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Dissecting *in vitro* host cell infection by *Plasmodium* sporozoites using flow cytometry

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Summary

The study of the liver stage of malaria has been hampered by limitations in the experimental approaches required to effectively dissect and quantify hepatocyte infection by *Plasmodium*. Here, we report on the use of flow cytometry, in conjunction with GFP-expressing *Plasmodium* sporozoites, to assess the various steps that constitute a successful malaria liver infection: cell traversal, hepatocyte invasion and intrahepatocyte parasite development. We show that this rapid, efficient and inexpensive method can be used to overcome current limitations in the independent quantification of those steps, facilitating routine or large-scale studies of host–pathogen molecular interactions.

Introduction

The liver stage of the life cycle of *Plasmodium* is the first, obligatory step in any natural malaria infection. During this asymptomatic phase, the sporozoites injected into the mammalian host through the bite of an infected mosquito reach the liver, where they traverse several hepatocytes before invading a final one with formation of a parasitophorous vacuole (PV) (Mota *et al*., 2001; Frevert *et al*., 2005). Inside hepatocytes, the parasites develop and multiply, forming a so-called exoerythrocytic form (EEF). During this process, which takes 5–7 days for the human parasite *Plasmodium falciparum* and *c.* 42 h for the rodent *Plasmodium berghei* parasite, the EEF grows in size and eventually releases 20 000–30 000 merozoites into the blood, where they will invade erythrocytes and cause the symptoms of the disease (reviewed in Prudencio *et al*., 2006).

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Although the liver stage of malaria constitutes an ideal target for prophylactic intervention, only recently have the processes that take place during this stage begun to be understood. This is largely due to practical issues hampering the study of what goes on when sporozoites invade and develop inside hepatic cells. In fact, unlike blood stages that can be easily obtained from frozen-infected blood, sporozoites need to be freshly extracted from the salivary glands of infected mosquitoes, which poses constraints to their availability. Moreover, studies in human volunteers or in endemic populations are not feasible due to ethic constraints and to the fact that this stage of disease is asymptomatic. This leaves researchers with a few important tools to study liver stage malaria, namely, *in vivo* animal models, *ex vivo* primary hepatocytes and *in vitro* hepatoma cell lines. The latter constitute a valuable resource to study the fundamental aspects of EEF formation and development and to evaluate drug candidates that may interfere with these processes.

Recently, genetic manipulation of *Plasmodium* has allowed the generation of parasites that express green fluorescent protein (GFP) (Kadekoppala *et al*., 2001; Natarajan *et al*., 2001; Franke-Fayard *et al*., 2004; Tarun *et al*., 2006; Ono *et al*., 2007), which enables cells infected with these parasites to be analysed by fluorescence-activated cell sorting (FACS). This technique has been successfully used to separate infected hepatocytes or hepatoma cells (Natarajan *et al*., 2001; Tarun *et al*., 2006) as well as to monitor infection of red blood cells in mice (Janse *et al*., 2006; Ono *et al*., 2007).

In this report we describe how flow cytometry can be employed to obtain crucial quantitative information regarding the hepatic stage of malaria. Our results show that this technique can be used to determine the efficiency of infection of a hepatoma cell line by the GFP-expressing rodent malaria parasite *P. berghei* (PbGFP). In particular, it enables the quantitative assessment of the cell traversal process that precedes invasion, as well as of both the invasion and development processes essential for the establishment of a successful infection.

