

A Microscale Human Liver Platform that Supports the Hepatic Stages of *Plasmodium falciparum* and *vivax*

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SUMMARY

The *Plasmodium* liver stage is an attractive target for the development of antimalarial drugs and vaccines, as it provides an opportunity to interrupt the life cycle of the parasite at a critical early stage. However, targeting the liver stage has been difficult. Undoubtedly, a major barrier has been the lack of robust, reliable, and reproducible in vitro liver-stage cultures. Here, we establish the liver stages for both *Plasmodium falciparum* and *Plasmodium vivax* in a microscale human liver platform composed of cryopreserved, micropatterned human primary hepatocytes surrounded by supportive stromal cells. Using this system, we have successfully recapitulated the full liver stage of *P. falciparum*, including the release of infected merozoites and infection of overlaid erythrocytes, as well as the establishment of small forms in late liver stages of *P. vivax*. Finally, we validate the potential of this platform as a tool for medium-throughput antimalarial drug screening and vaccine development.

INTRODUCTION

Despite major advances in the prevention and treatment of malaria, this disease continues to be a major global health problem in human populations, with ~250 million cases and nearly 1 million deaths every year (World Health Organization, 2010). Malaria is transmitted by *Plasmodium* sporozoites after they are injected by an infected mosquito. Uninucleate sporozoites travel to and invade liver hepatocytes, where they mature and multiply to form liver-stage schizonts. These schizonts eventually release pathogenic merozoites into the blood, where they invade erythrocytes and lead to the major clinical symptoms,

signs, and pathology of the disease. Human malaria is primarily caused by four species of *Plasmodium* parasites. *Plasmodium falciparum* (*P. falciparum*) is the most virulent and causes the vast majority of deaths. *Plasmodium vivax* (*P. vivax*) is less deadly but highly disabling. Notably, some *P. vivax* sporozoites develop into dormant hypnozoites, which remain in the liver and serve as a *P. vivax* reservoir that gives rise to a chronic, relapsing infection and causes significant added clinical and financial burden (Price et al., 2007). Currently, there is a renewed interest and focus on global malaria eradication, and it is now widely recognized that existing tools are insufficient to meet this goal (Alonso et al., 2011a, 2011b).

In particular, the clinical options that target the liver stages of the parasite life cycle are inadequate. There is only one licensed drug that eliminates hypnozoites, only a few drugs that target liver-stage parasites, and no licensed malaria vaccines. This problem has been exacerbated by the emergence of drug resistance and the inability to treat some populations with primaquine, the only currently approved drug with antihypnozoite activity (Wells et al., 2010). The *Plasmodium* liver stage is an attractive therapeutic target for the development of both antimalarial drugs and vaccines, as it provides an opportunity to interrupt the life cycle of the parasite at a critical early stage. Therefore, screening platforms that model the in vivo *Plasmodium* liver stage could be used to advance the pipeline for antimalarial drug development and to validate promising liver-stage vaccine candidates (Epstein et al., 2011; Plowe et al., 2009).

Studies of rodent *Plasmodium* pathogens (*P. berghei* and *P. yoelii*) have provided important insights, due in part to the capacity to conduct both in vitro and in vivo assays (Hoffman et al., 1989; Rodrigues et al., 2008; Silvie et al., 2007). Nonetheless, there are essential differences between the rodent and human parasites, such as their antigenic variation and mechanisms of host cell invasion (Carlton et al., 2002; McCutchan et al., 1985). To date, our understanding of the liver stage of human malaria, mainly *P. falciparum* and *P. vivax*, is based in large part on the infection of human hepatoma cell lines (Chattopadhyay et al., 2010; Epstein et al., 2011; Hollingdale et al., 1983;

Karnasuta et al., 1995; Sattabongkot et al., 2006). These cell lines, however, display abnormal proliferation, aberrant signaling, dysregulated gene expression, altered host responses to infection, and inadequate CYP450 and drug metabolism activity and thus do not accurately recapitulate human hepatocyte biology. Furthermore, in situ observation of pathogen development in liver cell lines is typically obscured after 6 days in culture, due to the continued proliferation of infected cells (Yokoo et al., 2004). Primary cultured human hepatocytes that exhibit more physiologic human liver functions have been studied previously, albeit less frequently than cell lines, and can support the development of the liver forms of *P. falciparum* and *P. vivax* (Mazier et al., 1984, 1985; Rodrigues et al., 2008; van Schaijk et al., 2008; Yalaoui et al., 2008). Nonetheless, in the 25 years since these findings were first published, primary hepatocyte systems are rarely employed and difficult to translate to screening platforms due to limited cell availability and challenges in maintaining their functional phenotype over extended periods of time in vitro (Bhatia et al., 1999).

Two recent advances may help overcome the deficiencies of existing in vitro liver models. First, several groups have developed methods and novel culture platforms that support the maintenance of primary human hepatocytes (Bhatia et al., 1999; Guguen-Guillouzo and Guillouzo, 2010; LeCluyse et al., 2012). In particular, our group has developed a microliver platform, which leverages bioengineering techniques to organize primary human hepatocytes among supportive stromal cells (Khetani and Bhatia, 2008). Hepatocytes in these micropatterned cocultures (MPCCs) exhibit human-specific drug metabolism, retain drug responsiveness and hepatic energy metabolism, secrete of liver-specific proteins, polarize, and do not proliferate. Importantly, the hepatocytes in MPCCs maintain a functional phenotype for up to 4–6 weeks and are compatible with medium-throughput drug screening methods and automated data collection. This platform has been found to be more predictive than existing in vitro liver models for generating and identifying human drug metabolites and drug-induced liver toxicity and supports the persistent replication of hepatitis C virus (Jones et al., 2010; Ploss et al., 2010; Wang et al., 2010). Furthermore, the availability of large cryopreserved lots of human primary hepatocytes means that donor-dependent interexperimental variability can be minimized. Nonetheless, reproducible access to sporozoites is also critical to achieve a practical system. Cryopreservation of large batches of aseptic sporozoites has also now been established for both *P. vivax* (Chattopadhyay et al., 2010) and *P. falciparum* (Epstein et al., 2011) sporozoites.

In this resource report, we demonstrate the feasibility of integrating cryopreserved human hepatocytes in MPCCs with cryopreserved *P. falciparum* and *P. vivax* sporozoites to form an in vitro platform that supports the liver stages of human malaria infection. This platform offers the potential for automation based on several factors, including preselection of cryopreserved human hepatocyte and sporozoite batches to standardize infection rate, a machine-learning algorithm that enables an automated imaging-based readout of immunofluorescent staining, and the capacity to generate a positive Z factor in response to drug exposure. The 96-well, medium-throughput format requires fewer reagents (drugs, sporozoites) than larger footprint in vitro assays, or than most in vivo assays, including humanized mouse

models (Vaughan et al., 2012). Collectively, our data document the development and characterization of a highly-reproducible, medium-throughput microscale human liver platform that may aid in the development of safe and efficacious antimalarial drugs and liver-stage vaccines.

RESULTS

Functional Characterization of Micropatterned Cocultures from Cryopreserved Primary Human Hepatocytes

In order to establish an MPCC in vitro culture of primary human hepatocytes (Figure 1A) suitable for *Plasmodium* infection, we screened cryopreserved hepatocytes from several individual patient donors to identify those that met the following criteria: (1) selective adhesion to collagen type I; (2) maintenance of a functional hepatocyte phenotype for up to 3 weeks as assessed by albumin expression, urea production, and CYP450 activity; and (3) expression of the previously identified *Plasmodium* host entry factor, CD81 (Silvie et al., 2003). We identified eight donor sources of cryopreserved human hepatocytes that were plateable on collagen I and displayed typical hepatocyte morphology, including the presence of bile canaliculi (Figure S1 available online). Seven of these sample sets demonstrated functional capacity in culture, as quantified by their production of albumin and urea, and also exhibited CYP450 activity for up to 3 weeks (Figure 1B). We next quantified the expression of the host entry factor CD81 on hepatocytes from each of the seven donors at the day of infection. Four of these samples (donors 2, 3, 7, and 8) expressed high levels of CD81 by immunofluorescence (IF) (Figure 1D).

Infection of Primary Human Hepatocyte MPCCs with *P. falciparum*

To test whether primary hepatocytes stabilized by culturing in MPCCs can be infected with *P. falciparum*, we exposed MPCCs to cryopreserved *P. falciparum* sporozoites (NF54) (Figures 2A and 2B). We confirmed productive infection in all seven donors by staining for HSP70 expression. However, a wide range of infection rates were observed between lots (Figure 1C, left panel). Specifically, at day 3 postinfection, the number of HSP70-positive, *P. falciparum*-infected hepatocytes was much higher in samples from donors 7 and 8 relative to the other tested lots. Hepatocytes from donors 7 and 8 were selected for further characterization of the model. We also examined the susceptibility of the same donor samples to productive infection by two different rodent species of *Plasmodium*. As shown in Figure 1C (middle panel), donors 7 and 8 also exhibit the highest levels of *P. yoelii* infection. However, this correlation was not observed in *P. berghei* (Figure 1C, right panel). Overall, infection with *P. berghei* was higher compared with *P. falciparum* and *P. yoelii*, with donor 1 showing slightly higher infection with *P. berghei* than the other donors. Interestingly, high CD81 expression was necessary, but not sufficient, to support robust infection by *P. falciparum* (Figures 1C and 1D). We next evaluated three different batches of cryopreserved *P. falciparum* sporozoites. As seen in Figure 1F, this experiment confirmed productive infection using sporozoites from three cryopreserved batches. However, infection efficiencies varied across batches.

