A toolbox to study liver stage malaria

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The first obligatory phase of mammalian infection by *Plasmodium* parasites, the causative agents of malaria, occurs in the liver of the host. This stage of *Plasmodium* infection bears enormous potential for anti-malarial intervention. Recent technological progress has strongly contributed to overcoming some of the long-standing difficulties in experimentally assessing hepatic infection by *Plasmodium*. Here, we review appropriate infection models and infection assessment tools, and provide a comprehensive description of recent advances in experimental strategies to investigate the liver stage of malaria. These issues are discussed in the context of current challenges in the field to provide researchers with the technical tools that enable effective experimental approaches to study liver stage malaria.

Malaria and the life cycle of *Plasmodium* parasites

Malaria is endemic in several areas of the world, particularly in Southeast Asia and sub-Saharan African regions, where it is responsible for over 200 million infections yearly and a death toll of nearly 800 000 (http://www.who.int/malaria/). The disease is caused by apicomplexan parasites of the *Plasmodium* genus, which infect their mammalian host through the bite of a female *Anopheles* mosquito vector. Five different species of *Plasmodium* can cause malaria in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*. Whereas *P. falciparum* accounts for most of the mortality associated with malaria, *P. vivax* infection constitutes an increasingly documented concern [1].

Mosquito salivary gland-resident *Plasmodium* parasites, termed sporozoites at this stage of their lifecycle (Figure 1a, left), are injected into the skin of their mammalian host during the blood meal of the mosquito. *Plasmodium* sporozoites use their capacity to traverse or migrate through host cells [2] to reach a blood vessel [3], and possibly to cross the liver sinusoids [4], as well as several hepatocytes, before infecting a final one in a process termed productive invasion, which occurs with the formation of a parasitophorous vacuole (PV). Inside the PV, *Plasmodium* parasites multiply, generating exoerythrocytic forms (EEFs) that grow in size as *Plasmodium* replicates to generate thousands of new parasites (Figure 1a, right). The liver stage of infection is an obligatory step in the life cycle of the parasite (Box 1) and culminates in the release of parasite-filled vesicles, termed merosomes, into the blood stream [5]. Merosomes eventually burst, presumably in the microvasculature of the lungs [6], releasing erythrocyte-infective parasites called

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Glossary

Axenic development: process of sporozoite differentiation into early exoerythrocytic forms in the absence of host cells.
Cell traversal: process of sporozoite migration through cells with disruption of their plasma membrane and without formation of a parasitophorous vacuole.
DE-52: weak anion exchanger chromatographic resin based on the diethylaminoethyl (DEAE) tertiary amine functional group.
Exflagellation: the extrusion of rapidly waving flagellum-like microgametes from microgametocytes; in the case of human malaria parasites, this occurs in the *Anopheles* mosquito vector after ingestion of the infected blood.
Exoerythrocytic forms (EEFs): intrahepatic forms of the *Plasmodium* parasite, which develop inside a parasitophorous vacuole to generate first-generation merozoites.
Fluorescence-activated cell sorting (FACS): a technique to rapidly separate cells in a suspension on the basis of size and the wavelength of their fluorescence.
Gliding motility: a form of substrate-dependent locomotion used by apicomplexan invasive stages, powered by a parasite actin/myosin motor.
Hypnozoites: liver-resident parasite forms that can stay dormant for long periods of time before resuming development and eventually cause relapses in disease symptoms.
ImageJ: public domain, image processing program developed at the National Institutes of Health that can display, edit, analyze, process, save, and print several image formats.
Immortalized cell line: population of cells from a multicellular organism which would normally not proliferate indefinitely but, because of mutation, can keep undergoing division rather than undergoing normal cellular senescence.
Intravital microscopy: the direct real-time imaging of biological phenomena in exposed tissues.
Liver stages: stages of *Plasmodium* development that occur between sporozoite invasion of hepatocytes and merosome release.
Merosomes: merozoite-filled vesicles that bud-off infected hepatic cells into the blood stream.
Merozoites: the erythrocyte-infective forms of *Plasmodium* parasites.
Parasitophorous vacuole (PV): a vacuole within the host cell inside which the parasite resides.
Primary hepatocytes: parenchymal liver cells that perform all the functions ascribed to the liver.
Productive invasion: infection of a hepatic cell by *Plasmodium* sporozoites with formation of a parasitophorous vacuole inside which the parasite replicates and grows.
Reflection interference contrast microscopy (RICM): microscopy method that uses polarized monochromatic light to illuminate an object. The reflected light interferes and generates patterns that allow distinguishing how close a part of the object is to the substrate.
Relapse: the appearance of hypnozoite-derived parasites in the blood after the initial blood infection has been cleared.
RNA interference (RNAi): gene silencing method whereby expression of targeted mRNAs is inhibited by complementary double-stranded RNA chains.
Sinusoids: small, fenestrated blood vessels found in the periphery of the lobules of the liver. Sinusoids are lined with endothelial and Kupffer cells.
Sporozoites: the salivary gland-resident, liver-infective forms of *Plasmodium* parasites.
Total internal reflection fluorescence microscopy (TIRF): technique that allows investigating fluorescent objects that are placed within about 100 nm above a substrate surface by reflecting a laser beam from the bottom of that substrate (usually a glass slide), thus generating a limited so-called evanescent field above the surface.
Traction force microscopy (TFM): technique that allows calculating the forces exerted by objects/cells on the substrate. To this end, cells are plated on a flexible gel (e.g. polyacrylamide) that contains fluorescent beads. When the cell pulls on the substrate, the beads are displaced and indicate the direction and strength of the applied force, which can be determined computationally.
Widefield epifluorescence microscopy: fluorescence microscopy where the excitatory light is passed through the objective lens and then onto the specimen, giving rise to emitted light which is focused on the detector by the same objective that is used for the excitation.

