Review

In vivo imaging in NHP models of malaria: Challenges, progress and outlooks

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ABSTRACT

Animal models of malaria, mainly mice, have made a large contribution to our knowledge of host–pathogen inter-actions and immune responses, and to drug and vaccine design. Non-human primate (NHP) models for malaria are admittedly under-used, although they are probably closer models than mice for human malaria; in particular, NHP models allow the use of human pathogens (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium knowlesi*). NHPs, whether natural hosts or experimentally challenged with a simian *Plasmodium*, can also serve as robust pre-clinical models. Some simian parasites are closely related to a human counterpart, with which they may share a common ancestor, and display similar major features with the human infection and pathology. NHP models allow longitudinal studies, from the early events following sporozoite inoculation to the later events, including analysis of organs and tissues, particularly liver, spleen, brain and bone marrow. NHP models have one other significant advantage over mouse models: NHPs are our closest relatives and thus their biology is very similar to ours.

Recently developed *in vivo* imaging tools have provided insight into malaria parasite infection and disease in mouse models. One advantage of these tools is that they limit the need for invasive procedures, such as tissue bi-opsies. Many such technologies are now available for NHP studies and provide new opportunities for elucidating host/parasite interactions. The aim of this review is to bring the malaria community up to date on what is current-ly possible and what soon will be, in terms of *in vivo* imaging in NHP models of malaria, to consider the pros and the cons of the various techniques, and to identify challenges.

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1. Introduction

Malaria parasites infect a wide range of animals, including reptiles, birds, rodents and NHPs, in addition to humans. Four species, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium falciparum* and *Plasmodium vivax*, are recognized as natural malaria parasites of humans. More recently, it has emerged that a fifth species, *Plasmodium knowlesi*, that morphologically resembles *P. malariae* and consequently has undoubtedly been misdiagnosed in the past, is a human pathogen [1]. The main simian *Plasmodium* parasites infecting Old World primates include *P. knowlesi*, *Plasmodium coatneyi*, *Plasmodium fragile*, *Plasmodium cynomolgi*, *Plasmodium inui*, *Plasmodium fieldi*, and *Plasmodium simiovale*, in South East Asia, as well as *Plasmodium gonderi* in Africa, while simian *Plasmodium* parasites infecting New World primates comprise *Plasmodium brasilianum* and *Plasmodium simium* in South America [2].

Simian *Plasmodium* parasites sharing similarities with human parasites were reported at the beginning of the XXth century [3]. Deciphering the evolutionary history of *Plasmodium* parasites and their primate hosts' restrictions is a matter of intensive research, to determine the origin of human *Plasmodium* parasites and when they emerged.

Plasmodium parasites show a large propensity to "jump" from NHPs to humans and the reverse. The origin of *P. falciparum* was recently determined. African Apes are likely a reservoir and the species has been found in Gorillas [4,5], Bonobos [6] and Chimpanzees [7]. For *P. vivax* and *P. malariae*, which are genetically closely related to neotropical *P. simium* and *P. brasilianum*, the mechanisms of the host switch, from New World primates to humans (zoonosis) or the reverse (anthroponosis), have not been completely unraveled [8]. Some New World monkeys are susceptible to human *P. falciparum*, *P. vivax* and *P. malariae*. Current simian *Plasmodium* species known to naturally infect humans include not only *P. knowlesi*, but also, although very rarely, *P. simium* in South America and *P. cynomolgi* in Asia [3,9].

This close relatedness between human and NHP hosts and between human and simian *Plasmodium* parasites, makes NHP models of choice for human malaria for the biomedical research community.

2. A plethora of animal models are available to study *Plasmodium* biology and pathogenesis, malaria immunity, and vaccine and drug efficacy

In contrast to other pathogens such as HIV, both in-field trials and experimental human challenges are feasible with *Plasmodium* [10]. Human subjects are mostly used to assess vaccine-induced protection. Nonetheless, animal models remain highly valuable, not only to avoid ethical problems with work on humans, but also to control the experimental conditions. Indeed the choice of the pathogen form (species, strain, isolate or clone) and the timing of infection allow the study of different aspects of the disease. Furthermore, the type of infection, using infected red blood cells (iRBCs) or sporozoites, leads to study more specifically the blood-stage or the transmission and the liver-stage, respectively.

Animal models allow longitudinal studies from the early events following sporozoite inoculation, analysis of clinically-silent stages and collections of organs and tissues. Confounding factors, such as previous infections, co-infections or malnutrition can be avoided. However, there is not a single animal model that perfectly reproduces the spectrum of human *Plasmodium* infection, malaria and immunity. Differences between parasite–host combinations may indeed be viewed as an asset, because they may reflect the heterogeneity of human malaria, as highlighted in a recent debate on animal models in malaria research [11]. Each model has different strengths, limitations and degree of relevance, and thus models should be carefully selected according to the scientific issues and hypotheses addressed.

2.1. Limitations of mice and humanized mouse models for malaria

Mice are extensively used for modeling malaria [12–16]. Human *Plasmodium* parasites are unable to infect mice, so rodent malaria parasites may be used. The four most widely used species are *Plasmodium berghei*, *Plasmodium yoelii*, *Plasmodium chabaudi* and *Plasmodium vinckei*, and they are used with inbred or outbred mice. They were isolated from their natural hosts, such as the African rats for *P. berghei*, and then adapted to laboratory mice. Rodent *Plasmodium* infections in mice display some, but not all, of the main features of the human infection and disease.

Complex humanized mouse models have been developed to overcome the host species barriers to the use of *P. falciparum* in small laboratory animals [17]. Briefly, mice with a reconstituted human hematopoietic system, producing human RBCs, susceptible to several *P. falciparum* strains and supporting long-lasting parasitemia and gametocyte development, would be ideal for studying the blood-stage. However, no such perfect model is available. Only transient models have been reported, involving perfusion of infected RBCs into immunodeficient mouse strains, such as NOD/SCID/IL2Rγ- [18–20].

For transmission and liver-stage studies, mice with engrafted human hepatocytes have been generated and there is evidence that following infection with *P. falciparum* sporozoites, liver-stage schizonts develop [21,22]. The complete development of liver-stage parasites was recently reported, with liver-stage schizonts exhibiting exoerythrocytic merozoite formation and merosome release, and even with the transition from *P. falciparum* liver-stage infection to a blood-stage infection with gametocyte development [23]. However, the main drawback of these humanized mice is clearly their low to medium throughput. They are also inappropriate for the study of *P. vivax*, which requires human reticulocytes and thus human erythropoiesis. Finally, these mouse models are not adapted for the assessment of specific immunity which requires a mature and fully competent human immune system.

2.2. Advantages of NHPs over mice and humans for the study of parasite biology, infection dynamics and pathogenesis

There are also several well-established NHP models available, although they are not widely used. This is probably because NHP studies require appropriate specific facilities (possibly with associated mosquito facilities), and more rigorous ethical justification than other animal models. They also require a close collaborative work between primatologists and malariologists, and specially trained personnel.

Safety issues are raised and BSL2 or BSL3 animal facilities are required when using mosquito-borne *Plasmodium* species infectious for humans, such as human *Plasmodium* parasites and some of the macaque parasites, including *P. cynomolgi* and *P. inui*.

NHP studies are also particularly costly. Nevertheless, many believe that such models are worth the effort and they are committed to promote the use of NHPs, because these models of malaria closely reproduce human malaria. Indeed, continued investment in NHP systems was recognized as a priority at a recent meeting on animal models for research on severe malaria [15].

According to the molecular timescale for Vertebrate evolution, mice and humans are separated by ~40 million years. Over this period, their immune systems have been subjected to very different selection pressures and there have been divergent host/pathogen adaptations. The *Macaca* genus is more closely related to humans, the most recent common ancestor being ~25 million years ago [24]. The phylogenetic proximity of humans and NHPs is associated with, for instance, comparable dendritic cell subsets and similar panoplies of pattern recognition receptors [25–27]. Both are central to the study of malaria immunology and to vaccine development.

In vivo malaria liver-stage studies in humans are virtually impossible, whereas NHPs provide access to asymptomatic stages, including the journey of sporozoites from skin to liver and the dormant hypnozoite forms in the liver, as seen with *P. vivax* or *P. cynomolgi*. In addition,

various genes are conserved among primate parasites (*P. falciparum*, *P. vivax* and *P. knowlesi*) but are absent from rodent parasites (*P. berghei*, *P. yoelii* and *P. chabaudi*) [28]. The potential protective efficacy of antigens corresponding to these genes or of new genetically attenuated sporozoites cannot be assessed in mice. The degree of attenuation resulting from the deletion of any particular gene may also differ between *Plasmodium* species [29].

The recent whole-genome sequencing of rhesus macaque (*Macaca mulatta*) and cynomolgus macaque (*Macaca fascicularis*) [30–32] makes these species even more valuable for modeling human diseases. Also, as spin-offs of the extensive use of these NHP species in HIV/AIDS research, numerous tools for detailed characterization of their physiology and immune responses, in characterized genetic backgrounds (MHC, restriction factors, and KIR genetics) are available [33–37]. In rhesus, cynomolgus and pigtailed macaques (*Macaca nemestrina*), tetramer staining to quantify antigen-specific CD8+T cells is feasible, since MHC class I typing is possible.