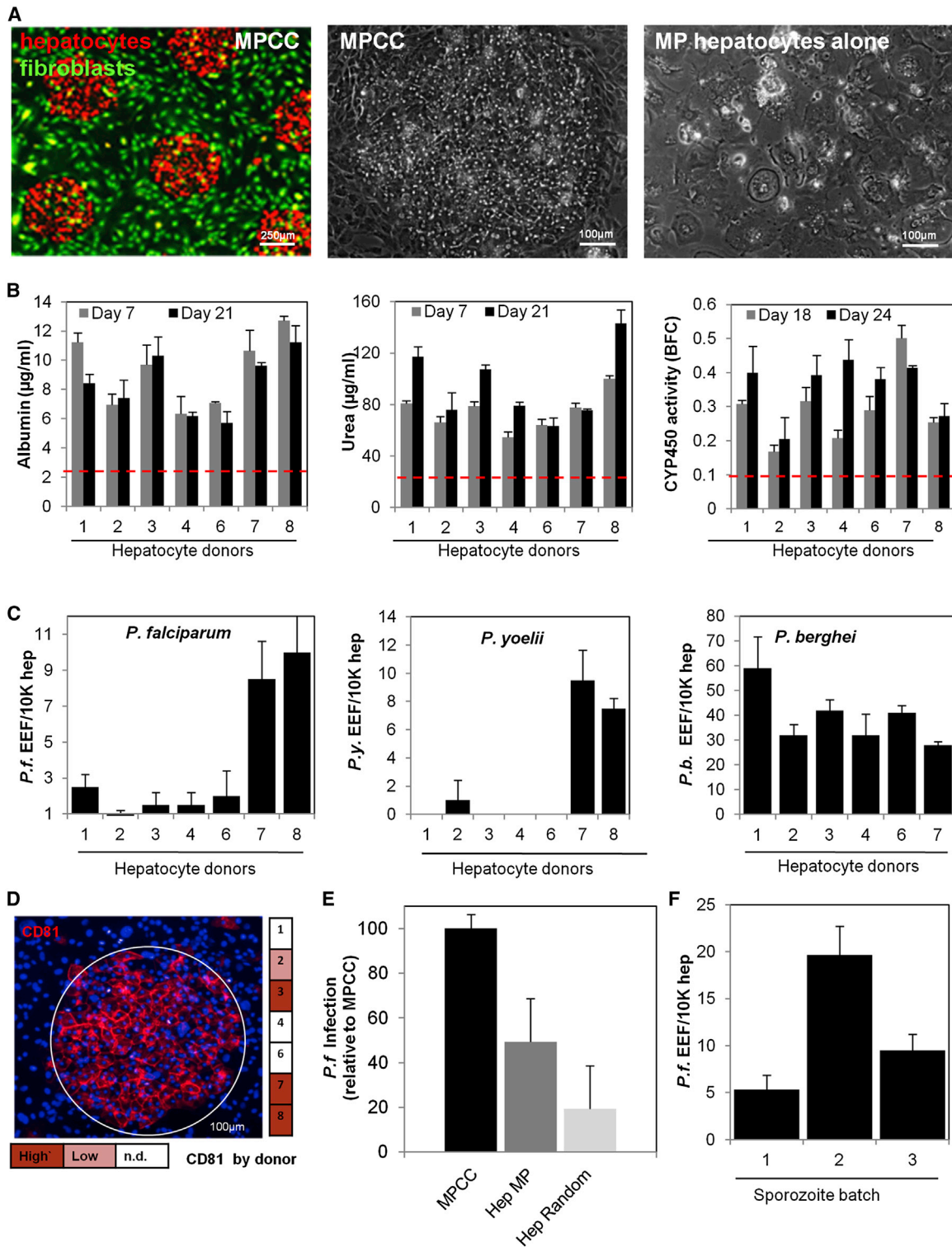


Figure 1. Functional Characterization of Cryopreserved Human Hepatocytes in Micropatterned Cocultures and Cryopreserved *Plasmodium falciparum* Sporozoites

(A) Morphology of primary human hepatocytes in micropatterned cocultures (left; hepatocytes, red and fibroblasts, green). Representative coculture of hepatocytes with (middle) and without (right) fibroblasts 18 days postseeding.

(B) Albumin secretion, urea synthesis, and CYP450 activity in MPCCs of different donors. Red dashed lines indicate the average level observed in 6-day hepatocyte monocultures (SD = 0.9, 5.4, and 0.06 for the left, middle, and right panels, respectively).

(C) *P. falciparum*, *P. yoelii*, and *P. berghei* infection across donors.

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Based on higher infection levels, sporozoites from batch 2 were selected for further characterization of the model. Importantly, we observed higher infection rates when hepatocytes were cultured in the MPCC format as opposed to standard, unpatterned (randomly distributed) monocultures (Figure 1E, Hep Random). MPCCs remained susceptible to *P. falciparum* infection for many weeks after they were patterned; however, infection rates are optimal at day 2 after patterning (data not shown).

Recapitulation of the Entire *P. falciparum* Liver Stage in MPCCs

Having established that *P. falciparum* can infect MPCCs, we next sought to establish whether the entire liver stage could be reproduced in vitro. The viability of the cryopreserved *P. falciparum* sporozoites was evaluated by assessing their gliding motility (Figure 2C) and by their capacity to traverse cells using a cell-wounding assay (Mota et al., 2001). On average, the addition of 37,000 motile cryopreserved *P. falciparum* sporozoites to 10,000 patterned hepatocytes resulted in 3% rhodamine-positive traversed cells (Figure 2D). To quantify the ability of the *P. falciparum* sporozoites to invade hepatocytes, *P. falciparum*-treated MPCCs were fixed 3 hr postinfection (Rénia et al., 1988). On average, 10% of hepatocytes contained intracellular cryopreserved *P. falciparum* sporozoites (Figure 2E). The human hepatocyte infection rate, based on the percentage of cells containing HSP70-expressing parasites at day 3 postinfection, was 0.2% (Figure 2J). Notably, at this same time point, fresh sporozoites achieved infection rates that were 7- to 13-fold higher (Figure 5A). Representative images of the HSP70-expressing parasites at day 3 and day 5 are shown in Figures 2F and 2G.

The maturation of parasites derived from cryopreserved *P. falciparum* was assessed using three parameters: (1) immunostaining of the infected cultures with antibodies against two proteins first expressed during late liver stages, PfEBA-175 (*P. falciparum* erythrocyte-binding antigen, 175 kDa) and PfMSP-1 (merozoite surface protein 1), which indicate full development of the respective schizonts inside the hepatocytes into merozoites; (2) the size of HSP70-expressing schizonts relative to previous reports of *P. falciparum* schizont size during liver-stage development; and (3) the progression rate, calculated as the percentage of schizont-bearing cells at day 6 relative to day 3. At day 5 postinfection, cryopreserved sporozoites express both PfEBA-175 and PfMSP-1 (Figure 3C). The parasites we observed were similar in size to what has been reported in other in vitro settings (~10–15 μm at day 5) (Mazier et al., 1985; van Schaijk et al., 2008) but smaller than those reported in vivo (Shortt et al., 1951; Shortt and Garnham, 1948; Vaughan et al., 2012) (Figures 2L and 3B). Importantly, we demonstrated that the rate at which parasites progressed to the schizont stage between day 3 and day 6 postinfection was higher in infected MPCCs (33%) relative to the commonly used hepatoma line HC04 (14%) (Figure 2K).

Finally, to demonstrate that hepatic schizonts derived from cryopreserved *P. falciparum* sporozoites could achieve full maturity and release infective merozoites, red blood cells (RBCs) were added to the hepatocyte cultures at day 6 postinfection. After 10 days, Giemsa staining revealed infection of erythrocytes by *P. falciparum* merozoites (Figures 2H and 2I).

Assessing Progression of Attenuated *P. falciparum* for Vaccine Applications

The ability of the MPCCs to recapitulate the entire liver stage of *P. falciparum* in vitro highlights the potential to use this platform to study the biology of *P. falciparum*-infected hepatocytes. For example, this capability should enable the assessment of candidate pre-erythrocyte malarial vaccines that are based on live-attenuated parasites (Annoura et al., 2012; Epstein et al., 2011; Mueller et al., 2005; van Schaijk et al., 2008).