Results and discussion

Quantification of cell traversal by migrating sporozoites

When sporozoites traverse hepatocytes before the final invasion, they disrupt the membrane of the host cell,

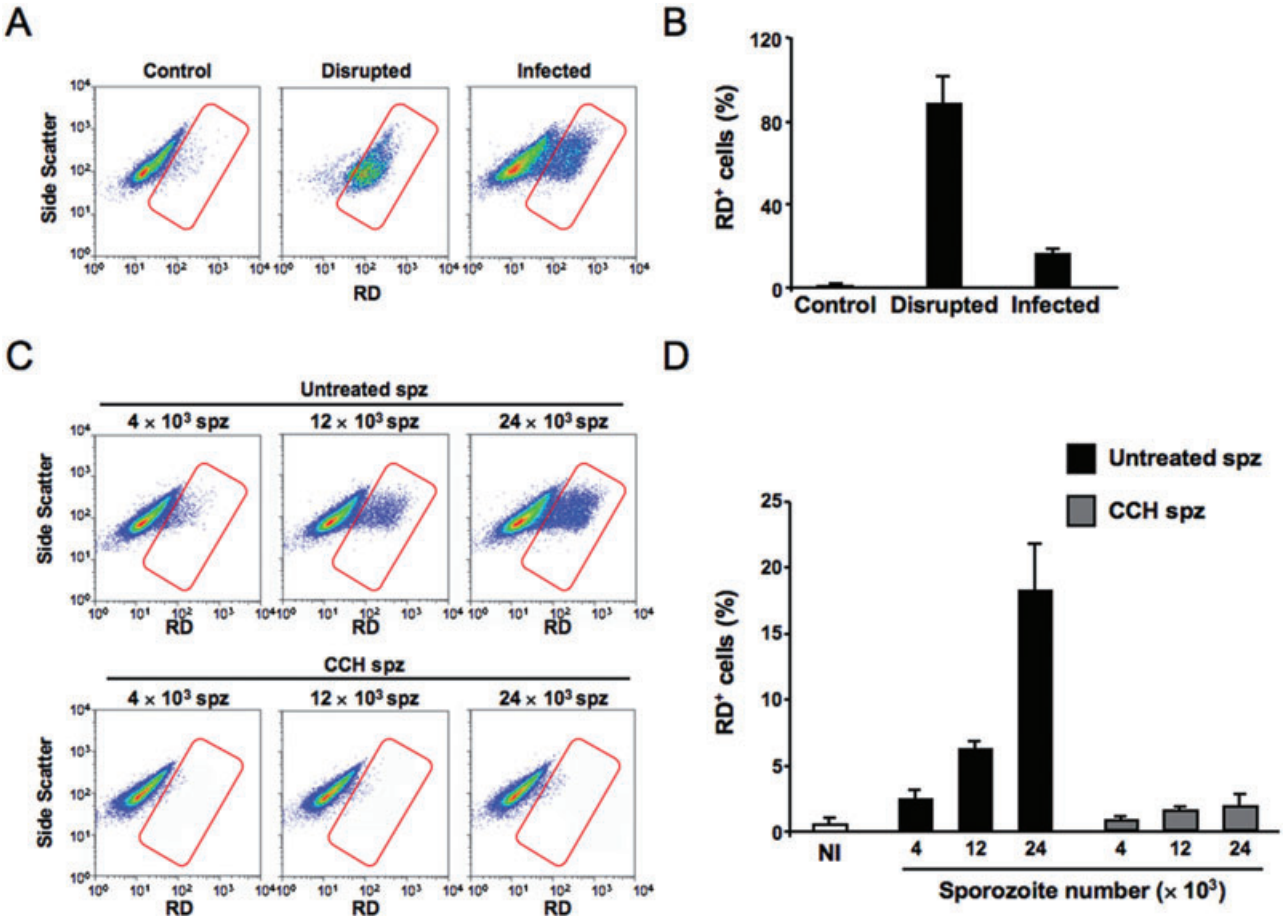

Fig. 1. FACS assessment of cell wounding during sporozoite traversal.
A. Cell wounding was assessed in a RD-based assay by fluorescence intensity. Dot plots represent undisrupted-uninfected cells (control), mechanically disrupted cells and cells incubated with sporozoites (15×10^3) for 2 h. The gate contains RD^+ cells.
B. Bar plot represents the percentage of RD^+ cells in the conditions in (A) ($n = 3$). Error bars represent SD.
C. Cell wounding by sporozoites was assessed in a RD-based assay by fluorescence intensity. Dot plots represent cells incubated for 2 h with different numbers of sporozoites (spz), previously treated with cytochalasin (CCH) or not (Untreated). The gate contains RD^+ cells. Non-infected cells were used as control.
D. Bar plot represents the percentage of RD^+ cells in the conditions in (C) ($n = 3$). NI corresponds to non-infected cells used as control. Error bars represent SD.