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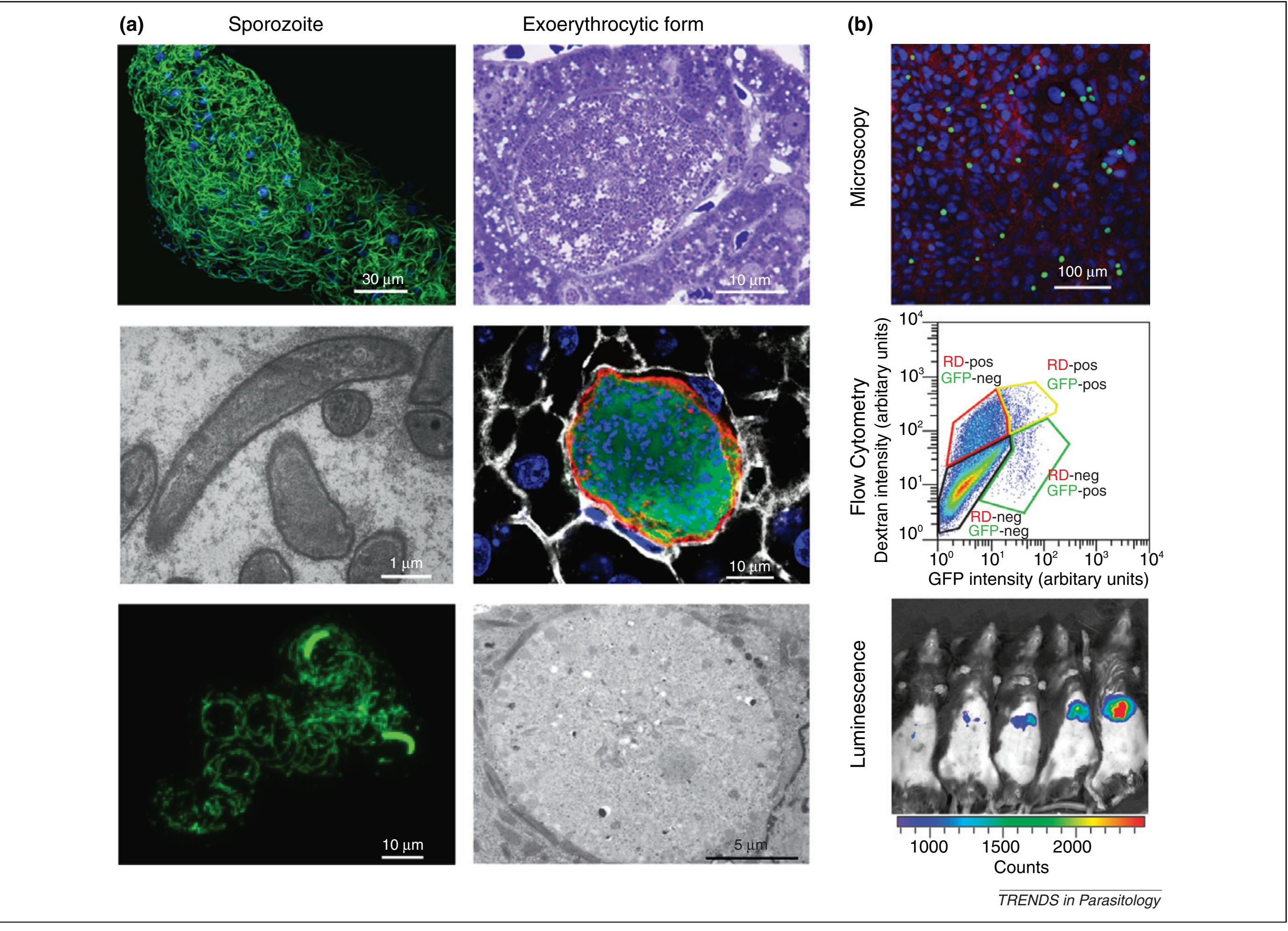

Figure 1. Visualization and quantification of *Plasmodium* liver infection. **(a)** Visualization of pre-erythrocytic *Plasmodium* parasites. (top-left) GFP-tagged *P. berghei* sporozoites inside mosquito salivary glands; (middle-left) electron micrograph of *P. berghei* sporozoites inside mosquito salivary glands; (bottom-left) circumsporozoite protein trails of gliding *P. berghei* sporozoites; (top-right) semi-thin epon section of *P.yoelii* in mouse liver 52 hours post-infection (hpi) (reprinted with permission from [6]); (middle-right) immunofluorescence staining of *P. berghei* EEF in the liver 48 hpi of C57/Bl6 mice (green: PbHsp70; red: PbUIS4; white: filamentous actin; blue: nuclei); (bottom-right) electron micrograph of a *P. falciparum* EEF developing in primary human hepatocytes 48 hpi (reprinted with permission from [98]). **(b)** Quantification of *Plasmodium* hepatic infection. (top) EEFs developing in Huh7 cells imaged 24 hpi with *P. berghei* sporozoites (green: PbHsp70; red: filamentous actin; blue: nuclei); (middle) flow cytometry analysis of Huh7 cells 2 hpi with GFP-expressing *P. berghei* parasites, in the presence of rhodamine Dextran; (bottom) luminescence analysis following luciferin injection of C57/Bl6 mice 44 hpi with different amounts of luciferase-expressing sporozoites.

Box 1. The significance of understanding *Plasmodium* liver stage infection

Whereas *Plasmodium* blood stages have been recognized since the 1880s, it was not until 1948 that liver forms of the parasite were first identified [70]. Our understanding of the detailed biology of *Plasmodium* hepatic stages is limited, and important gaps subsist in our knowledge of the interactions that occur at the *Plasmodium*–hepatic cell interface. Besides (or perhaps because of) the fundamental biological questions that remain unanswered, the potential of the liver stage of infection for anti-malarial intervention remains underexploited. In fact, despite its asymptomatic nature, the hepatic stage of *Plasmodium* is an obligatory phase of the developmental journey of the parasite from the mosquito into erythrocytes, and one during which parasite numbers increase by four orders of magnitude [5]. These features make the liver stage an attractive target for the development of malaria prophylaxis strategies. Moreover, *P. ovale* and *P. vivax* are able to generate cryptic forms called hypnozoites that may remain dormant in the liver for long periods of time, eventually causing relapses in disease symptoms. Hypnozoites represent a reservoir of liver parasites that can resume their developmental process towards forming infectious merozoites. This constitutes a very significant cause of morbidity in various regions of the world, particularly in areas ranging from temperate through the subtropical and tropical zones of the world where *P. vivax* is endemic [1]. Thus, intervention during this stage of infection is essential to achieve radical cure, the elimination of hypnozoites before they can cause blood infection. As such, in a period where malaria eradication is emerging as a desirable if audacious goal, it is clear that this goal will not be attained without the development of effective hypnozoiticides.

