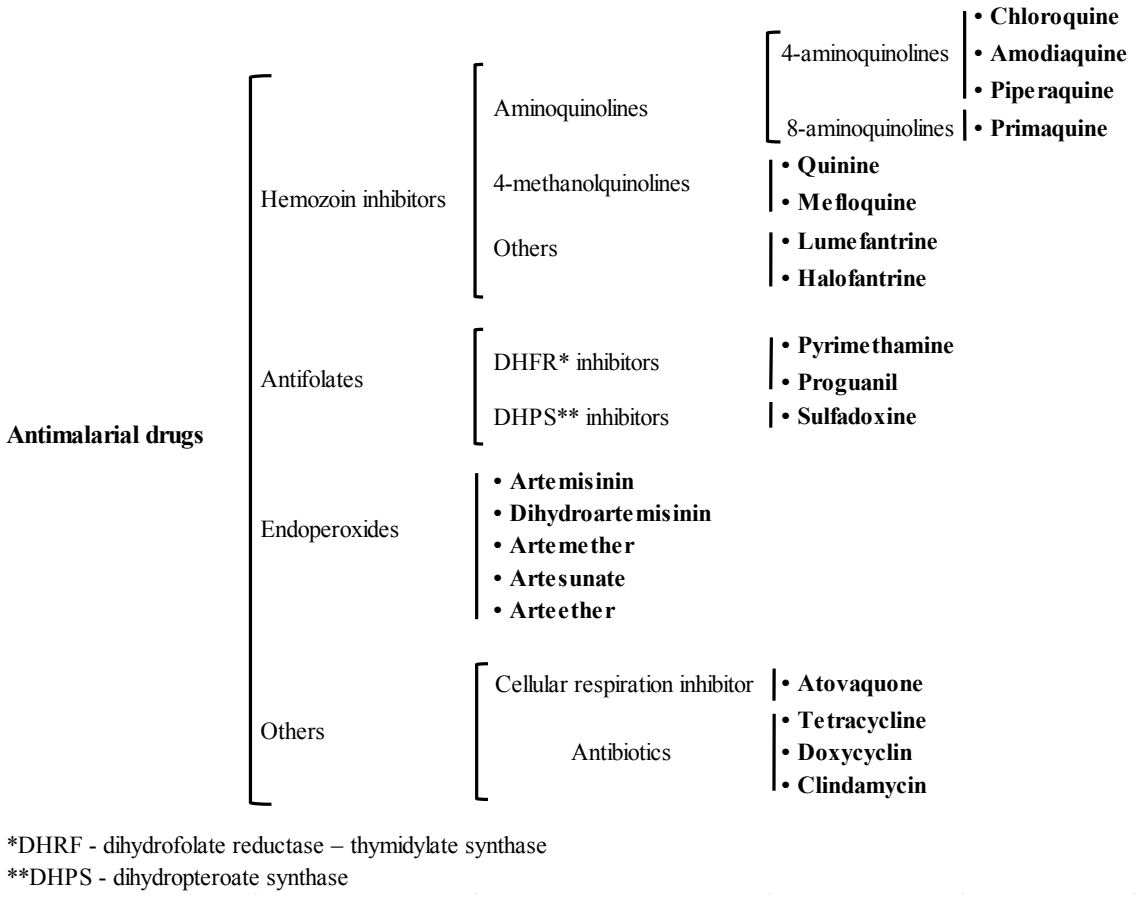


Figure 3. The main antimalarial drugs assembled according to the chemical classes they belong to or their function.
Hemozoin inhibitors are drugs that interfere with hemozoin formation which are mainly quinoline-related drugs. Antifolates are drugs that interfere with folic acid synthesis which are divided by DHFR inhibitors or DHPS inhibitors according with the inhibited enzyme. The class of endoperoxides are related to artemisinin and its derivatives which mode of action remains poorly understood. There are other compounds with antimalarial effects which are antibiotics, frequently by acting on protein and nucleic acid synthesis and atovaquone which interferes with parasite mitochondria blocking cellular respiration. Adapted from [11].

2.1 History of the principal antimalarial drugs

The history of the discovery of antimalarial drugs was often directly related to the history of wars [12,13]. Malaria had a great influence on the outcome of several wars since, in some cases, it caused a greater health risk to military than the battlefield injuries [12]. The constant search for new compounds to treat and prevent malaria was due to several reasons: limited access to the existent drugs in certain areas, the need for large-scale production, the emergence of resistance, the cost, ineffectiveness, or the secondary effects caused by

some drugs. All these aspects make for a long history that begins with herbal treatments which were gradually replaced by purified chemical compounds and later synthetic drugs.

Quinine, a natural alkaloid extracted from the cinchona tree bark was among the first compounds used as a treatment for malaria [12,14]. It has been suggested that quinine interferes with heme detoxification inhibiting hemozoin formation and consequently, killing the parasite by accumulation of cytotoxic free heme [9]. It belongs to the category of "blood schinzonticide" drugs (Figure 1). By the 19th century quinine was still the only known antimalarial agent and became a standard therapy worldwide [15]. Before the formulation of quinine as a drug, the extract of the bark was used but it had a bitter taste. The bark was administrated prophylactically as a daily routine in suspensions of wine or spirits which led to the well-known gin and tonic recipe (nowadays the tonic water has only a small amount of quinine) [12]. Following the scientific developments, it was possible to isolate the active compound of cinchona tree in 1820 [9]. This makes malaria one of the first diseases to be treated by a pure chemical compound [12].

During the Second World War the global supply of quinine was cut off after Japanese took over Java, where 97% of the total amount of quinine was produced. It was essential to find a solution that provided large-scale production of antimalarial therapy, unlimited by the supply or location of natural resources. After the large advances in synthetic organic chemistry, it became finally possible to synthetize quinine in 1944. However, its production was difficult and economically inviable [12]. Scientists cooperated and tested many compounds in order to find prototypes for new antimalarial drugs. These efforts culminated in the discovery of chloroquine as a powerful antimalarial drug almost one decade after it had been synthetized. This drug proved to be the most effective antimalarial drug at that time and was the main choice in eradication and control programs [13]. Curiously, one strategy of prophylactic mass distribution introduced at the time was putting chloroquine into common cooking salt [12]. The intensive use throughout the world led to the emergence of *P. falciparum* parasites resistant to chloroquine in 1957 [16]. Like the others quinoline derivatives, the mechanism of action of chloroquine is thought to be the interference with hemozoin formation. Chloroquine binds hemozoin to prevent further biocrystallization of heme and binds to heme forming a highly toxic complex. This complex along with free heme results in cell lysis and autodigestion of the parasite [9].

Other drugs were developed owing to the Second World War efforts. Before the "chloroquine era" an 8-aminoquinoline compound named plasmoquine was synthetized which was a precursor for development of primaquine [12]. This drug remains very important currently because of its capacity in preventing relapses of *P. vivax* and *P. ovale* acting as a hypnozoitocide (Figure 1) [17]. In 1945 and in 1952, respectively, two inhibitors of the dihydrofolate reductase (proguanil and pyrimethamine) were also synthetized [13]. Later in the 1980s, proguanil was combined with a hydroxynaphthoquinone compound (atovaquone) which is safe and highly effective antimalarial combination therapy.

The waning efficacy of quinoline related antimalarial drugs along with the rapid development of resistance, especially the failure of chloroquine, increased malaria cases in many parts of the world, especially in Africa and Southeast Asia [18]. During the Vietnam War, even though all troops received prophylaxis with chloroquine and primaquine, malaria was the main cause of fatalities. Due to this situation, new compounds were developed which are structurally related to quinoline antimalarial drugs, such as mefloquine,

halofantrine, lumefantrine and piperaquine [12]. The Chinese government also decided to screen traditional remedies for antimalarial drug activities [18].

"Back to nature", the plant *Artemisia annua* was tested and proved to have potent antimalarial activity. In 1972, artemisinin was purified and was demonstrated to be a very effective antimalarial drug [18]. The unprecedented structure, the fast and effective acting, the fact that is well tolerated and safe, and the short life time of this drug made it distinct from the other antimalarial drugs [18]. Although it has been the subject of much discussions, the mode of action of artemisinin remains poorly understood. Certainly, antimalarial activity depends on an endoperoxide bridge that characterizes the structure of artemisinins [19,20]. This structure can be modified in order to improve the solubility of the drug. In fact, the parent drug of artemisinin was slightly soluble in water or oil. If its carbonyl group is reduced it is obtained dihydroartemisinin (DHA) and its derivatives artesunate (water soluble), artemether and arteether (both oil soluble) [19]. These drugs act against asexual stages, particularly young ring forms, inhibiting their development to mature forms. However, they are also effective against sexual stages. Because they are among the most potent antimalarial agents, artemisinin-based combination therapy is the first line treatment recommended by WHO for uncomplicated and severe malaria [21].

2.2 Treatment of malaria

To give the appropriate treatment to a patient with malaria, the clinician has to take several aspects into consideration: it is important to know what species of *Plasmodium* is involved and the respective parasitemia; the geographic and travel history of the patient in order assess if the infection was acquired in an area of drug resistance, the efficacy, availability, cost and side effects of antimalarial drugs, and the clinical status of the patient to determine the route of administration [6]. The WHO, taking into account these factors, established guidelines for the treatment and chemoprevention of malaria which are briefly outlined in Table 1.

Table 1. Summary of the current guidelines for malaria treatment according to WHO [21]**.**

Treatment of:	WHO guidelines
Uncomplicated *P. falciparum* malaria	3 days treatment with an ACT: • artemether + lumefantrine • artesunate + amodiaquine • artesunate + mefloquine • dihydroartemisinin + piperaquine • artesunate + sulfadoxine-pyrimethamine
Uncomplicated malaria caused by *P. vivax*, *P. ovale*, *P. malariae* or *P. knowlesi*	**Areas with chloroquine-susceptible infections** • Chloroquine or ACT (except first trimester of pregnancy) **Areas with chloroquine-resistant infections** • ACT • Quinine for pregnant women in their first trimester **For preventing relapse in *P. vivax* or *P. ovale* malaria** • Primaquine (different administration depending on G6PD status of the patient)
Severe malaria	• At least 24h of intravenous or intramuscular artesunate + 3 days of an ACT

Uncomplicated versus severe malaria

The clinical symptoms of malaria are caused by the erythrocytic stage. Many waste compounds are developed during the parasite maturation which are released into the bloodstream when infected RBCs lyse. Some of these compounds stimulate macrophages and other cells to produce cytokines that are going to produce fever and other symptoms and signs. Usually, malaria is classified as uncomplicated malaria or severe malaria. The major clinical features of uncomplicated malaria include fever, chills, sweats, headaches, nausea and vomiting and body aches. It may cause an enlarged spleen, enlargement of the liver and mild anemia [6]. When the progression of the disease is fulminant it may evolve to impaired consciousness (cerebral malaria), prostration, multiple convulsions, acidosis, hypoglycemia, severe anemia, renal impairment, jaundice, pulmonary edema, significant bleeding, shock or hyperparasitemia (>10%). If a patient infected with *P. falciparum* has one or more of these clinical features he is considered to have severe malaria [21]. Uncomplicated malaria is caused by all the five species of *Plasmodium* that can infect humans whereas severe malaria is caused mostly by *P. falciparum*. Differently from the other four species, *P. falciparum* parasites grow in RBCs of all ages which means that high parasitemias can occur easily. Furthermore, contrarily to the other species, RBCs infected with mature stages of *P. falciparum* exhibit cytoadherence meaning that they adhere to the endothelium of venular blood vessel walls instead of circulating in the blood stream which has pathological consequences [22]. It is thought that parasite sequestration occurs in cerebral microvasculature and may be a leading cause of neural dysfunction [23]. Although it is rare, occasionally *P. vivax* and *P. knowlesi* can also cause severe malaria. The clinical status

of the patient determines if drug administration is parenteral (intravenous or intramuscular) or oral medication. Only quinine and artesunate are available for intravenous treatment [21].

Combination therapy

Another important factor to take into account in malaria therapy are the associated problems with each antimalarial drug. Over the years, approaches and guidelines to minimize these problems were developed. The main approach was the use of combination therapy instead of monotherapy. Combination therapy is the use of two or more classes of antimalarial drugs with unrelated mechanism of action. In the case of artemisinin-based combination therapy (ACT) this is the combination of an artemisinin derivate with other antimalarial which has a different mode of action and a longer half-life. The key reason of combination therapy is the lower probability of the parasites to develop resistance to both drugs [19]. Even drugs to which resistance has been described, may remain useful in combination and remain effective if the prevalence of resistance to each agent is low enough [24].

Combination therapy also addresses the problem that artemisinin has a very short half-life which is associated with high rates of recrudescence if used alone. Recrudescence is a recurrence of asexual parasitemia with the genetically same parasite after a treatment that was ineffective to clear asexual parasites completely. It is different from re-infection and relapse which are a recurrence after drug treatment by a new infection of a genetically different parasite or by dormant forms (only in *P. vivax* and *P. ovale* infections), respectively. By combining artemisinin with a long-lasting drug, it is possible to rapidly reduce parasite densities to very low levels which are then cleared by longer-acting drugs [25]. In this way, clearance of the parasite is achievable and the recrudescence is prevented, even with a shortened treatment regimen of 3 days ACT instead of the 7 days of monotherapy [26].

The combination therapy is also a good approach for drugs which are less efficient as single therapy, may it be either by combination with a more powerful drug like artemisinin or by synergic effects with another drug. For example, the antifolate drugs as pyrimethamine and the antibiotic sulphonamides as sulfadoxine act inhibiting different enzymes of the folic acid synthesis. Thus, there is a strong synergy between these two classes when they are used in combination increasing their efficacy. However, in this case, as they have similar mechanisms of action, there is less protection from resistance [26].

Prophylaxis

Chemoprophylaxis is defined by the use of antimalarial drugs for prophylaxis and preventive treatment. If the drug inhibits liver stage development it is causal prophylaxis, whereas if the drug acts by killing the asexual blood stages it is suppressive prophylaxis. For travelers, it is important to start chemoprophylaxis before travelling to endemic areas to test for tolerability and to ensure that therapeutic levels during the stay are reached. In causal prophylaxis, the drug can be stopped soon after returning from endemic areas. In

suppressive prophylaxis it should be prolonged until 2-4 weeks, so that in case of exposure parasites have time to emerge and become asexual forms and thus the drug can act on preventing malaria [21].

2.3 Resistance

Perhaps, being among the first diseases to be treated by a pure chemical compound as well as by a synthetic compound, malaria parasites were also among the first pathogenic microbes to become resistance to these compounds. According to WHO, antimalarial drug resistance is "the ability of a parasite strain to survive and/or multiply despite administration and absorption of an antimalarial drug given in doses equal to or higher than those usually recommended, provided that drug exposure is adequate" [21].

Resistance arises from point mutations or gene amplifications that confer a survival advantage in the presence of antimalarial drugs. The mutations trigger essentially two main mechanisms of resistance: (i) the reduction of drug availability at the site of action (mutations in transporter genes) or (ii) the modification of the drug target (mutations in genes of the target) [21]. After the genetic event, especially when exposed to sub-therapeutic drug concentrations, there is a selection of the mutant parasites that are less susceptible [27]. Consequently, a preferential transmission and spread of the mutant parasites occurs.