This drive for progress is well illustrated by Primate Info Net, a library and information service which gathers key information about various primate species and provides links to resources about NHPs in biomedical research (http://pin.primate/wisconsin.edu), or the Nonhuman Primate Reagent Resource whose goals are to facilitate the use of NHPs by producing specific reagents such as antibodies and recombinant cytokines, and by sharing information on cross-reactivity of immunological reagents and assays (http://nhpreagents.org). Similarly, a new research center dedicated to preclinical research programs on host–pathogen interactions, human vaccines and antimicrobial treatments in NHPs was recently launched in France: the Center for Infectious Disease Models and Innovative Therapies (IDMIT) (http://idmitcenter.fr) provides the scientific community with a NHP facility, with the cynomolgus macaques/*P. cynomolgi* model currently being implemented, combined with the most recent technologies, including imaging technologies (*in vivo* fluorescent microscopy and MRI are already available and PET–CT will be in 2017). To optimize the use of NHPs, particular emphasis is given to assay standardization and harmonization, and both a reagent repository and a biobank have been created.

2.3. Various complementary NHP models of human malaria

Several NHP/parasite combinations have been described. They are variously useful to study *Plasmodium* biology, transmission, immunology and/or pathology, acute and chronic infection, or severe malaria, cerebral malaria, placental malaria, and other malaria-associated pathologies (liver, lung, kidney or spleen involvement and thrombocytopenia, hemorrhage, and anemia). Very useful reviews have been published recently providing summaries of NHP models for the study of hepatic infections and pathogenesis [15,38]. The various isolates/strains adapted to NHPs, their history and their characteristics (*e.g.* natural *vs.* experimental host, virulence, lethality, capacity to produce infective gametocytes, resistance to anti-malarial drugs, complete or partial development) have been exhaustively listed elsewhere [2,39]. Here, we present only the most widely used models.

2.3.1. P. falciparum and P. vivax in neotropical primates

There are few *in vivo* models for human malaria using human parasites. The host specificity of malaria parasites is narrow such that the only suitable NHP species that can be infected with *P. falciparum*, *P. vivax* or *P. malariae* are some neotropical primates: South American owl monkeys (*Aotus* spp.) and squirrel monkeys (*Saimiri* spp.) [40,41]. Furthermore, the parasites must be adapted to grow in these nonnatural hosts. Only *P. ovale* cannot be adapted to these New World monkeys. The susceptibility to infection by sporozoites and/or iRBCs, the production of viable gametocytes, the characteristics of the bloodstage infection and the requirement for splenectomy all depend on the host/strain combination. The Colombian Night Monkey (*Aotus lemurinus griseimembra*) may emerge as one of the best NHP malaria

models because of its high susceptibility to infection both by blood forms and by sporozoites of both *P. falciparum* and *P. vivax* [42]. *Saimiri sciureus* is used to investigate *P. falciparum* blood-stage human parasites, but splenectomy is required for reproducible high parasitemia after injection of parasitized RBCs [43]. *Saimiri boliviensis* is considered as the most reliable model for *P. vivax* [41].

Chimpanzees are also susceptible to *P. falciparum* and *P. vivax*, as well as *P. malariae* and *P. ovale*. However, their use is limited for ethical reasons. Indeed, the European Union has now banned the use of great apes for research purposes (Chimpanzees, Bonobos, Gorillas and Orangutans) and such research is also now highly restricted in the US [44].

The obvious advantage of New World primates over Old World primates as models for human malaria is that human parasites can be used. However, the advantages of using human parasites are counterbalanced by the limited supply of neotropical primates. There are fewer breeding colonies than for macaques, for example, and export of some of them from their countries of origin is prohibited. A recent workshop on neotropical primates in biomedical research [45] led to the conclusion that efforts should be pursued to develop new reagents and to support breeding colonies, including by improving colony and veterinary management. Finally, less blood for experimental purposes can be collected from these species than from macaques.

2.3.2. P. knowlesi in macaques

P. knowlesi is a primate malaria parasite endemic in some South East Asian species, for example cynomolgus and pig-tailed macaques. *P. knowlesi* is also the fifth malaria species naturally infecting humans and causing life-threatening disease [46]. Experimental infections have been described in a wide range of NHPs, from Old World primates rhesus macaques and baboon (*Papio* spp.) to New World primates, such as squirrel and *Aotus* monkeys and the common marmoset (*Callithrix jacchus*). Interestingly, there are two opposed asexual-blood stage outcomes in rhesus and cynomolgus macaques: *P. knowlesi* causes severe disease, inevitably lethal if untreated, after inoculation of rhesus macaques with sporozoites or iRBCs, whereas parasite development is controlled and chronic infection develops in its natural host, cynomolgus macaques. Parasitemia needs to be monitored daily because *P. knowlesi* has an asexual life cycle of about 24 h and the parasites can increase quickly to detrimental levels. The model of *P. knowlesi* in macaques is mainly used for investigating parasite biology, physiopathology and immunology, and for vaccine studies [47–49].

2.3.3. Simian Plasmodium species in macaques

Human *Plasmodium* species, other than *P. knowlesi*, do not infect Old World monkeys. However, there are many simian *Plasmodium* species naturally infecting macaques, and some are used as experimental models of human malaria.

P. coatneyi and *P. fragile*, which naturally infect cynomolgus macaques, and bonnet (*Macaca radiata*) and toque macaques (*Macaca sinica*), respectively, are used as models of *P. falciparum* in various macaques (*M. mulatta*, *M. fascicularis*, *M. nemestrina* or *Macaca fuscata*), with sequestration, rosetting, and severe disease, including cerebral malaria [50–52]. Both parasites are very virulent after sporozoite inoculation of rhesus macaques and Japanese macaques (*M. fuscata*) [53,54].

P. cynomolgi, which naturally infects a wide variety of macaque hosts, including cynomolgus macaques, is a model for studying the biology, immunology and pathology of *P. vivax*. It mimics the biology and pathogenesis of *P. vivax*, with the dormant relapsing parasite forms, hypnozoites, and similar RBC infection features (reticulocytes) [48,55–57].

Although *P. fieldi* and *P. simiovale*, which naturally infect cynomolgus and pigtailed macaques and toque macaques, respectively, share some characteristics with *P. ovale*, they are reproducing better features of *P. vivax* infection. Both are used as models of *P. vivax* in rhesus and cynomolgus macaques, but not as frequently as *P. cynomolgi* [49].

P. inui naturally infects a large variety of macaque species, and is considered as a macaque model for *P. malariae*, as it displays persistence and a similar kidney pathology [58].

Some macaque species are natural hosts, and consequently there has been evolutionary co-adaptation [59]. Note also that animals may have been exposed to malaria before experimental infection. It may be preferable to use naive animals: captive-born, or from areas where the prevalence of malaria is low, such as cynomolgus macaques from Mauritius.

In malaria endemic regions, there are other pathogens which may interfere with the host immune responses to *Plasmodium*. Macaques are also used to study a wide range of infections with human pathogens or their simian counterparts, as well as vaccines [60,61]. Thus, it is possible to model co-infections in macaques, such as SIV/*Plasmodium* [62–65] and *Schistosoma*/*Plasmodium* [66] and to study interference with vaccine-induced immune responses [67].

To conclude, NHP models of human malaria present several advantages over mouse models, which justify the efforts to facilitate and improve their use. This is especially true as *in vivo* imaging technologies, which have revolutionized our understanding of malaria in the last few years using mouse models, are actually not limited to small laboratory animals. However, insufficient access to adequate structures and equipment for imaging NHPs associated with suitable confinement for class 2 and 3 pathogens, in large part explains the relatively limited exploration of pathogen transmission and dissemination using *in vivo* imaging in NHPs. Nevertheless, *in vivo* imaging is entirely feasible in larger animals such as monkeys [68,69]; even though adult male Aotus monkeys and rhesus macaques weigh about 1 and 8 kg on average, respectively, and are about 35 and 55 cm long, respectively [70] (mice weigh 20–30 g and are 6 cm long).

3. *In vivo* imaging and infectious diseases in NHPs

Early events in the transmission and dissemination of pathogens in the host organism through mucosal barriers are still poorly understood in most cases. A better characterization of these early steps of infection is needed to develop and improve prevention strategies and therapy.

In vivo imaging can be used for non-invasive and longitudinal studies, allowing the use of animals to be minimized and refined. Molecular imaging techniques such as optical imaging (fluorescence, bioluminescence), PET (positron emission tomography) and SPECT (single-photon emission tomography) can document molecular and cellular events. These methods are also complementary, as different techniques are appropriated for different biological investigations in different organs. The strengths and weaknesses of various imaging techniques have been reviewed elsewhere [71,72].

Fluorescence imaging and bioluminescence imaging involve the detection of visible light and are therefore limited to the study of superficial organs; they have mainly been used in small animals. The feasibility of using bioluminescence to visualize infection by, and dissemination of, various viruses, such as murine herpes virus, mouse hepatitis virus, coronavirus, or Chikungunya virus, has been demonstrated in murine models [73–75]. The relative ease of use and the relatively low cost of these imaging techniques make them useful for the exploration of superficial organs even in large animals. Furthermore, with adapted endoscopic and intraoperative techniques, fluorescence could be used to study deeper organs. Traditional confocal microscopy can image at depths up to 100 μm. Intravital multiphoton imaging with multiphoton excitation can extend this limit up to a maximum of 1 mm in living animals [76], which is a huge advantage, especially when excision of the specimen is not possible. Recently, two-photon microscopy has been used for imaging and analyzing immune responses in single cells in intact tissues and for time-lapse imaging of living tissues. Indeed, the use of a pulsed infrared laser for fluorescent dye excitation was a major advance [77]: the high excitation wavelength allows deep tissue imaging and its relatively low energy, constrained to the focal plane, limits phototoxicity. As a consequence, two-photon microscopy has

become the technique of choice to follow the dynamic behavior of immune cells *in vivo*; single cells can be studied in murine models of tumors, skin graft or infection [78–80]. For instance, intravital two-photon microscopy of the popliteal lymph nodes in mice was used to investigate how fluorescently labeled inactivated vesicular stomatitis virus (VSV) is captured from the lymph and transported into the B cell compartment to induce humoral immunity [81]. It has also been used to investigate the dynamic behavior of T cells infected with green fluorescent protein- (GFP-) expressing HIV in the stromal environment of lymph nodes [82]. Intravital microscopy of the skin or surgically exposed internal organs offer excellent resolution for studying individual cells or even subcellular structures and microorganisms [83,84], although this is not strictly noninvasive. However, this technique has only been applied to murine models.