The symptomless character of liver infection by *Plasmodium* and the obvious difficulties in obtaining human samples from infected livers have earned this parasite life cycle stage the label ‘silent.’ These difficulties were further compounded by technical limitations to the experimental investigation of hepatic infection by *Plasmodium*. We now know that, despite being clinically silent, the liver stage of *Plasmodium* infection is not only immunologically active [71] but also one where a rich array of host–parasite interactions takes place [61,62,72]. This realization has prompted efforts towards developing tools that enable a deeper understanding of its biology and the exploitation of its potential for intervention.

merozoites (reviewed in [7,8]). Merozoite release marks the beginning of the blood stage of infection, when *Plasmodium* cyclically invades and ruptures erythrocytes, giving rise to disease symptoms. During this phase, some parasites differentiate into male and female gametocytes that can be ingested by a mosquito. The sexual phase of the life cycle of the parasite takes place in the mosquito midgut and eventually results in the formation of sporozoites that reach the salivary glands, completing the cycle.

Addressing *Plasmodium* liver infection experimentally

Sporozoite-stage parasites

Sporozoites are the liver-infective forms of *Plasmodium* parasites. They are formed following the sexual phase of the life cycle of the parasite and therefore can be obtained from the salivary glands of infected *Anopheles* mosquitoes (Figure 1a, left), the definitive hosts for *Plasmodium* parasites, and an essential component of malaria research. Although sporozoites have been produced *in vitro* in low numbers [9], an effective *in vitro* culture system for sporozoites remains a major hurdle in this field of research. Thus, the maintenance of mosquito colonies is a prerequisite for all laboratory work requiring completion of *Plasmodium* sporogonic development. Parasite genotype-vector genotype interactions are a major determinant of infection outcome [10]. Therefore, selection of an appropriate *Anopheles–Plasmodium* combination is imperative. Various combinations are commonly used in the laboratory, allowing high infection rates. The two most commonly used vector species are *A. gambiae* and *A. stephensi*, but *Anopheles freeborni* and *Anopheles arabiensis* are also used. These different species present different permissiveness for infection with cultured *P. falciparum,* resulting in different infection levels: *A. freeborni* >> *A. gambiae* = *A. arabiensis* > *Anopheles stephensi* [11]. Likewise, permissiveness to the most common rodent *Plasmodia*, *Plasmodium berghei* and *Plasmodium yoelii* is different even for the same mosquito species.

The use of mosquito-borne human-infective *Plasmodium* species in the laboratory poses obvious safety concerns as well as several technical challenges. For these reasons, although both *P. falciparum* and *P. vivax* sporozoites have been used experimentally, most liver stage malaria research has employed parasite species that infect other mammals, namely rodents and non-human primates (Figure 2 and Supplementary Table S1). Among the rodent models of *Plasmodium*, *P. berghei* and *P. yoelii* have been the most commonly employed in liver stage research. Conversely, the non-human primate parasite *Plasmodium cynomolgi* is capable of generating persisting EEFs that produce relapses [12] and has been proposed to mimic the *Plasmodium* species that can generate dormant forms in human livers [13].

Infection can be carried out either through parasite delivery by mosquito bite or by injection of sporozoites isolated from the salivary glands of infected mosquitoes. Unfortunately, isolated sporozoites have limited viability, and strategies for their preservation are not generally available. Following collection of the salivary glands [14], sporozoites can be obtained by mechanical disruption of the salivary gland material, followed by filtration or by centrifugation at low speed to remove debris. Highly pure sporozoite suspensions can be obtained through chromatography separation on an anion exchange column [15,16] but with relatively low yields.

Host cell models

Liver-stage malaria research has made use of several cell lines, primary hepatocyte cultures, and animal models to study the processes that occur in the infected liver (Figure 2 and Supplementary Table S1). Among the main advantages of cell lines are their easy maintenance and propagation in the laboratory, a well-defined genome and transcriptome, and their amenability to genetic manipulation. Several cell lines have been employed in *in vitro* studies of hepatic infection by *Plasmodium*. Most commonly, rodent *Plasmodia* are used with HepG2 [17], Huh7 [18], or Hepa1-6 [19] cell lines, whereas *P. vivax* can infect HepG2 [20] and HC04 [21] cells, and the latter is the only cell line known to support *in vitro* growth of *P. falciparum* [21] (Figure 2 and Supplementary Table S1). For high-throughput, microscopy-based applications, the use of Huh7 may be preferred to HepG2 cells because the latter form less uniform monolayers [22,23]. One drawback of cell lines, however, is the fact that they are modified, immortalized cells that may have lost some of the features of the cells they were derived from. Thus, an alternative approach relies on the *ex vivo* use of primary hepatocyte cultures, which retain more, although not all [24], the characteristics of liver hepatocytes. Primary hepatocytes can either be acquired commercially or purified in the laboratory following established, if relatively complex, procedures [25]. A wide array of primary hepatocytes of human, non-human primates and rodent origins with different *Plasmodium* specificities and infection outcomes has been described (Figure 2 and Supplementary Table 1).

Cells in culture are infected by adding isolated *Plasmodium* sporozoites to the cell monolayer. Increased efficiency and synchronization of the infection can be achieved by centrifugation [26]. It is worth mentioning cell density as a possible pitfall in *in vitro* or *ex vivo* infection studies. In fact, intracellular parasite development is partly dependent on cell confluence with EEFs growing to a lesser extent when the latter is high. This is particularly relevant when employing continuously replicating cell lines to address late time points of EEF development, or when the procedure requires that proliferating cells be seeded several days before infection. It is generally accepted that sporozoites should be added to ~60–70% confluent cell monolayers, at a mean of infection of 1:3–1:5 sporozoites per cell [18]. Even under these conditions, the percentage of parasitized cells obtained in *in vitro* or *ex vivo* infections is relatively low (< 5%) [18]. Increasing the number of added sporozoites does not yield a linear enhancement of infection and may lead to greater cell death, presumably because of increased cell traversal and the inability of some cells to reseal their membrane after they have been traversed [2]. Low infection rates are a familiar problem for liver stage malariologists. One strategy to overcome this limitation involves the stimulation of sporozoite, apical-regulated exocytosis by addition of calcium ionophores [27], or of uracyl derivatives or forskolin [28], which decrease