which then quickly reseals (Mota *et al*., 2001). Cell traversal can be monitored and quantified by fluorescence microscopy by a cell wounding assay employing rhodamine dextran (RD), which, if present in the medium upon sporozoite addition, is trapped within the cell before resealing occurs (Mota *et al*., 2001). Generally, such quantification is laborious and time-consuming, as it involves the visual inspection of RD-positive (RD^+) cells and their quantification. A very recent report uses FACS to compare cell wounding by mutant and wild-type sporozoites but does not provide any controls to show that this technique can be used to quantify cell traversal (Labaied *et al*., 2007). We have sought to establish whether FACS can be used to quantitatively assess cell traversal by sporozoites. RD was added to the cells immediately prior to sporozoite addition and cells were collected and analysed by FACS 2 h later. Non-infected cells incubated with RD during the same period as the infected ones and cells that were mechanically disrupted in the presence of RD were used as controls (Fig. 1A and B). The percentage of RD^+ cells in untreated cultures is minimal, when compared with those in which cells were either incubated with sporozoites or mechanically disrupted, showing that membrane wounding by traversing sporozoites can be detected by this approach (Fig. 1B). In order to determine whether the technique can give quantitative information about cell traversal, cells were incubated with different amounts of sporozoites for 2 h (Fig. 1C and D). Cells incubated with sporozoites treated with cytochalasin (CCH), whose motility is impaired (Stewart *et al*., 1986), and uninfected cells, were used as controls (Fig. 1C and D). The results obtained show that the percentage of RD^+

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cells correlates with the number of sporozoites added to cells (Fig. 1D). When cells are incubated with a fixed number of sporozoites for periods of time between 15 min and 2 h, the percentage of RD^+ cells correlates with the time of incubation (data not shown). These results show that flow cytometry can indeed be used to successfully quantify hepatocyte traversal by sporozoites.

Quantification of invasion

Invasion of hepatoma cells by sporozoites is usually assumed to be complete within the first 2 h following parasite addition to the cells (Mota *et al*., 2001). Thus, by determining the extent of infection 2 h after sporozoite addition, one can, conceivably, assess the efficiency of the invasion process. Quantification of invasion by any of the techniques traditionally employed to study hepatocyte infection is difficult. Until now, it has relied on a parasite surface molecule double immunostaining, performed before and after cell permeabilization (Sinnis, 1998). This method distinguishes between parasites attached to the cells and invading ones, but it is a time-consuming procedure requiring pre-labelled, parasite-specific antibodies. Another approach that can be employed to determine infection levels *in vitro* is quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), using primers specific for the parasite in parallel with primers for a housekeeping gene to allow normalization of the results, as described for the quantification of *in vivo* liver infections (Bruna-Romero *et al*., 2001). However, quantification by qRT-PCR is not only a time-consuming approach but also an expensive one, rendering it unsuitable for large-scale or routine studies. Now, we demonstrate that these limitations can be overcome by using FACS to analyse cells 2 h after sporozoite addition.

We infected Huh7 cells with fixed numbers of PbGFP sporozoites and analysed them by FACS 2 h later. Non-infected cells were used as control (Fig. 2A and B). Our results show that the infected cell population can be successfully quantified using this technique and that, as expected, the percentage of PbGFP-containing cells depends on the number of sporozoites added (Fig. 2B). CCH treatment of sporozoites does not impair their ability to attach to cells but renders them unable to invade (Stewart *et al*., 1986). Cells incubated with CCH-treated sporozoites for 2 h and then analysed by FACS show only negligible amounts of GFP fluorescence, when compared with that of cells incubated with untreated sporozoites and similar to those displayed by non-infected cells (Fig. 2A and B). This shows that tripsinization of cells prior to FACS analysis ensures that all GFP-positive (GFP^+) events observed correspond to parasite-containing cells and not to cells with sporozoites attached to their surface. In a parallel experiment, we incubated cells with the supernatant of infected cells, collected 2 h after parasite addition. Two hours later, cells to which this supernatant was added were collected and analysed by FACS, revealing only residual amounts of infected cells (data not shown). This result is in agreement with the notion that successful infection is accomplished within the first 2 h that follows sporozoite addition.