Positron emission tomography (PET), magnetic resonance imaging (MRI) and single photon emission computed tomography (SPECT) can also be used in both small and large animals. Indeed, they are routinely used for clinical purposes [72]. These techniques can reveal molecular and cellular events. The sensitivity of PET is high, in the range of 10^{-11} to 10^{-12} mol/L, and is independent of the location, or depth, of the reporter probe. Combining morphological/anatomical and molecular imaging methods, using multimodality hardware and/or co-registration post-acquisition processing, allows whole-body exploration of the early events in the transmission of pathogens through mucosal barriers, assessment of the dynamics of pathogen biodistribution, evaluation of the dynamics of early events following vaccinations, and determination of the pharmacokinetics and pharmacodynamics of new drugs in infected hosts. CT (computed tomography) and MRI can be combined with PET or SPECT to provide anatomical references [85]. MRI gives good spatial resolution allowing both anatomical imaging and molecular imaging, if associated with contrast agents, for cell tracking, characterization of inflammation or cancer cell detection [86–88]. [^{18}F]-labeled fluoro-2-deoxy-2-D-glucose ([^{18}F]FDG) has been widely used for PET imaging of activated cells (such cells show increased glucose metabolism). [^{18}F]-labeled 3′-fluoro-3′-deoxy-thymidine ([^{18}F]FLT) was designed as a tracer for cell proliferation and is increasingly being exploited in oncology.

The location and replication of pathogens can be followed by PET reporter gene imaging, for example using herpes simplex virus-1 thymidine kinase (HSV1-tk). HSV1-tk phosphorylates a broad range of nucleoside analogs, and its expression can be imaged with several substrate analogs including 2′-deoxy-2′-^{18}F-fluoro-5-ethyl-1-β-D-arabinofuranosyl-uracil (^{18}F-FEAU) and acycloguanosine derivatives, such as 9-(4-^{18}F-fluoro-3-[hydroxymethyl]butyl)guanine (^{18}F-FHBG). This strategy has been widely used in various animal models including NHPs, but not yet in the malaria context [89–92]. PET imaging has also been used to detect immune activation of T cells in lymphoid organs in the context of antitumor immunity using an [^{18}F]-labeled 2′-deoxycytidine analog in a mouse model and [^{18}F]FLT in humans after anti-tumor vaccination [93,94].

The pharmacokinetics, biodistribution, metabolism and toxicity of diagnostic doses of novel radiolabeled or contrast imaging agents have been studied in NHPs to facilitate translation to clinical applications [68,95]. *In vivo* brain imaging by PET and/or MRI has been developed for diverse experimental models of neurodegenerative studies. For instance, imaging techniques, including PET, can directly visualize the concentration and localization of amyloid deposits, the pathological marker of Alzheimer disease, throughout the brain [96–98].

The immune response to viral infection in SHIV-infected macaques has also been explored by SPECT [99] and delivery of a botulism vaccine has been studied by a combination of PET and MRI [69]. Also, [^{18}F]FDG PET/CT imaging has also been used to assess novel combinations of new drugs in NHP models of tuberculosis [100].

The variety of imaging technologies available for the examination of living material is increasing rapidly. The choice of imaging technology for a study should be based on the biological issues to be resolved and the organ(s) or tissues to be explored. For *Plasmodium* infection and

malaria studies, the organs and tissues of interest (*e.g.* skin, blood, lymph, liver, spleen, lungs or brain) depend on the malaria stage, transmission, and liver- and blood-stages. Similarly, the forms of the pathogen differ during parasitic development. Mosquitoes inject sporozoites into the skin. Sporozoites are characterized by their size (10–15 μm in length and 1 μm in diameter), their mobility, and their characteristics of migration from skin to liver, including the speed, the gliding motility and the cell traversal. After productive invasion of hepatocytes, parasites develop in the parasitophorous vacuole until the egress of merosomes from liver cells, with or without dormant hypnozoite formation, depending on the *Plasmodium* spp. After merosomes burst in the microvasculature, merozoites (1.5–2.5 μm in length and 1.0–2.0 μm in diameter) are released and invade RBCs. The resulting iRBCs can bind non-infected RBCs (rosetting), and adhere to endothelial cells of the microvasculature (sequestration). The rupture of schizont-infected erythrocytes releases new merozoites. Some parasites eventually differentiate into male and female gametocytes (up to 10 μm in length and 2.5 μm in diameter) that can be ingested by mosquitoes. Therefore, the strategy for *in vivo* imaging of *Plasmodium* infection and malaria, and thus the choice of imaging techniques, should be adapted to the resolution, depth of penetration, and sensitivity required. Multi-modal imaging approaches are entirely feasible.

4. *In vivo* imaging of malaria in NHPs

4.1. Clinical severe malaria: imaging of brain and spleen in NHPs

In vivo imaging studies of malaria have mainly focused on clinical features of the disease using various animal models, including NHP models. In particular, the features of cerebral malaria in NHPs have been explored using *in vivo* imaging.

Cerebral malaria is a major complication of severe human malaria and is defined as acute encephalopathy caused by *P. falciparum* infection [101]. Imaging analysis of the brain is currently the preferred technique for the evaluation and diagnosis of impaired cerebral function. There have been numerous reports of brain imaging (CT, MRI) in patients with cerebral malaria [102–105], revealing brain swelling, small hemorrhagic lesions, and focal lesions in the cerebrum and brainstem. However, the correlation between pathological changes and altered metabolic activity, which may directly reflect neurological symptoms in cerebral malaria patients, is not clearly understood [106].

MRI and magnetic resonance spectroscopy (MRS) have been used to study experimental cerebral malaria and to identify its early markers in mice infected with *P. berghei* ANKA [107–109]. Platelet accumulation, which occurs in the microvasculature of patients with cerebral malaria, has also been investigated by MRI in the *P. berghei* ANKA/murine model [110].

PET imaging with [18F]FDG has been used in a primate model of severe human malaria (Japanese macaques infected with *P. coatneyi*) to evaluate cerebral glucose metabolism [53,54,106,111]. The value of the *P. coatneyi* Japanese macaque model for the study of cerebral malaria was demonstrated by the identification of cytoadherence of infected erythrocytes to brain endothelial cells within microvessels *in vivo*, similar to observations in human cerebral malaria [53,54]. Indeed, FDG-PET imaging studies demonstrated that pathological findings in infected monkeys were similar to those in human cases of *P. falciparum* malaria [106].

The group that studied cerebral glucose metabolism in Japanese macaques infected with *P. coatneyi* also investigated the relationship between glucose uptake by the spleen determined by FDG-PET and histopathological changes in spleen tissue: FDG uptake was higher in infected than control animals. This may reflect the activation of host splenic clearance systems or glucose consumption by congested malarial parasites themselves, or both [112].

Imaging analysis of the progression of pathological processes in NHP models can be informative about the mechanisms of malaria. The use of *in vivo* imaging in NHP models of experimental malaria may help the development of imaging strategies that could be transferred to studies for

humans. However, murine models of malaria, and particularly as concerns the transmission, liver-stage and blood stage of the parasite, have been much more extensively used than NHP models for *in vivo* imaging.

4.2. Transmission, liver- and blood-stages: imaging mainly in mice

The techniques of *in vivo* imaging can contribute to parasitology by providing dynamic images of host–pathogen interactions *in vivo*, thereby overcoming the limitations of post-mortem investigation and *in vitro* studies (reviewed in [113–115]). Most imaging studies in murine models of malaria have used fluorescence, including wide-field fluorescence microscopy, intravital microscopy, and bioluminescence. To analyze the migration of parasites, transgenic parasites expressing GFP [116,117], red fluorescent protein (RFP) [118] or a GFP–luciferase fusion protein [119] were used. Fluorescent *P. berghei* sporozoites have been used to study mosquito and mammalian host interactions in the dermis with malaria parasites by *in vivo* microscopy [116,117,120–122]. Not all sporozoites reach the liver. Some enter lymph vessels and accumulate in the draining lymph node, where they appear to reside for some time before being degraded [120]; others remain in the skin. It is possible to observe and count the number of fluorescent parasites that are ejected from the mosquito over time [123]. This has revealed that there is a limited pool of *Plasmodium* sporozoites available for ejection within the vector's salivary glands.

RFP- and GFP-expressing parasites and various *in vivo* microscopy techniques have been used for *in vivo* investigations of the behavior of *P. berghei* parasites in the hepatic tissue of the murine host. These studies have provided useful information about the primary infection process and about parasite interactions with the host immune cells in the liver [118,124–126].

