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A rapid and robust tri-color flow cytometry assay for monitoring malaria parasite development

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Microscopic examination of Giemsa-stained thin blood smears remains the gold standard method used to quantify and stage malaria parasites. However, this technique is tedious, and requires trained microscopists. We have developed a fast and simple flow cytometry method to quantify and stage, various malaria parasites in red blood cells in whole blood or *in vitro* cultured *Plasmodium falciparum*. The parasites were stained with dihydroethidium and Hoechst 33342 or SYBR Green I and leukocytes were identified with an antibody against CD45. Depending on the DNA stains used, samples were analyzed using different models of flow cytometers. This protocol, which does not require any washing steps, allows infected red blood cells to be distinguished from leukocytes, as well as allowing non-infected reticulocytes and normocytes to be identified. It also allows assessing the proportion of parasites at different developmental stages. Lastly, we demonstrate how this technique can be applied to antimalarial drug testing.

Malaria remains one of the most devastating parasitic diseases and is responsible for ~250 million cases of disease and nearly one million deaths per year, mostly in Africa[1]. The etiologic agent is a protozoan of the genus *Plasmodium* with a complex life cycle. The blood stage phase is responsible for the symptoms and the pathologies of the infection[2]. Microscopic examination of Giemsa's stained slides remains the 'gold standard' method for the detection, quantification, speciation and staging of blood stage malaria parasites[3]. However, it is tedious, time-consuming and relies on the skills of microscopists who have to be trained to correctly identify the different parasite species and stages [4]. This final point is of greatest concern, as this inter-reader viability, gives rise to the common criticism that microscopy counts are relatively subjective. Parasite quantification based on flow cytometry has been proposed with the goals of increasing precision and objectivity[5]. These methods are generally based on the detection of *Plasmodium* double-stranded DNA in the infected erythrocytes [6]. Different permeable dyes such as Hoechst [7], DRAQ-5 [8], SYBR Green [9], thiazole orange [10], acridine orange [11], hydroethidine [12], SYTO-16 [13], propidium iodide [14] or non-permeable dyes such as YOYO-1 [15] or SYTOX-Green [16] have been used successfully in these different studies. However, these techniques lose accuracy when applied to samples directly isolated from infected individuals [17]. This is because malaria often induces anaemia and reticulocytosis in humans and in animals. These pathologic conditions are characterized by an efflux of erythroblasts and normoblasts into the blood. These nucleated cells can be stained by DNA/RNA dyes and as such can be confounded with infected red blood cells (IRBC) [16]. However, they also express the CD45 pan-leukocyte marker, which we have exploited to develop a new and robust flow cytometry method for parasitemia quantification.

Here we show how this novel tri-colour method (TCM) can be used to quantify and stage parasites in small amounts of fresh whole blood (~1μL) from mice (i.e. *P. yoelii or P. berghei*) or humans (*P. falciparum* or *P. vivax*), or in *in vitro* cultures of *P. falciparum*. This method also allows for the assessment of white blood cell and reticulocyte frequencies in parallel with parasitemia determination. We demonstrate the utility of this technique as applied to an *in vitro* assay of *P. falciparum* drug sensitivity.

Results

***Ex-vivo* determination of *P. yoelii* parasitemia in mice.** The strategy for evaluation of parasitemia *in vivo* was based on the quantification of the different cell populations (uninfected normocytes, uninfected reticulocytes,