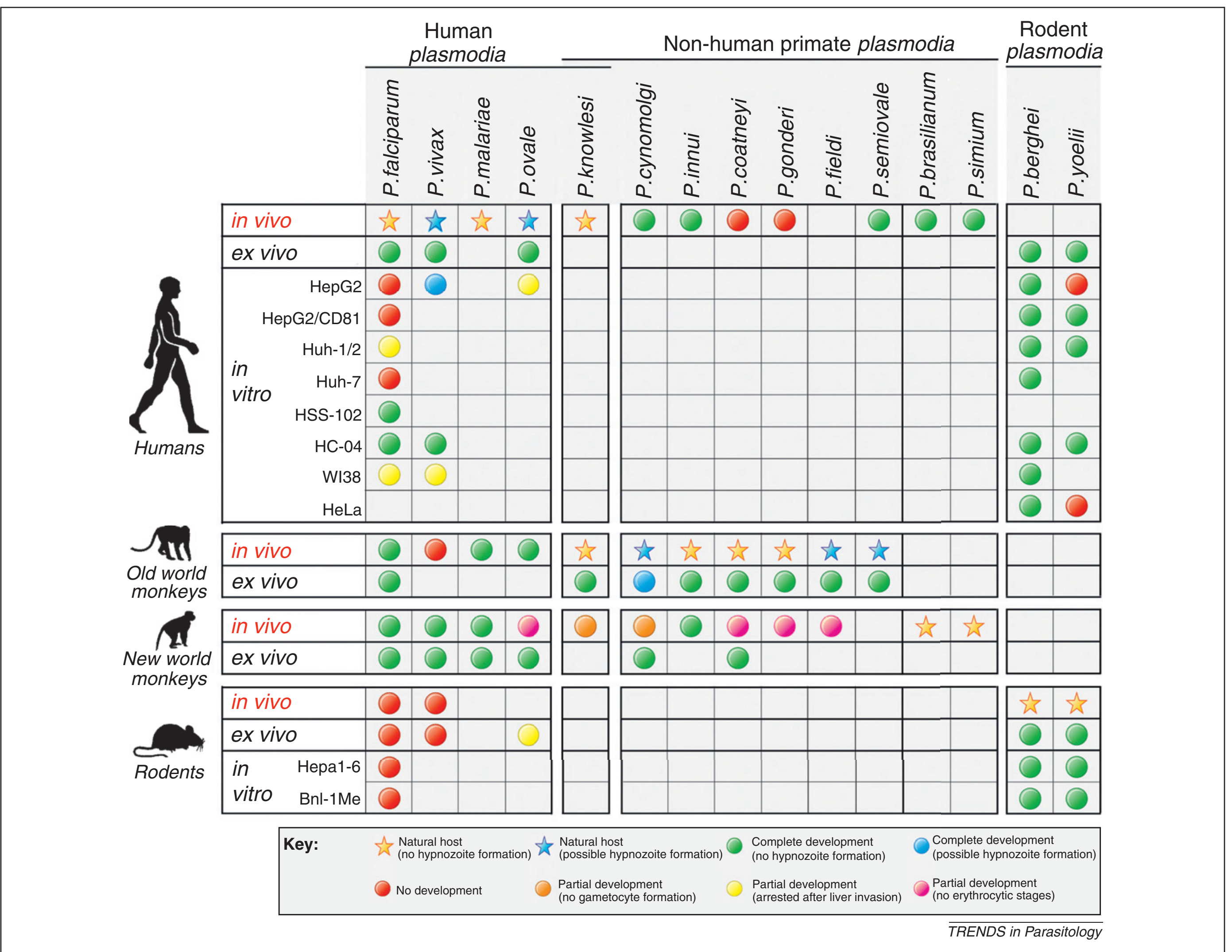

Figure 2. Host–parasite combinations of *Plasmodium* hepatic infection models. The most common combinations of *Plasmodium* and mammalian host hepatic models are shown. Blank cells correspond to combinations for which no information is available. Please see also Supplementary Table S1 for a fully referenced account of *Plasmodium* hepatic infection models.

migration through cells and enhance infectivity. Such treatments may, however, perturb the experimental measurements and confound the results, and therefore their use should be carefully considered.

Animal models

Rodents and non-human primates are relevant animal models for malaria research, as they can be infected by a variety of *Plasmodia* that mimic human parasites [29] (Figure 2 and Supplementary Table S1). They are particularly useful when investigating the response of the organism as a whole to infection, as is the case with immune response and drug delivery or toxicity studies. Increasing restrictions to the use of non-human primates in research, coupled with high costs, make rodents the *in vivo* model of choice in many laboratories around the world. Infection of anesthetized animals can be carried out through the bite of infected mosquitoes [30], or by intramuscular [31], subcutaneous [31], intradermal [31], intrahepatic [32], intravenous [31] or even oral [33] administration of isolated sporozoites. On average, only a few sporozoites are delivered in a single infected mosquito bite [34], the most physiologically relevant route of parasite transmission. These numbers can be greatly increased if sporozoites are administered intravenously, arguably the preferred delivery method for isolated sporozoites, leading to higher liver parasite loads [35]. The magnitude of hepatic infection varies greatly depending on animal and *Plasmodium* species. In any event, of the total hepatocytes in the liver, only a small proportion is parasitized following sporozoite administration.

Humans ultimately constitute the ideal system to investigate infection by *Plasmodium* parasites. Human subjects have been used not only in the past but also in recent studies to answer fundamental questions on the protection conferred by *Plasmodium* liver stages [36]. Nevertheless, given the obvious ethical constraints to studies with humans, animal models remain the closest surrogates of human infection available to researchers.

Investigating hepatic infection

The accurate assessment of *Plasmodium* infectivity of hepatic cells is a pivotal requirement for liver stage malaria research. With advances in microscopy-based

techniques, the advent of quantitative reverse transcriptase PCR, and the availability of transgenic parasites, researchers now have a plethora of techniques at their disposal to gauge infection. Whereas some of these have evolved from the early days of liver-stage malaria research, others, such as the use of radioactive DNA probes, have been largely abandoned.