The use of bioluminescence imaging has been described for *in vitro* screening of inhibitors and chemicals for antimalarial activity against blood stages of *P. berghei* [127], and for *in vitro* and *in vivo* assays to analyze *Plasmodium* liver stage development using transgenic *P. berghei* (PbGFP-Luccon) or *P. yoelii* (Py-GFP-luc) [128] parasites, which express the bioluminescent reporter protein, luciferase. In these assays, parasite development in hepatocytes can be visualized and quantified by real-time bioluminescence imaging both in culture and in live mice. The luminescence signal is a measure of the parasite load in the liver parasite [129,130]. PbGFP-Luccon can therefore be used for the evaluation of protective immunity against malaria, following immunization with either radiation attenuated sporozoites or wild-type sporozoites under chloroquine prophylaxis [131].

These transgenic parasites are straightforward and valuable tools for investigating the biology and immunology underlying the mechanisms of pathogen transmission and dissemination, and the mechanisms of protection against malaria. However, findings in murine models require confirmation in NHP models. Rodents and primates differ in their sites and mechanisms of erythropoiesis, making NHP research for blood-stage malaria valuable. Furthermore, work with NHP models would contribute to a more complete understanding of the mechanisms of infection of some parasites, such as *P. vivax*. This species has a silent liver phase in humans which could not been explored in murine models since rodent parasites do not have a dormant liver-stage form.

4.3. Tools required facilitating in vivo imaging of malaria in NHP models

Progress in various areas would allow the use of NHP models of human malaria to be extended: improvement of techniques for culture and laboratory maintenance of *Plasmodium*, completion of their genome sequences and generation of transgenic strains [132,133]. These issues are not specific to the *in vivo* imaging field. Advances in these areas are in line with the research agenda for malaria eradication (malERA), recently established by the consultative group on Basic Science and Enabling Technologies [134].

Table 1

Several malaria NHP models have been developed to study host/pathogen interactions, disease pathogenesis, malaria immunity, drugs and vaccines. New World primates (*Aotus* and *Saimiri*) are used for experimental infections with either human malaria species (except *P. ovale*) or simian malaria species. They are the only animal models available for the study of vaccine efficacy or drug susceptibility of human malaria parasites *P. falciparum* and *P. vivax*. Macaques (Old World primates), when infected with simian malaria parasites, can also be used to study the biology of the human malaria and to explore mechanisms and treatments for severe pathology associated with malaria infections. Only the main models are listed below [2,11,38,39].

	Plasmodium	NHP	Model	Remarks	References
Human *Plasmodium*	*P. falciparum*[a]	*Aotus lemurinus griseimembra*	Human infection with *P. falciparum*, severe malaria and vaccine efficacy	No cerebral malaria	[42]
	P. vivax	*Aotus lemurinus griseimembra* *Saimiri boliviensis*	Human infection with *P. vivax*, severe malaria and vaccine efficacy	For protective efficacy testing of sporozoite and liver stage vaccines but not blood-stage vaccines (unless strains further adapted in spleen-intact animals)	[41,42]
	P. knowlesi[a]	*Macaca mulatta*	Human infection with *P. knowlesi*, severe malaria and malaria immunity	Including cyto-adherence, parasite sequestration and Ag variation with Ag switching *in vivo*; renal failure	[47–49]
		Macaca fascicularis	Natural control of parasite development and chronic infection (natural host)		
Simian *Plasmodium*	*P. coatenyi*	*Macaca mulatta* *Macaca fuscata*[b]	Human infection with *P. falciparum* and severe malaria	Including cerebral malaria, cytoadherence, parasite sequestration and Ag variation, anemia, placental malaria	[50,51,53,54,66]
	P. cynomolgi[a]	*Macaca mulatta* *Macaca fascicularis*	Human infection with *P. vivax* and malaria immunity	Including relapse-infections resulting from reactivation of hypnozoites and unique iRBC features	[48,55–57,64]
	P. fragile	*Macaca mulatta*	Human infection with *P. falciparum* and severe malaria	Including cerebral malaria; cytoadherence, parasite sequestration and Ag variation	[52,62,63,65]

[a] The only transgenic parasites encoding an imaging reporter gene that could be used for *in vivo* fluorescent microscopy and bioluminescence are *P. falciparum*, *P. knowlesi* and *P. cynomolgi*. So far, they have only been used *in vitro*.

[b] To date, only cerebral malaria in the NHP model of *M. fuscata/P. coatneyi* was studied using *in vivo* imaging (PET).

Currently, it is not possible to culture *Plasmodium* throughout its complete life cycle. Long-term *in vitro* blood-stage culture of *P. vivax* and *P. cynomolgi* is not possible, in contrast to *P. falciparum* and *P. knowlesi* [135]. If such culture techniques were available, it would be possible to study at least blood-stage parasites more easily, to screen drugs, and to feed female *Anopheles* mosquitoes, without having to use infected donor NHPs for a continuous source of parasites. Unfortunately, some *in vitro*-adapted strains lose their capacity to produce sexual stages, like for *P. knowlesi* [135].

Bites from infected mosquitoes or sporozoite injection can be used for experimental sporozoite inoculation. Sporozoites isolated from salivary glands of mosquitoes have a limited *ex vivo* viability [136,137], and methods to improve their preservation would be useful.

Mosquito colonies need to be maintained near the NHP facilities to facilitate transmission and liver-stage studies in NHP models due to the lack of an effective *in vitro* sporozoite culture system, including for the transformation of gametocytes into ookinetes and the sporogonic development into sporozoites occurring in mosquitoes.

Whole genome sequences of *P. falciparum* [138], *P. vivax* [139] and *P. knowlesi* have been reported [140]. Draft sequences for three

Table 2

Available tools for *in vivo* imaging of *Plasmodium* parasites and host response in NHPs. So far these transgenic parasites expressing an imaging reporter gene have only been used for *in vitro* studies, and should be developed for other parasites such as *P. vivax* to explore the biology of *Plasmodium* by *in vivo* imaging.

Imaging the pathogen		
Available transgenic *Plasmodium* parasites	**Imaging techniques**	**References**
• Transgenic GFP and GFP–Luc *P. falciparum* with a constitutive expression	Fluorescence Bioluminescence	[145,158,23]
• Transgenic *P. falciparum* with a blood-stage expression		
• Transgenic GFP–mCherry *P. cynomolgi* with a constitutive expression	Fluorescence	[55]
• Transgenic GFP *P. knowlesi* with a blood-stage expression	Fluorescence	[159]

Imaging the host		
Model	**Imaging techniques**	**References**
Cerebral/splenic glucose metabolism in *P. coatneyi*/Japanese macaque models	[^{18}F]FDG-PET imaging MRI	[106,111,112]

P. cynomolgi strains were published recently [141]. Improved and additional genomes for each species are in progress.

Genetic manipulations (gene disruption, mutation, tagging or introduction) were initially developed in *P. berghei* [142] and then in *P. falciparum* [143–145]. The generation of transgenic parasites that contain an imaging-reporter gene, either a fluorescent reporter or an enzymatic reporter, such as luciferase or tk, is essential for *in vivo* imaging. Exogenous genes can be introduced either on a plasmid or by homologous recombination of a linear DNA fragment into the parasite genome, targeted to a silent chromosomal locus without alteration of the phenotype of the parasite after disruption, such as the dispensable Pf47 locus in *P. falciparum*. Episomal plasmids containing origins of replication derived from bacteria do replicate in *Plasmodium*; they are not equally segregated into daughter cells during mitosis and they may be lost. Stable episomal transfection is however possible, and differs from transient episomal transfection by the presence of a selection marker in the transfected DNA. Multiple rounds of drug treatment allow selecting parasites with crossover integration of episomes. For comprehensive reviews and detailed protocols, see [146–149]. Briefly, the asexual stage has a haploid genome and thus is the preferred stage for the generation of transgenic parasites because it requires modification of only a single allele. Optimal conditions have been defined for electroporation and for nucleofection ensuring the access of exogenous DNA to the parasite nucleus while preserving viability. Transfection efficiencies differ according to the developmental stage of the parasite and the *Plasmodium* species. Infected erythrocytes containing ring-stage parasites (*P. falciparum*) or mature schizonts (*P. berghei*) can be used for genetic manipulations, and transfected (*e.g.* resistant or fluorescent) parasites can be selected and cloned either *in vivo* or *in vitro*. Various promoters, both homologous and heterologous, with different strengths and timing of activity, *e.g.* stage-specific, conditional or constitutive, are available for use in these species.

The third *Plasmodium* parasite for which transfection has been reported is *P. knowlesi* [132,150–152]. Stable transfection of *P. vivax* has not yet been described: only transient transfection of *P. vivax* blood-stage parasites using *P. vivax*-infected reticulocytes from splenectomized monkeys has been reported [153,154]. Stable transfection of *P. cynomolgi* has been demonstrated [155,156].

There is no standardization in generating and reporting genetically modified primate malaria parasites, in contrast to rodent transgenic parasites [157]. There are several transgenic *P. falciparum* expressing an imaging reporter gene during blood-stage. They are used for *in vitro* assays,

likely because of the lack of gametocyte production. Theoretically, RBCs infected with these transgenic parasites could be used for *in vivo* imaging of blood-stage. Transgenic *P. falciparum* expressing an imaging reporter gene throughout the entire parasite life cycle include *P. falciparum* expressing GFP (3D7HT-GFP) [158], deposited at the Malaria Research and Reference Reagent Resource Center (MR4) and tested for liver-stage using *ex vivo* human primary hepatocytes infection; and *P. falciparum* expressing a luciferase–GFP fusion (NF54HT-GFP–Luc) tested for liver-stage using human liver chimeric mice [23]. They are available for *in vivo* imaging studies in NHP models of malaria. Recently, a transgenic *P. cynomolgi* with two expression cassettes for constitutive expression of GFP and mCherry was developed. *Ex vivo* infection of primary hepatocytes from rhesus monkey was performed and developing liver stage and hypnozoites forms were documented by live fluorescent microscopy [55]. Finally, the adaptation of a *P. knowlesi* line expressing GFP throughout the asexual blood-stage cycle to continuous culture in human erythrocytes has been recently demonstrated, providing potential tools for studying blood-stage in NHPs [159].