The traversal and invasion processes can be simultaneously assessed in RD-treated cell samples infected with PbGFP sporozoites (Fig. 2C and D). By carefully gating the various cell populations, FACS can be used to determine the percentage of cells whose membrane has been disrupted (RD^+) and of those containing a parasite at the time of measurement (GFP^+). Furthermore, a double-positive population (RD^+GFP^+) consisting of cells either that contain parasites 'in transit' or that were traversed and subsequently invaded can be identified (Fig. 2D). Approximately 50% of the GFP^+ cell population is RD-negative and therefore these cells have been unequivocally invaded. On the other hand, invasion with PV formation may or may not have occurred in the remaining 50% of the GFP^+ cell population, which corresponds to RD^+GFP^+ cells. Hence, data from double-fluorescence FACS measurements should be taken into account when the estimation of the percentage of invaded, non-traversed cells is of relevance.

Quantification of EEF development

By itself, flow cytometry information collected 2 h after sporozoite addition is useful to evaluate the ability of parasites to invade cells in culture, but provides little insight into the extent of their intracellular development. The latter can be assessed by quantifying infection at later time points after sporozoite addition. Traditionally, this was done by immunofluorescence staining of the parasite and EEF quantification by microscopy. This is a time-consuming approach, made easier by developments in automatic image acquisition and treatment techniques, but is still impractical for the assessment of the early developmental stages of infection. As an alternative to microscopy, qRT-PCR can be used to quantify the number of parasite copies at specific times following sporozoite addition. However, in addition to the disadvantages mentioned earlier, qRT-PCR has limitations regarding an effective differentiation between the invasion and development processes, if samples are analysed at a single time point of EEF development. This can be circumvented by analysing samples at different times after sporozoite addition, but this further increases both the price and time of data acquisition. In order to overcome these drawbacks, we sought to determine whether FACS can be used to reliably estimate the extent of intrahepatocyte parasite development.