infected red blood cells and white blood cells) present in whole blood preparations. First, 1 µl of blood from an uninfected mouse was diluted in 100 µl of PBS and the solution was stained with dihydroethidium and Hoechst together with anti-CD45 mAb coupled to APC for 20 min. The samples were next analyzed by flow cytometry using the laser configuration described in Materials and Methods (Figure 1A and supplementary Table 1). Events were gated according to their FSC-A/SSC-A profile (gate G1) to exclude debris. The doublets corresponding to cell aggregates were also excluded by the FSC-A/FSC-H profile and single cells (in gate G2) were further analyzed. A dot plot of CD45/Hoechst was used to quantify white blood cells (WBC) and nucleated erythroblasts (in gate G3) that are positive for the CD45 marker and are stained by Hoechst DNA dye. Dihydroethidium is converted to Ethidium in viable cells, where it stains both DNA and RNA. A dot plot of Ethidium/Hoechst was used to identify cell populations containing DNA and RNA (in gate G4). In uninfected blood, there is nearly a 1:1 correspondence between the events in gates G3 and G4. Next, we used the same protocol with blood samples taken from mice infected with *P. yoelii* 17X (Figure 1B). The same gating procedures were applied and the parasitemia was determined by subtracting the percentage of WBC/erythroblasts (CD45 and Hoechst double-positive, gate G3) from the percentage of cells containing DNA and RNA (positive for Ethidium and Hoechst, gate G4) since IRBC are CD45 negative. The lower limit of detection for parasitemia was determined using blood from uninfected mice (Figure 1A) and was always 0.02-0.04%. In the experiment presented in Figure 1B, the *P. yoelii* parasitemia calculated by the flow cytometry method was 13.15%, similar to the 12.77% determined by microscopy. Using the TCM, we were also able to identify the reticulocyte population since these denucleated but RNA-retaining cells can be identified as being Hoechst-negative, Ethidium-positive, and CD45-negative (Figure 1A). In samples from mice infected with *P. yoelii* 17X clone 1.1, the uninfected reticulocytes disappeared. This reflected the consumption of this cell population by a parasite strain known for its selective tropism for reticulocytes [18].

***Ex-vivo* determination of *P. berghei* parasitemia in mice.** When applied towards another rodent parasite, *P. berghei* ANKA, we showed that the TCM method can also successfully assess