5. Conclusions and perspectives

In vivo imaging is an enormously powerful approach to deciphering host/*Plasmodium* interactions in mouse models, and NHP models are extremely valuable models of human malaria. Consequently, it is unsurprising that *in vivo* imaging is increasingly linked to primate parasitology, particularly as it becomes clear that *in vivo* imaging is feasible with larger animals. However, so far, studies using *in vivo* imaging approaches in NHPs are rare. They have been limited to PET imaging to document inflammation and cerebral malaria in the *M. fuscata*/*P. coatneyi* model [106,111,112]. Admittedly, facilities where the dual NHP/malaria expertise is available, and have both the desired model up-and-running and appropriate *in vivo* imaging devices can be difficult to find (Table 1). Nevertheless, the list of transgenic parasites encoding an imaging reporter gene usable in NHP models is growing (Table 2). To our knowledge, they have not yet been tested *in vivo*.

There is no doubt that the complexity of the interaction between pathogens and the host immune system can only be properly understood when studied in the native tissue environment [160]. Indeed the *in vivo* dynamic imaging techniques can reveal differences with *in vitro* data such as the CD8+T cell-mediated elimination of malaria liver stages [161]. Development of these new imaging tools that are applicable for NHP models will allow a better understanding of the biology of *Plasmodium* and of the host immune responses against parasites and parasitized cells as well as the development of novel therapeutic strategies. To conclude, *in vivo* imaging in NHP models of malaria could be very helpful to explore the following top four topics: the study of the early events in transmission to characterize the dynamic of skin stage and liver infection, the longitudinal characterization of hypnozoite forms and infection relapses, the time-lapse imaging of the invasion of red blood cells, and the dynamic imaging of host-parasite interactions and immunity.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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References

[1] Collins WE. *Plasmodium knowlesi*: a malaria parasite of monkeys and humans. Annu Rev Entomol 2012;57:107–21.
[2] Galinski MR, Barnwell JW. Nonhuman primate models for human malaria research. In: Elsevier, editor. Nonhuman primates in biomedical research:diseases. 3rd ed. Academic Press; 2012. p. 299–323.
[3] Coatney GR. The simian malarias: zoonoses, anthroponoses, or both? Am J Trop Med Hyg 1971;20:795–803.
[4] Prugnolle F, Ollomo B, Durand P, Yalcindag E, Arnathau C, Elguero E, et al. African monkeys are infected by *Plasmodium falciparum* nonhuman primate-specific strains. Proc Natl Acad Sci U S A 2010;108:11948–53.
[5] Liu W, Li Y, Learn GH, Rudicell RS, Robertson JD, Keele BF, et al. Origin of the human malaria parasite *Plasmodium falciparum* in gorillas. Nature 2010;467:420–5.
[6] Krief S, Escalante AA, Pacheco MA, Mugisha L, Andre C, Halbwax M, et al. On the diversity of malaria parasites in African apes and the origin of *Plasmodium falciparum* from Bonobos. PLoS Pathog 2010;6:e1000765.
[7] Duval L, Fourment M, Nerrienet E, Rousset D, Sadeuh SA, Goodman SM, et al. African apes as reservoirs of *Plasmodium falciparum* and the origin and diversification of the *Laverania* subgenus. Proc Natl Acad Sci U S A 2011;107:10561–6.
[8] Tazi L, Ayala FJ. Unresolved direction of host transfer of *Plasmodium vivax* v. *P. simium* and *P. malariae* v. *P. brasilianum*. Infect Genet Evol 2011;11:209–21.
[9] Deane LM. Simian malaria in Brazil. Mem Inst Oswaldo Cruz 1992;87(Suppl. 3):1–20.
[10] Sauerwein RW, Roestenberg M, Moorthy VS. Experimental human challenge infections can accelerate clinical malaria vaccine development. Nat Rev Immunol 2011;11:57–64.
[11] Langhorne J, Buffet P, Galinski M, Good M, Harty J, Leroy D, et al. The relevance of non-human primate and rodent malaria models for humans. Malar J 2011;10:23.
[12] Renia L, Belnoue E, Landau I. Mouse models for pre-erythrocytic-stage malaria. Methods Mol Med 2002;72:41–55.
[13] Sanni LA, Fonseca LF, Langhorne J. Mouse models for erythrocytic-stage malaria. Methods Mol Med 2002;72:57–76.
[14] Wykes MN, Good MF. What have we learnt from mouse models for the study of malaria? Eur J Immunol 2009;39:2004–7.
[15] Craig AG, Grau GE, Janse C, Kazura JW, Milner D, Barnwell JW, et al. The role of animal models for research on severe malaria. PLoS Pathog 2012;8:e1002401.
[16] Stephens R, Culleton RL, Lamb TJ. The contribution of *Plasmodium chabaudi* to our understanding of malaria. Trends Parasitol 2012;28:73–82.
[17] Vaughan AM, Kappe SH, Ploss A, Mikolajczak SA. Development of humanized mouse models to study human malaria parasite infection. Future Microbiol 2012;7:657–65.
[18] Arnold L, Tyagi RK, Meija P, Swetman C, Gleeson J, Perignon JL, et al. Further improvements of the *P. falciparum* humanized mouse model. PLoS One 2011;6:e18045.
[19] Moore JM, Kumar N, Shultz LD, Rajan TV. Maintenance of the human malarial parasite, *Plasmodium falciparum*, in scid mice and transmission of gametocytes to mosquitoes. J Exp Med 1995;181:2265–70.
[20] Moreno Sabater A, Moreno M, Moreno FJ, Eguiluz C, van Rooijen N, Benito A. Experimental infection of immunomodulated NOD/LtSz-SCID mice as a new model for *Plasmodium falciparum* erythrocytic stages. Parasitol Res 2005;95:97–105.
[21] Morosan S, Hez-Deroubaix S, Lunel F, Renia L, Giannini C, Van Rooijen N, et al. Liver-stage development of *Plasmodium falciparum*, in a humanized mouse model. J Infect Dis 2006;193:996–1004.
[22] Sacci Jr JB, Alam U, Douglas D, Lewis J, Tyrrell DL, Azad AF, et al. *Plasmodium falciparum* infection and exoerythrocytic development in mice with chimeric human livers. Int J Parasitol 2006;36:353–60.
[23] Vaughan AM, Mikolajczak SA, Camargo N, Lakshmanan V, Kennedy M, Lindner SE, et al. A transgenic *Plasmodium falciparum* NF54 strain that expresses GFP–luciferase throughout the parasite life cycle. Mol Biochem Parasitol 2012;186(2):143–7.
[24] Kumar S, Hedges SB. A molecular timescale for vertebrate evolution. Nature 1998;392:917–20.
[25] Lore K. Isolation and immunophenotyping of human and rhesus macaque dendritic cells. Methods Cell Biol 2004;75:623–42.
[26] Ketloy C, Engering A, Srichairatanakul U, Limsalakpetch A, Yongvanitchit K, Pichyangkul S, et al. Expression and function of Toll-like receptors on dendritic cells and other antigen presenting cells from non-human primates. Vet Immunol Immunopathol 2008;125:18–30.
[27] Gujer C, Sundling C, Seder RA, Karlsson Hedestam GB, Lore K. Human and rhesus plasmacytoid dendritic cell and B-cell responses to Toll-like receptor stimulation. Immunology 2011;134:257–69.
[28] Frech C, Chen N. Genome comparison of human and non-human malaria parasites reveals species subset-specific genes potentially linked to human disease. PLoS Comput Biol 2011;7:e1002320.
[29] Annoura T, Ploemen IH, van Schaijk BC, Sajid M, Vos MW, van Gemert GJ, et al. Assessing the adequacy of attenuation of genetically modified malaria parasite vaccine candidates. Vaccine 2012;30:2662–70.
[30] Gibbs RA, Rogers J, Katze MG, Bumgarner R, Weinstock GM, Mardis ER, et al. Evolutionary and biomedical insights from the rhesus macaque genome. Science 2007;316:222–34.
[31] Yan G, Zhang G, Fang X, Zhang Y, Li C, Ling F, et al. Genome sequencing and comparison of two nonhuman primate animal models, the cynomolgus and Chinese rhesus macaques. Nat Biotechnol 2011;29:1019–23.
[32] Higashino A, Sakate R, Kameoka Y, Takahashi I, Hirata M, Tanuma R, et al. Whole-genome sequencing and analysis of the Malaysian cynomolgus macaque (*Macaca fascicularis*) genome. Genome Biol 2012;13:R58.

[33] Kaizu M, Borchardt GJ, Glidden CE, Fisk DL, Loffredo JT, Watkins DI, et al. Molecular typing of major histocompatibility complex class I alleles in the Indian rhesus macaque which restrict SIV CD8 + T cell epitopes. Immunogenetics 2007;59:693–703.