One of the major causes of resistance is the inappropriate use of antimalarial drugs. Especially in cases of high parasitemias, it is important to prescribe an adequate treatment and the patient should follow it strictly. Though, other factors contribute to development and spread of drug resistance, such as the mutation rate of the parasite, the degree of resistance conferred by the mutation, intrinsic characteristics of the drug (e.g., quality, life-time) or immunity of the human host [2,26].

According to WHO recommendations there are some considerations for monitoring the efficacy of therapeutics, such as: confirmation of the quality of antimalarial drugs; distinguishing re-infection from recrudescence by molecular genotyping to establish the possibility of treatment failure, and eventually to identify genetic markers of drug resistance; evaluate susceptibility of the parasite *in vitro* and measure exposure drug levels in cases of slow therapeutic response or treatment failure [28].

Currently, *P. falciparum* resistance has been described for all antimalarial drug classes [25]. For some antimalarial drugs, resistance occurred very quickly after their introduction (Figure 4). This is a major public health problem by destabilizing the efforts to control malaria. In fact, drug resistance has been implicated in the spread of malaria to new areas and re-emergence in areas where it had been eliminated [25]. The biggest concern is chloroquine resistance which has been described everywhere in the world for *P. falciparum,* except in Central America and the Caribbean. There are only few new alternatives that are safe and particularly important in Africa, economically accessible. Artemisinin is one of them [18].

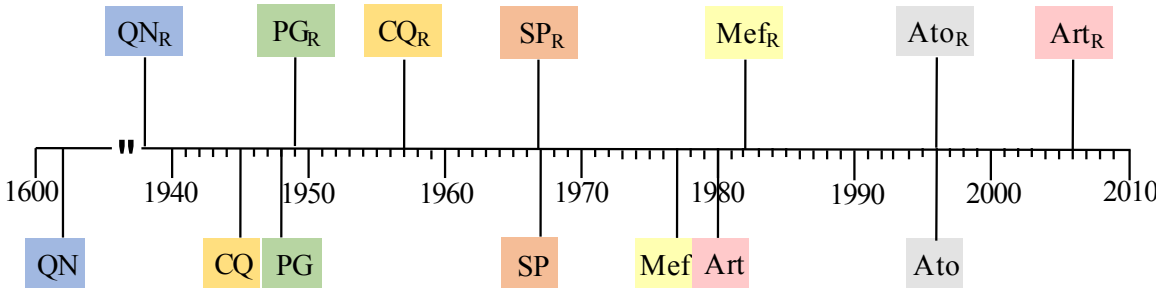


Figure 4. Timeline with approximate dates of introduction and first reported resistance to antimalarial drugs. Quinine (QN) has been used since 1632 without losing effectiveness until the early 20th century. Currently, it is only used as a second line drug. Since QN, no antimalarial drug was used successfully for a such a long period. Chloroquine (CQ) resistance was reported after 12 years of being introduced, mefloquine (Mef) after 5 years and proguanil (PG) after only 1 year. There are also cases were the year of introduction matches with the year of the first resistance. This is the case for sulfadoxine-pyrimethamine (SP) and atovaquone (Ato). Despite the existence of resistance, these antimalarials continue to be used. SP was used to combat CQ resistance in Africa. Ato and PG are used in combination (Malarone). Artemisinin (Art) was introduce in the 1980s and a slowed parasite clearance after the treatment was reported recently. The definition of artemisinin resistance is, for that reason, different from the other drugs. However, Art is now used only in combinations. R-resistance. Adapted from [29,16].

Partial resistance to artemisinin

The existence of artemisinin "resistance" has been subject of much discussions. This is because "resistance" has been defined by a slowed parasite clearance after the drug treatment. In the first report of a possible resistance to artemisinin, Dondorp *et al.* described a delayed parasite clearance of 84h in patients from Pailin (western Cambodia) in comparison with the 48h time clearance on average in patients from north-western Thailand [30]. Beyond Cambodia, this artemisinin resistance is now prevalent in the Lao People's Democratic Republic, Myamar, Thailand and Vietnam [21]. This is of particular concern due to the fact that artemisinin derivatives are the drug of choice as first line treatment. What makes artemisinin better and different from the other drugs is the efficient and fast therapeutic responses [21]. As a consequence of resistance this advantage is lost, which translates into the delayed parasite clearance and an increase of ACT failure rates. Along with this, there is a selective pressure on the partner drugs which resistance is also increasing [21]. This is a worrying situation, especially because local treatment guidelines cannot be changed without solid evidence. However, standard *in vitro* tests fail to detect this artemisinin resistance since they do not show significant alterations in IC50 values (drug concentration that inhibits parasite growth by 50%) [30,31]. Methods to detect artemisinin resistance as well as studies to determine the best treatments for artemisinin-resistance malaria are needed.

3. Antimalarial sensitivity assays

With the rapid development of antimalarial drug resistance, the surveillance of parasites drug sensitivity became a high priority. To limit the spread of resistance it is crucial to have adequate strategies for treatment according with the resistance pattern of the parasites. Consequently, sensitivity tests have become very important tools in controlling malaria. *In vitro* sensitivity tests are also important to screen new antimalarial compounds as well as to evaluate synergic effect between existent drugs for new therapy combinations. Moreover, they can be essential to validate genetic markers of drug resistance identified by molecular genotyping. Currently, there are several sensitivity tests available which can be performed in patients, *ex-vivo* or *in vitro*.

3.1 Therapeutic efficacy trial - *in vivo*

One approach is the assessment of therapeutic responses in patients, that is a therapeutic efficacy trial. This was originally defined in terms of parasite clearance with four different categories: sensitive and three levels of resistance [32]. However, this can be problematic in highly endemic areas where the existence of asymptomatic parasitemia is common. Thus, the WHO modified this protocol taking into account the clinical outcome instead of the parasite clearance. In areas with intensive transmission the prime objective of treatment is resolution of clinical features so each case is classified as: adequate clinical response, early treatment failure and late treatment failure [33]. Recently, the protocol was updated and has also been validated for low-to-moderate transmission regions (Table 2) [34].

Table 2. Classifications of *in vivo* antimalarial sensitivity test outcomes according to WHO protocol [34]**.**

Classification of responses to treatment (2009)	
Early treatment failure (ETF)	• danger signs or severe malaria on day 1, 2 or 3, in the presence of parasitemia; • parasitemia on day 2 higher than on day 0, irrespective of axillary temperature; • parasitemia on day 3 with axillary temperature ≥ 37.5 °C; and • parasitemia on day 3 ≥ 25% of count on day 0.
Late clinical failure (LCF)	• danger signs or severe malaria in the presence of parasitemia on any day between day 4 and day 28 (day 42) in patients who did not previously meet any of the criteria of early treatment failure; and • presence of parasitemia on any day between day 4 and day 28 (day 42) with axillary temperature ≥ 37.5 °C in patients who did not previously meet any of the criteria of early treatment failure.
Late parasitological failure (LPF)	• presence of parasitemia on any day between day 7 and day 28 (day 42) with axillary temperature < 37.5 °C in patients who did not previously meet any of the criteria of early treatment failure or late clinical failure.

Adequate clinical and parasitological response (ACPR)	• absence of parasitemia on day 28 (day 42), irrespective of axillary temperature, in patients who did not previously meet any of the criteria of early treatment failure, late clinical failure or late parasitological failure.

Basically, to perform these tests, a group of individuals with a symptomatic parasitemia is selected which are treated with a given known concentration of the drug. Subsequently, these patients are followed during 28 days for drugs with a half-life of less than 7 days and for 42 days for drugs with longer half-lives [35]. This "*in vivo"* approach is, however, a reflection of interactions between drugs, parasites and the host response, while *in vitro* tests do not consider host responses. For that reason, the treatment response can be diminished by factors other than drug resistance, such as drug absorption and metabolism or the host immune system. Efficacy trials tests can be performed in the field without special equipment and results are more easily to interpret than *in vitro* assays. However, has several disadvantages as ethical problems, the long duration of the assay making the patient monitoring difficult, outcome interference by host factors and does not allow a quantitative assessment.

3.2 *In vitro* assays

The *P. falciparum* continuous culture method, developed by Trager and Jensen in 1976, was an important breakthrough in malaria research [36]. With techniques that enabled scientists to grow erythrocyte stages of *P. falciparum* in continuous culture it was possible to have access to large quantities of human malaria parasites without resorting to blood taken directly from humans and allowed human malaria research in non-endemic areas. Along with this, parasite cultures permitted the development of *in vitro* tests for testing antimalarial drug effects. By excluding the interaction of the drug with the human host, these tests are more objective to quantify the sensitivity to the drug without any risk to the patient. However, there are some important considerations regarding a couple of aspects: the initial culture condition, such as parasite density, hematocrit and the parasite stage where the drug in question acts. *In vitro* tests are basically a mix of cultured parasites with growth medium and different concentrations of a certain drug. Parasite growth over time is measured and compared with a drug-free control. Although basic aspects tend to be the same in all *in vitro* tests, parasite growth is measured in several different ways.

Microscopy

Commonly known as "WHO test", the microtest was developed after the introduction of continuous culture in order to overcome disadvantages of the anterior macrotest [37]. The macrotest was among the first *in vitro* tests and used schizont maturation as a measure of parasite growth. Basically, these tests consist in evaluating the percentage of parasites that develop into schizonts when exposed to an increasing drug concentration. For that, schizonts are counted versus the total number of parasites by optical microscopy on thick films prepared from the cultured samples after 24-30h of incubation [38]. Although it requires little technical equipment, it is a labor-intensive procedure and very time-consuming. To limit individual

subjectivity in counting and distinguishing developmental stages, it requires well trained personnel. Furthermore, using schizont maturation with 24-30h of incubation as an indicator of parasite growth may be misleading in some situations: parasites which grow until the late trophozoite stage count as the same as parasites that do not develop at all [39]. Thus, drugs that slow down the parasite growth may have the same outcome as drugs that inhibit growth completely. Furthermore, it might be difficult to measure the effects of drugs that act on late stages of the parasite. Thus, specific indicators of parasite growth are required for an assay system that provides an objective measure and can be used for large numbers of samples.

Radioisotope methods

Another approach to access parasite maturation is the use of radiolabeled precursors of nucleic acids, proteins and phospholipids. These radiolabeled precursors are incorporated in actively dividing cells present in cultures, which means the parasites. Measuring isotopic precursors quantitatively is an indirect measure of metabolic activity of the parasite. [H^3]hypoxanthine is a purine base, a DNA precursor, and it is considered the preferred radioisotope for labelling parasite DNA in *in vitro* drug sensitivity tests [14]. The incorporation of [H^3]hypoxanthine is directly proportional to the number of *P. falciparum* infected erythrocytes when the initial parasitemia rages between 0.1% and 1% [40]. Developed by Desjardins *et al.* in 1979, this method overcomes the variability of microscope techniques, being the first radioisotope semi-automated assay [41]. For that reason, it is a rapid, sensitive and accurate determination of drug inhibiting effects. The disadvantages of using this method are the use of radioactive products which implies a special disposal system and the very expensive equipment.

Antigen detection methods

Parasite lactate dehydrogenase

Parasite lactate dehydrogenase (pLDH) is an important enzyme in the final step of glycolysis pathway [42]. By playing an important role in glycolysis metabolizing pyruvate to lactic acid, its production is a good indicator of parasite viability. Monitoring enzymatic activity of pLDH determines indirectly drug effects [43]. There is a decrease of pLDH levels (corresponding to parasite density) when drug exposure occurs comparing with initial values. pLDH activity is distinguishable from host LDH activity by using a coenzyme called APAD that is an analogue of NAD. In the presence LDH and APAD, pyruvate is formed from L-lactate resulting in the formation of reduced APAD. Consequently, reduced APAD reduces blue tetrazolium forming a blue formazan product that can be measured by spectrophotometry [39]. Although being a rapid method, the relatively low sensitivity (requiring 1-2% of initial parasitemia) makes it inadvisable for routine field application. To overcome this limitation a new pLDH approach was developed in which monoclonal antibodies specific for pLDH were used [44]. Thus, this double-site enzyme-linked LDH immunodetection (DELI) assay is considerably more sensitive and can be applicable in the field, however monoclonal antibodies supplies are limited.

Histidine-rich protein II

Similar to the DELI assay, there is another immunodetection assay which is based on *P. falciparum* histidine-rich protein II (HRP II) quantification [45,46]. HRP II is produced as a consequence of parasite growth and multiplication. Although its function remains unknown it has been associated with hemozoin formation. Thus, inhibition of parasite growth by a drug means inhibition of HRP II production. This production can be measured by a simple commercial ELISA kit that includes HRP II-specific monoclonal antibodies [47]. One of the main disadvantages is the incubation time required, because HRP II remains at low concentrations during the first 48h of incubation and only after 60-72h reach high levels for detection [14].

Flow cytometry

Flow cytometry is used to measure several properties of the cell and can also be used to measure drug effects on malaria parasites [48,49,50]. The basics of this method consists in detecting DNA content of infected RBCs using DNA-binding dyes. This is possible due to the fact that practically only parasite DNA is present in the culture since host erythrocytes have no DNA content and therefore do not show fluorescence. Monitoring DNA content provides information on the differentiation of asexual stages by its amount of DNA (namely fluorescence intensity), a parameter increasing proportionally to parasite maturation. There are several fluorochromes that can be used, such as thiazole orange, acridine orange, Hoechst 33258, Hoechst 33342, SYBR® Green and others. Between all these, SYBR® Green is the dye of choice for performing sensitivity tests since it does not require an ultra-violet light source which is more expensive [49]; and it is specific for double-stranded DNA which excludes the possibility of labelling incorrectly RNA from reticulocytes [48]. By the end of the incubation period, cultures are fixed and stained with one of these dyes and the fluorescence intensity is measured. Flow cytometric approaches are rapid, accurate, highly sensitive, automated and non-radioactive. However, it requires expensive equipment, specialized personnel and therefore this procedure is difficult to apply in the field.

Fluorometric assay

The staining principles behind fluorometric assay are the same as those of the flow cytometric approach [51,52]. However, instead of using flow cytometer to measure fluorescence intensity a minifluorometer, a spectrophotometer or a fluorescence-activated microplate reader is used. This difference makes fluorometric assays more accessible economically but it also makes them less sensitive than other methods.