To illustrate the potential use of MPCCs in vaccine development, we compared the infection capacity of a pre-erythrocytic malaria vaccine candidate comprised of cryopreserved, live-attenuated *P. falciparum* sporozoites to that of cryopreserved nonattenuated (wild-type) *P. falciparum* sporozoites. Entry of the attenuated parasites was evaluated relative to the function of nonattenuated parasites. As seen in Figure 3A, entry by both groups of parasites was similar. Late liver-stage development was evaluated at day 5 postinfection. Immunofluorescence staining with EBA-175 and MSP-1 antibodies detected mature schizonts only in MPCCs infected with the nonattenuated sporozoites, whereas MPCCs infected with the live-attenuated sporozoites were positive only for the early liver-stage antigen, LSA-1 (Figure 3C). As expected, schizonts that were established by the nonattenuated sporozoites were larger than the immature forms established by the attenuated sporozoites where the hepatic-stage development is arrested. Figure 3B shows a scatter plot of the range of exoerythrocytic form (EEF) sizes generated by each group.

Development of a Semiautomated, Medium-Throughput Platform for Antimalarial Applications

We next explored the utility of the MPCCs as a potential antimalarial drug screening platform. The hepatocyte serves as both the site of antimalarial drug metabolism (or bioactivation) and the host for the parasite. Thus, phenotypic stability of the hepatocyte and a full drug metabolism repertoire has the potential to capture a full range of drug responses such as efficacy, drug-drug interaction, and toxicity (Ploss et al., 2010). MPCCs were established in a 96-well format, infected with *P. falciparum*, and treated with the canonical malaria drug, primaquine. The impact of the drug was evaluated based on its ability to reduce parasite infection relative to control cultures in a multiday dose. As seen in Figures 4A and 4B, the half maximal inhibitory concentration (IC₅₀) for primaquine was approximately 1 μM for *P. falciparum* during 3 days in culture. The IC₅₀ for *P. falciparum* in the MPCC was lower compared to the IC₅₀

(D) Representative CD81 immunofluorescence staining at day 4 postseeding (left). Heatmap indicates relative CD81 expression per donor as measured by IF (right; n.d., not detected).

(E) *P. falciparum* infection in hepatocyte monocultures, micropattern (MP), or randomly distributed (Random) relative to infection in MPCCs. 10,000 hepatocytes were plated in each case.

(F) Levels of infection by three sporozoite batches in a single hepatocyte donor. Error bars represent SD. See also Figure S1.

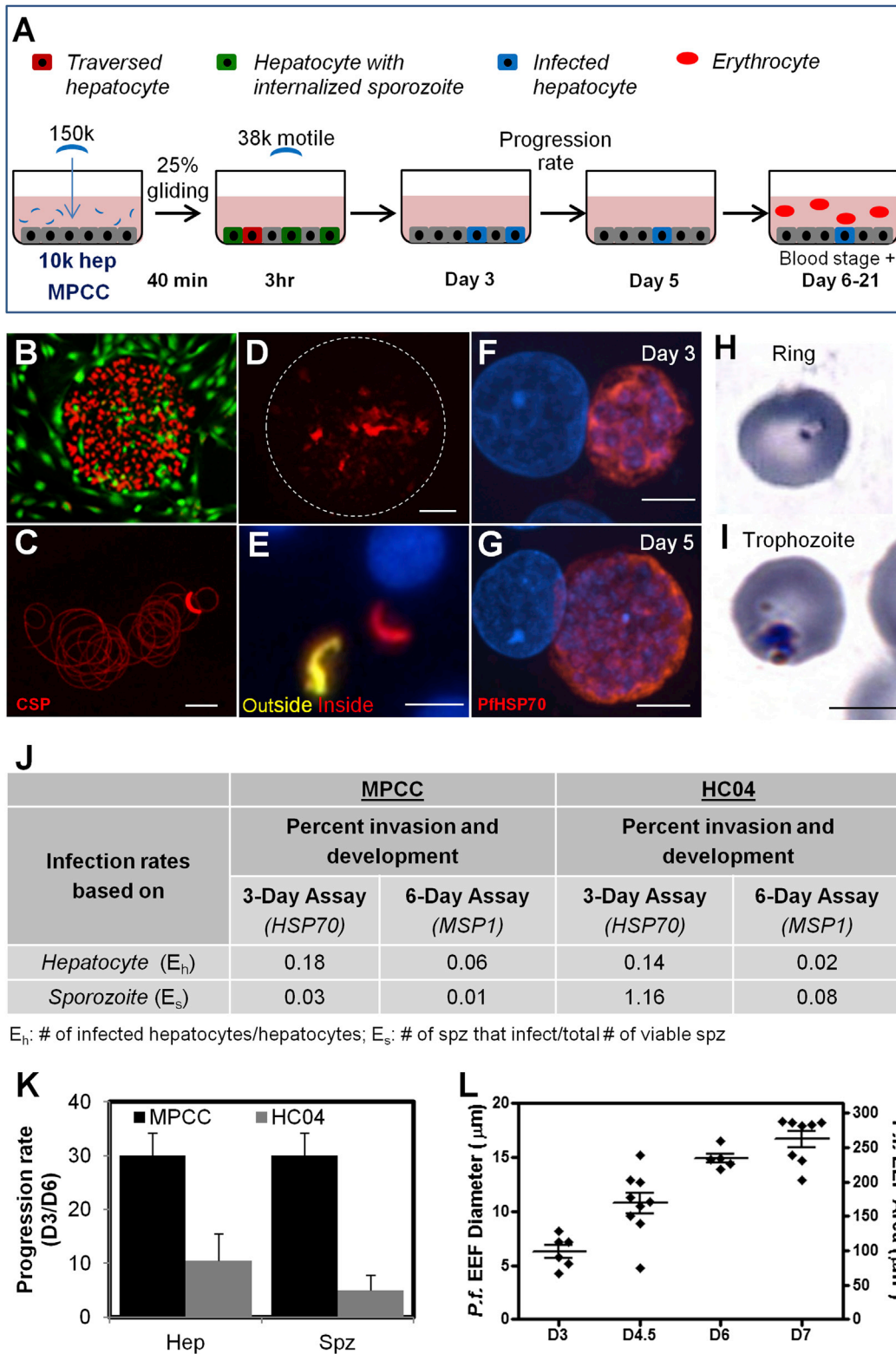


Figure 2. Liver-Stage Recapitulation in Primary Human Hepatocyte MPCCs

(A) Schematic of *P. falciparum* infection assay.

(B) Typical morphology of primary human hepatocytes in MPCCs (hepatocytes, red; fibroblasts, green).

(C) Representative image of *P. falciparum* sporozoites gliding. CSP immunostaining was used to visualize trails. Quantification based on the average fraction of sporozoites that perform at least one circle.

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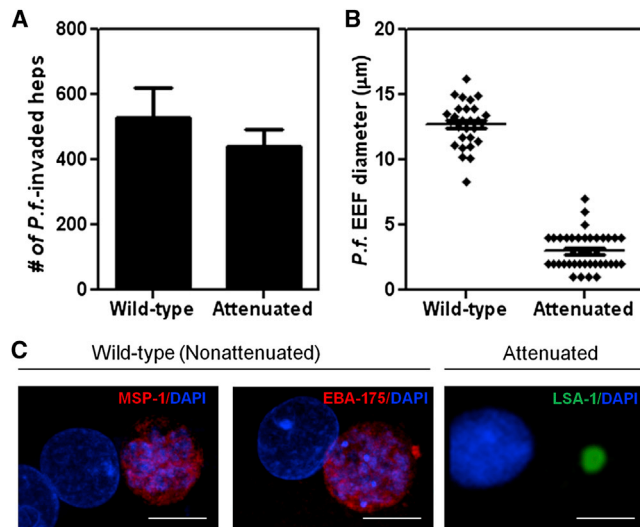


Figure 3. Comparison of Live-Attenuated versus Wild-Type Parasites for Candidate Vaccine Evaluation

(A) Number of infected hepatocytes observed after wild-type (nonattenuated) and attenuated cryopreserved sporozoite infection.
 (B) Size distribution of wild-type and attenuated parasites in MPCCs after 5 days of culture.
 (C) Representative images of parasites at day 5 postinfection. Wild-type parasites are identified by anti-MSP-1 and anti-EBA-175 staining. Attenuated parasites are identified by anti-LSA-1 staining. Nuclei are visualized with DAPI (blue). Scale bar = 10 μm . Error bars represent SEM.

obtained using unpatterned, monocultures of the hepatoma cell line HC04 (Figures 4A and 4B). Primaquine is known to require bioactivation in the liver in order to produce active daughter metabolites that mediate drug action (Pybus et al., 2012). Thus, the clearance of the parent compound can be used as a proxy for the level of bioactivation in culture. We monitored depletion of primaquine in MPCCs, patterned monocultures of primary hepatocytes only (Hep MP), and the HC04 cell line over 2 days by high-performance liquid chromatography and found that MPCCs most efficiently cleared primaquine over this time frame (Figure 4C). We next compared the expression level of 83 human-specific drug metabolism genes in order to assess whether the observed macroscopic differences in parent compound depletion correlates with the drug metabolism repertoire of these model systems. In general, the bulk of hepatocyte drug metabolism genes were expressed at higher levels in the MPCCs (Figure 4E). Of the three major enzymes involved with primaquine metabolism, two of the three were expressed at

higher levels in MPCC than the other systems. In particular, monoamine oxidase A (MAO-A), which is thought to account for over 75% of primaquine metabolism, was expressed at a level 4-fold higher than hepatoma cell lines, suggesting that the platform may offer a more predictive system with which to assay candidate drug performance, including studies of mechanisms of action.