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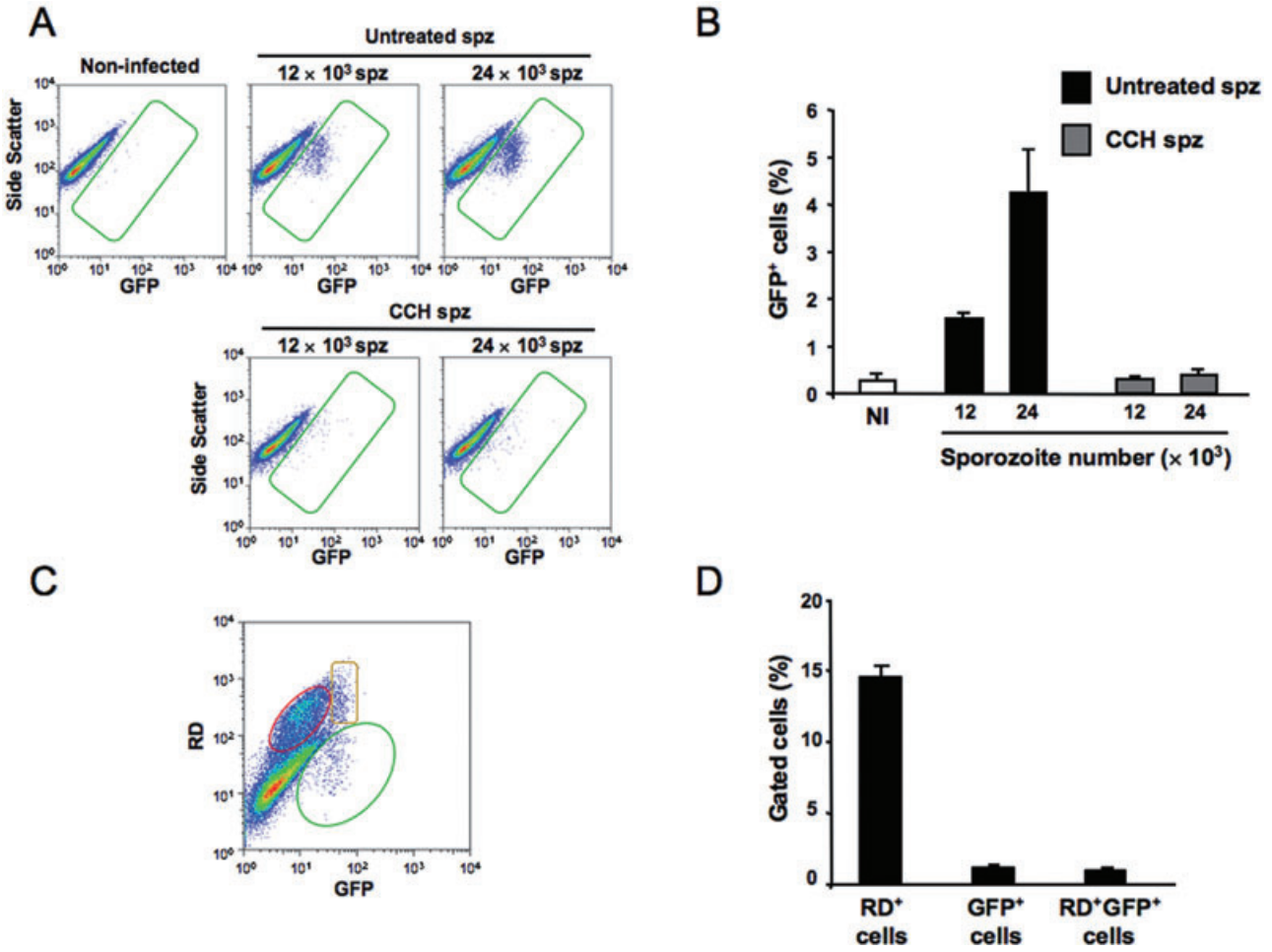

Fig. 2. FACS assessment of cell invasion.
A. Cell invasion was quantified by determining the proportion of GFP⁺ cells in Huh7 cells incubated with PbGFP sporozoites. Dot plots represent non-infected cells and cells incubated for 2 h with different numbers of PbGFP sporozoites (spz), previously treated with cytochalasin (CCH) or not (Untreated). The gate contains GFP⁺ cells.
B. Bar plot represents the percentage of GFP⁺ cells in the conditions in (A) ($n = 3$). NI corresponds to non-infected cells used as control. Error bars represent SD.
C. Traversal versus invasion were assessed in cells incubated with PbGFP sporozoites (15×10^3) for 2 h. The gates contain RD⁺ (red) GFP⁺ (green) or RD⁺GFP⁺ (yellow) cells.
D. Bar plot represents the percentages of RD⁺, GFP⁺ or RD⁺GFP⁺ cells ($n = 3$). Error bars represent SD.

We infected Huh7 cells with a fixed number of PbGFP sporozoites and analysed them by FACS at various time points post infection (Fig. 3A and B). As control, we used radiation-attenuated PbGFP sporozoites (RASPbGFP), which are known to retain their ability to invade cells but fail to develop and multiply (Suhrbier *et al.*, 1990). As expected, the percentage of PbGFP-infected cells decreases for later time points of infection, as a result of the multiplication of cells in culture (Fig. 3A). When the FACS data are plotted as a histogram, it becomes apparent that the maximum fluorescence of the PbGFP-infected cells increases as the infection is allowed to proceed for longer (Fig. 3B). These data show that although the relative proportion of PbGFP-infected cells is decreasing as the time post infection increases, the fluorescence emitted by these cells is increasing during the same period, as a result of EEF development. When cells are infected with RASPbGFP sporozoites, the percentage of infected cells also decreases with time (Fig. 3A). However, the maximum fluorescence of RASPbGFP-infected cells does not change significantly in the same period (Fig. 3B), in agreement with the knowledge that development of irradiated parasites is impaired (Suhrbier *et al.*, 1990).