Molecular techniques

All the methods mentioned above are methods where drug susceptibility was tested phenotypically. Molecular techniques, such as real-time polymerase chain reaction (PCR), restriction fragment length

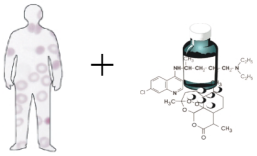
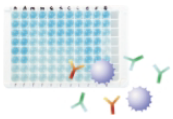
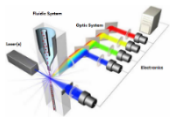
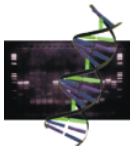
polymorphism and probe hybridization assess genotypic information. These tests are based on the identification of mutations in parasite genes that confer resistance to the drug. Several markers are being associated with antimalarial drug resistance. Mutations of the dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*) genes are respectively related to pyrimethamine and sulfadoxine resistance [28]. On the other hand, mutations in the chloroquine resistance transporter (*pfcrt*) gene is a determinant for chloroquine resistance [53]. It has also been suggested that multidrug resistance gene 1 (*pfmdr1*) is implicated in *P. falciparum* resistance to several blood schizonticides including not only chloroquine but also mefloquine, quinine and artemisinin [28, 54]. Recently, alterations in the kelch13 gene were linked to delayed parasite clearance in artemisinin-treated patients [55]. The *exo-E415G* and *plasmepsin 2-3* are also markers that have been recently implicated in piperaquine resistance [56]. These methods do not depend on viable parasites and allow analysis of several samples in a short period, but require sophisticated equipment and trained personnel. More importantly, the number of validated molecular markers is still limited.

Ring Stage Assay – Detecting artemisinin resistance

Artemisinin resistance has been associated with decreased susceptibility of ring stage parasites and, in some lines, of mature trophozoite stage. Currently, there is no validated molecular marker or *in vitro* test to identify artemisinin resistance accordingly to the Global Plan for Artemisinin Resistance Containment [57]. So far, no consistent and significant correlations between parasite clearance half-life and readouts from the common *in vitro* susceptibility assays (*eg*, elevated IC_{50} value), have been shown. One suggested reason for this is that parasites are exposed to very low concentrations of dihydroartemisinin for 48-72 h in theses assays, whereas *in vivo* they are exposed to much higher concentrations for only 1-2h. Consequently, a novel *in vitro* assay was developed, particularly for detection of artemisinin resistance [31]. This, so-called ring stage survival assay (RSA) measures the susceptibility of 0-3 h post-invasion rings when exposed to a short pulse of high-dose DHA. The percent survival of these parasites is calculated at 72h relative to a control of dimethyl sulfoxide (DMSO) exposed parasites. The percent survival calculation is manually performed by counting the number of viable parasites in 10,000 erythrocytes in a blood smear. Although microscopy remains cheap and reliable, the method is laborious and time-consuming for application in the field and for large-scale epidemiological studies. Alternative ways of measurement have to be developed and adapted for use with ring stages under field-based conditions. Despite these limitations, RSA provided *in vitro* evidence for the reduced susceptibility to artemisinin in *P. falciparum* ring-stage parasites from western Cambodia, which until then had only been documented in *in vivo* studies and by parasite population genetic evidence [31].

Although all antimalarial sensitivity tests can be used, there is no ideal one yet for standard use, as each of them has advantages and limitations (Table 3). In the future, with improvement of existent assays or development of new ones it is expected that worldwide guidelines may be stablished as has occurred with antimicrobial susceptibility tests (Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines).

Table 3. Advantages and limitations of the available sensitivity assays for *P. falciparum* [14,15,25,39].

Sensibility Test	Advantages	Limitations
Drug efficacy trials 	• Reflects the real biological nature of treatment response • Reflects epidemiological situation • Results are easy to interpret • Require little equipment	• Long duration (14-28 days) • Host factors can interfere with the outcome • Does not allow quantitative assessment • Ethical and organizational problems
In vitro **Microscopy** 	• Requires little equipment	• Labor-intensive procedure • Very time-consuming • Highly trained personnel • Require 24-30h of incubation
Radioisotope methods 	• Semi-automated • Rapid, sensitive and accurate	• Radioactive • Expensive equipment • Required initially high parasitemia • Require 48h of incubation
Antigen detection 	• Rapid and reliable • Equipment relatively cheap	• Limited supplies of monoclonal antibodies • Require 48-72h of incubation
Flow cytometry 	• Rapid and accurate • Highly sensitive • Automated	• Expensive equipment • Highly specialized personnel • Require 48-72h of incubation
Fluorometric assay 	• Equipment more economical than flow cytometry	• Low sensitivity • Require 48-96h of incubation
Molecular techniques 	• Do not depend on viable parasites • Allows rapid analysis of several samples	• Sophisticated equipment • Highly trained personnel • Limited validated resistance markers

4. Novel flow cytometric hemozoin detection assay for antimalarial drug testing

4.1 The malaria pigment - Hemozoin

A distinct characteristic of the infected RBCs is the presence of hemozoin. Actually, this was the pigment that Laveran "followed" leading to the discovery of the malaria parasites within the RBCs. The dark color of this pigment also contributed to localize parasites in the gut of mosquitos enabling to complete the life cycle of malaria. When Hz was associated to malaria it was thought that it was melanin but this idea was later refuted by Brown who showed that malaria pigment was in fact hematin rather than melanin [58].

During the intra-erythrocytic life cycle, as the parasite grows, the degradation of the RBC contents increases. This occurs because *de novo* biosynthesis of amino acids is limited for the parasite and there are not sufficient free amino acids within the RBCs to sustain the synthesis of plasmodial proteins. As a consequence, the main source of amino acids for the parasite is the hemoglobin present in the RBCs. Besides the amino acids, the digestion of hemoglobin also releases free heme which the parasite converts by a crystallization process into hemozoin [59]. This conversion is so fast that the amount of free toxic heme never exceeds 1% of the total heme in the parasite when the hemoglobin digestion occurs at a constant rate [60].

Studies about the mechanism of Hz formation *in vivo* has been the cause of much discussion. There are several hypotheses proposed for the Hz crystallization but the most convincing is protein and lipid activity. Some believed that HRP II was the best candidate for catalyzing hemozoin formation [61]. However, it has been found that HRP II is present only in a small quantity within the parasite food vacuole where the Hz is formed [62]. Furthermore, Hz formation occurs normally in mutant clones and certain species of *Plasmodium* lacking genes for HRP II and HRP III [63]. Other studies have implicated lipids in Hz formation due to their ability to solubilize hematin [64]. However, the answer to the question how Hz is formed remains unclear. Currently, it is believed that Hz is formed by a biocrystalization process which implies both proteins and lipid membranes. Biocrystalization is a term used for Hz instead of biomineralization which is the process where inorganic salts were formed by the deposition of low molecular inorganic materials in living organisms [58].

Hz plays an essential role in malaria research. This pigment has specific features due to its crystalline nature. Of great importance is the capacity of Hz to depolarize light and its paramagnetic properties, because both facilitate its detection by optical methods [65, 66].

4.2 Hemozoin detection by flow cytometry

A flow cytometer is a commonly used instrument that allows to analyze several parameters of a particle in a fluid. It is composed by three main systems: the fluidics, the optics and the electronics. The purpose of the fluidic system is to transport particles in a stream to the laser beam one at the time. The optical system is made of one or more lasers which illuminate the particles present in a stream as they pass through. Optical filters and beam splitters are responsible to direct the light to the appropriate detectors. Then, electronic system converts the light signals into electronic pulses which can be processed by a computer. The basic principle of flow cytometry consists in detecting the refraction and scattering of light when a cell passes through the laser. This allow to measure parameters as the size, granularity/complexity and fluorescence of a cell. Light that is scattering in the forward direction is collected by a Forward Scatter (FCS) detector that converts intensity in voltage and gives the size of the cell. Light that is scattered to the side is detected by Side Scatter (SSC) and corresponds to granularity and cell complexity.

As a consequence of being birefringent, Hz is capable of rotating the plane of polarized light, a process called depolarization. Laser light, commonly used in flow cytometers, produce polarized light. To detect Hz, Frita *et al.* described a flow cytometer modification based on a creation of a second SSC that measures the depolarization of light. In front of this second SSC detector (depolarized SSC) a polarization filter was placed which is perpendicularly positioned to the incident polarized laser beam [67]. Therefore, depolarized SSC will not capture any light unless the analyzed cell contains Hz capable to depolarize the light. This modification was applied to the optical bench of the flow cytometer CyFlow (Figure 5) to develop a flow cytometric Hz detection assay for real-time sensitivity testing of *P. falciparum* [66].

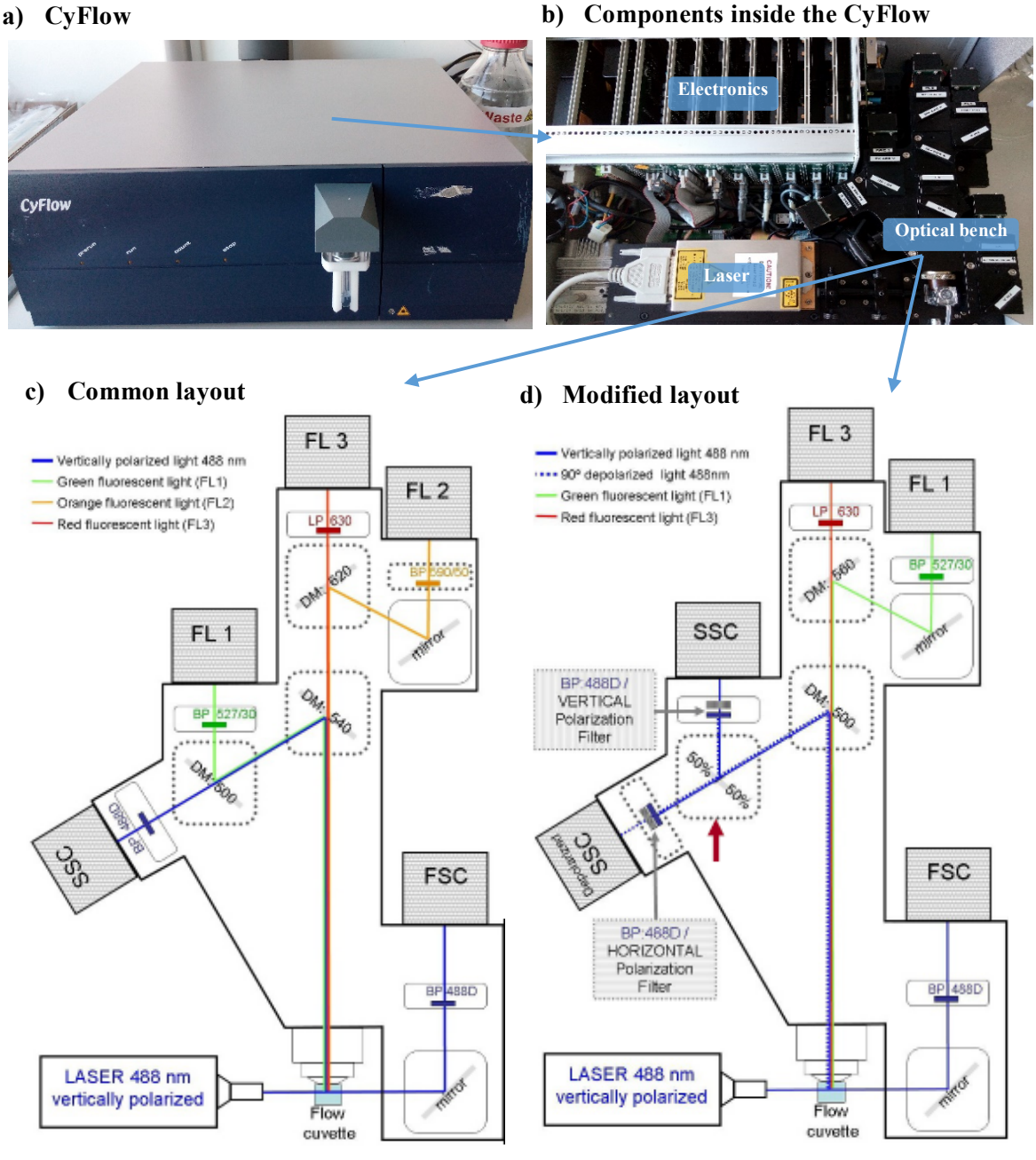


Figure 5. Flow cytometry modifications to detect light depolarization.
a) Image of the portable flow cytometer CyFlow Blue (Partec, Germany). The lid of the CyFlow can be easily removed. b) Inside the instrument: the electronic system, the laser and the optical bench. c) Optical bench common layout for detection of forward scatter (FSC), side-scatter (SSC), green (FL1), orange (FL2) and red (FL3) fluorescence. The optical bench can be easily modified by the operator. d) The modification layout to detect depolarized light instead of FL2 detection. Red squares represent dichroic mirrors that were changed, red arrow shows 50%/50% beam splitter placed between the two SSC each of them with a polarization filter in front (vertical for SSC and horizontal for depolarized SSC). Images c) and d) adapted from [67].

4.3 Using hemozoin detection by flow cytometry for antimalarial drug testing

Since the amount of Hz increases accordingly with the parasite maturation, it is possible to assess the parasite stage based on the amount of Hz that iRBCs contains. Consequently, if *P. falciparum* cultures were incubated over time with a certain drug, inhibitory effects could be detected measuring Hz present in the cells at different times of incubation. It was demonstrated that drug effects could be observed after only 18h of incubation for quinolines and endoperoxides in ring stage synchronized cultures with 1% parasitemia, while other assays require between 48 and 72 hours [66]. Other methods may work with lower parasitemias which is important in *ex-vivo* samples that can have very low parasitemias. One of the main advantages of this method is the earlier detection of drug effects and determination of IC50 values, which is done after only 24h of incubation [66].

Besides the speed, the fact that is reagent-free and does not require extensive sample preparation makes this method particularly interesting for the field. However, Hz levels in early ring forms circulating in blood of patients are below the threshold of the flow cytometer. Thus, it cannot be detected unless it is measured in bulk after a rigorous lysis of RBCs. Free synthetic Hz is easily detectable by flow cytometry but the detection of free hemozoin after a blood sample lysis was not investigated with this method so far [68].

Nevertheless, this novel method was already investigated *ex vivo* in a six-month study conducted in Gabon, Lambaréné [69]. Cultures of parasites obtained from blood samples of malaria patients were incubated with chloroquine, artesunate and artemisinin. Drug inhibitory effects were noticed in 39/46 (85%) of the samples tested of which 25 were detected at 24h and 14 at 48h of incubation [69]. The IC50 values obtained allowed the conclusion that chloroquine-resistant parasites are present in that region, although they remain sensitive to artesunate and artemisinin [69]. The Hz detection assay also proved to be an alternative assay for artemisinin testing *ex vivo*. However, the potential of the method to detect artemisinin resistance was not investigated yet, neither *in vitro* or *ex vivo*. The fact that the method detects drug effects much earlier than the other phenotypic assays can be relevant for artemisinin where the forms responsible for resistance are the very young parasites.

The study in Gabon confirmed the easy implementation and the success of this method under field conditions and gave support to the idea that a flow cytometer might not be an impediment to field application worldwide. Certainly, cytometers that collect light trough fiber optics cables such as BD LSRFortessa or FACSaria are more difficult to implement as they require other modifications to detect depolarization. However, a variety of flow cytometers exist now which can be easily modified, such as the Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA, USA), Accuri C6 (BDBiosciences, La Jolla, USA) and Cyflow Cube (Partec, Münster, Germany) [68].