[34] Karl JA, Wiseman RW, Campbell KJ, Blasky AJ, Hughes AL, Ferguson B, et al. Identification of MHC class I sequences in Chinese-origin rhesus macaques. Immunogenetics 2008;60:37–46.

[35] Wiseman RW, Karl JA, Bimber BN, O'Leary CE, Lank SM, Tuscher JJ, et al. Major histocompatibility complex genotyping with massively parallel pyrosequencing. Nat Med 2009;15:1322–6.

[36] Lim SY, Rogers T, Chan T, Whitney JB, Kim J, Sodroski J, et al. TRIM5alpha modulates immunodeficiency virus control in rhesus monkeys. PLoS Pathog 2010;6: e1000738.

[37] Blokhuis JH, van der Wiel MK, Doxiadis GG, Bontrop RE. The mosaic of KIR haplotypes in rhesus macaques. Immunogenetics 2010;62:295–306.

[38] Prudencio M, Mota MM, Mendes AM. A toolbox to study liver stage malaria. Trends Parasitol 2011;27:565–74.

[39] Collins WE. Nonhuman primate models. I. Nonhuman primate host–parasite combinations. Methods Mol Med 2002;72:77–84.

[40] Collins WE. South American monkeys in the development and testing of malarial vaccines—a review. Mem Inst Oswaldo Cruz 1992;87(Suppl. 3):401–6.

[41] Collins WE. Nonhuman primate models. II. Infection of Saimiri and Aotus monkeys with Plasmodium vivax. Methods Mol Med 2002;72:85–92.

[42] Herrera S, Perlaza BL, Bonelo A, Arevalo-Herrera M. Aotus monkeys: their great value for anti-malaria vaccines and drug testing. Int J Parasitol 2002;32:1625–35.

[43] Contamin H, Behr C, Mercereau-Puijalon O, Michel J. Plasmodium falciparum in the squirrel monkey (Saimiri sciureus): infection of non-splenectomised animals as a model for exploring clinical manifestations of malaria. Microbes Infect 2000;2:945–54.

[44] Altevogt BM, Pankevich DE, Pope AM, Kahn JP. Research agenda. Guiding limited use of chimpanzees in research. Science 2012;335:41–2.

[45] Tardif SD, Abee CR, Mansfield KG. Neotropical primates in biomedical research. ILAR J 2011;52:386–92.

[46] Collins WE, Barnwell JW. Plasmodium knowlesi: finally being recognized. J Infect Dis 2009;199:1107–8.

[47] Weiss WR, Jiang CG. Protective CD8 + T lymphocytes in primates immunized with malaria sporozoites. PLoS One 2012;7:e31247.

[48] Praba-Egge AD, Montenegro S, Cogswell FB, Hopper T, James MA. Cytokine responses during acute simian Plasmodium cynomolgi and Plasmodium knowlesi infections. Am J Trop Med Hyg 2002;67:586–96.

[49] Collins WE, Skinner JC, Broderson JR, Filipski VK, Morris CM, Stanfill PS, et al. Susceptibility of Macaca fascicularis monkeys from Mauritius to different species of Plasmodium. J Parasitol 1992;78:505–11.

[50] Moreno A, Cabrera-Mora M, Garcia A, Orkin J, Strobert E, Barnwell JW, et al. Plasmodium coatneyi in rhesus macaques replicates the multisystemic dysfunction of severe malaria in humans. Infect Immun 2013;81:1889–904.

[51] Aikawa M, Brown A, Smith CD, Tegoshi T, Howard RJ, Hasler TH, et al. A primate model for human cerebral malaria: Plasmodium coatneyi-infected rhesus monkeys. Am J Trop Med Hyg 1992;46:391–7.

[52] Fujioka H, Millet P, Maeno Y, Nakazawa S, Ito Y, Howard RJ, et al. A nonhuman primate model for human cerebral malaria: rhesus monkeys experimentally infected with Plasmodium fragile. Exp Parasitol 1994;78:371–6.

[53] Kawai S, Aikawa M, Kano S, Suzuki M. A primate model for severe human malaria with cerebral involvement: Plasmodium coatneyi-infected Macaca fuscata. Am J Trop Med Hyg 1993;48:630–6.

[54] Kawai S, Kano S, Suzuki M. Rosette formation by Plasmodium coatneyi-infected erythrocytes of the Japanese macaque (Macaca fuscata). Am J Trop Med Hyg 1995;53:295–9.

[55] Voorberg-van der Wel A, Zeeman AM, van Amsterdam SM, van den Berg A, Klooster EJ, Iwanaga S, et al. Transgenic fluorescent Plasmodium cynomolgi liver stages enable live imaging and purification of malaria hypnozoite-forms. PLoS One 2013;8:e54888.

[56] Ylostalo J, Randall AC, Myers TA, Metzger M, Krogstad DJ, Cogswell FB. Transcriptome profiles of host gene expression in a monkey model of human malaria. J Infect Dis 2005;191:400–9.

[57] Dembele L, Gego A, Zeeman AM, Franetich JF, Silvie O, Rametti A, et al. Towards an in vitro model of Plasmodium hypnozoites suitable for drug discovery. PLoS One 2011;6:e18162.

[58] Collins WE, Warren M, Sullivan JS, Barnwell JW. Plasmodium inui shortii: studies in old world and new world monkeys. Am J Trop Med Hyg 2009;80:160–4.

[59] Bonhomme M, Blancher A, Jalil MF, Crouau-Roy B. Factors shaping genetic variation in the MHC of natural non-human primate populations. Tissue Antigens 2007;70:398–411.

[60] Gardner MB, Luciw PA. Macaque models of human infectious disease. ILAR J 2008;49:220–55.

[61] Shedlock DJ, Silvestri G, Weiner DB. Monkeying around with HIV vaccines: using rhesus macaques to define 'gatekeepers' for clinical trials. Nat Rev Immunol 2009;9:717–28.

[62] Trott KA, Richardson A, Hudgens MA, Abel K. Immune activation and regulation in simian immunodeficiency virus—Plasmodium fragile-coinfected rhesus macaques. J Virol 2013;87:9523–37.

[63] Ryan-Payseur B, Ali Z, Huang D, Chen CY, Yan L, Wang RC, et al. Virus infection stages and distinct Th1 or Th17/Th22 T-cell responses in malaria/SHIV coinfection correlate with different outcomes of disease. J Infect Dis 2011;204:1450–62.

[64] Koehler JW, Bolton M, Rollins A, Snook K, deHaro E, Henson E, et al. Altered immune responses in rhesus macaques co-infected with SIV and Plasmodium cynomolgi: an animal model for coincident AIDS and relapsing malaria. PLoS One 2009;4:e7139.

[65] Trott KA, Chau JY, Hudgens MG, Fine J, Mfalila CK, Tarara RP, et al. Evidence for an increased risk of transmission of simian immunodeficiency virus and malaria in a rhesus macaque coinfection model. J Virol 2011;85:11655–63.

[66] Semenya AA, Sullivan JS, Barnwell JW, Secor WE. Schistosoma mansoni infection impairs antimalaria treatment and immune responses of rhesus macaques infected with mosquito-borne Plasmodium coatneyi. Infect Immun 2012;80:3821–7.

[67] Yin J, Vahey MT, Dai A, Lewis MG, Arango T, Yalley-Ogunro J, et al. Plasmodium inui infection reduces the efficacy of a simian immunodeficiency virus DNA vaccine in a rhesus macaque model through alteration of the vaccine-induced immune response. J Infect Dis 2012;206:523–33.

[68] Nishii R, Tong W, Wendt III R, Soghomonyan S, Mukhopadhyay U, Balatoni J, et al. Pharmacokinetics, metabolism, biodistribution, radiation dosimetry, and toxicology of 18F-fluoroacetate (18F-FACE) in non-human primates. Mol Imaging Biol 2012;14:213–24.

[69] Yuki Y, Nochi T, Harada N, Katakai Y, Shibata H, Mejima M, et al. In vivo molecular imaging analysis of a nasal vaccine that induces protective immunity against botulism in nonhuman primates. J Immunol 2010;185:5436–43.

[70] Cawthon Lang KA. Primate factsheets: taxonomy, morphology and ecology; 2005.

[71] Pysz MA, Gambhir SS, Willmann JK. Molecular imaging: current status and emerging strategies. Clin Radiol 2010;65:500–16.

[72] James ML, Gambhir SS. A molecular imaging primer: modalities, imaging agents, and applications. Physiol Rev 2012;92:897–965.

[73] Raaben M, Prins HJ, Martens AC, Rottier PJ, De Haan CA. Non-invasive imaging of mouse hepatitis coronavirus infection reveals determinants of viral replication and spread in vivo. Cell Microbiol 2009;11:825–41.

[74] Milho R, Smith CM, Marques S, Alenquer M, May JS, Gillet L, et al. In vivo imaging of murid herpesvirus-4 infection. J Gen Virol 2009;90:21–32.

[75] Ziegler SA, Nuckols J, McGee CE, Huang YJ, Vanlandingham DL, Tesh RB, et al. In vivo imaging of chikungunya virus in mice and Aedes mosquitoes using a Renilla luciferase clone. Vector Borne Zoonotic Dis 2011;11(11):1471–7.

[76] Helmchen F, Denk W. Deep tissue two-photon microscopy. Nat Methods 2005;2:932–40.

[77] Cahalan MD, Parker I, Wei SH, Miller MJ. Two-photon tissue imaging: seeing the immune system in a fresh light. Nat Rev Immunol 2002;2:872–80.