Next, to further adapt the micro-liver platform for medium-throughput screens, we characterized several critical parameters. As seen in Figures 5A and 5B, we found that day-to-day variability of hepatocyte infection using the same batch of cryopreserved sporozoites could be minimized (coefficient of variation [CV] = 10%) when compared to the range of infection rates obtained using three independent batches of fresh sporozoites (CV = 42%). Next, a positive Z factor ($Z' > 0$), which indicates confidence in separating two normally distributed populations, was obtained when primaquine-treated wells (10 μM) were compared to control cultures (Figure 5C). In addition, the reproducibility of a phenotypic assay was assessed by establishing consistent dose-dependent inhibitory effects of two different drugs at three different concentrations in two independent experiments (atovaquone and primaquine) (Figure 5D). Furthermore, the magnitude of the inhibitory impact of primaquine was consistent across two independent human hepatocyte donors (Figure S2). Finally, we established and validated an automated image analysis readout using this platform, following the infection of MPCCs with two different doses of sporozoites. As shown in Figures 5E and S3, the number of parasites detected by automated image acquisition and analysis closely matches the counts obtained using conventional manual fluorescence microscopy.

Infection of MPCCs with *Plasmodium vivax*

Plasmodium vivax differs from *P. falciparum* in several important ways. A key feature of *P. vivax* that underlies its persistence in the population is that the liver acts as a reservoir for dormant hypnozoites or small forms (Cogswell, 1992; Krotoski et al., 1982). These hypnozoites can reactivate after weeks, months, or even years, depending on the strain of *P. vivax* (Dao et al., 2007; Durante Mangoni et al., 2003; Garnham et al., 1975). However, due to a lack of model systems available to investigate this elusive organism, the biology of the dormant form of *P. vivax* is underexplored.

Based on our ability to establish the liver stage of *P. falciparum* in MPCCs and maintain the hepatocyte phenotype for 4–6 weeks and the observation that some strains of *P. vivax* can reactivate over a similar timescale, we next explored the

(D) Cell traversal ability of *P. falciparum* sporozoites as visualized by dextran-positive staining of primary human hepatocytes.

(E) Representative double immunofluorescence stain (anti-PfCSP, both before and after cell permeabilization) of *P. falciparum*-infected MPCCs. Extracellular and intracellular sporozoites are labeled with yellow and red, respectively. Nuclei are visible with blue DAPI stain.

(F and G) Representative images of *P. falciparum* in human primary hepatocytes at days 3 (F) and 5 (G) postinfection. Parasites are identified by anti-PfHSP70 staining (red).

(H and I) Infection of human RBCs by merozoites released from infected liver-stage culture. Representative images of Giemsa-stained RBCs in the ring stage (H) and the trophozoite stage (I).

(J) Infection rates using MPCC primary human hepatocytes or HC04 hepatoma cells, calculated based on the plated number of sporozoites or hepatocytes.

(K) Progression rate from day 3 to day 6 in MPCC and HC04 calculated as infection rate of day 6 \div infection rate of day 3 \times 100 (based on hepatocytes and sporozoites).

(L) Schizont size distribution at days 3, 4.5, 6, and 7. Scale bar = 5 μm (F–I), 10 μm (C and E), or 100 μm (D). Error bars represent SEM.

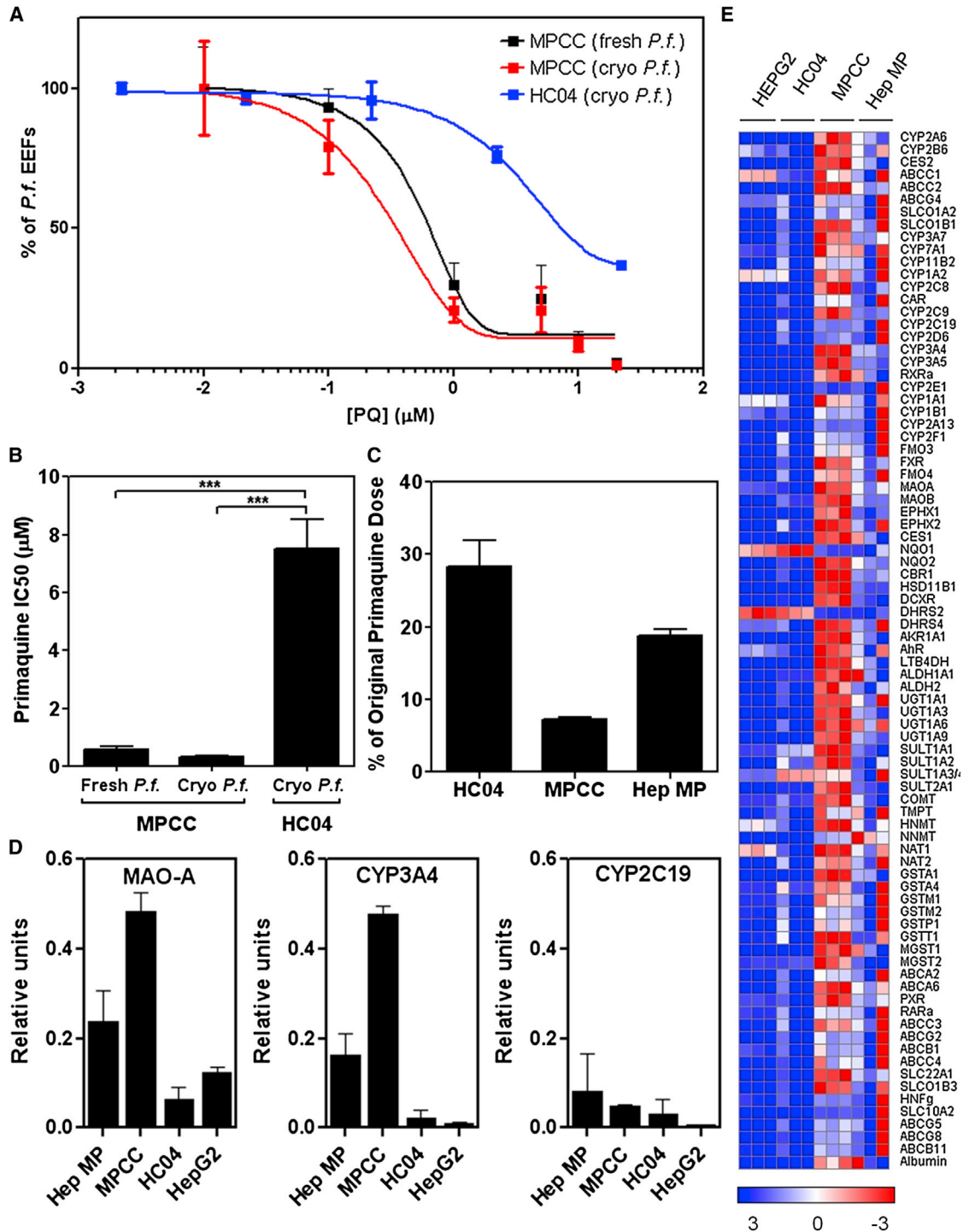


Figure 4. Utility of Medium-Throughput Human Hepatocyte Platform to Identify Lead Compounds

(A) Primaquine treatment of MPCCs or HC04 infected with fresh or cryopreserved sporozoites.

(B) IC₅₀ of primaquine in MPCCs versus HC04 ($p = 0.0002$ by one-way ANOVA; $***p < 0.001$ by Tukey's multiple comparison test).

(C) Primaquine metabolism by HC04, MPCCs, and patterned monocultures of primary human hepatocytes (Hep MP) quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

(D) Relative expression of three putative metabolism genes implicated in primaquine metabolism.

(E) Heatmap displays of LMA-Luminex analysis for 83 human-specific drug metabolism genes. Columns represent triplicate loadings of RNA extracted from HEPG2, HC04, MPCC, and Hep MP. Gene expression relative to average of control gene transferrin, and heatmaps are row normalized. Error bars represent SEM. See also Figure S2.