Although it already surpasses some of other methods limitations, further optimizations can improve the usefulness of this method to possibly become the one considered ideal for antimalarial sensitivity testing.

5. Objectives and thesis outline

Considering all the facts mentioned in the introductory chapter, this thesis has two main objectives: 1) Explore the effects of lysing samples before depolarized side-scatter measurement and 2) Investigate artemisinin resistance *in vitro* by Hz detection assay using a different protocol.

The thesis is organized in five chapters. The following section is composed of the materials and methods that were used in the development of this thesis.

The results obtained are presented in the third chapter. The results section is sub-divided in two main parts, each of one inherent to the proposed objectives. The first part consists in: non-lysed and lysed samples detection of hemozoin by depolarized side-scatter, the assessment of inhibitory effects of chloroquine, piperaquine and dihydroartemisinin and the respective comparison of IC50 values obtained, and lastly, the determination of the limit of detection with lysed samples. The second part is composed by the detection of *in vitro* artemisinin-resistant parasites with the respective comparison of survival rates obtained and the detection of a delayed growth in the artemisinin resistant strain.

In the chapter four, the results are discussed and compared with previous data reported in the literature. Possible explanations for the observed results are presented.

Finally, the last chapter integrates the conclusions, highlights the unanswered questions and proposes what should be done in the future to clarify some aspects.

II. Materials and methods

All reagents were obtained from Sigma Aldrich (St Louis, US.), unless stated otherwise.

Uninfected human red blood cells were obtained from buffy coats that were collected weekly from the *Instituto Português do Sangue e da Transplatação*, Lisbon without any personal information. This study, including the origin and use of human blood, was approved by the Ethical Committee of the Faculty of Medicine, University of Lisbon.

1. Reagents and culture medium

1.1. Complete malaria culture medium (CMCM)

Complete malaria culture medium was prepared according to the recommendations of the Malaria Research and Reference Reagent Resource Centre (MR4) [70]. The culture medium consisted on 500 mL RPMI 1640 with $NaHCO_3$ and without L-glutamine (Gibco, Carlsbad, US.) supplemented with 500 µL of gentamicin 50 mg/mL, 5 mL of L-glutamine 200 mM, 12 mL of Hepes 1 M and 50 mL of Albumax II solution.

1.2. Albumax II solution

A volume of 500 mL of Albumax II solution was prepared with 5.2 g RPMI with L-glutamine and without $NaHCO_3$ (Gibco, Carlsbad, US.), 500 µL of gentamicin 50 mg/mL, 2.98 g of Hepes, 1.67 g $NaCO_3$, 1 g of glucose, 0.1 g of hypoxanthine (pre-diluted with a small amount of ddH_2O and a drop of 10 nM NaOH) and 25 g of Albumax II (Gibco, Carlsbad, US.). The pH of the solution was adjusted between 7.2 and 7.4. Albumax II solution was then filter sterilized (filter sterile 0.2 um), aliquoted in 50 mL tubes and stored at -20ºC to be thawed whenever CMCM was prepared.

1.3. Cryoprotective solution (Glycerolyte)

Glycerolyte was prepared with 57% of glycerol, 16 g/L of sodium lactate (VWR, Radnor, US.), 300 mg/L of potassium chloride and 25 mM of sodium phosphate (pH 6.8)

1.4. Sorbitol solution

Sorbitol was prepared by dissolving 5 g of sorbitol in 100 mL of distilled water and then filter sterilized.

1.5. Lysis solution

The lysis solution was prepared with 20 mM of NaOH and Triton X-100 at 0.063%. Stocks solutions of 475 mM NaOH and 10% Triton X-100 were previously prepared with distilled water. To prepare 1 L of lysis solution, 42 mL and 6.3 mL of NaOH and Triton X-100 stock solutions were mixed and distilled water was added until a total volume of 1 L was reached.

2. *Plasmodium falciparum* strains

All strains were provided by the Malaria Research and Reference Reagent Resource Centre (MR4) for distribution by BEI Resources NIAID, NIH.

3D7 – Laboratory-adapted susceptible strain contributed by Daniel J. Carucci;

IPC5188/MRA-1239 – Artemisinin-sensitive strain contributed by Didier Ménard;

IPC5202/MRA-1240– Artemisinin-resistant strain contributed by Didier Ménard.

3. *In vitro* culturing of *Plasmodium falciparum*

P. falciparum parasites were cultured and maintained in a tissue culture flask with daily exchanges of CMCM containing 5% of recently collected human erythrocytes under an atmosphere of 5% O_2 and 5% CO_2, at 37ºC. As uninfected controls, red blood cells from healthy donors were also cultured as described above. The level of parasitemia was assessed daily by microscopy and maintained at values lower than 2% by diluting parasitized erythrocytes with uninfected erythrocytes.

3.1. Preparation of uninfected erythrocytes

In a sterile process, the content of the buffy coats was transferred to 50 mL tubes and then centrifuged (Eppendorf Centrifuge 5810 R with Rotor A-4-62) at 1800 rpm for 5 minutes, without brake. The supernatant (plasma and white blood cells) was removed and RPMI-1640 (Gibco, Carlsbad, US.) was added to wash the cells by centrifuging them again at the same conditions as described above. Washing steps were repeated until the supernatant was clear. The pellet of red blood cells was aliquoted and store at 4ºC and used up to 4-6 days.

3.2. Sorbitol synchronization

Sorbitol synchronization was performed when *P. falciparum* continuous cultures reached a parasitemia of more than 2% with a minimum of 50% rings. First, the culture was centrifuged at 1800 rpm for 5 minutes and the culture medium was discarded. Then, a volume of 5% (w/v) sorbitol corresponding to 10x the volume of the pellet was added to the pelleted cells and incubated at 37ºC for 10 minutes. Next, the culture was washed twice in RPMI-1640 by centrifugation at 1800 rpm, for 5 minutes. Finally, CMCM was added to the pellet and the synchronized culture was maintained at the conditions described above.

3.3. Tight synchronization

Tight synchronization was performed as a sequence of sorbitol treatments with purification by Percoll gradient as represented in Figure 6. After three sorbitol synchronizations, synchronous schizonts were incubated for 15 minutes at 37ºC in RPMI-1640 supplemented with 15 U/mL of sodium heparin (Rotexmedica, Luitre, France). Then, the schizonts culture was purified on a 35%/75% (v/v) Percoll gradient, washed in RPMI-1640 and cultured with fresh uninfected erythrocytes. After 3 hours, cultures were treated again with sorbitol to eliminate the remaining schizonts. Finally, the hematocrit was adjusted to 2% and to 1%. These steps were performed immediately before the beginning of the Ring-Stage-Assay (see section 10).

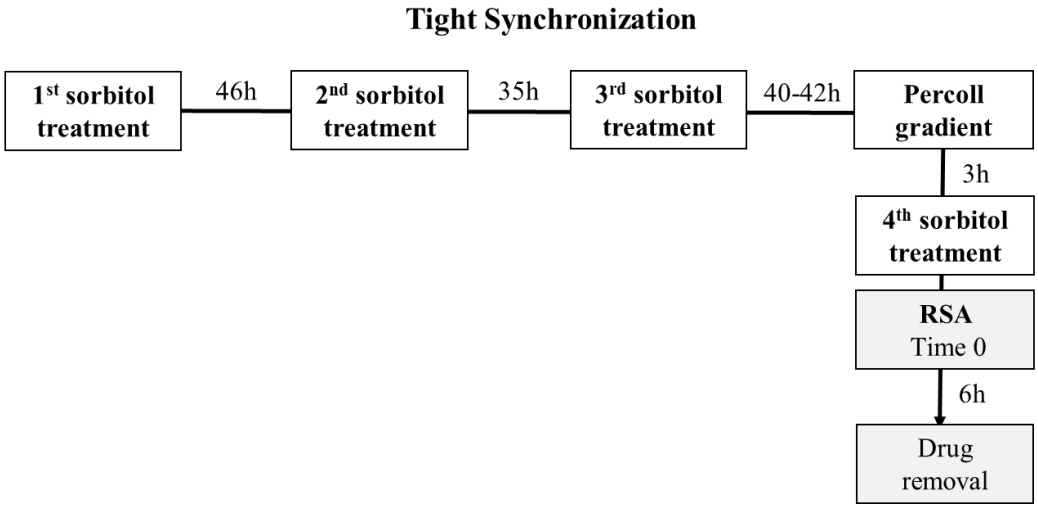


Figure 6. Schematic representation of tight synchronization protocol.
Tight synchronization was performed by a sequence of three sorbitol treatments separated by indicated intervals of time. These intervals between sorbitol treatments were stablished based on the microscopic observation of blood smears to determine when the new generation of ring form parasites started to appear. Percoll gradient was performed when the majority of schizont forms were segmenting. A fourth sorbitol treatment was done before the Ring-Stage Assay was performed. Synchronized parasites were incubated for 6 hours with the drug (dihydroartemisinin). After that period, the drug was removed.

3.4. Preparation of frozen stocks of *P. falciparum*

To prepare frozen stocks, highly parasitized cultures containing mainly ring form parasites were selected. Each culture was centrifuged at 1800 rpm for 5 minutes to remove the supernatant. The volume of the pellet was measured and the same volume of glycerolyte solution was gently added drop by drop while swirling the tube. Around 500-700 µL of the preparation was distributed into microtubes properly identified with parasite strain and date. Microtubes were then stored at -80ºC.

3.5. Thawing frozen stocks of *P. falciparum*

When required, frozen stocks were removed from cold storage and thawed at 37ºC for 1-2 minutes. The content of the microtube was transferred to a 50 mL falcon tube and the blood volume (V) was measured. Then, 0.1V of a 12% NaCl sterile solution was slowly added while swirling the tube. After 5 minutes, 10V of 1.6% NaCl sterile solution was carefully added dropwise, as previously described. The tube was then centrifuged at 1800 rpm for 5 minutes to remove the supernatant and pelleted cells were washed with 10V of RPMI-1640, again slowly, dropwise and while swirling the tube. After the centrifugation, supernatant was discarded and the volume of pelleted cells was measured and transferred into a culture flask with CMCM. The hematocrit was adjusted to 5% and the culture was incubated at 5% O_2, 5% CO_2, at 37ºC, and maintained as described in section 3.

4. Microscopy

Parasitemia, parasite maturation and synchronicity of the culture was assessed by the observation of blood smears stained with Giemsa (Merck, Darmstadt, Germany). Smears were fixed in absolute methanol and

stained with 10% Giemsa-solution prepared in 0.4% PBS for 20 minutes. The parasitemia was estimated by counting the number of total red blood cells and infected red blood cells, by bright field microscopy under oil immersion with a 100x objective. Microscopy pictures were taken using a Leica DM2500 (Leica, Solms, Germany).

5. SYBR green staining

A volume of 5 µL (~ 800 000 cells) of each culture sample was stained with 1x of a DNA-specific dye SYBR green I (Invitrogen, Carlsbad, USA). Samples were stained for 20 minutes, in the dark, after which a volume of 1 ml of PBS was added and samples were analyzed using a 535/45 nm bandpass filter.

6. Flow cytometer and the modification (depolarized side-scatter detection)

The Cyflow® Blue (Partec, Münster, Germany) is a portable five parameter flow cytometer with a blue laser excitation (488 nm) and detectors for: forward scatter (FSC), side scatter (SSC), green fluorescence (FL1), orange fluorescence (FL2) and red fluorescence (FL3).

For this study, the flow cytometer was modified as described elsewhere [68]. Basically, a second SSC detector was created with a 50%/50% beam splitter between the new and the pre-existent SSC detector. A polarizer was placed 90 degrees to the polarization plane of the laser light, in front of one of the SSC detectors, allowing detection of depolarized light. The setup is shown in Figure 5 (I. Introduction section).

7. Flow cytometric analysis

Flow cytometry data was analyzed using FlowJo software (version 9.0.2, Tree Star Inc., Oregon, US.). The gating strategy is shown in figure 8 (III. Results section). The red blood cells were detected by their characteristic forward (FCS) and side-scatter (SSC) (Figure 8a and 8b).

Flow cytometry modification allowed to assess parasite maturation by measuring the percentage of depolarizing events. These events were defined in plots of SSC versus depolarized-SSC (Figure 8c and 8d). The uninfected control was always used as a mean of comparison to exclude any background signal.

Parasitemia was assessed by analyzing the percentage of SYBR green positive cells. Plots green fluorescence (FL1) versus red fluorescence (FL3) were used to allow the differentiation between weakly stained cells and auto-fluorescence cells. Selection of positive events was done at each time-point using the uninfected samples stained with SYBR green, from corresponding time-point, as negative control (Figure 8e and 8f). Parasitemia was determined by quantifying the percentage of SYBR green positive cells.

8. Preparation of drugs

The final working solutions of all drugs were done on the day of the experiment. They had to be prepared twice as concentrated because they were posteriorly added to the same volume of the culture. The chosen drugs concentrations were selected based on previous experiments and/or IC50s values described in the literature [66,69].

8.1. Chloroquine (CQ)

A stock solution was prepared by dissolving powdered CQ in Milli-Q water. The stock solution was stored at -4ºC. Working solutions of 12, 25, 50, 100 and 200 nM of CQ were prepared by diluting the stock solution in CMCM.

8.2. Piperaquine (PQ)

Piperaquine was kindly provided by Sigma-tau (Pomezia, Italy). A stock solution was prepared by dissolving powdered PQ in Milli-Q water. The stock solution was stored at -4ºC. On the day of the experiments working solutions of 12, 36, 108, 324 and 972 nM were prepared by diluting the stock solution with CMCM.

8.3. Dihydroartemisinin (DHA)

A stock solution was prepared by dissolving powdered DHA in DMSO. The stock solution was aliquoted and stored up to 6 months at -20ºC. Working solutions of 0.24, 1, 4, 16, 64 nM were prepared for the lysis experiments. A working solution at 1400 nM was prepared and used for RSA.

9. Hemozoin detection sensitivity assay

9.1. Standard protocol

A volume of 100 µL of a blood suspension from a ring-stage synchronized culture at 2.5% hematocrit and approximately 1% parasitemia were incubated with the same volume of increasing concentrations of antimalarial drugs or with CMCM (for the drug free control and uninfected controls) in 96 well-plates, for 48 or 72 hours, at 37ºC in a 5% CO_2 atmosphere.

At each time-point, 5 µL of the blood suspension present in each well was used and stained with SYBR green as described above. Samples were analyzed in triplicates and in each measurement approximately 100,000 events were analyzed.

At specific time-points, 50% inhibitory concentrations (IC50) were calculated based on a nonlinear regression model (sigmoidal dose-response/variable slope) using the SigmaPlot – Systat Software (Chicago, US.).