[78] Ng LG, Mrass P, Kinjyo I, Reiner SL, Weninger W. Two-photon imaging of effector T-cell behavior: lessons from a tumor model. Immunol Rev 2008;221:147–62.

[79] Celli S, Albert ML, Bousso P. Visualizing the innate and adaptive immune responses underlying allograft rejection by two-photon microscopy. Nat Med 2011;17:744–9.

[80] Konjufca V, Miller MJ. Two-photon microscopy of host–pathogen interactions: acquiring a dynamic picture of infection in vivo. Cell Microbiol 2009;11:551–9.

[81] Junt T, Moseman EA, Iannacone M, Massberg S, Lang PA, Boes M, et al. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. Nature 2007;450:110–4.

[82] Murooka TT, Deruaz M, Marangoni F, Vrbanac VD, Seung E, von Andrian UH, et al. HIV-infected T cells are migratory vehicles for viral dissemination. Nature 2012;490:283–7.

[83] Deguine J, Breart B, Lemaitre F, Di Santo JP, Bousso P. Intravital imaging reveals distinct dynamics for natural killer and CD8+ T cells during tumor regression. Immunity 2010;33:632–44.

[84] Bousso P, Robey EA. Dynamic behavior of T cells and thymocytes in lymphoid organs as revealed by two-photon microscopy. Immunity 2004;21:349–55.

[85] Neuner I, Kaffanke JB, Langen KJ, Kops ER, Tellmann L, Stoffels G, et al. Multimodal imaging utilising integrated MR-PET for human brain tumour assessment. Eur Radiol 2012;22(12):2568–80.

[86] de Vries IJ, Lesterhuis WJ, Barentsz JO, Verdijk P, van Krieken JH, Boerman OC, et al. Magnetic resonance tracking of dendritic cells in melanoma patients for monitoring of cellular therapy. Nat Biotechnol 2005;23:1407–13.

[87] McAteer MA, Sibson NR, von Zur Muhlen C, Schneider JE, Lowe AS, Warrick N, et al. In vivo magnetic resonance imaging of acute brain inflammation using microparticles of iron oxide. Nat Med 2007;13:1253–8.

[88] Hadjipanayis CG, Machaidze R, Kaluzova M, Wang L, Schuette AJ, Chen H, et al. EGFRvIII antibody-conjugated iron oxide nanoparticles for magnetic resonance imaging-guided convection-enhanced delivery and targeted therapy of glioblastoma. Cancer Res 2010;70:6303–12.

[89] Najjar AM, Nishii R, Maxwell DS, Volgin A, Mukhopadhyay U, Bornmann WG, et al. Molecular-genetic PET imaging using an HSV1-tk mutant reporter gene with enhanced specificity to acycloguanosine nucleoside analogs. J Nucl Med 2009;50:409–16.

[90] Soghomonyan S, Hajitou A, Rangel R, Trepel M, Pasqualini R, Arap W, et al. Molecular PET imaging of HSV1-tk reporter gene expression using [18F]FEAU. Nat Protoc 2007;2:416–23.

[91] Yaghoubi SS, Gambhir SS. PET imaging of herpes simplex virus type 1 thymidine kinase (HSV1-tk) or mutant HSV1-sr39tk reporter gene expression in mice and humans using [18F]FHBG. Nat Protoc 2006;1:3069–75.

[92] Paneda A, Collantes M, Beattie SG, Otano I, Snapper J, Timmermans E, et al. Adeno-associated virus liver transduction efficiency measured by in vivo [18F]FHBG positron emission tomography imaging in rodents and nonhuman primates. Hum Gene Ther 2011;22:999–1009.

[93] Radu CG, Shu CJ, Nair-Gill E, Shelly SM, Barrio JR, Satyamurthy N, et al. Molecular imaging of lymphoid organs and immune activation by positron emission tomography with a new [18F]-labeled 2′-deoxycytidine analog. Nat Med 2008;14:783–8.

[94] Aarntzen EH, Srinivas M, De Wilt JH, Jacobs JF, Lesterhuis WJ, Windhorst AD, et al. Early identification of antigen-specific immune responses in vivo by [18F]-labeled 3′-fluoro-3′-deoxy-thymidine ([18F]FLT) PET imaging. Proc Natl Acad Sci U S A 2011;108:18396–9.

[95] Tian M, Wen X, Jackson EF, Ng C, Uthamanthil R, Liang D, et al. Pharmacokinetics and magnetic resonance imaging of biodegradable macromolecular blood-pool contrast agent PG-Gd in non-human primates: a pilot study. Contrast Media Mol Imaging 2011;6:289–97.

[96] Trojanowski JQ, Vandeerstichele H, Korecka M, Clark CM, Aisen PS, Petersen RC, et al. Update on the biomarker core of the Alzheimer's Disease Neuroimaging Initiative subjects. Alzheimers Dement 2010;6:230–8.

[97] Villemagne VL, Ong K, Mulligan RS, Holl G, Pejoska S, Jones G, et al. Amyloid imaging with ^{18}F-florbetaben in Alzheimer disease and other dementias. J Nucl Med 2011;52:1210–7.

[98] Brockschnieder D, Schmitt-Willich H, Heinrich T, Varrone A, Gulyas B, Toth M, et al. Preclinical characterization of a novel class of ^{18}F-labeled PET tracers for amyloid-beta. J Nucl Med 2012;53(11):1794–801.

[99] Di Mascio M, Paik CH, Carrasquillo JA, Maeng JS, Jang BS, Shin IS, et al. Noninvasive in vivo imaging of CD4 cells in simian-human immunodeficiency virus (SHIV)-infected nonhuman primates. Blood 2009;114:328–37.

[100] Lin PL, Coleman T, Carney JP, Lopresti BJ, Tomko J, Fillmore D, et al. Radiologic responses in cynomolgous macaques for assessing tuberculosis chemotherapy regimens. Antimicrob Agents Chemother 2013;57(9):4237–44.

[101] Molyneux ME. Impact of malaria on the brain and its prevention. Lancet 2000;355:671–2.

[102] Looareesuwan S, Warrell DA, White NJ, Sutharasamai P, Chanthavanich P, Sundaravej K, et al. Do patients with cerebral malaria have cerebral oedema? A computed tomography study. Lancet 1983;1:434–7.

[103] Kampfl AW, Birbamer GG, Pfausler BE, Haring HP, Schmutzhard E. Isolated pontine lesion in algid cerebral malaria: clinical features, management, and magnetic resonance imaging findings. Am J Trop Med Hyg 1993;48:818–22.

[104] Rasalkar DD, Paunipagar BK, Sanghvi D, Sonawane BD, Loniker P. Magnetic resonance imaging in cerebral malaria: a report of four cases. Br J Radiol 2011;84:380–5.

[105] Patankar TF, Karnad DR, Shetty PG, Desai AP, Prasad SR. Adult cerebral malaria: prognostic importance of imaging findings and correlation with postmortem findings. Radiology 2002;224:811–6.

[106] Sugiyama M, Ikeda E, Kawai S, Higuchi T, Zhang H, Khan N, et al. Cerebral metabolic reduction in severe malaria: fluorodeoxyglucose-positron emission tomography imaging in a primate model of severe human malaria with cerebral involvement. Am J Trop Med Hyg 2004;71:542–5.

[107] Penet MF, Viola A, Confort-Gouny S, Le Fur Y, Duhamel G, Kober F, et al. Imaging experimental cerebral malaria in vivo: significant role of ischemic brain edema. J Neurosci 2005;25:7352–8.

[108] Penet MF, Kober F, Confort-Gouny S, Le Fur Y, Dalmasso C, Coltel N, et al. Magnetic resonance spectroscopy reveals an impaired brain metabolic profile in mice resistant to cerebral malaria infected with Plasmodium berghei ANKA. J Biol Chem 2007;282:14505–14.

[109] Saggu R, Faille D, Grau GE, Cozzone PJ, Viola A. In the eye of experimental cerebral malaria. Am J Pathol 2011;179:1104–9.

[110] von Zur Muhlen C, Sibson NR, Peter K, Campbell SJ, Wilainam P, Grau GE, et al. A contrast agent recognizing activated platelets reveals murine cerebral malaria pathology undetectable by conventional MRI. J Clin Invest 2008;118:1198–207.

[111] Kawai S, Sugiyama M. Imaging analysis of the brain in a primate model of cerebral malaria. Acta Trop 2010;114:152–6.

[112] Kawai S, Ikeda E, Sugiyama M, Matsumoto J, Higuchi T, Zhang H, et al. Enhancement of splenic glucose metabolism during acute malarial infection: correlation of findings of FDG-PET imaging with pathological changes in a primate model of severe human malaria. Am J Trop Med Hyg 2006;74:353–60.

[113] Heussler V, Doerig C. In vivo imaging enters parasitology. Trends Parasitol 2006;22:192–5 [discussion 195–196].

[114] Singer M, Frischknecht F. Understanding parasite transmission through imaging approaches. Methods Enzymol 2012;506:19–33.

[115] Chen Q, Wang H. Implications of imaging malaria sporozoites. Trends Parasitol 2008;24:106–9.

[116] Natarajan R, Thathy V, Mota MM, Hafalla JC, Menard R, Vernick KD. Fluorescent Plasmodium berghei sporozoites and pre-erythrocytic stages: a new tool to study mosquito and mammalian host interactions with malaria parasites. Cell Microbiol 2001;3:371–9.