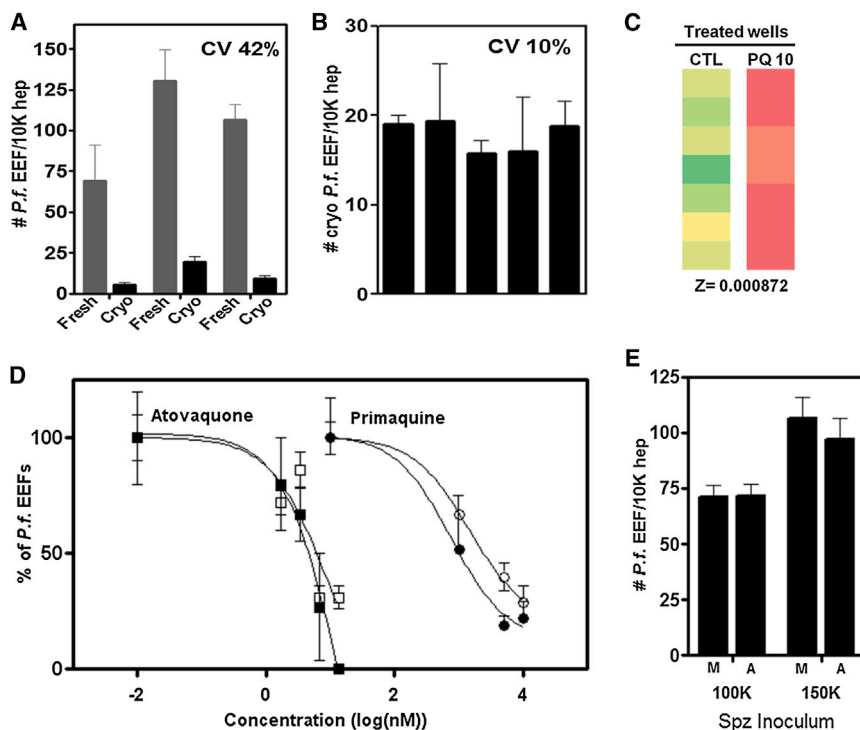


Figure 5. Adapting the Format to Drug Screening

(A) Interexperimental variability measured by the coefficient of variation (CV) and infection rate using fresh and cryopreserved sporozoites from three different batches.

(B) Interexperimental variability measured by the coefficient of variation (CV) and infection rate using cryopreserved sporozoites from the same batch.

(C) Heatmap indicating levels of infection (green, highest EEF numbers; red, lowest EEF numbers) observed in seven representative control or primaquine-treated wells. Comparison yields positive Z factor.

(D) *P. falciparum* infection with primaquine or atovaquone in two independent experiments performed on different days.

(E) *P. falciparum* infection in MPCCs following two doses of fresh sporozoites determined by manual counts (indicated by M) or image analysis automation (indicated by A). Error bars represent SEM. See also Figure S3.

feasibility of establishing the liver stages of *P. vivax* in MPCCs over time. As for the *P. falciparum* experiments, the viability of the cryopreserved *P. vivax* sporozoites was evaluated by assessing their gliding motility prior to each experiment (Figure 6A). We explored several strains of *P. vivax* including Chesson, a strain known to efficiently form dormant forms and reactivate at shorter timescales (Hollingdale et al., 1986; Krotoski et al., 1986). Cultures were infected and then fixed at various time points and stained for circumsporozoite protein (CSP). Using immunofluorescence microscopy, we analyzed the size distribution and localization of the *P. vivax* forms over a 3-week period. We readily observed *P. vivax* liver forms in MPCC, including mature, liver-stage schizonts larger than 20 μm found at day 6 postinfection (Figures 6C, 6B, S4A, and S4B). In addition, a population of smaller *P. vivax* CSP (PvCSP)-positive forms (<5 μm) was detected within MPCCs at all time points, particularly identifiable at 6 and 21 days postinfection (Figures 6A, 6B, and S4A–S4C). The infection efficiency, based on the percentage of hepatocytes containing large and small PvCSP-positive forms at day 6 postinfection, was 0.013% and 2%, respectively (Figure S4D). Similar numbers of small forms were observed up to day 21 (data not shown). Importantly, 85% of the small forms observed were intracellular, based on immunostaining performed before and after permeabilization (Figure S4C). In contrast, cultures infected with *P. falciparum* contained very few small forms after 15 days in culture, and those were predominantly extracellular (Figure S4E). These *P. vivax* small forms may represent the dormant hypnozoite stage of the parasite life cycle, which is responsible for clinical relapses in *P. vivax* malaria patients; however, further characterization will be required to substantiate this hypothesis. Both small and large forms were observed when other strains of fresh

and frozen *P. vivax* sporozoites were examined (Figure S4A and data not shown). Finally, we demonstrated that the MPCC system can support maturation of hepatic *P. vivax* schizonts, based on the detection of the late-stage antigen PvMSP-1 at day 12 postinfection (Figure S4B).

DISCUSSION

In this report, we describe an in vitro cell-based platform that recapitulates the human liver stage of *P. falciparum* and *P. vivax* infection. Although some attempts to infect cryopreserved human primary hepatocytes have been described in the past (Meis et al., 1985; Silvie et al., 2004), this source of hepatocytes has not been routinely adopted by the field to date. The advantages of screening individual donor-derived, cryopreserved hepatocytes is paralleled by the successful production of purified, vialled, cryopreserved sporozoites (Chattopadhyay et al., 2010; Epstein et al., 2011; Hoffman et al., 2010). These cryopreserved resources overcome the donor-to-donor variability seen in primary cultured human hepatocytes as well as infectivity rate variability introduced by different batches of mosquitoes or sporozoites. In addition, the use of cryopreserved components in this platform allows for a reliable source of reagents for use in longitudinal experiments, including screening and subsequent validation. Furthermore, since cell culture of patterned hepatocytes using the MPCC platform can maintain individual patient samples for 4 and 6 weeks (Khetani and Bhatia, 2008), it is feasible to perform long-term monitoring of both liver- and blood-stage *Plasmodium* infections, analyze genetic changes acquired inside *Anopheles* mosquitoes, assess the safety of attenuated sporozoite vaccine candidates, and characterize *P. vivax* hypnozoites in vitro using this system. Notably, development of vaccines and drugs against this stage of *P. vivax* has been identified as a critically important goal for research to eradicate malaria (Alonso et al., 2011a, 2011b).

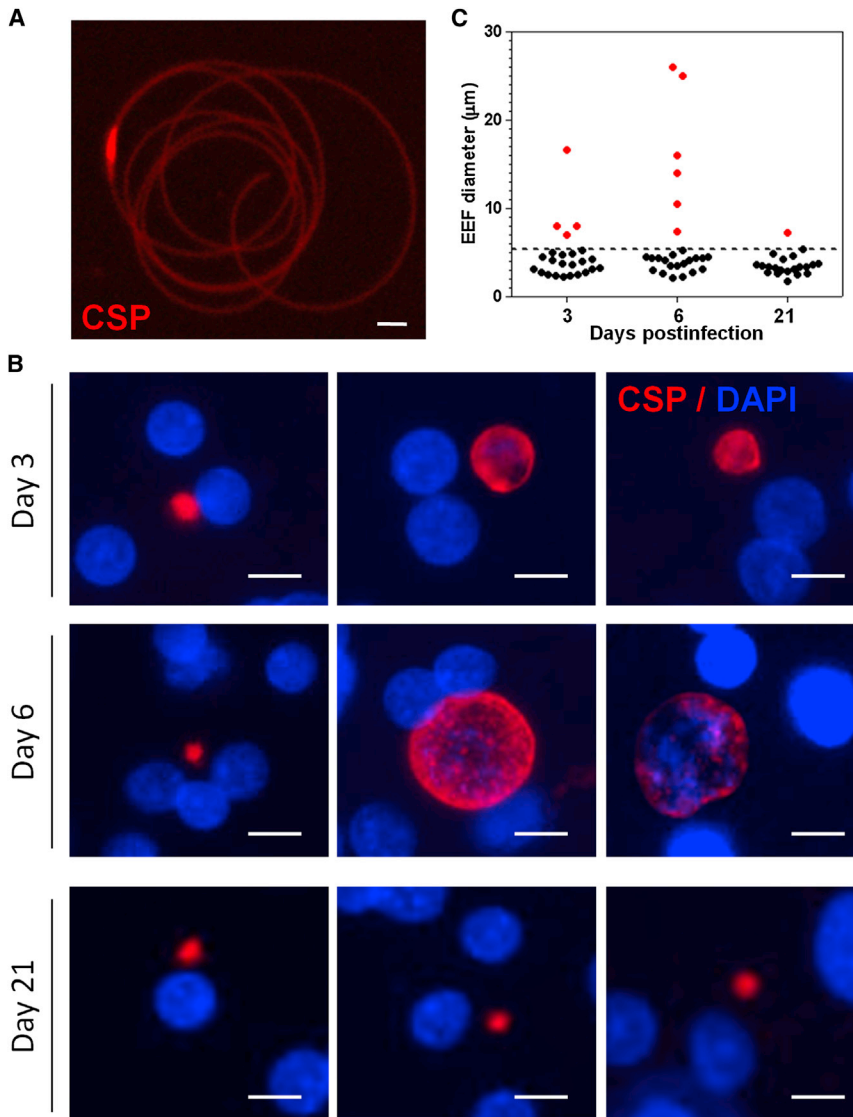


Figure 6. Infection with *Plasmodium vivax*
 (A) Representative image of PvCSP-stained *P. vivax* (Chesson) sporozoites gliding.
 (B) Representative images of PvCSP-positive parasites over time. Scale bar = 10 μm .
 (C) Size distribution of *P. vivax* parasites in MPCCs over time. Red, all observed parasites bigger than 5 μm ; black, 20 representative forms smaller than 5 μm . See also Figure S4.