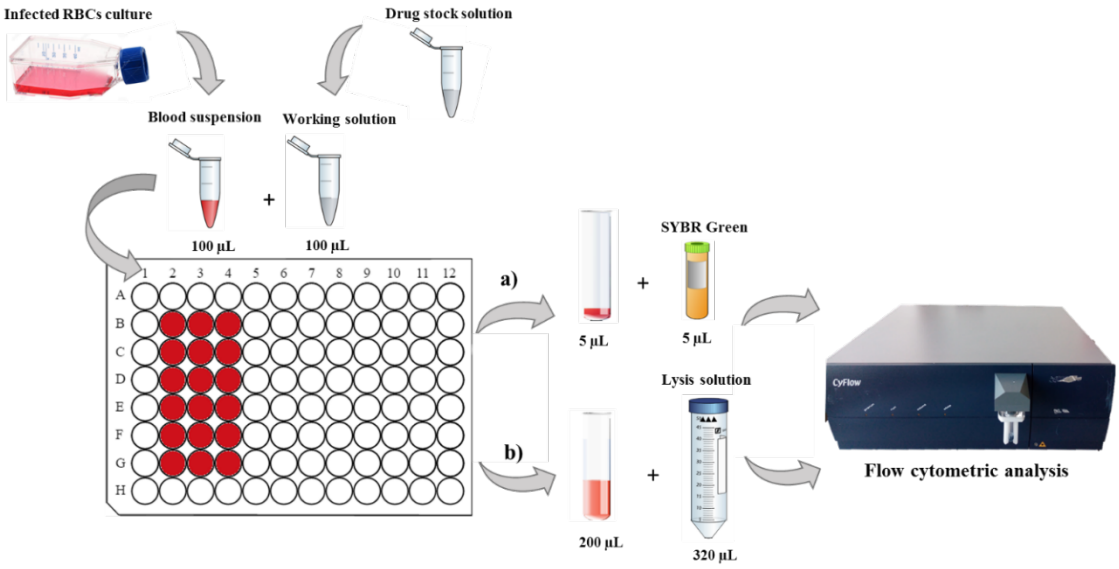


Figure 7. Schematic representation of hemozoin detection assay general protocol.
At each time-point, samples followed the steps represented in a) for standard protocol and in b) for the lysis protocol. Modified images from [71].

9.2. Lysis protocol

For lysis experiments, the same standard protocol was followed as described above but with an additional step of lysing the samples before the flow cytometric measurement was performed. Samples were lysed by adding to the content of each well 320 µl of lysis solution. For these experiments, individual plates were prepared for every time-point, because at each measurement the whole well volume of the well was lysed.

The limit of detection of the assay using lysed samples was measured using a schizont culture at 5% parasitemia and 5% hematocrit. The parasitemia was then diluted to 1%, 0.5%, 0.1%, 0.05% and 0.01% by adding uninfected RBCs.

10. Ring stage assay protocol

Tightly synchronized ring form cultures were incubated with 700 nM of DHA for 6 hours, as described elsewhere (Figure 6) [31]. After that period, the drug was washed away by performing three centrifugations at 1800 rpm for 5 minutes with RPMI-1640. Washed cells were put back in culture with CMCM. Parasite maturation and growth was assessed by flow cytometry at every 6 hours during a whole life-cycle (48h) and then at 72h of incubation. Microscopy was used to assess to parasitemia and parasite survival at 72h of incubation. Parasite survival was determined by counting the proportion of viable parasites (in approximately 2000 erythrocytes) that developed into second-generation rings or trophozoites with normal morphology at 72h after drug removal. The parasite survival rate was expressed as a ratio of viable parasites exposed to DHA in comparison to viable parasites in the drug free control.

III. Results

1. Lysis protocol optimization

1.1 Depolarized side scatter detects hemozoin in non-lysed and lysed samples

The flow cytometric analysis of non-lysed and lysed samples is shown in figures 8 and 9, respectively. Uninfected and infected erythrocytes were analyzed. For both non-lysed and lysed samples, depolarizing events are easily detectable in the infected samples in comparison with the uninfected control (Figure 8c, 8d, 9c and 9d). For non-lysed samples, the percentage of depolarizing events were 0.08% and 0.57% for the uninfected and infected cultures, respectively. After lysis of the exact same cultures, the difference in the percentage of depolarizing events between the uninfected and the infected samples was more evident with 0.3% and 8.2% of depolarizing events, respectively. Non-lysed samples were stained for the presence of DNA using SYBR Green. This allowed to confirm which depolarizing events found in the infected samples were indeed parasitized RBCs. Of note, the gain values and scales had to be adjusted for analyzing lysed samples since they had inherently different characteristics. All subsequent results are based on depolarizing events expressed as a percentage of all events analyzed.

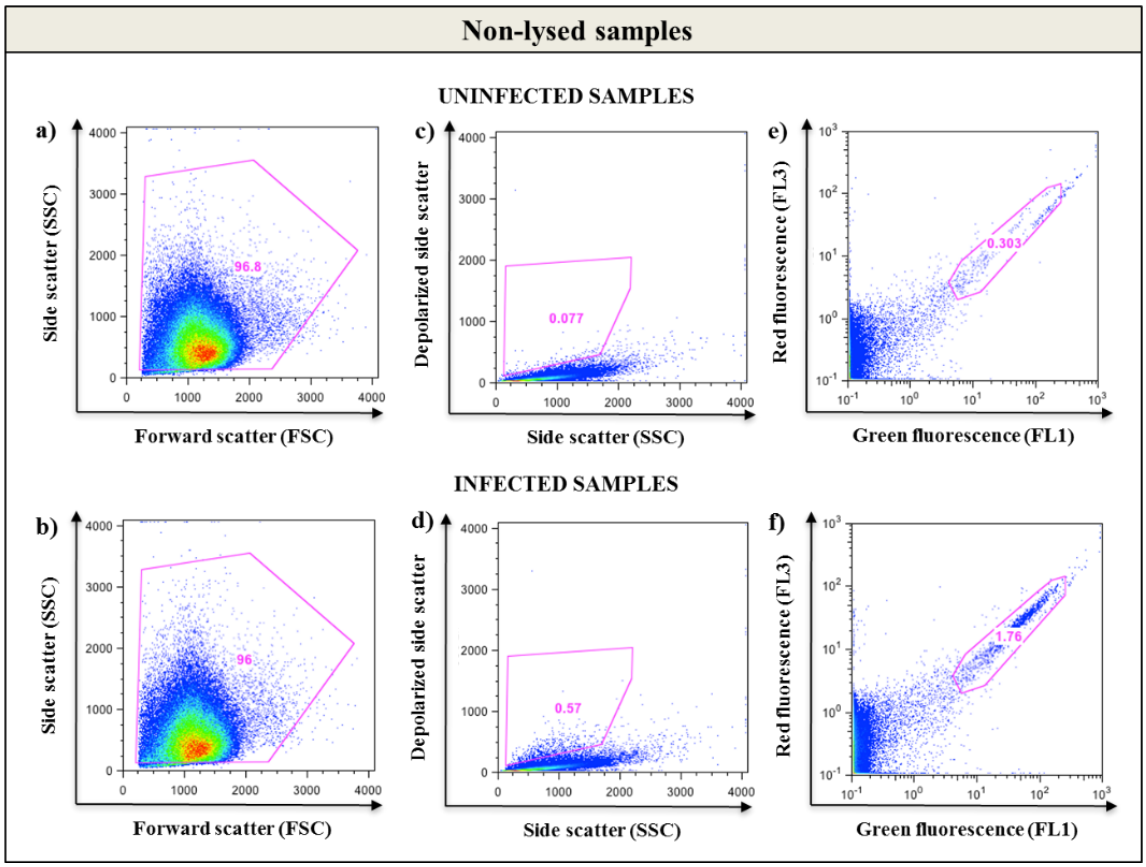


Figure 8. Gating strategy to select depolarizing parasitized red blood cells in a non-lysed *P. falciparum* culture. Flow cytometric analysis of an uninfected and an infected synchronized culture of *P. falciparum* (3D7) with 1% parasitemia, stained with SYBR Green, after 24h of incubation. Plots of forward vs. side scatter for the uninfected and infected cultures appear in figures a) and b). Corresponding plots of side scatter vs depolarized side scatter appear in figures c) and d). Figures e) and f) illustrate gates defining SYBR Green-positive cells.

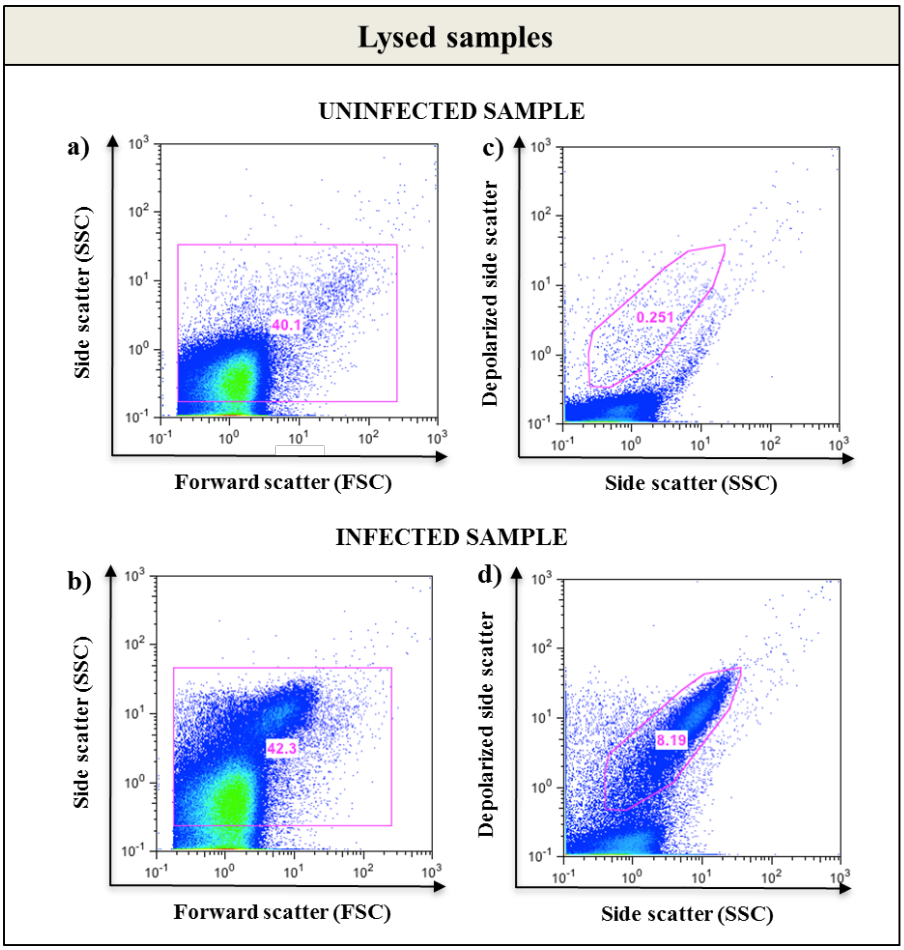


Figure 9. Gating strategy to detect depolarizing events in a lysed *P. falciparum* culture.
Flow cytometric analysis of an uninfected and an infected synchronized culture of *P. falciparum* (3D7) with 1% parasitemia, after 24h of incubation and subjected to the lysis procedure. Plots of forward vs. side scatter for the uninfected and infected cultures appear in figures a) and b). Corresponding plots of side scatter vs depolarized side scatter appear in figures c) and d). Gain values had to be adjust and scale was altered for logarithmic. A much higher number of depolarizing events is observed when compared to non-lysed sample and, consequently, a bigger difference between depolarizing events of uninfected and infected samples is detectable. It is of note that is also perceptible an increase of background events when compared with non-lysed samples.

1.2 Detection of antimalarial drug effects using lysed samples

Lysing the samples allowed to detect free hemozoin and to distinguish between infected and uninfected samples. In order to verify if drug effects could be detected earlier in lysed samples than in non-lysed ones, *P. falciparum* 3D7 parasites were incubated with several drugs and parasite growth was assessed at different times over a whole 48-hour life cycle. Measurements were performed every two hours between 6-14 hours of incubation to detect the first signs of drug effects. Simultaneously, non-lysed samples were measured, as a control, by hemozoin detection and SYBR Green I fluorescence at 24h and 48h of incubation for all antimalarial drugs tested. Time curves of *P. falciparum* 3D7 in the presence of chloroquine, piperaquine and dihydroartemisinin are shown in figure 10, 11 and 12, respectively.

Chloroquine

When exposed to increasing concentrations of chloroquine, the difference between inhibiting and non-inhibiting concentrations in *P. falciparum* 3D7 strain started to be visible after 8 hours of incubation, with the largest difference being observed at 36 hours (Figure 10b). Taking into account the standard deviations, chloroquine inhibitory effect could be clearly detected at 10h of incubation. At 12 hours the higher concentrations showed only 50% of the depolarizing effects obtained by the drug free control (Figure 10a). Corresponding values of 100 nM and 50 nM were 0.3% and 0.5% respectively, while drug free control corresponds to 1.1%.

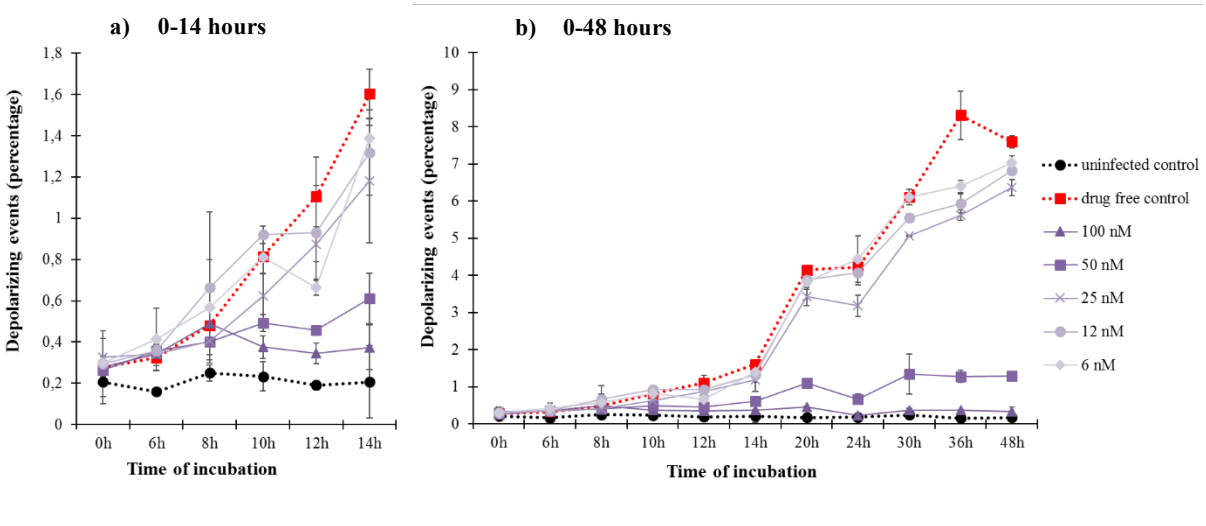


Figure 10. Time curve of *P. falciparum* in the presence of different concentrations of chloroquine.
Uninfected control and a ring-stage synchronized culture (0.5% parasitemia) of *P. falciparum* 3D7 strain were incubated for 48h with increasing concentrations of chloroquine (6 nM, 12 nM, 25 nM, 50 nM and 100 nM). In a) the time curve corresponding to the first 14 hours of incubation is represented. In b) the time curve until 48 hours of incubation time is represented. The samples were lysed before the measurement. Each time-point represents the mean value of triplicate measurements (± one SD).