[117] Franke-Fayard B, Trueman H, Ramesar J, Mendoza J, van der Keur M, van der Linden R, et al. A Plasmodium berghei reference line that constitutively expresses GFP at a high level throughout the complete life cycle. Mol Biochem Parasitol 2004;137:23–33.

[118] Frevert U, Engelmann S, Zougbede S, Stange J, Ng B, Matuschewski K, et al. Intravital observation of Plasmodium berghei sporozoite infection of the liver. PLoS Biol 2005;3:e192.

[119] Franke-Fayard B, Janse CJ, Cunha-Rodrigues M, Ramesar J, Buscher P, Que I, et al. Murine malaria parasite sequestration: CD36 is the major receptor, but cerebral pathology is unlinked to sequestration. Proc Natl Acad Sci U S A 2005;102:11468–73.

[120] Amino R, Thiberge S, Martin B, Celli S, Shorte S, Frischknecht F, et al. Quantitative imaging of Plasmodium transmission from mosquito to mammal. Nat Med 2006;12:220–4.

[121] Amino R, Thiberge S, Blazquez S, Baldacci P, Renaud O, Shorte S, et al. Imaging malaria sporozoites in the dermis of the mammalian host. Nat Protoc 2007;2:1705–12.

[122] Vanderberg JP, Frevert U. Intravital microscopy demonstrating antibody-mediated immobilisation of Plasmodium berghei sporozoites injected into skin by mosquitoes. Int J Parasitol 2004;34:991–6.

[123] Frischknecht F, Baldacci P, Martin B, Zimmer C, Thiberge S, Olivo-Marin JC, et al. Imaging movement of malaria parasites during transmission by Anopheles mosquitoes. Cell Microbiol 2004;6:687–94.

[124] Sturm A, Amino R, van de Sand C, Regen T, Retzlaff S, Rennenberg A, et al. Manipulation of host hepatocytes by the malaria parasite for delivery into liver sinusoids. Science 2006;313:1287–90.

[125] Thiberge S, Blazquez S, Baldacci P, Renaud O, Shorte S, Menard R, et al. In vivo imaging of malaria parasites in the murine liver. Nat Protoc 2007;2:1811–8.

[126] Tavares J, Formaglio P, Medvinsky A, Menard R, Amino R. Imaging sporozoite cell traversal in the liver of mice. Methods Mol Biol 2013;923:401–10.

[127] Lin JW, Sajid M, Ramesar J, Khan SM, Janse CJ, Franke-Fayard B. Screening inhibitors of P. berghei blood stages using bioluminescent reporter parasites. Methods Mol Biol 2013;923:507–22.

[128] Miller JL, Murray S, Vaughan AM, Harupa A, Sack B, Baldwin M, et al. Quantitative bioluminescent imaging of pre-erythrocytic malaria parasite infection using luciferase-expressing Plasmodium yoelii. PLoS One 2013;8:e60820.

[129] Annoura T, Chevalley S, Janse CJ, Franke-Fayard B, Khan SM. Quantitative analysis of Plasmodium berghei liver stages by bioluminescence imaging. Methods Mol Biol 2013;923:429–43.

[130] Meister S, Plouffe DM, Kuhen KL, Bonamy GM, Wu T, Barnes SW, et al. Imaging of Plasmodium liver stages to drive next-generation antimalarial drug discovery. Science 2011;334:1372–7.

[131] Ploemen I, Behet M, Nganou-Makamdop K, van Gemert GJ, Bijker E, Hermsen C, et al. Evaluation of immunity against malaria using luciferase-expressing Plasmodium berghei parasites. Malar J 2011;10:350.

[132] Kocken CH, Zeeman AM, Voorberg-van der Wel A, Thomas AW. Transgenic Plasmodium knowlesi: relieving a bottleneck in malaria research? Trends Parasitol 2009;25:370–4.

[133] Thompson J, Millington OR, Garside P, Brewer JM. What can transgenic parasites tell us about the development of Plasmodium-specific immune responses? Parasite Immunol 2008;30:223–33.

[134] malERA. A research agenda for malaria eradication: basic science and enabling technologies. PLoS Med 2011;8:e1000399.

[135] Zeeman AM, der Wel AV, Kocken CH. Ex vivo culture of Plasmodium vivax and Plasmodium cynomolgi and in vitro culture of Plasmodium knowlesi blood stages. Methods Mol Biol 2013;923:35–49.

[136] Vaughan JA, Scheller LF, Wirtz RA, Azad AF. Infectivity of Plasmodium berghei sporozoites delivered by intravenous inoculation versus mosquito bite: implications for sporozoite vaccine trials. Infect Immun 1999;67:4285–9.

[137] Hegge S, Kudryashev M, Barniol L, Frischknecht F. Key factors regulating Plasmodium berghei sporozoite survival and transformation revealed by an automated visual assay. FASEB J 2010;24:5003–12.

[138] Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, et al. Genome sequence of the human malaria parasite Plasmodium falciparum. Nature 2002;419:498–511.

[139] Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, et al. Comparative genomics of the neglected human malaria parasite Plasmodium vivax. Nature 2008;455:757–63.

[140] Pain A, Bohme U, Berry AE, Mungall K, Finn RD, Jackson AP, et al. The genome of the simian and human malaria parasite Plasmodium knowlesi. Nature 2008;455:799–803.

[141] Tachibana S, Sullivan SA, Kawai S, Nakamura S, Kim HR, Goto N, et al. Plasmodium cynomolgi genome sequences provide insight into Plasmodium vivax and the monkey malaria clade. Nat Genet 2012;44:1051–5.

[142] van Dijk MR, Waters AP, Janse CJ. Stable transfection of malaria parasite blood stages. Science 1995;268:1358–62.

[143] Crabb BS, Cowman AF. Characterization of promoters and stable transfection by homologous and nonhomologous recombination in Plasmodium falciparum. Proc Natl Acad Sci U S A 1996;93:7289–94.

[144] Wu Y, Kirkman LA, Wellems TE. Transformation of Plasmodium falciparum malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. Proc Natl Acad Sci U S A 1996;93:1130–4.

[145] VanWye JD, Haldar K. Expression of green fluorescent protein in Plasmodium falciparum. Mol Biochem Parasitol 1997;87:225–9.

[146] de Koning-Ward TF, Janse CJ, Waters AP. The development of genetic tools for dissecting the biology of malaria parasites. Annu Rev Microbiol 2000;54:157–85.

[147] Carvalho TG, Menard R. Manipulating the Plasmodium genome. Curr Issues Mol Biol 2005;7:39–59.

[148] Limenitakis J, Soldati-Favre D. Functional genetics in Apicomplexa: potentials and limits. FEBS Lett 2011;585:1579–88.

[149] Rug M, Maier AG. Transfection of Plasmodium falciparum. Methods Mol Biol 2012;923:75–98.

[150] van der Wel AM, Tomas AM, Kocken CH, Malhotra P, Janse CJ, Waters AP, et al. Transfection of the primate malaria parasite Plasmodium knowlesi using entirely heterologous constructs. J Exp Med 1997;185:1499–503.

[151] Kocken CH, Ozwara H, van der Wel A, Beetsma AL, Mwenda JM, Thomas AW. Plasmodium knowlesi provides a rapid in vitro and in vivo transfection system that enables double-crossover gene knockout studies. Infect Immun 2002;70:655–60.

[152] Ozwara H, van der Wel A, Kocken CH, Thomas AW. Heterologous promoter activity in stable and transient Plasmodium knowlesi transgenes. Mol Biochem Parasitol 2003;130:61–4.

[153] Pfahler JM, Galinski MR, Barnwell JW, Lanzer M. Transient transfection of Plasmodium vivax blood stage parasites. Mol Biochem Parasitol 2006;149:99–101.

[154] Sanchez CP, Pfahler J, Del Portillo HA, Lanzer M. Transient transfection of Plasmodium vivax blood-stage parasites. Methods Mol Biol 2012;923:151–9.

[155] Akinyi S, Hanssen E, Meyer EV, Jiang J, Korir CC, Singh B, et al. A 95 kDa protein of Plasmodium vivax and P. cynomolgi visualized by three-dimensional tomography in the caveola-vesicle complexes (Schuffner's dots) of infected erythrocytes is a member of the PHIST family. Mol Microbiol 2012;84:816–31.

[156] Kocken CH, van der Wel A, Thomas AW. *Plasmodium cynomolgi*: transfection of blood-stage parasites using heterologous DNA constructs. Exp Parasitol 1999;93:58–60.

[157] Khan SM, Kroeze H, Franke-Fayard B, Janse CJ. Standardization in generating and reporting genetically modified rodent malaria parasites: the RMgmDB database. Methods Mol Biol 2012;923:139–50.

[158] Talman AM, Blagborough AM, Sinden RE. A *Plasmodium falciparum* strain expressing GFP throughout the parasite's life-cycle. PLoS One 2012;5:e9156.

[159] Moon RW, Hall J, Rangkuti F, Ho YS, Almond N, Mitchell GH, et al. Adaptation of the genetically tractable malaria pathogen *Plasmodium knowlesi* to continuous culture in human erythrocytes. Proc Natl Acad Sci U S A 2013;110:531–6.

[160] Coombes JL, Robey EA. Dynamic imaging of host–pathogen interactions in vivo. Nat Rev Immunol 2010;10:353–64.

[161] Cockburn IA, Amino R, Kelemen RK, Kuo SC, Tse SW, Radtke A, et al. In vivo imaging of CD8 + T cell-mediated elimination of malaria liver stages. Proc Natl Acad Sci U S A 2013;110:9090–5.