Silvie et al., 2003); and (3) MPCCs fabricated from different human hepatocyte donors enable direct comparison of host factors that impact entry of different sporozoite species and strains (e.g., CD81 in *P. yoelii*, *P. berghei*, and *P. falciparum*), whereas hepatoma cells are typically limited to one or very few donor genotypes. We demonstrated that maintenance of hepatocyte function and expression of the host entry factor CD81 (Silvie et al., 2003) were necessary, but not sufficient, to obtain adequate levels of infection by *P. falciparum*. This finding suggests the existence of molecular differences among donors that determine their permissiveness to *Plasmodium* infection. Furthermore, most antimalarial drug development leads identified in RBC-based high-throughput screens do not require metabolic activation. Thus, in such cases, screening via the MPCC format might yield the same, higher, or lower IC_{50} predictions, should the candidate compound be cleared rapidly or bio-activated via metabolic pathways (Gamo et al., 2010; Guiguemde et al., 2010).

The infection rates reported in this study using cryopreserved sporozoites

Recent publications have highlighted that existing candidate vaccines continue to underperform in clinical trials, and significant “blood breakthrough” of presumably attenuated parasites formulations has been observed (Annoura et al., 2012).

In our experience with disease modeling of drug-induced liver injury and hepatitis C infection, establishing platforms that better reflect host biology is an important first step to determining where existing model systems were lacking (e.g., P450 activity and interferon signaling, respectively). In this case, we have already observed three advantages over in vitro hepatoma cultures: (1) sporozoites appear to progress through the parasite life cycle more efficiently in MPCCs relative to infected hepatoma cells, offering the potential to improve studies of drugs and sporozoite attenuation strategies that act in the second half of the liver life cycle; (2) MPCCs are able to predict differences in infection rates of sporozoites in vivo that result from cryopreservation, likely reflecting the presence of critical host factors that are altered in hepatoma cells (Albuquerque et al., 2009; Epiphonio et al., 2008; Prudêncio et al., 2008; Rodrigues et al., 2008;

are comparable or higher than those previously documented with fresh sporozoites, and they are an additional ~ 10 -fold higher when fresh sporozoites are used in this platform (Mazier et al., 1984, 1985; Sattabongkot et al., 2006; van Schaijk et al., 2008). The infection rates of hepatocytes in MPCCs are similar in comparison to the infection rates recently reported in HC04 cells, which range from 0.4% to 0.06% (Epstein et al., 2011; Sattabongkot et al., 2006). However, in our system, the progression rate from one stage of the life cycle to the other was much higher than in HC04, offering the potential for studying later stages of the liver life cycle more efficiently. Nonetheless, our MPCC infection rates remain low relative to those recorded in in vivo settings (Shortt et al., 1951; Shortt and Garnham, 1948). Experiments done in mice with *P. yoelii* (Conteh et al., 2010) and nonhuman primates with *P. knowlesi* (Jiang et al., 2009) have demonstrated that intravenous inoculation of only a few noncryopreserved sporozoites (10 sporozoites [SPZI]) can lead to a productive malaria infection that results in detectable parasitemia in the blood stage. Varying hypotheses have been put forward to

explain the discrepancy between model systems. For example, our *in vitro* systems do not provide the host hepatocytes with potentially necessary, physiologically relevant cellular components such as Kupffer cells or sinusoidal endothelial cells. Further, the MPCC platform conformational cues may be important for EEF growth (e.g., two-dimensional versus three-dimensional). These hypotheses will be explored further.

In conclusion, the ability to support the liver stages of *P. falciparum* and *P. vivax* parasites in a medium-throughput format offers promise to improve our fundamental understanding of the liver stages of human malaria as well as accelerate the development of drugs and vaccines to aid in its eradication.

EXPERIMENTAL PROCEDURES

Micropatterned Cocultures

12 mm coverslips that were placed into tissue culture polystyrene 24-well plates or glass-bottomed 96-well plates were coated homogeneously with rat tail type I collagen (50 $\mu\text{g}/\text{ml}$) and subjected to soft lithographic techniques (Ploss et al., 2010) to pattern the collagen into microdomains (islands of 500 μm) that mediate selective hepatocyte adhesion. To create MPCCs, cryopreserved primary human hepatocytes were pelleted by centrifugation at $100 \times g$ for 6 min at 4°C , assessed for viability using trypan blue exclusion (typically 70%–90%), and then seeded on collagen-micropatterned plates. The cells were washed with medium 2–3 hr later and replaced with human hepatocyte culture medium. 3T3-J2 murine embryonic fibroblasts were seeded (40,000 cells per well of a 24-well plate and 10,000 cells per well of a 96-well plate) in fibroblast medium 3 days later. Fibroblast medium was replaced with human hepatocyte medium 24 hr after fibroblast seeding and subsequently replaced daily (Khetani and Bhatia, 2008).

P. falciparum and *P. vivax* Sporozoites

Mosquitoes were fed on Pf- and Pv-infected blood as previously described (Chattopadhyay et al., 2010; Epstein et al., 2011). Briefly, *P. falciparum* and *P. vivax* sporozoites were extracted from infected mosquitoes by dissection of their salivary glands and passing the glands back and forth through a 26G needle fitted to a 1 ml syringe. Following extraction, sporozoites were purified from mosquito salivary gland material contamination and cryopreserved in liquid nitrogen vapor phase (LNVP) (Epstein et al., 2011; Hoffman et al., 2010). Live-attenuated *P. falciparum* sporozoites were attenuated by exposure of PfSPZ-infected mosquitoes to 150 Gy (Epstein et al., 2011; Hoffman et al., 2010).

Infection of MPCCs with *P. falciparum* and *P. vivax*

Typically, infection of MPCCs is conducted 2 days after hepatocyte seeding, but it can also be initiated after longer culture periods. Cryovials containing *P. falciparum* or *P. vivax* were warmed for 30 s at 37°C , 200 μl of human hepatocyte medium was added, and the cryovials were centrifuged for 2 min at $14,000 \times g$. Next, 200 μl of the supernatant was aspirated, and the sporozoite pellet was resuspended and diluted accordingly. Each well was infected with a ratio of 3:1 (infective sporozoites:hepatocytes). After incubation at 37°C and 5% CO_2 for 3 hr, the wells were washed once, and fresh medium was added. Medium was replaced daily. Samples were fixed on days 3 and 5 postinfection with *P. falciparum*, and days 3, 6, 12, and 21 postinfection with *P. vivax*.

Cell Wounding and Membrane Repair Assay

Sporozoite migration through cells can be quantified by the detection of sporozoite-wounded hepatocytes using a cell-impermeant fluorescent tracer macromolecule as previously described (Mota et al., 2001). Briefly, MPCCs were infected with *P. falciparum* in the presence of 1 mg/ml of 10,000 Da tetramethylrhodamine-dextran (lysine-fixable) (Sigma). At 3 hr postinfection, MPCCs were washed thrice with PBS, fixed with 1% paraformaldehyde at room temperature for 20 min, and mounted on glass slides. Migration of

sporozoites through cells is quantified by the number of dextran-positive hepatocytes per island.

Double-Staining Assay for Sporozoite Entry

At 3 hr postinfection, primary human hepatocytes or MPCCs were fixed and stained using a double-staining protocol as previously described (Rénia et al., 1988). Briefly, to label extracellular sporozoites, the samples were fixed with 4% paraformaldehyde for 10 min at room temperature, blocked with 2% BSA in PBS, incubated with a primary mouse anti-PfCSP (1:100, Sanaria) (Nardin et al., 1982), washed thrice in PBS, and incubated with a secondary goat anti-mouse Alexa Fluor 488 conjugate. This was followed by permeabilization with -20°C methanol for 10 min at 4°C , incubation with the same primary mouse anti-PfCSP, washing thrice with PBS, and incubation with a secondary goat anti-mouse Alexa Fluor 594 conjugate. This second step labels both intracellular and extracellular sporozoites. In case of MPCCs, the samples were counterstained with Hoechst and mounted on glass slides as described above. The number of invaded sporozoites (only green) in primary human hepatocytes was counted using Acumen Explorer.

Gliding Assay

Motility of cryopreserved sporozoites was determined in each batch to define the number of infective sporozoites. Sporozoite gliding was evaluated with 20,000 sporozoites for 40 min in complete Dulbecco's modified Eagle's medium (DMEM), at 37°C on glass coverslips covered with anti-CSP monoclonal antibody (clone 2A10 for *P. falciparum* and 210 clone NSV3 for *P. vivax*). Sporozoites were subsequently fixed in 4% paraformaldehyde (PFA) for 10 min and stained with anti-CSP. The percentage of sporozoites associated with CSP trails was visualized by fluorescence microscopy. Quantification was performed by counting the average percentage of sporozoites that perform at least one circle.

Primaquine Treatment of *P. falciparum* EEFs in MPCCs

Infected MPCCs were incubated with media containing primaquine diphosphate (Sigma) ranging from 0.5–20 μM . Fresh primaquine-containing medium was added daily until the samples were fixed at days 3 and 5 postinfection.