Piperaquine

The inhibitory effects of piperaquine in lysed samples started to be detectable at 10 hours of incubation being more evident at 12h (Figure 11a). The percentage of depolarizing events at 12h of incubation for the concentrations of 486 nM, 162 nM and 54 nM were of 1.2%, 1.5% and 1.6% respectively, compared to the 2.5% of the drug-free control. A peak of depolarizing events occurs at 30h, and the highest peak at 48h of incubation where the highest difference between drug-free control and uninfected control was observed (Figure 11b).

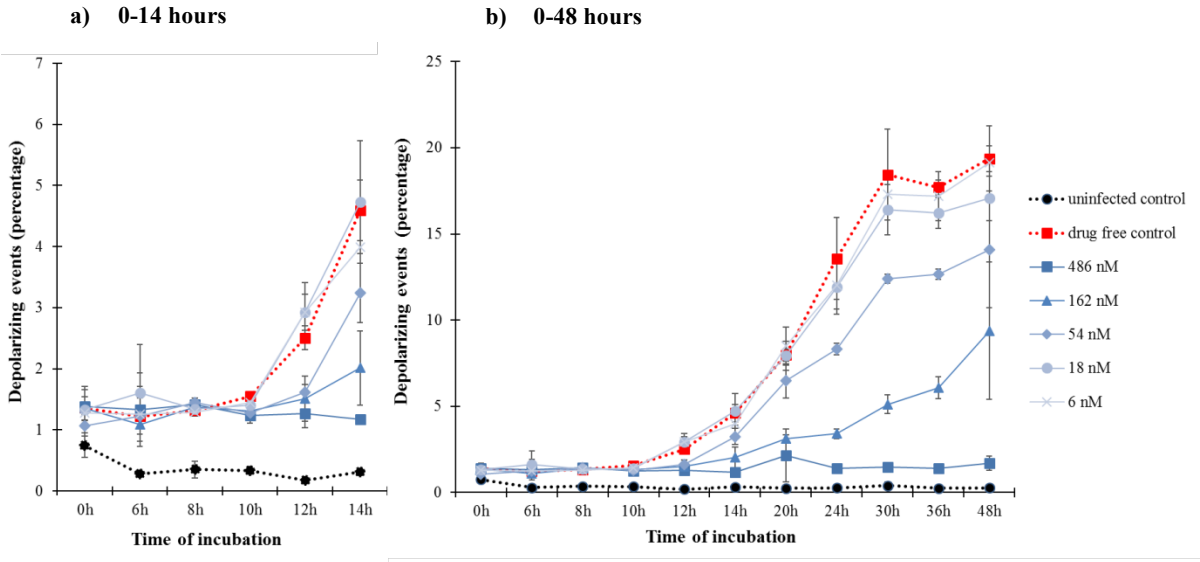


Figure 11. Time curve of *P. falciparum* in the presence of different concentrations of piperaquine.
Uninfected control and a ring stage synchronized culture (1% parasitemia) of *P. falciparum* 3D7 strain were incubated for 48h with increasing concentrations of piperaquine (6 nM, 18 nM, 54 nM, 162 nM and 486 nM). In a) the time curve corresponding to the first 14 hours of incubation is represented. In b) the time curve until 48 hours of incubation time is represented. The samples were lysed before the measurement. Each time-point represents the mean value of triplicate measurements (± one SD).

Dihydroartemisinin

Regarding dihydroartemisinin, the difference between inhibiting concentrations and drug free control could be detected at 12 hours of incubation but was more evident at 14 hours, considering the standard deviations (Figure 12a). At the 14h time-point, the percentage of depolarizing events corresponding to the highest concentrations of 32 nM and 8 nM were 2.2% and 2% contrasting with 3.7% for the drug free control. This variation is even more evident at 20h where, at concentrations of 32 nM and 8 nM, the percentage of depolarizing cells was 1.4% and 2.7%, while in the drug free control is 8.5%. The drug-free time curve showed two peaks of depolarization that occurred at 30h and at 48h of incubation. (Figure 12b).

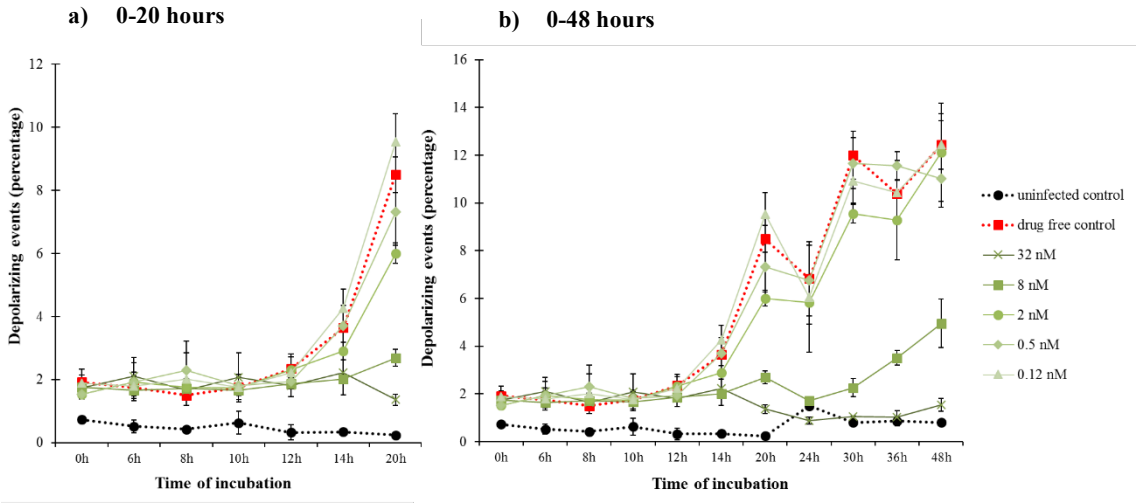


Figure 12. Time curve of *P. falciparum* in the presence of different concentrations of dihydroartemisinin.
Uninfected control and a ring stage synchronized culture (0.8% parasitemia) of *P. falciparum* 3D7 were incubated for 48h with increasing concentrations of dihydroartemisinin (0.12 nM, 0.5 nM, 2 nM, 8 nM and 32 nM). In a) the time curve corresponding to the first 20 hours of incubation is represented. In b) the time curve until 48 hours of incubation time is represented. The samples were lysed before the measurement. Each time-point represents the mean value of triplicate measurements (± one SD).

1.3 Comparison of IC50 values

The analysis of the *P. falciparum* sensitive-strain (3D7) in the presence of chloroquine, piperaquine and dihydroartemisinin, shows that it was possible to observe the first signs of drug effects after only 8h of incubation for chloroquine, 10h for piperaquine and 12h for dihydroartemisinin. The IC50 values were calculated at different incubation times and compared in Table 4 to determine the earliest time-point that would allow to reliably calculate IC50 values. Some values could not be determined because at these time-points, the maximum concentration of the drug tested did not inhibit 50% of the parasite population.

The IC50 values calculated for chloroquine were approximately the same for all incubation times tested, except for the 10h time-point which was higher. Although piperaquine showed similar values for 14h and 20h, the 12h time-point revealed a much higher value than the other incubation times. Even though it was only possible to calculate IC50 values for 20h and 24h, the values obtained for dihydroartemisinin were approximately the same. For all the drugs tested, the lowest IC50 value was obtained for the later time-point while the highest value was calculated for the earlier incubation period.

Table 4. IC50 values of antimalarial drugs tested against P. falciparum (3D7) at different time-points measuring lysed samples.

Drug	IC50 - Lysed samples				
	10h	12h	14h	20h	24h
Chloroquine	94,7 nM	42,9 nM	41,4 nM	42,2 nM	34 nM
Piperaquine	*	330,2 nM	119,6 nM	116,8 nM	79,3 nM
Dihydroartemisinin	*	*	*	4,6 nM	3,5 nM

*values were not determined because the maximum concentration of the drug tested did not inhibit 50% of the parasite population.

1.4 Determination of the lower Hz assay detection limit using the lysis protocol

The comparison between an uninfected lysed sample with an infected lysed sample showed a depolarizing population that could be identified and gated. Additionally, measuring lysed samples provided an earlier detection of antimalarial drug effects. Therefore, the limit of detection of Hz detection assay using lysis protocol was investigated. Five different parasitemias of 0.01%, 0.05%, 0.1%, 0.5% and 1% were investigated (Figure 13). The higher parasitemia tested (1%) corresponded to the higher percentage of depolarizing events (28.3%). The lower parasitemia tested (0.01%) showed no evident difference from the uninfected control. However, the depolarizing events present in the sample with a 0.05% parasitemia were clearly above (3.8%) the uninfected control (0.3%).

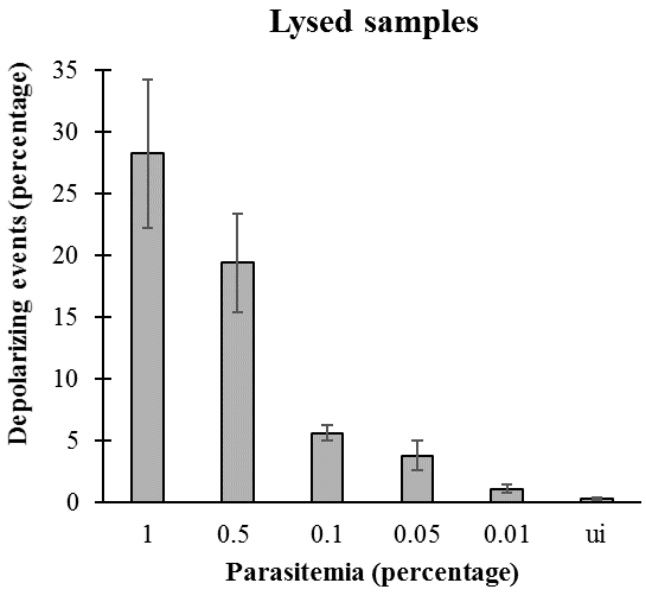


Figure 13. Testing the lower limit of detection of the hemozoin assay using the lysis protocol.
Flow cytometric analysis of the total percentage of depolarizing events measuring different parasitemias of a *P. falciparum* culture (3D7) with a majority of schizont forms. Results are acquired immediately after the samples were lysed and each parasitemia was tested in triplicates. ui – uninfected control

2. The potential of the Hemozoin assay to detect artemisinin resistance *in vitro*

2.1 Detection of *in vitro* artemisinin-resistant parasites – Ring Stage Assay

The potential of the hemozoin assay to detect artemisinin resistance *in vitro* was explored. After a rigorous synchronization of the cultures, it was investigated if it was possible to distinguish an artemisinin resistant parasite from a sensitive parasite when exposed to the drug concerned (Figure 14).

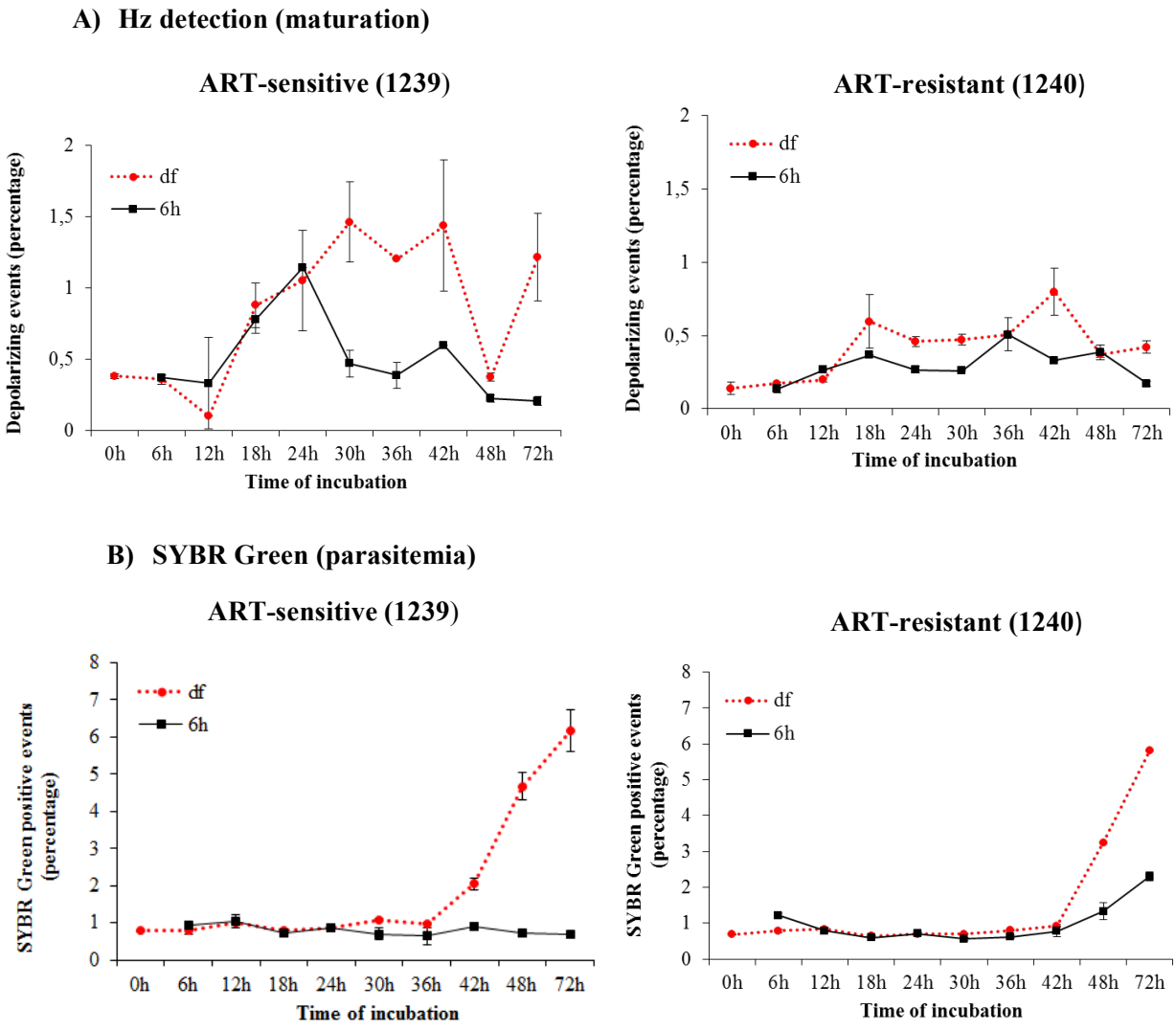


Figure 14. Comparison of inhibitory effects of DHA on sensitive and resistant parasites.
A drug free control (red spotted line) and tightly synchronized cultures of *P. falciparum* 1239 and 1240 strains (black lines) were incubated for 72h in the absence of drug treatment and after a 6h pulse of DHA 700 nM, respectively. Effects were evaluated by A) flow cytometric Hz detection assay and B) SYBR Green analysis. The measurements were performed every 6h until the 48h and then at 72h of incubation. Each time-point represents the mean value of triplicate measurements (± one SD).