Microscopy

Microscopy-based techniques have played a key role in the investigation of liver stage malaria from its onset. Optical microscopy was the basis for the initial identification of *Plasmodium* exoerythrocytic stages [37] and has been used in numerous studies since then. Observations of schizonts in fixed liver sections (Figure 1a, right) were instrumental in identifying and describing malaria liver infection and defining appropriate models to understand it. In parallel, electron microscopy (EM) (Figure 1a) provided an ultrastructural basis to inspect liver schizonts [38] and the structural changes that accompany the transformation of sporozoites into EEFs [39,40]. Finally, by coupling EM with the use of appropriate antibodies (immuno-EM), it became possible to pinpoint the localization of specific parasite proteins in isolated sporozoites [41] or infected cells [42].

Sporozoite gliding motility is an essential feature of the ability of *Plasmodium* to infect cells [43]. Gliding motility can be visualized live in a light-field microscope but can also be easily quantified by counting the number of trails produced by a given number of sporozoites on a glass slide for a defined period of time. During gliding, sporozoites release several parasite surface proteins, including the circumsporozoite (CS) protein. Staining of these trails can be easily carried out to enable gliding quantification (Figure 1a, bottom left). Gliding motility can also be quantified by live microscopy and image processing [44] or by measuring the number of traversed cells [2,45]. Besides, techniques such as reflection interference contrast microscopy (RICM) [46], traction force microscopy (TFM) [46] and total internal reflection fluorescence microscopy (TIRF) [47] have been recently used to investigate the molecular mechanisms that govern gliding motility.

Immunofluorescence-based techniques have enabled the use of a variety of imaging methods to analyze *Plasmodium* liver stages, which were recently reviewed by Rankin *et al.*, and include widefield epifluorescence, and spinning disc and laser scanning confocal microscopy [48]. These techniques have been used to visualize parasites *in vitro*, *ex vivo* or *in vivo* and have been applied to either fixed or live samples (Figure 1a). An array of *Plasmodium*-specific antibodies can be employed to visualize the parasite throughout its developmental stages inside hepatic cells (Figure 3). Immunostaining analyses of fixed samples can be carried out on infected cell lines or primary cells, as well as on liver sections. Transgenic parasites engineered to express fluorescent proteins have opened new avenues for visualization of host–parasite interactions, for example [49,50], as well as to investigate *Plasmodium* features such as organelle segregation [51]. When employed on fixed samples, the fixation procedure may lead to loss of endogenous fluorescence intensity. However, the signal can be amplified through the use of a (conjugated) antibody against the engineered fluorophore. Fluorescent *Plasmodia* can also be used in live imaging studies, as high-speed live video microscopy provides a window through which to view the dynamics of hepatic infection. Under controlled temperature and atmospheric conditions, parasites can be visualized inside live cells in culture at specific intervals over several hours [52], as well as used in intravital observations of infection of animal model livers [53,54].

Microscopy-based techniques have been widely used to visualize and investigate infected cells or liver slices as well as to quantify infection [22,23] (Figure 1b, top). Measurement of infection loads by microscopy is time-consuming, although its efficacy can be improved by automated image acquisition and treatment protocols [22,23]. The fact that only relatively small numbers of parasites are effectively imaged makes it necessary to ensure that the acquired data are statistically solid. One advantage of the method, however, is that it simultaneously provides not only visual information about parasite morphology but also, through the use of appropriate antibodies or dyes, an insight into host nuclear and cellular features (Box 2). Infection quantifications are achieved by determining the number and the area of imaged EEFs and normalizing the data against cell numbers or confluency. The latter can be reasonably estimated by counting the number of nuclei or actin density, respectively, per unit area [22,23]. Computation of EEF/nuclei numbers and EEF areas can be greatly aided by using ImageJ (http://rsbweb.nih.gov/ij/). To distinguish intracellular from internalized sporozoites imaged at early time points, double-staining methods, in which parasites are successively stained either twice with an anti-CS antibody [55], or with a peroxidase- and a fluorescein-labeled antibody [56], can be employed.

Quantitative real-time PCR

Quantitative real-time reverse transcriptase PCR (qRT-PCR) is the gold standard method for measurement of parasite loads in cultured infected cells [23] or in livers of animal models [30]. In all cases, primers are used to amplify a *Plasmodium*-specific target, typically 18S ribosomal RNA [30], in a pool of cDNA synthesized using the total (parasite and non-parasite) RNA in the sample lysate as template. The correct assessment of infection by this method requires the use of an additional reaction employing a set of primers targeting a suitable housekeeping gene, for normalization purposes. The main drawbacks of this technique are that it is costly, time-consuming and requires expensive equipment. However, besides its high accuracy and sensitivity, it presents the added advantage that, through the use of appropriate primers, it enables gauging the expression of other *Plasmodium* or host genes of interest in the infected cell or liver extracts. Likewise, qRT-PCR can also be used to measure the expression of a given *Plasmodium* gene in isolated sporozoites [40].

Flow cytometry

Flow cytometry has emerged as an attractive means of measuring infection in cell lines infected with fluorescent transgenic parasites, whose fluorophore is expressed under the control of a housekeeping promoter [49]. Among the