In order to determine whether there is a correlation between the position of the GFP⁺ curve and the number of copies of parasite at a given time post infection, we analysed cells infected under similar conditions as those described above by qRT-PCR (Fig. 3C). We observed that, as expected, the number of parasite copies in PbGFP-infected cells increases up until 36 h post infection and is

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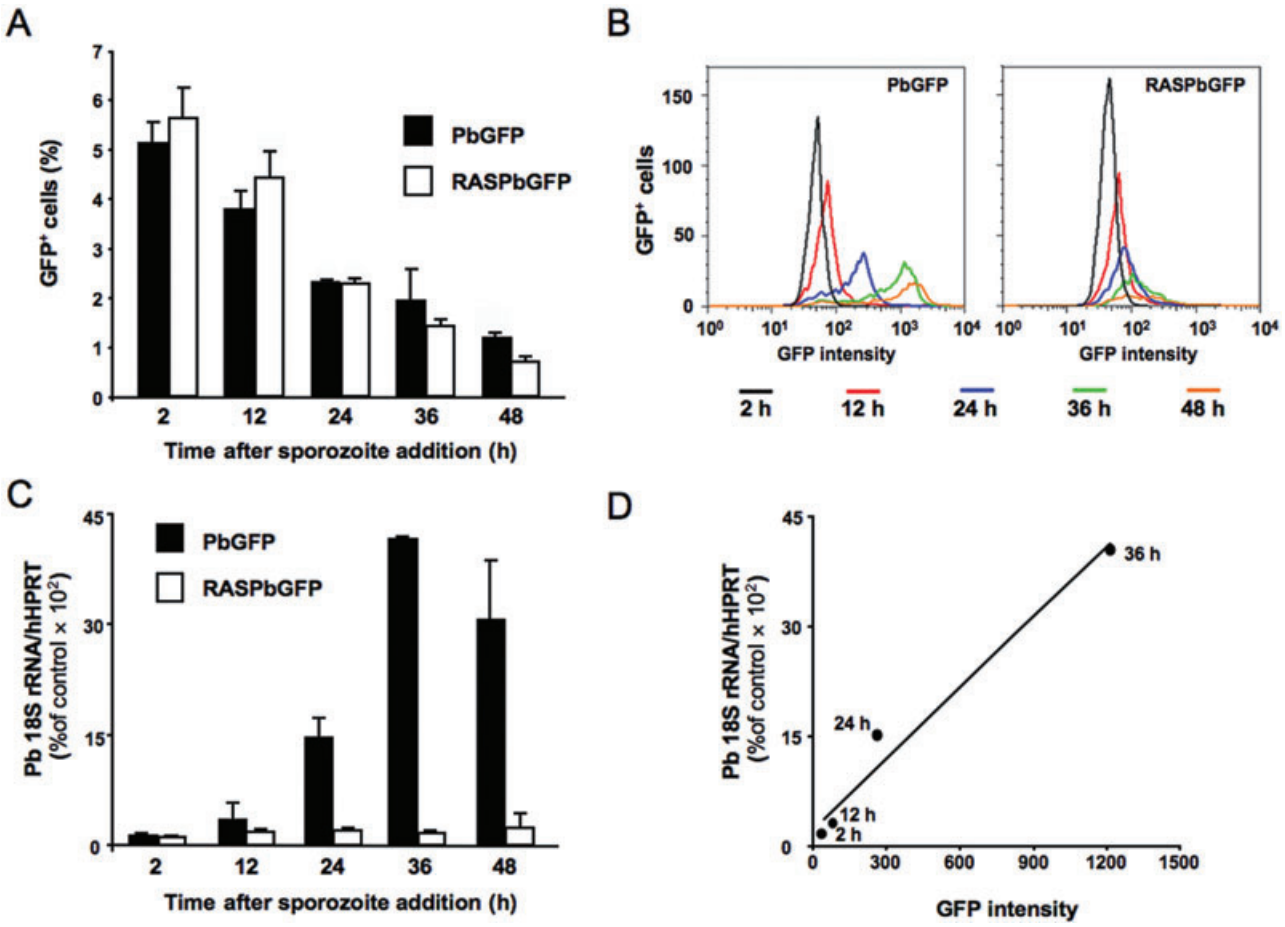