An assay with a high-dose pulse of DHA for 6h was performed (RSA) where two strains were tested: an artemisinin sensitive strain (1239) and an artemisinin resistant strain (1240). Parasite development was assessed by the Hz detection method (Figure 14A) and simultaneously by SYBR Green (Figure 14B), at every 6h during a 48h life cycle. With the Hz detection method, the percentage of depolarizing events decreased at 24h in the sensitive parasites while in the resistant parasites the percentage of depolarizing events was similar to the drug free control. With SYBR Green it was possible to clearly distinguish the resistant strain, after 42hours of incubation, when the parasitemia increased similar to the curve of the drug free control; while the sensitive strain maintained, approximately, the initial parasitemia.

2.2 Comparison of survival rates between artemisinin-resistant and artemisinin-sensitive parasites using different methods

In the RSA survival rates are calculated instead of IC50 values, because only a concentration of the drug is tested. The survival rates of three different *P. falciparum* strains (3D7, 1239 and 1240) were calculated using different methods: 1) Hz detection assay: measured maturation at 30h of incubation (Figure 15A); 2) SYBR Green analysis: measured parasitemia at 48h of incubation (Figure 15B); and 3) Microscopy: assessed parasitemia at 72h of incubation (Figure 15C). All the methods showed a higher percentage of survival in the artemisinin resistant parasites in comparison to the lower survival rates detected in the artemisinin sensitive parasites. However, the difference between resistant and sensitive strains was higher when survival rates were determined by microscopy. In the Hz detection assay, resistant parasites showed a survival rate of 54% while the sensitive 1239 strain had 30% of survival.

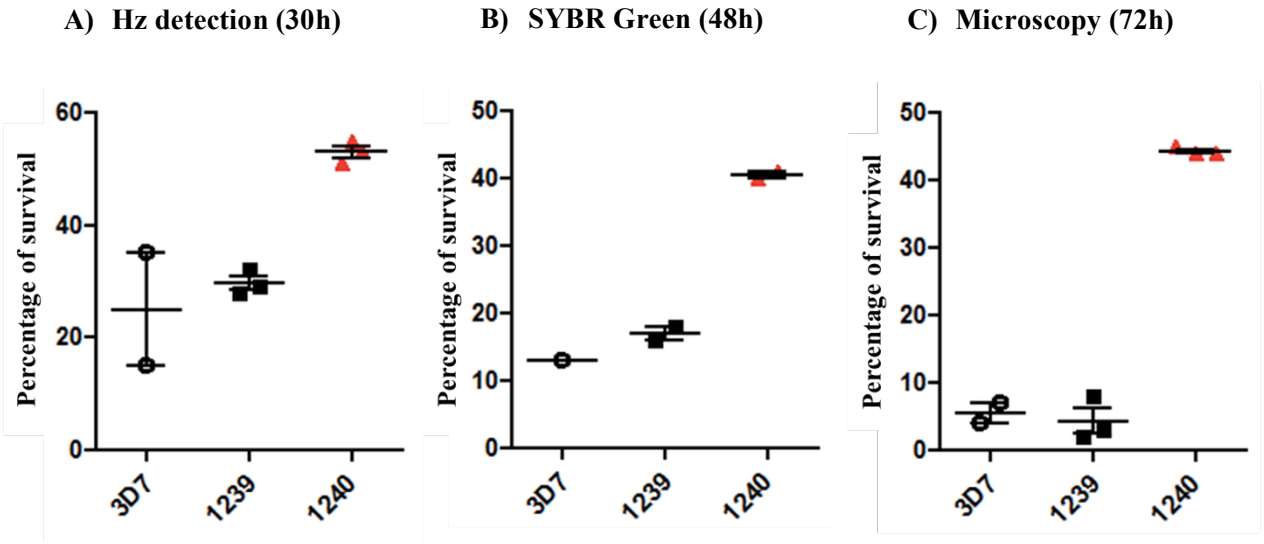


Figure 15. Artemisinin-resistant and artemisinin-sensitive parasites survival rates.
Survival rates obtained by A) Hz detection at 30h, B) SYBR Green at 48h and C) Microscopy at 72h. Each symbol represents one experiment and the respective percentage of survival obtained. Black circles and squares corresponds to sensitive strains (3D7 and 1239) while the red triangles correspond to resistant strain (1240). For each strain, the mean value of survival rates of different experiments is presented.

Similarly the same was observed by SYBR Green analysis where the percentage of survival were of 46% and 18% for artemisinin resistant (1240) and artemisinin sensitive parasites (1239), respectively. The difference was more evident by microscopy where the median survival rates determined for resistant parasites were 44% and for sensitive parasites were 5%. The survival rates of the sensitive culture-adapted 3D7 strain were comparable to the ones obtained for the 1239 artemisinin-sensitive strain.

2.3 Detection of delayed growth in artemisinin-resistant parasites

The development pattern of artemisinin-sensitive and artemisinin-resistance parasites was monitored *in vitro* over 48h. The parasite development of three different *P. falciparum* strains was analyzed by hemozoin detection (Figure 16A), by SYBR Green (Figure 16B) and by microscopy (Figure 16C). Parasites were tightly synchronized and their development in the absence of drug treatment was assessed every 6 hours during a whole life cycle of 48 hours and at 72h of incubation.

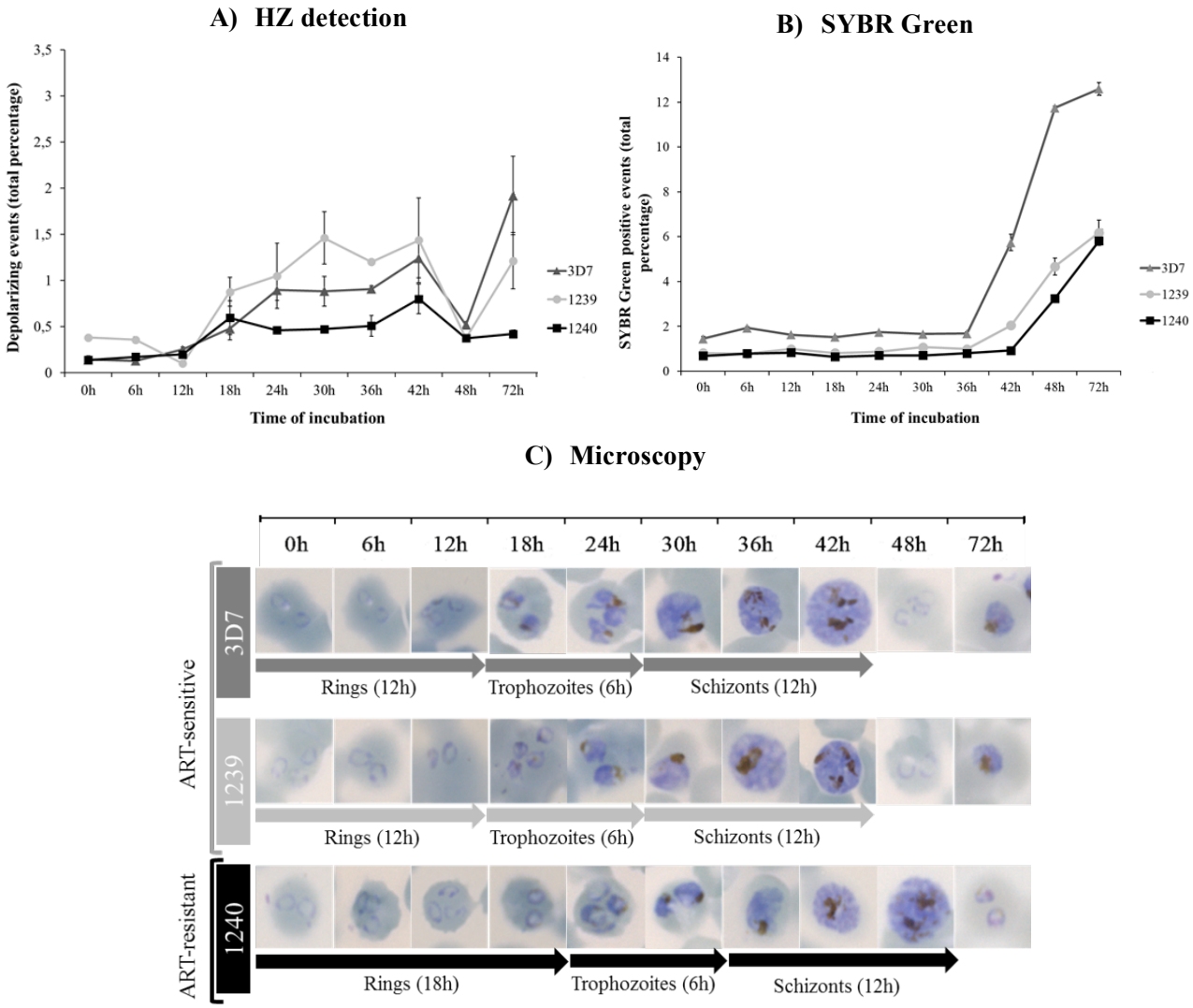


Figure 16. Assessment of parasite development by different methods.
Strains represented in grey corresponds to artemisinin-sensitive parasites (3D7 and 1239) while artemisinin resistance strain (1240) corresponds to the black color. Parasites development was assessed by A) flow cytometric Hz detection assay analysis B) SYBR Green analysis and C) microscopy observation of blood smears. In A) the percentage of depolarizing events (hemozoin-containing cells) is shown which is related to parasite maturation over time. In B) the SYBR Green positive events are shown which correspond to parasitemia. Each time-point represents the mean value of triplicate measurements (± one SD). In C) pictures of blood smears representatives of each time-point show the maturation forms of parasites.

All methods showed that artemisinin resistant parasites had a longer cycle in comparison to the artemisinin sensitive strains. With SYBR Green and microscopy it was observed that artemisinin-resistant parasites have an approximately 6h delay in comparison to artemisinin-sensitive ones (Figure 16B and 16C). Microscopy showed that artemisinin resistant parasites had a ring-stage phase of 18 hours instead of 12 hours observed in the artemisinin sensitive strains. The other stages of development seem to have the same duration in all strains. The difference between resistant and sensitive strains could only be detected after 72 hours of incubation by the Hz detection assay.

IV. Discussion

Currently, there are different approaches for the assessment of *P. falciparum* susceptibility *in vitro*, as reviewed elsewhere [15,39]. These approaches are essential tools in basic research, drug development, epidemiology of drug resistance and importantly, to provide guidelines for public health policies. Although several methods exist, the limitations of each of them makes it difficult to determine a single ideal method. The WHO microtest and the isotopic assays have been used for more than two decades and have proven their reliability in the field and in well-equipped research laboratories, respectively [39]. However, both have limitations: the WHO microtest is laborious and results are subjective; and the isotopic assays has low sensitivity and involves radioactive material [39]. Flow cytometry is also a reliable technique to assess the parasite maturation and sensitivity to antimalarial drugs. However, it usually relies on the use of expensive reagents and may even require more sophisticated flow cytometers with different light sources [39]. Another important assay is based on the detection of the parasite HRPII which is easily perform with a simple commercial ELISA kit, and even has a low limit of detection. Despite these advantages, it requires expensive reagents, such as antibodies [39]. Furthermore, all these methods have long turn around-times (48-72h) [14]. Altogether, these limitations are a driving force for the continuous development of new and more accurate and rapid sensitivity assays.

The presented method is based on a flow cytometer modification that allows the detection of Hz without the need for additional reagents. The use of Hz as a maturation indicator dates back to the 1980s when a simple visual agglutination test was designed [10]. The amount of dark-pigmented precipitates (Hz aggregations) produced by parasites within the RBCs became visible to the naked eye after a lysis process. However, this was not very reliable due to the interference of leukocytes with precipitate formation [14]. Recently, an improvement of this method was reported where Hz is measured by a colorimetric assay [72]. It also involves lysing the cells to liberated Hz which is then transformed into heme and the absorbance was read. However, the multiple manipulation steps involved may introduce some variability in results.

What makes Hz attractive as a marker, besides being a product of the parasite maturation, is the optical and magnetic properties of this crystal which can be measured using different approaches. For example, a novel magneto-optical technology (MOT) is under development and was tested in our laboratory [73]. This method consists in detecting Hz via the magnetically induced rotational motion of the crystals. For that, a rigorous lysis of the RBCs is performed to release all the intracellular hemozoin. The advantage of this method is the very low limit of detection reported to be capable of detecting 0.0008% parasitemia [73]. However, it is complex and each measurement is time-consuming.

The optical properties allow Hz to depolarize light which can be detected by microscopy or flow cytometry. In 1999, it was reported that Hz could be detected within leucocytes with a flow cytometry based full-blood-count analyzer Cell Dyn® (Abbott, Santa Clara, CA) [74]. Studies about detecting Hz inside infected RBCs were posteriorly reported using the same equipment [75, 76]. Based on these findings, Frita *et al.* described a simple way to modify a standard bench-top flow cytometer to allow depolarized Side-Scatter measurements, and they showed that Hz could be detected within *P. berghei* infected RBCs [67].

Depolarized SSC allowed not only to detect infected RBCs easily, but also to distinguish different maturation stages of the parasite and detect drug effects.

These results were the basis for the development of the novel Hz detection assay which already proved to be reliable in detecting Hz within the intact RBCs and assessing to inhibitory drug effects on *P. falciparum* [66,77]. In this study, the effects of lysing samples were explored in some aspects: detection of drug effects, respective calculation of IC50 values and improvement of the limit of detection. In addition, the potential of the method to detect artemisinin resistance was also investigated. First, a different protocol of cultures synchronization and drug incubation was tested. The main objective was to determine if using the Hz detection method, it would be possible to differentiate between artemisinin-resistant and artemisinin-sensitive strains. Survival rates of resistant and sensitive parasites were calculated and compared between other methods also performed alongside with Hz assay. Lastly, a growth curve of the two strains was performed in order to investigate if Hz assay is capable to detect a delay growth of resistant parasites in the absence of the drug as it has been described.

Blood sample lysis allow to detect inhibitory effects of antimalarial drugs earlier

A previous study concluded that free crystals of synthetic Hz can be easily analyzed by the Hz detection assay showing a wide range of depolarized side-scatter [68]. Based on this, it appeared interesting to investigate the detection of free Hz obtained after a blood sample lysis. A simple experiment was performed using the same protocol and the same lysis solution used in the MOT method [73]. The results showed that free Hz from a *P. falciparum* lysed culture can also be easily detected with the Hz assay. Similar to synthetic Hz, it also showed a wider distribution of depolarizing events comparing to non-lysed samples (Figures 8 and 9). Unsurprisingly, due to the strong cell lysis, background events also increased and sometimes it was difficult to separate depolarizing population from non-depolarizing. However, it was easy to differentiate infected from uninfected lysed samples (Figures 9c and 9b)

Then, a step of blood lysis was included in a standard experiment to detect antimalarial drug effects. Rebelo *et al.* described that drug effects can be consistently detected at 18 hours of incubation in a standard non-lysis protocol [66]. The results obtained in this study suggest that drug effects could be clearly detected at 10h for chloroquine, 12h for piperaquine and 14h for dihydroartemisinin (Figures 10, 11 and 12). This means that the lysis procedure decreased the time of incubation needed to observe inhibitory effects by 8 to 4 hours depending on the drug tested. Interestingly, the first sign of drug effects for chloroquine could be detected starting at 8 hours of incubation which was never reported by other methods. Although 10-14 hours is still a long period, it comes closer to the ideal of a “same-day” assay read-out.