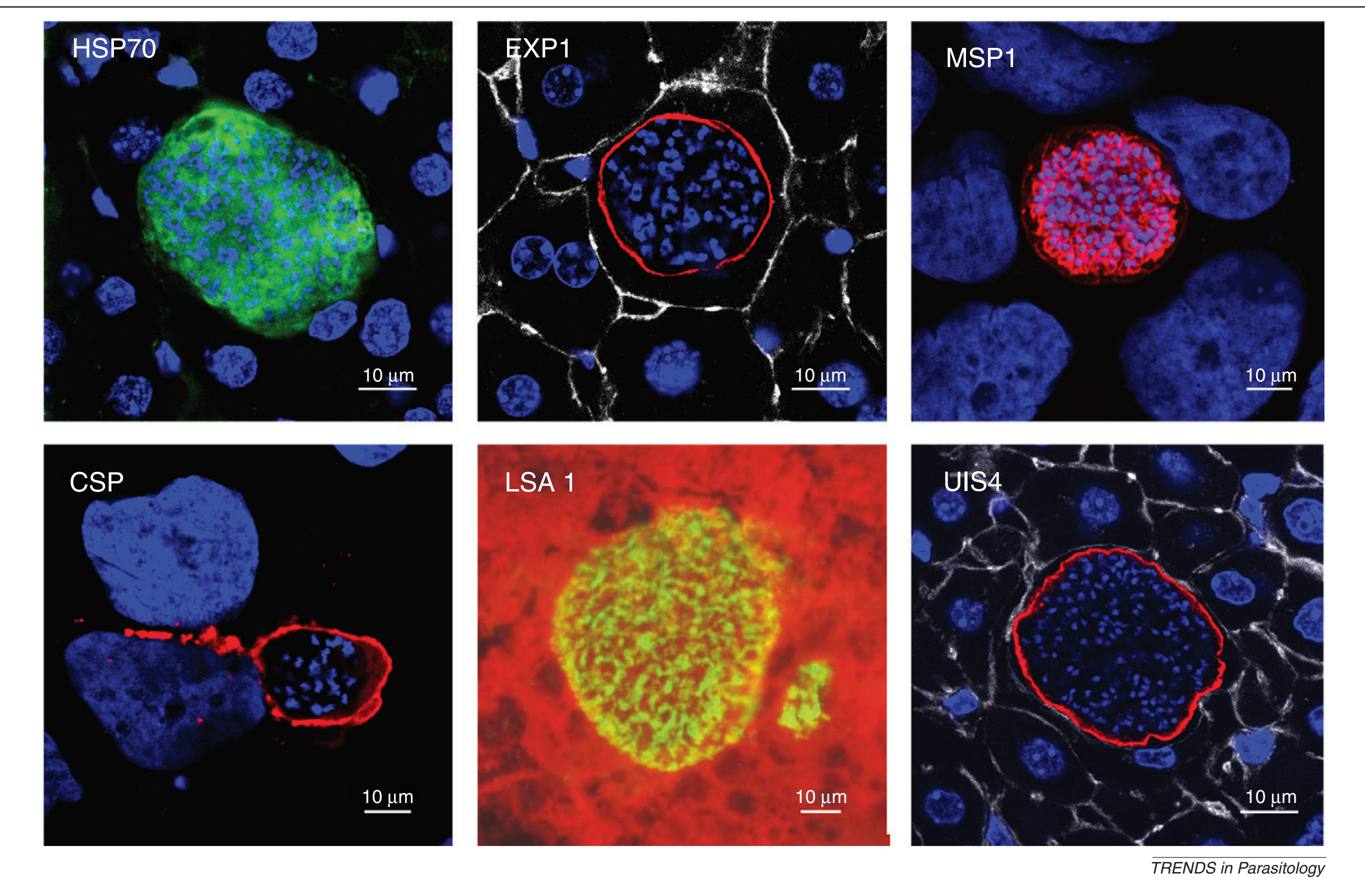

Figure 3. Immunofluorescence microscopy observation of pre-erythrocytic *Plasmodium* proteins. Representative immunofluorescence microscopy images of immunostaining analyses using *Plasmodium*-specific antibodies commonly employed to visualize intrahepatic parasites. When shown, nuclei are depicted in blue and filamentous actin in white; remaining colors depict the parasite molecule indicated on the top left corner of the image. For the LSA1 image, the parasite molecules are depicted in green and the host hepatocytes in red. HSP70: *P. berghei* heat-shock protein 70 in a C57/Bl6 mouse liver section, 46 hours post-infection (hpi); EXP1: *P. berghei* exported protein 1 in C57/Bl6 mouse liver section, 46 hpi; MSP1: *P. berghei* merozoite surface protein 1 in Huh7 cells, 63 hpi; CSP: *P. berghei* circumsporozoite protein in Huh7 cells, 48 hpi; LSA1: *P. falciparum* liver stage antigen 1 in human liver hepatocytes grown in chimeric mice, 6 days hpi (reprinted with permission from [99]); UIS4: *P. berghei* upregulated in infective sporozoites 4 in C57/Bl6 mouse liver section, 46 hpi.

Box 2. Addressing host–parasite interactions during *Plasmodium* hepatic infection.

The amazing replication rate attained by *Plasmodium* in the mammalian liver is clearly suggestive of hepatocytes providing a unique micro-environment for parasite development. A vast array of interactions takes place between the parasite and the infected cell, whose intricacies we are only beginning to unravel.

Although *Plasmodium* sporozoites can undergo the very first steps of development in the absence of host cells (axenic development) [73], it is increasingly recognized that host factors must play a crucial role during infection of hepatic cells by *Plasmodium* parasites, and several host molecules have been shown to influence the progression and fate of liver stages [22,23,71,74,75]. Their identification has been directed by existing experimental observations [71] or has stemmed from unbiased approaches like yeast two-hybrid [74] or RNA interference (RNAi) screens [22,23,76]. Further understanding of the role of host factors during infection has relied on genetic tools like RNAi, but also on cell biology techniques employing antibodies [23,74], fluorescent probes [77], and agonists or antagonists of target molecules or pathways [78]. Transcriptomics approaches have also been employed to profile changes that occur in the host during infection of hepatoma cells [62] and mouse livers [61]. Finally, patch-clamping has been employed to investigate the role of host ion transporters in infected cells [59]. The elucidation of host-mediated processes during the *Plasmodium* liver stage has been further aided by *in vivo* infection models, particularly rodents, in which the expression of a given gene has been knocked-down, deleted or modified to help clarify the role of the protein encoded by that gene [22,23,71,75,79]. Recently, luciferase- and GFP-tagged strains of *Plasmodium berghei* were used to differentiate blood from liver stage parasites and to show that blood-stage parasitaemia impairs the growth of a subsequent sporozoite infection of liver cells and protects the host from the risk of superinfection [80].