RNA Isolation and LMA-Luminex Analysis

Total RNA from three wells per condition was purified using TRIzol (Invitrogen) and RNeasy Mini kit (QIAGEN) and pooled for analysis. LMA-Luminex procedures and probes are previously described (Chen et al., 2011). Briefly, data for triplicate loadings, expressed in mean fluorescent intensity of at least 100 beads per sample, were scaled to the human transferrin gene and row normalized for heatmap representation using GenePattern open-source software (Broad Institute).

Image Automation

Images of 96-well plates were acquired using high-content screening microscopes (ImageXpress Micro XL, Molecular Device) and then analyzed by CellProfiler and CellProfiler Analyst (Broad Institute) (Carpenter et al., 2006; Jones et al., 2008). Parasites were visualized through immunofluorescent staining of the HSP70 protein and can be distinguished from imaging artifacts by their proximity to a hepatocyte nucleus (within 30 pixels) and lack of autofluorescence (no signal in unlabeled channels). We developed an automated image analysis pipeline to identify every infection in every image and measure hundreds of features (e.g., shape, area, texture) of each parasite. These features are then used to train machine learning algorithms to identify and count the number of parasites in each image using CellProfiler Analyst.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.chom.2013.06.005>.

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Cell Host & Microbe, Volume 14

Supplemental Information

A Microscale Human Liver Platform

that Supports the Hepatic Stages

of Plasmodium falciparum and vivax

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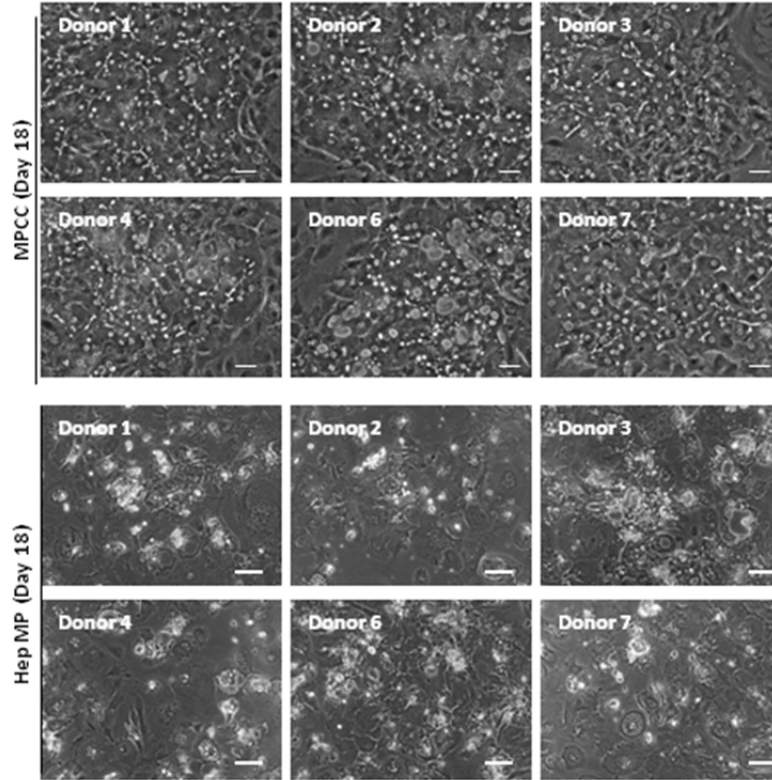


Figure S1. Cryopreserved Human Hepatocytes Morphology, Related to Figure 1

Morphology of cryopreserved human hepatocytes from different donors in micropatterned cocultures (day 18 post-seeding).

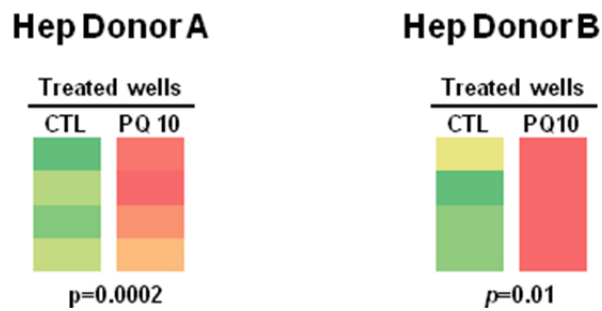


Figure S2. Inhibitory Effect of Primaquine, Related to Figure 4

Inhibitory effect of primaquine (PQ) using two different hepatocyte donors.

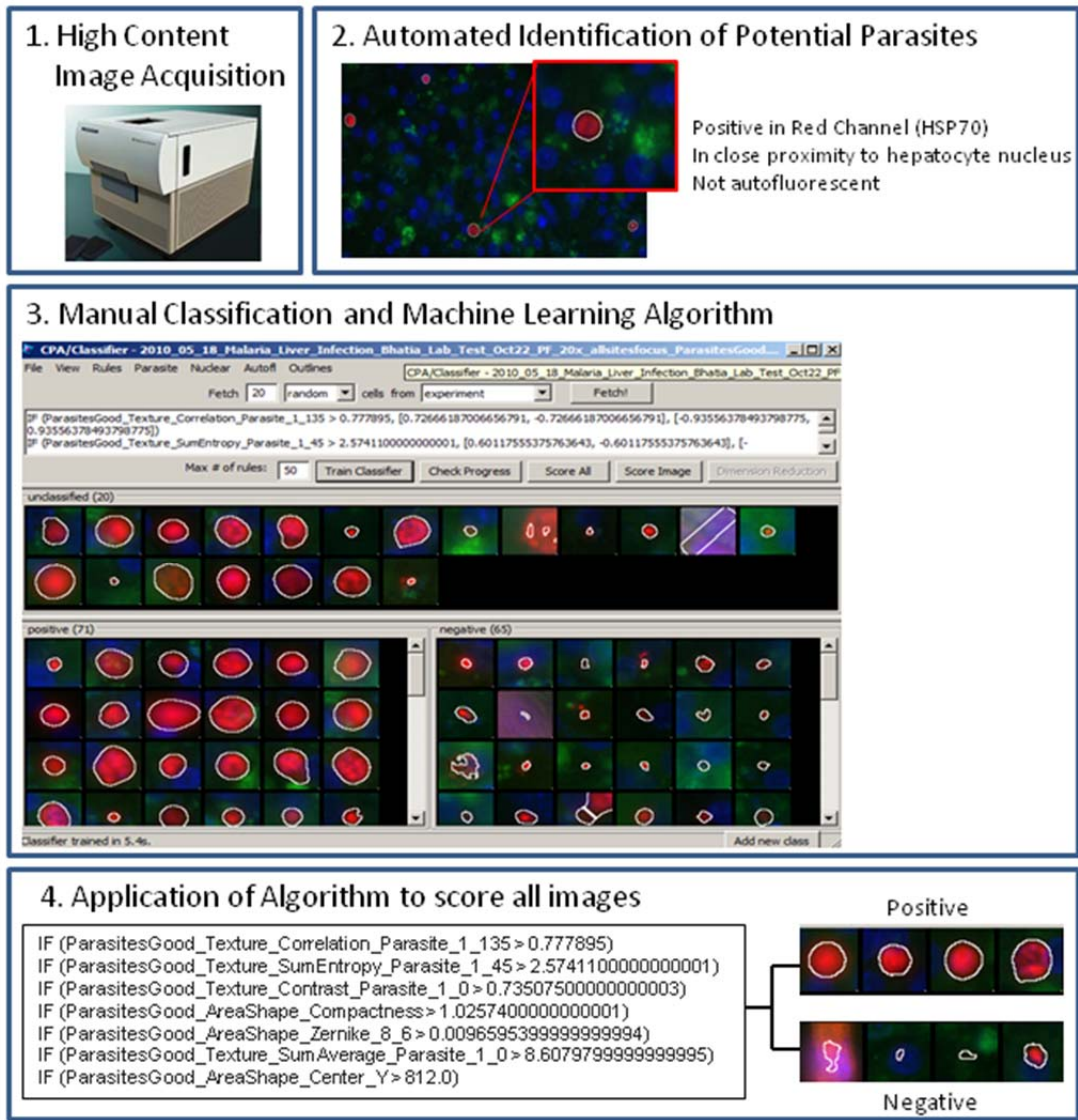
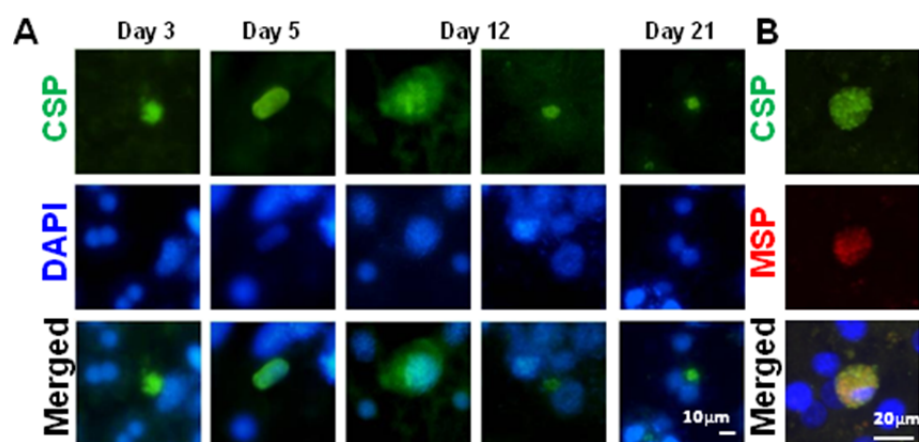
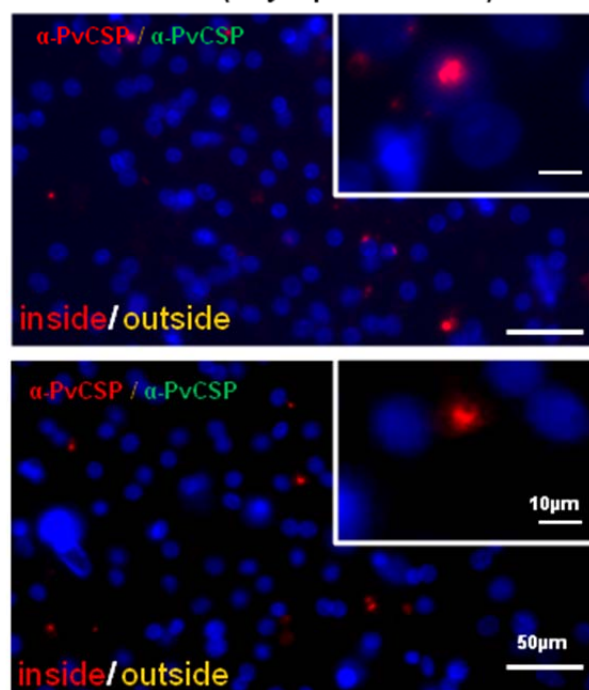