Fig. 3. Assessment of infection at various times after sporozoite (30×10^3) addition to cells.
A. Proportion of infected cells was quantified during infection with PbGFP (black bars) or RASPbGFP (white bars) ($n = 3$).
B. EEF development was assessed by fluorescence intensity of GFP⁺ cells. The graphs show one representative data set of triplicate samples.
C. Infection was quantified by parasite-specific qRT-PCR 2, 12, 24, 36 and 44 h post infection with PbGFP or RASPbGFP ($n = 3$). Infection is expressed as percentage of control (2 h time point of cells infected with RASPbGFP). Error bars represent SD.
D. Positive correlation ($R^2 = 0.98$) between the parasite load measured by qRT-PCR and the maximum intensity of the GFP⁺ band in the FACS histograms.

reduced at 48 h post infection. The number of parasite copies in RASPbGFP-infected cells increases slightly between 2 and 12 h post infection and then remains roughly constant up until 48 h after sporozoite addition, a result that is in agreement with previous observations (Suhrbier *et al.*, 1990). Our results also indicate that the number of PbGFP copies decreases between 36 and 48 h after infection, possibly due to detachment of infected cells or release of merozoites (Sturm *et al.*, 2006). This is not observed for RASPbGFP-infected cells, suggesting that *Plasmodium* release by the host cells is dependent on the development of the parasites within them.

The correlation between the number of parasite copies and the intensity of the GFP⁺ band in PbGFP-infected cells was further established by plotting the former against the latter (Fig. 3D). This shows that intracellular parasite development takes place exponentially until parasite release starts to occur. Moreover, it shows that the fluorescence corresponding to the maximum of the GFP peak in a FACS histogram correlates linearly with the number of copies of parasite in the cells and can thus be used as a quick and effective way of estimating the intracellular development of the parasite.

Finally, we assessed parasite development in cells that are either RD⁻GFP⁺ or RD⁺GFP⁺, by independently plotting the histograms of each of these cell populations at time points between 2 and 48 h following sporozoite addition. Our results reveal that RD⁻GFP⁺ cells contain EEFs that develop normally during this period, showing that the parasites in these cells have undergone produtive invasion with formation of a PV. On the contrary, parasites in RD⁺GFP⁺ cells develop poorly if at all and their GFP intensity decreases at the later time points assessed (data not shown).

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The method presented in this report has various important applications as it constitutes an efficient way of assessing *Plasmodium* infection of hepatocytes under different experimental conditions. Compared with other available methods to address this, it presents advantages both in terms of speed of data acquisition and in terms of cost-effectiveness. Furthermore, it distinguishes between invasion and developmental stages of *Plasmodium* within a hepatocyte, overcoming limitations presented by other available techniques in this respect. The method can, for instance, be used to address the effectiveness of a given drug in impairing the invasion and/or the development of the parasite in the liver. It can also serve to study the role of specific host genes in *Plasmodium* invasion and/or development, either by employing genetically modified cell lines or by using RNA interference to down-modulate the expression of the gene(s) of interest. Finally, the method provides a quick way to address the potential invasion and/or developmental consequences of introducing specific gene mutations or deletions in the parasite. The methodology described here greatly extends the potential of combining GFP-expressing pathogens with flow cytometry to address host–pathogen interactions.