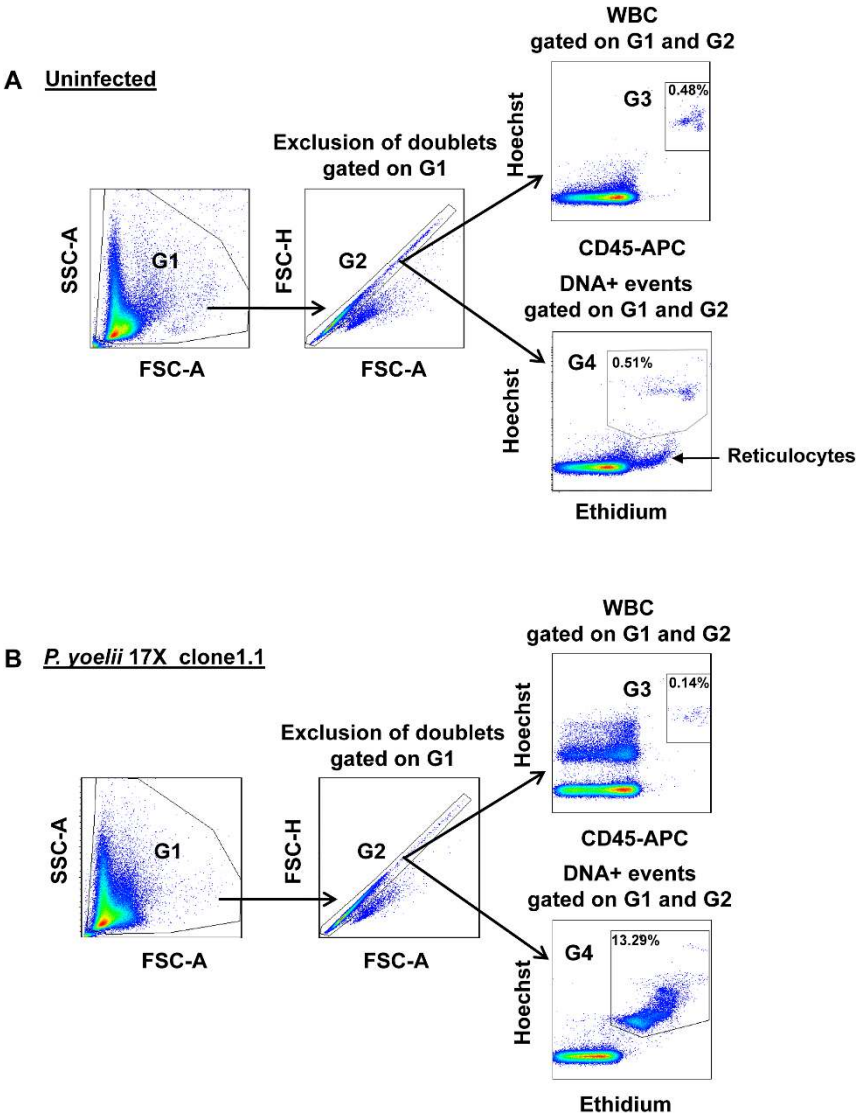

Figure 1 | Flow cytometry gating strategy for *P. yoelii* parasitemia determination. The TCM was performed with blood from (A) an uninfected C57BL/6J mouse and (B) a mouse infected 5 days previously with *P. yoelii* 17X clone 1.1. Red blood cells and white blood cells are first gated (G1) on a forward scatter/side scatter (FSC-A/SSC-A) dot plot. The G1 events are visualized using a FSC-A/FSC-H dot plot and the singlets (single cells) are gated on gate G2. Cells on G2 are simultaneously displayed on both CD45/Hoechst and Ethidium/Hoechst dot plots. CD45-positive white blood cells (WBCs) appear in gate G3 and are subtracted from all DNA- and RNA-containing cells (gate G4) to calculate the parasitemia.

parasitemia (Figure 2). We noted that the DNA-positive events were mostly distributed in two main clusters on the Hoechst/Ethidium dot plot (Figure 2B) and sought to determine by flow cytometry cell sorting if they represented different stages of parasite maturation. Cells in Gate 4 were sorted into either Hoechst^{low} Ethidium^{low} (1) or Hoechst^{high} Ethidium^{high} (2) populations, smeared on glass slides and stained with Giemsa. Rings and trophozoites were found in the Hoechst^{low}Ethidium^{low} population whereas multi-rings, schizonts and gametocytes were found in the Hoechst^{high} Ethidium^{high} population.

To further confirm the reliability of the TCM for parasitemia measurement, we took advantage of the fact that the *P. berghei* ANKA parasites used in this study express GFP, allowing direct comparison of GFP fluorescence-derived parasitemia and those obtained by the TCM method. There was an excellent linear correlation between the two methods when assessed by the Spearman rank correlation test (slope = 1.044; R^2 = 0.999) (Figure 3A). Bland–Altman analysis [19] shows good agreement between the TCM method and Flow cytometry method using GFP or from microscopy for measuring parasitemias below 10% parasitemia (Figure 3B and 3C). We observed a discrepancy between the TCM and the microscopy at parasitemias greater than 10%. However, they were no systematic bias between these methods (Bias=−0.09%). The poor method agreement observed at >10% parasitemia are most likely due to inaccuracies in microscopic counts (commonly noted at these high parasitemias). It is worth noting that most human parasitemias rarely exceed 4% and at these levels the agreement between TCM and microscopy is acceptable. We also observed a systemic but acceptable bias when TCM was compared to GFP determination (Bias=−0.21%). This is due to the fact that at high parasitemia many leukocytes are GFP positive, possibly after phagocytising live parasites.