As expected, parasite proteins also play a crucial role during hepatic infection. Their identification and characterization has relied mostly on the use of transgenic parasites where a specific gene has been deleted or fused with a gene encoding a fluorescent probe [66]. Genetic modification of various *Plasmodium* species has been reported [43,81,82]. Such genetically modified parasites have been instrumental in furthering our understanding of, for example, parasite motility [43], cell traversal [4], infectivity [83,84], development [42], merozoite formation [85] and egress [86]. More recently, two *Plasmodium* molecules, PUF2 and IK2, have also been implicated in the transformation from slender, motile and cell-cycle arrested sporozoites into round, non-motile and replicating exo-erythrocytic liver stage forms [40,87], an essential step in the transmission of the parasite from the mosquito vector to the vertebrate host.

Inhibitor compounds have also been used to identify *Plasmodium* or host factors at play during infection [58,88,89]. However, care should be employed when ascribing the effects of a given inhibitor to either a parasite or a host molecule, because it can be difficult to exclude the possibility of the compound acting (also) on the other.

main advantages of this technique is the fact that it can be used to gauge the three main phases of hepatic cell infection: traversal, invasion and parasite development [18] (Figure 1b, middle). Following cell processing [18], infected cells can be distinguished from their non-infected counterparts on the basis of the fluorescence they emit, which, in turn, is proportional to the number of parasite copies in the cell. Thus, invasion rates can be measured by determining the percentage of fluorescence-positive cells at early time points after sporozoite addition (i.e. before parasite replication has started) [18,22,23,57]. At later time points, fluorescence intensity is an effective measure of parasite copy numbers in cells (i.e. EEF development) [18,23,57–59] and of infected cell survival rates [18]. Finally, by incubating cells with a fluorescent dye, such as rhodamine dextran (RD), cell traversal can be measured as the percentage of RD-positive cells [18,57] (Figure 1b, middle). Another advantage of the method is it can analyze a large number of cells per sample and absolute numbers of infected or traversed cells can be determined by including a defined number of fluorescent beads in a sample of known volume. Finally, the technique is cheap and sample acquisition is fast, enabling medium-to-high throughput analyses. There are, however, disadvantages to be considered. Besides the high cost of the equipment involved, the main disadvantage relates to cell autofluorescence, which may make it difficult to differentiate infected from non-infected cells, particularly at early time points of infection and when analyzing primary hepatocytes.

Flow cytometry of cells infected with fluorescent parasites can also be used to sort infected cells from among a majority of non-infected ones. Fluorescence-activated cell sorting (FACS) of *Plasmodium*-infected cells has been employed to sort infected cells cultured *in vitro* or from infected mouse livers [60–62]. This strategy has been used to sort infected cells at different time points of infection to determine the transcriptional profile of the host hepatoma cells throughout infection [62] or to survey the transcriptome and proteome of liver-stage parasites in infected mouse livers [61]. A similar strategy can be envisaged to enrich an infected cell population to be used in, for example, lipidomics or metabolomics analyses.

Luminescence

Chemiluminescent *Plasmodium* parasites expressing the luciferase gene under the control of a constitutive promoter have recently become valuable tools for hepatic infection measurements [63,64]. These parasites can be used to assess infection *in vitro* and *in vivo* with important advantages over other existing methods. *In vitro*, parasite loads in infected cultured cells can be determined by cell lysis followed by addition of the luciferase substrate, luciferin. The luciferase-catalyzed reaction that ensues results in the emission of an amount of light proportional to the parasite burden in the lysate [63]. The method is relatively inexpensive, quick, and requires only the availability of a luminescence microplate reader. One drawback is that it does not distinguish parasite invasion from development. Another is that it does not provide information about host cell viability or death at the time of infection measurement. The latter hindrance can, however, be minimized through the use of a dye such as AlamarBlue® to assess cell confluency before lysis. The speed and simplicity of luciferase-based infection measurement makes it well-suited for high-throughput studies where large numbers of samples need to be analyzed. This is the case, for instance, of screens to identify drugs targeting *Plasmodium* hepatic stages, as have been proposed for the parasite blood stages [65], or of RNAi screens, instead, or to complement previously employed microscopy-based approaches [22,23].

Luciferase-expressing parasites have opened new avenues for the measurement of *Plasmodium* infection *in vivo*.

Box 3. The liver stage of *Plasmodium* as a target for malaria control

The hepatic stage of the *Plasmodium* life cycle presents unique features that make it appropriate for anti-malarial intervention at the levels of prophylaxis, treatment and vaccination. First, the relatively low numbers of parasites that infect hepatocytes, coupled with the asymptomatic nature of this phase of infection, make the liver an attractive target for prophylactic measures [7]. Second, the existence of *Plasmodium* species capable of forming cryptic forms that remain dormant in the liver for long periods of time demands effective ways of clearing hepatic parasites before they can cause disease relapses [1]. Third, the liver stage of infection is immunologically active and the sole known source of effective generation of sterile immunity [67].

Prophylactic and therapeutic action against malaria requires the use of drugs that can effectively impair or eliminate hepatic stage parasites during their sojourn in the liver. Unfortunately, attempts to find efficient drugs against liver stages have been relatively limited. Presently, primaquine (PQ) is the sole drug licensed for clinical use against *Plasmodium* liver stages, and its administration is restricted to documented cases of *P. vivax* or *P. ovale* infections [90]. Moreover, PQ can produce complications such as hemolytic anemia, which is potentially lethal in people deficient in 6-glucose phosphate dehydrogenase (G6PD), a common trait in malaria-endemic regions [90]. Efforts towards the identification of new, effective drugs targeting *Plasmodium* liver stages can conceivably follow two types of strategies. One option is to employ a medicinal chemistry approach to modify existing compounds with known activity to improve their efficacy while reducing their adverse effects [91,92]. Another alternative is the use of high-throughput screens to discover novel active compounds. With the availability of fluorescent and luminescent transgenic parasites (see main text), such drug screens will probably become a reality in the short term.