Experimental procedures

Cell, parasites and infection

Huh7 cells, a human hepatoma cell line, were cultured in RPMI (Gibco/Invitrogen) medium supplemented with 10% fetal calf serum (FCS) (Gibco/Invitrogen), 1% non-essential amino acids (Gibco/Invitrogen), 1% penicillin/streptomycin (Gibco/Invitrogen), 1% glutamine (Gibco/Invitrogen) and 10 mM Hepes, pH 7 (Gibco/Invitrogen) and maintained at 37°C with 5% CO_2. GFP-expressing *P. berghei* (parasite line 259 cL2) sporozoites were obtained by disruption of the salivary glands of freshly dissected infected female *Anopheles stephensi* mosquitoes, bred at the Insectary of the Instituto de Medicina Molecular. Radiation-attenuated sporozoites were obtained by irradiation of sporozoites with 160 Gy on a Compagnie Oris Industrie IBL 437C irradiator, at room temperature, for approximately 80 min. Non-irradiated sporozoites used in development experiments were left at room temperature during the same period. Sporozoite motility was impaired by incubation with 10 μM cytochalasin (Calbiochem) for 10 min at room temperature prior to addition to cells. Non-treated sporozoites used in these experiments were left at room temperature during the same period. Cells (17.5×10^4 per well) were seeded on 24-well plates the day before infection. Cells were infected by addition of specific numbers of sporozoites, followed by centrifugation at 1700 *g* for 7 min at 37°C. For membrane disruption assessment, 2 mg ml^{-1} Dextran tetramethylrhodamine 10 000 MW, lysine fixable (fluoro-ruby) (Molecular Probes/Invitrogen) were added to the cells immediately prior to sporozoite addition. Cells used as positive controls for the membrane disruption assay were mechanically disrupted by passage through a 27 G syringe five times, in the presence of 2 mg ml^{-1} Dextran.

Fluorescence-activated cell sorting (FACS)

Cell samples for FACS analysis were washed with 1 ml of PBS, incubated with 150 μl of trypsin for 5 min at 37°C and collected in 400 μl of 10% FCS in PBS at the selected time points post sporozoite addition. Cells were then centrifuged at 0.1 *g* for 3 min at 4°C and re-suspended in 150 μl of 2% FCS in PBS. Cells were analysed on a Becton Dickinson FACScalibur with the appropriate settings for the fluorophores used. Data acquisition and analysis were carried out using the CELLQuest (version 3.2.1fl1, Becton Dickinson) and FlowJo (version 6.3.4, FlowJo) software packages respectively.

Quantification of parasite copy numbers by qRT-PCR

Cells were washed with 1 ml of PBS and collected at the selected time points in 150 μl of RLT buffer (Qiagen) containing 1% β-mercaptoethanol and immediately stored at −80°C until further processing. Total RNA was isolated from Huh7 cells with Qiagen's Micro RNeasy kit, following the manufacturer's instructions, and converted into cDNA using Roche's Transcriptor First Strand cDNA Synthesis kit, according to the manufacturer's protocol. The qRT-PCR reactions used Applied Biosystems' Power SYBR Green PCR Master Mix and were performed according to the manufacturer's instructions on an ABI Prism 7000 system (Applied Biosystems). Amplification reactions were carried out in a total reaction volume of 25 μl, containing 0.8 pmoles μl^{-1} or 0.16 pmoles μl^{-1} of the PbA- or housekeeping gene-specific primers respectively. Relative amounts of PbA mRNA were calculated against the Hypoxanthine Guanine Phosphoribosyltransferase (HPRT) housekeeping gene. PbA- and HPRT-specific primer sequences were 5′-AAG CAT TAA ATA AAG CGA ATA CAT CCT TAC-3′ and 5′-GGA GAT TGG TTT TGA CGT TTA TGT G-3′, and 5′-TGC TCG AGA TGT GAT GAA GG-3′ and 5′-TCC CCT GTT GAC TGG TCA TT-3′ respectively.

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