The differences observed between the antimalarial drugs tested may be explained for several reasons. First, the results of this proof-of-concept study are based on few experiments. Certainly, a larger number of repeated experiments are necessary to validate these results. Another reason for differences between the drugs might be inherent to the mechanism of action of each drug tested. However, it would be expected that chloroquine and piperaquine have a similar behavior since they belong to the same class of 4-aminoquinolines. Interestingly, these drugs allowed the earliest detection of inhibitory effects but they still

differed by two hours. The possible reason for the obtained results is based on the number of cells analyzed per sample where small variations can be introduced. In the standard protocol for each sample measurement only 5µL of the culture is used. The lysis protocol implies lysing the whole well of the culture, meaning that 200µL is used (Figure 7 in II. Materials and Methods section). Consequently, with the lysis protocol we analyze free Hz in bulk coming from the lysis of a number of cells forty times higher than the number of intact cells analyzed in the standard protocol. Therefore, when the standard protocol is performed, the amount of Hz within each cell may not be sufficient to be detectable in early time-points. However, if we lysed the samples even if the amount of Hz per cell is lower, the quantity of Hz coming from the total number of cells can be enough to be detected. Thus, the inhibitory effects of the drugs can be detected earlier.

Results from the standard protocol published before show that depolarizing events reached a peak at 30h which corresponds to the maximum maturation of the parasites [66]. When analyzing lysed samples, a peak at 30h or 36h (in the case of chloroquine) is observed and no accentuated decrease is detected at 48h, as it has been previously observed in non-lysed samples [66]. In this case, when lysed samples are analyzed the total amount of hemozoin present in the sample will be detected, therefore a cumulative effect of the Hz produced from different cycles is quantified. Unlike what happens in the standard protocol (non-lysed) where only the hemozoin that is present within each cell is being measured. Consequently, non-lysed samples growth curves showed a decrease on depolarizing events at 48h. Regarding the delayed peak of depolarization that was observed at 36h in the chloroquine experiment, this could be explained by the fact that parasites used in this assay were probably younger and thus their maximum of maturation was reached later than the ones observed in the other experiments.

IC50 values obtained with lysed samples are comparable with other assays

The IC50 values acquired performing the lysis protocol were comparable to the ones reported by other validated assays (Tables 4 and 5). Even though it was possible to detect drug inhibitory effects earlier, the analysis of IC50 values reported for the same strain and drugs, led us to consider that results which were more comparable with the literature were those obtained with the time-point of 24h of incubation (Tables 4 and 5). Overall, the IC50 values tend to decrease from the earlier to the later time-point.

For chloroquine, the IC50 value for the 24h lysed samples (34 nM) was identical to what was obtained by our controls (HZ detection standard assay and to SYBR Green). In the literature IC50 values for the same strain (3D7) have been described ranging from 6.5 nM to 40.5 nM with different methods (Table 5). However, even using the same assay there is a poor correlation between different studies, that was also common to the other drugs tested. For instance, [H3]hypoxanthine standard method shows values from 6.5 nM up to 33.5 nM for chloroquine (Table 5). Even though values obtained in this study were within the range already reported it is important to note that comparing IC50 can be misleading for several reasons. Owing to the peculiarities of each assay, it is impossible to perform them all in the same conditions. For instance, in our method parasitemias of around 1% were used and compared with methods that use lower parasitemias ranging from 0.25 to 0.5%, as the standard [H3]hypoxanthine method [41]. When a higher

number of parasites is inoculated the inhibitory drug concentration for artemisinin, artesunate, chloroquine and mefloquine tend to increase, which is called inoculum effect [78,79]. Our results suggest that this can also occurs for piperaquine. The IC50 value of 24h lysed samples for piperaquine (79.3 nM) was higher than the one obtained by our controls and the ones found in the literature, between 16.9 nM and 36.9 nM for the standard method of [H3]hypoxanthine incorporation (Table 5). On the other hand, IC50 values of lysed samples for dihydroartemisinin were closely paralleling those obtained by the other methods tested as well as with the results in the literature reviewed (Table 5).

Considering the table above and our results, it appears that, overall, our method obtains increased IC50 values. However, it is important to reinforce the idea that reliability of the assays can be influenced by the mechanism of action of the drug [80] as well as the initial parasitemia, the hematocrit and the end-point for measuring parasite growth [39,78].

Table 5. IC50 values of several antimalarial drugs against *P. falciparum* culture-adapted strain (3D7) determined by different in vitro sensitivity tests.

Drug	[H^3]hypoxanthine	WHO schizont maturation assay	SYBR Green	HRPII- assay	pLDH-assay
CQ	15.7 nM [54] 9.56 nM [80] 33.5 nM [81] 29.6 nM [82] 22.76 nM [83] 6.5 nM [84]	14.79 nM [80]	11.54 nM [80] 8.1 nM [85] 22.2 nM [86] 31.10 nM [87]	10.10 nM [80] 7.5 nM [85]	14.97 nM [80] 40.5 nM [81]
PQ	16.9 nM [82] 36.90 nM [83] 27 nM [84]	–	26.33 nM [87]	–	–
DHA	5.27 nM [54] 4.2 nM [82] 2.0 nM [84]	–	3.78 nM [86] 2.30 nM [87] 22.1 nM [88]	2.30 nM [89]	–

Lysing samples improves the limit of detection

Taking into account the results obtained it became interesting to evaluate if the limit of detection of the Hz assay would change when the samples were lysed. Although the advantages of the Hz detection assay regarding the other methods, one of the major limitations of the method is the higher detection limit of 0.3% parasitemia [66]. Particularly if the blood is taken directly from the patients (*ex-vivo*) which can have parasitemias lower than the method detection threshold. Results obtained in the current study demonstrated that it was possible to decrease the limit of detection when the blood samples are subjected to a rigorous cell lysis.

When the lower limit of detection with the lysis procedure was investigated, it was shown that a parasitemia of 0.05% is still detectable by depolarized SSC (Figure 13). This means that lysing the samples can improve

the limit of detection of the presented method from 0.3% to 0.05% parasitemia. Other commonly used assays as HRP II or WHO schizont maturation test can detect parasitemias as low as 0,01% [46]. The novel MOT method can even detect lower parasitemias [73]. However, 0.05% is below the limit threshold of the standard [H^3]hypoxanthine method (0.25-0.5%) [41] and SYBR Green (0.5-1%) assays [48, 84, 90].

The hemozoin detection assay can detect artemisinin resistant parasites

A reduction in the sensitivity of the parasite ring stage without a corresponding loss of sensitivity in mature stages can explain the failure of standard sensitivity tests to detect artemisinin resistance. Consequently, the Ring Stage Assay was developed and proved to be capable of detecting artemisinin resistance [31]. This assay differs from the others essentially in the conditions of the culture tested and in the way how we subjected them to the drug in cause. The output of the assay is expressed in survival rates rather than IC50 calculations. *In vitro* higher survival rates were correlated to slow-clearing parasites and lower survival rates to fast-clearing parasites [31].

The artemisinin resistant (1240) and sensitive (1239) strains were reported to have survival rates of 88% and 0.1%, respectively [91]. However, results obtained in this study using the same assay (RSA 0-3h) were not consistent to the ones described since the median survival rates determined were 44% for resistant and 5% for sensitive strain (Figure 15C). Even though there is evidence for heritability in artemisinin resistance, the phenotype appears unstable, so it could be partially lost over time [92]. However, in most studies survival rates of artemisinin resistance strains tend to range between 4%-30% [31,93,94].

Despite the success of the RSA in detecting artemisinin resistance, it requires a complex and meticulous synchronization protocol, and most importantly, it depends on tedious microscopy readouts. This entails the subjectivity of the results and is very laborious and time-consuming. By performing a 0-3h RSA, it was possible to distinguish at 30h of incubation a resistant parasite from a sensitive parasite in the presence of the drug with the Hz detection method (Figure 15A). Survival rates obtained with the Hz assay showed higher values for resistant parasites than for the sensitive strain (1239). However, the difference between the strains was lower than the values obtained by microscopy. The increased survival rates of both sensitive and resistant parasites determined by the Hz assay might be due to the fact only 30% of all parasites were typically detected by depolarization measurements as compared to microscopy or SYBR Green. There is no clear explanation for that but a possible reason is the fact that only parasites that contain a high amount of Hz were detected. Thus, a mature parasite with less Hz might not be considered within the depolarization population. Consequently, the drug free control used to calculate these survival rates may not represent the whole population of parasites.

Survival rates obtained by SYBR Green analysis showed increased values for resistant parasites although the difference between strains was also higher by microscopy. The increased survival rates obtained by SYBR Green might be explained by the fact that this method, contrarily to microscopy, does not distinguish a viable from a dead parasite because the stain binds to both. Accurate results between microscopy and flow cytometry-based analysis can be obtained by a different approach that requires the use of two stains

and two lasers in order to distinguish pyknotic from viable parasites. Still, the range of survival rates reported for artemisinin resistant strains were between 1% and 30% [95].

A controversial characteristic of the artemisinin resistance phenotype that has been described is the ability of the parasite to recover from a drug-induced dormancy when exposed to high concentrations of the drug [96]. Available tests are limited to distinguish a dormant from a dead parasite. For instance, DNA/RNA of dead parasites would still be present and the morphology of both are identical to be differentiated visually. All the mentioned tests point to a common mechanism of resistance which is the reduction of the ring stage susceptibility to artemisinin, however, they examine different aspects and thus can give different results.

Delayed growth of resistant parasites can be detected by the hemozoin assay

It has been reported that artemisinin resistant parasites in the absence of antimalarial drugs show a delayed growth *in vitro* in comparison to the sensitive parasites [97]. Hott *et al.*, described that this delayed growth is caused by a longer ring stage. However, the entire duration of the parasite development does not change since the parasite also exhibit a short trophozoite stage [97].

Results obtained in this study by microscopy corroborated the idea of a prolonged ring stage development of resistant parasites with 18h instead of the 12h observed for sensitive parasites (Figure 16C). However, contrarily to what was previously described for resistant parasites, the whole life cycle duration was increased by 6h since the trophozoite stage duration is the same between parasite strains. The alternative method of Hz detection also allowed to observe a delayed growth of resistant parasites but only at 72h of incubation where the total percentage of depolarizing events already increased in sensitive parasites while resistant parasites lag behind and remain with approximately the same percentage of 48h.

Concordantly, the Hz detection assay results expressed what was observed by microscopy at 72h where artemisinin sensitive parasites were in the trophozoite stage while the artemisinin resistance parasites were still ring forms (Figure 16). Concurrently, SYBR Green results showed that it was possible to detect a delayed growth at 42h of incubation since parasitemia increased from 36h onwards in sensitive parasites while in resistant parasites this was only verified at 42h.

The detection of a prolonged life cycle in resistant parasites was common to all the methods and was caused by a longer ring stage observed microscopically. It was already described that resistant parasite forms which are less susceptible to artemisinin were precisely the young ring forms [93]. Taking into account the short half-life of this drug of approximately 1 hour [2], the prolonged ring stage has been suggested as a parasite mechanism of survival [97]. The idea is that resistant parasites in the presence of artemisinins can extend the ring stage until the drug effect is lost so they can proceed their growth later on. Nonetheless, the delayed growth is not common to all resistant clones; and a case was already reported where the resistant clone had a shorter life cycle of 36 hours [97]. Furthermore, previous results obtained in field studies demonstrated that strains with comparable susceptibility profiles to a determined drug can have different growth patterns *ex vivo* [69]. Thus, if the detection of delayed growth to determine artemisinin resistance works remains unclear.

V. Conclusions and future work

The emergence of resistance to antimalarial drugs is a major impediment in malaria control. A method of easy implementation in the field, which is more rapid, practical, affordable and capable to detect artemisinin resistance is still needed. The Hz detection assay already proved to be an alternative method with most of the mentioned characteristics. The presented study gave support to the idea that Hz detection method can be further optimized.

Overall, the implementation of a lysis step in Hz detection assay makes the method more sensitive without compromising the ease of execution and application in the field. Even though the use of an additional reagent, the lysis solution is simple to prepare and inexpensive. The major limitation is the use of a bigger amount of the samples and the need of using a different well-plate for each time-point which turns out to be more expensive than the standard protocol. Although it was concluded that lysing samples detects earlier inhibitory effects of antimalarial drugs, further studies are needed to replicate the experiments and to validate the results with the new lysis protocol. Moreover, it would also be important to verify if inhibitory drug effects could be detected with an initial parasitemia equal to the minimal limit of detection determined with lysed samples instead of the 1% used.

The importance of drug efficacy trial data is undisputed. With a constant development of new techniques to complement or replace the ones that have been used, it is important to verify if there is consistency between the results and it is necessary to validate the assays. Although in general the values were slightly higher, IC50 values determined by lysing the RBCs were comparable to the others already reported. In the future, it would be interesting to perform the experiments with other antimalarial drugs available.

The fact that the Hz detection assay detects drug effects earlier than most assays is especially relevant for artemisinin resistance because the young forms of the parasite that are less susceptible to the drug. This study allowed to confirm that, in fact, flow cytometry could be a useful tool for artemisinin resistance testing. Nonetheless, the synchronization protocol used for the RSA is very time-consuming and laborious. The synchronization protocol should be simplified and optimized in future researches.

The survival rates obtained were increased in relation to microscopy, particularly in the artemisinin sensitive strain. However, it was possible to distinguish the resistant strain from the sensitive strain at 30h of incubation with Hz detection assay. Though, the depolarizing peak in drug free control occurs at 42h, in future experiments it would be interesting to measure parasite maturation at 42h instead of the 30h used. This might approximate the survival values to the ones obtained by microscopy. When the growth pattern of the strains tested was investigated in the absence of the drug it was confirmed that resistant parasites showed a delayed growth, as has been reported. The idea of detecting artemisinin resistance by the altered growth curve of resistant parasite was considered but still requires further validation.

Overall, although the results suggest that the Hz assay could be used for testing artemisinin resistance, more studies are required since we based our observations only on one resistant strain (MRA-1240). Further studies should include other culture-adapted resistant strains. Ideally, also a field-study should be conducted

in Southeast Asia, where artemisinin resistance has been reported, to evaluate the usefulness of Hz assay with strains obtained directly from patients.

Despite the remaining open questions, the results strongly suggest that the Hz detection assay may play an important role in antimalarial drug testing, including artemisinin, in the future.

VI. References

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