A vaccine capable of creating an immunological environment that stops *Plasmodium* infection at an early stage of its development inside hepatocytes is also highly desirable. Until now, the only demonstrably effective vaccine shown to confer a sterile and lasting protection against malaria is the inoculation of radiation-attenuated sporozoites (RAS), which can invade but do not fully develop inside hepatocytes [93]. However, this whole-organism-based vaccination strategy presents logistical and safety concerns that cannot be easily overcome. Thus, several efforts have concentrated on constructing a subunit vaccine based on *Plasmodium* liver stage antigens, as is the case of the circumsporozoite protein (CS)-based RTS,S vaccine [94]. Unfortunately, however, CS-based vaccine candidates do not provide sterile protection in malaria endemic areas [94]. Renewed hopes for a whole-organism vaccine arose from reports showing that sporozoites deficient in certain genes (genetically attenuated sporozoites, GAS) can confer long-lasting protection against subsequent infections (reviewed in [67,95]). Nevertheless, the use of GAS as a human vaccine poses safety problems, such as the possibility of breakthrough infections. Therefore, alternative whole-organism or subunit vaccination strategies aimed at the hepatic stage deserve further exploration. Meanwhile, efforts towards obtaining drug-based prophylactics and treatments and against *Plasmodium* liver stages must not be overlooked.

Following luciferin injection, anesthetized mice can be imaged by bioluminescent imaging, and the luminescent signal emitted by the parasites in the liver is proportional to the parasite load [63,64] (Figure 1b, bottom). This non-invasive method can be repeated several times throughout hepatic parasite development, and infection can be allowed to proceed to the blood stage in the same animals where liver burdens were previously assessed. Although the sensitivity of the method is not as high as that of qRT-PCR, this strategy represents a huge advance in terms of the continuous monitoring of liver infection and the reduction of the number of animals used experimentally [63]. The method is ideally suited for *in vivo* drug assays as the same sets of mice can be used to measure the impact of a given compound on liver parasites and on disease progression.

The infection analysis techniques outlined in the sections above complement rather than replace one another. When selecting one strategy, considerations such as available resources and access to transgenic parasites must be taken into account. Besides, aspects such as measurement speed, sample numbers, and the specific objectives of the experiment must be considered. For *in vitro* measurements, it can be argued that visual insight is always desirable, making microscopy an appropriate option to gain information that other methods cannot provide. However, large numbers of samples cannot be easily processed for microscopy-based infection measurements. Thus, flow cytometry and luminescence are well-placed alternatives for increased sample throughput in, for example, RNAi or drug-sensitivity screening assays (Box 3). *In vivo*, luciferase-expressing parasites constitute an attractive asset to assess parasite loads, but bioluminescence measurements fall short of providing detailed information that can only be gathered from immunofluorescence imaging of liver slices, and are less sensitive than qRT-PCR methods. Ideally, infection should be assessed by several methods rather than a single one.

Concluding remarks

The number of scientific articles published on liver stage malaria over the past decade roughly equals the total number of articles on this subject in the fifty years before that. Besides reflecting an increasing awareness of the significance of this stage of infection (Box 1), this fact has been made possible by the development of appropriate infection models and by important technical advances in, for example, immunofluorescence microscopy [48] and genetic manipulation strategies [66]. Major progress made in methods to visualize *Plasmodium* liver stages significantly advanced our understanding of the biology of infection, and novel strategies to quantify hepatic parasite loads paved the way for high-throughput approaches to efficacious infection measurements [22,23]. Meanwhile, genetic tools enabled the construction of mutant parasites that not only represent a possible vaccination strategy [67] but have also been instrumental in the elucidation of crucial aspects of host–*Plasmodium* liver stage interactions (Box 2).

It is now accepted that the goal of malaria eradication can only be achieved through a multidisciplinary approach that will produce hitherto unavailable intervention tools and incorporate them with existing ones [68,69]. Generation of such tools will inevitably depend on improving our understanding of the various facets of malaria infection. The liver stage of the *Plasmodium* life cycle remains the most understudied phase of infection and one about which important information is lacking. However, given its unique features, it is also the one that arguably holds the greatest and most under-exploited potential for intervention (Box 3). At a time when malaria eradication is back on the World agenda, established methods must concur with emerging ones to overcome current limitations and make the liver stage of malaria infection ever less silent (Box 4).

Box 4. Outstanding questions and future directions

Despite recent advances, several technical challenges limit a more mechanistic insight into the biology of host–*Plasmodium* interactions. As such, significant gaps persist in our knowledge of *Plasmodium* pre-erythrocytic stages and crucial challenges lay ahead in bridging those gaps. One simple but important technical limitation stems from the low infectivity levels obtained experimentally, which preclude detailed biochemical and molecular analyses. Another crucial shortcoming is the lack of established models to study *P. vivax* infection of hepatocytes. In fact, despite recent reports on the development of *in vitro* infection systems suitable for investigation of putative hypnozoites [13,96], the lack of cell systems that can be used long enough to allow visualization of hypnozoite establishment and possible reactivation precludes crucial experiments to study the biology of these dormant forms.

At the same time, although we now recognize some of the host and parasite molecules involved in infection, very little is known about their exact role. As such, several fundamental questions remain unanswered:

- What is the mechanism employed by *Plasmodium* to productively invade hepatic cells and what are the signals that induce the parasite to invade versus to migrate through cells [97]?
- How does *Plasmodium* acquire the nutrients it needs to replicate inside hepatocytes, and what are those nutrients?
- What are the crucial *Plasmodium* molecules necessary to enter dormancy, and what triggers entering and exiting dormancy?
- Is *Plasmodium* detected by the host while replicating (or during dormancy) inside hepatocytes and, if so, how does it elude the immune system of the host?
- What are the triggers for merosome release?

These are some of the fundamental biological questions whose answers need to be found if we are to fully understand this crucial stage of the parasite life cycle. Thus, although adequate responses to several crucial issues have, so far, eluded researchers and need to be further pursued, their elucidation will certainly employ available tools as well as drive the development of new ones.

Acknowledgements

The authors gratefully acknowledge Carina S. Gomes-Santos, Ghislain G. Cabal, Maurice A. Itoe, and Vanessa Zuzarte-Luís (Unidade de Malária, Instituto de Medicina Molecular, Portugal), Volker Heussler (Institute of Cell Biology, University of Bern, Switzerland), and Stefan H. Kappe (Seattle Biomedical Research Institute) for providing pictures used in this Review. M.P. holds a Ciência 2007 position of the Portuguese Ministry of Science and Technology and is funded by FCT grant PTDC/SAU-MII/099118/2008. A.M.M. is funded by grant OPP1025364 of the Bill & Melinda Gates Foundation. This work was developed under the scope of EVIMalaR (EC-FP7). We apologize to those authors whose work was not cited because of journal constraints on the number of references.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pt.2011.09.004.

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