Figure S3. Image Automation Workflow, Related to Figure 5

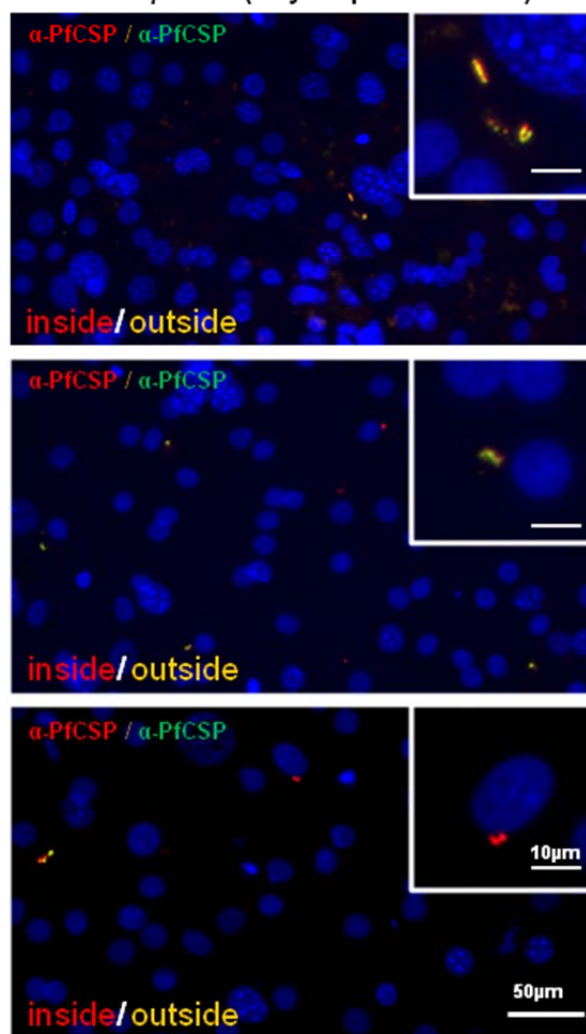
Images of 96 well plates are acquired using high content screening microscopes and analyzed by Cell Profiler (Broad Institute). Parasites are visualized through immunofluorescent staining of the HSP70 protein. Images of potential parasites are manually classified using Cell Profiler, which then learns the algorithm to score all images.



C *P. vivax* (Day 6 postinfection)



E *P. falciparum* (Day 15 postinfection)



D

	<i>P. vivax</i> (day 6 p.i.)
Total big forms [§] / 10K Hep	1.3 ± 0.2 (0.013%)
Total small forms observed / 10K hep [§]	200 ± 25 (2%)
% of all small forms inside	85%
% of all small forms outside	15%

[§] big forms defined as DAPI+ forms >10µm

[§] all small forms observed <5µm

Figure S4. *Plasmodium P. vivax* Infection Characterization, Related to Figure 6

Infection with *Plasmodium vivax* (India VII). **(A)** Representative images of parasites at day 3, 5, 12 and 21 post-infection. Parasites are identified by anti-PvCSP staining. Scale bar: 10µm. **(B)** Detection of MSP-1 protein at day 12 post-infection. **(C)** Representative double immunofluorescence stain (anti-CSP) of primary human hepatocytes infected with cryopreserved *P. vivax* at day 6 post-infection. Extracellular and intracellular parasites are labeled yellow and red, respectively. Nuclei visible with blue DAPI stain. **(D)** Quantification of small (<5µm) and big (>10µm) forms observed in *P.vivax* infected MPPC. **(E)** Representative double immunofluorescence stain (anti-CSP) of primary human hepatocytes infected with cryopreserved *P. falciparum* at day 15. Extracellular and intracellular parasites are labeled yellow and red, respectively. Nuclei visible with blue DAPI stain.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture

Cryopreserved primary human hepatocytes were purchased from vendors permitted to sell products derived from human organs procured in the United States by federally designated Organ Procurement Organizations. Vendors included CellzDirect and Celsis In vitro Technologies. Human hepatocyte culture medium was high glucose Dulbecco's Modified Eagle's Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) ITS™ (BD Biosciences), 7 ng/ml glucagon, 40 ng/ml dexamethasone, 15 mM HEPES, and 1% (v/v) penicillin-streptomycin. J2-3T3 murine embryonic fibroblasts (gift of Howard Green, Harvard Medical School) were cultured at <18 passages in fibroblast medium comprising of DMEM with high glucose, 10% (v/v) bovine serum, and 1% (v/v) penicillin-streptomycin. HC-04 cells were seeded in a 96-well plate (10,000 cells/well) or in a LabTek slide (40,000 cells/well).

Immunofluorescence Assay

MPCC were fixed with -20°C methanol for 10 min at 4°C, washed twice with PBS, blocked with 2% BSA in PBS and then incubated for 1h at room temperature with a primary antibody: mouse anti-CD81 (clone JS-81, BD Pharmingen; 1:100), rabbit anti-LSA-1 (Sanaria, 1:50), mouse anti-EBA-175 (Sanaria, 1:100) (Sim et al., 2011), mouse anti-PfHsp70 (clone 4C9, Sanaria; 1:100) (Tsuji et al., 1994), mouse anti-PfMSP-1 (Sanaria; 1:100), anti-rabbit PvMSP-1 (MRA16; MR4) or mouse anti-PvCSP (clone NVS3, Sanaria; 1:100) (Charoenvit et al., 1991). Samples were washed twice with PBS before incubation for 1 h at room temperature with secondary antibody: goat-anti-mouse Alexa Fluor 594 or Alexa 488, and goat anti-rabbit Alexa Fluor 488 (Invitrogen; 1:400). Samples were washed twice with PBS, with nuclei counterstained with Hoechst 33258 (Invitrogen; 1:1000), and then mounted on glass slides with Fluoromount G (Southern Biotech).

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For samples in 96-well plates, 50 μ L of Aquamount (Thermo-Scientific) was added per well after counter-staining with Hoechst. Images were captured on a Nikon Eclipse Ti fluorescence microscope.

Biochemical Assays

Cell culture supernatants were collected and stored at -20°C. Urea was measured by acid- and heat-catalyzed detection of diacetylmonoxime conversion to a colorimetric product (StanBio Labs). Albumin content was measured by an enzyme-linked immunosorbent assay using goat anti-human albumin antibody (Bethyl Labs) with horseradish peroxidase detection (Bethyl Labs) and 3,3',5,5'-tetramethylbenzidine (TMB, Pierce) development.

Coculture with Erythrocytes and Merozoite Invasion

To determine whether first-generation merozoites were released by infected hepatocytes, freshly prepared human O+ red blood cells (Research Blood Components) were added to each *P. falciparum*-infected well at a 2% hematocrit on day 6 after infection. Every week for two weeks, fresh red blood cells were added, and samples of the red blood cells from the previous

coculture were harvested. Thin blood smears were prepared, stained with Giemsa, and examined for asexual stage parasites.

Statistical Analysis

Experiments were repeated at least 2–3 times with duplicate or triplicate samples for each condition. Data from representative experiments are presented, whereas similar trends were seen in multiple trials. All error bars represent standard error of the mean (s.e.m).