***Ex-vivo* monitoring of *P. berghei* development in mice.** We next applied the TCM to monitor *P. berghei* GFP infection in C57BL/6J. The profile of parasitemia development was similar to profiles previously described and determined by microscopy [20]. As shown in

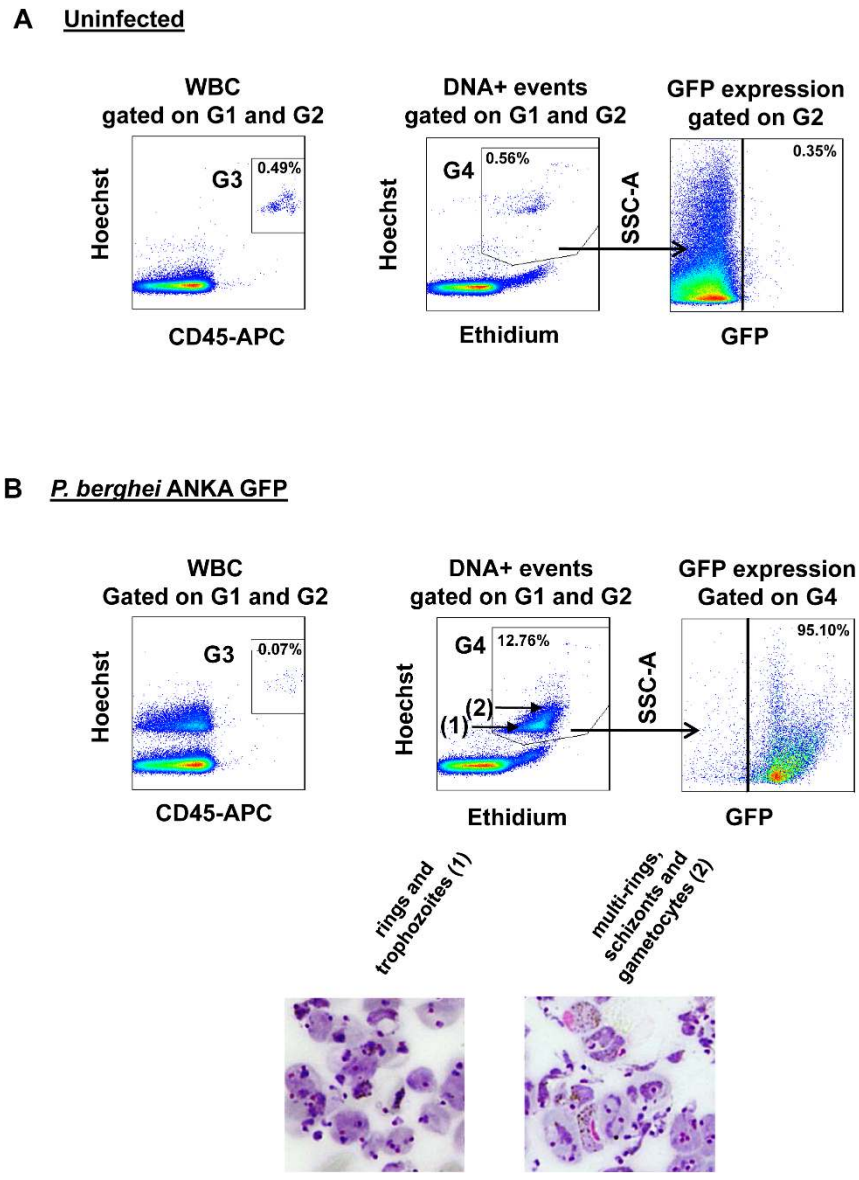

Figure 2 | Flow cytometry gating for *P. berghei* ANKA parasitemia determination. The TCM method was performed with blood from (A) an uninfected C57BL/6J mouse and (B) a mouse infected 7 days previously with *P. berghei* ANKA expressing GFP. The same flow cytometry gating strategy described in Figure 1 was used. On the extreme right panel, GFP expression for cells present in gate G4 was displayed on the SSC/GFP dot plot. In gate G4, the parasite population in (1) is composed of a mix of rings and trophozoites and in (2) of a mix of multi-rings, schizonts and gametocytes as shown in representative Giemsa-stained smears obtained after sorting by flow cytometry.

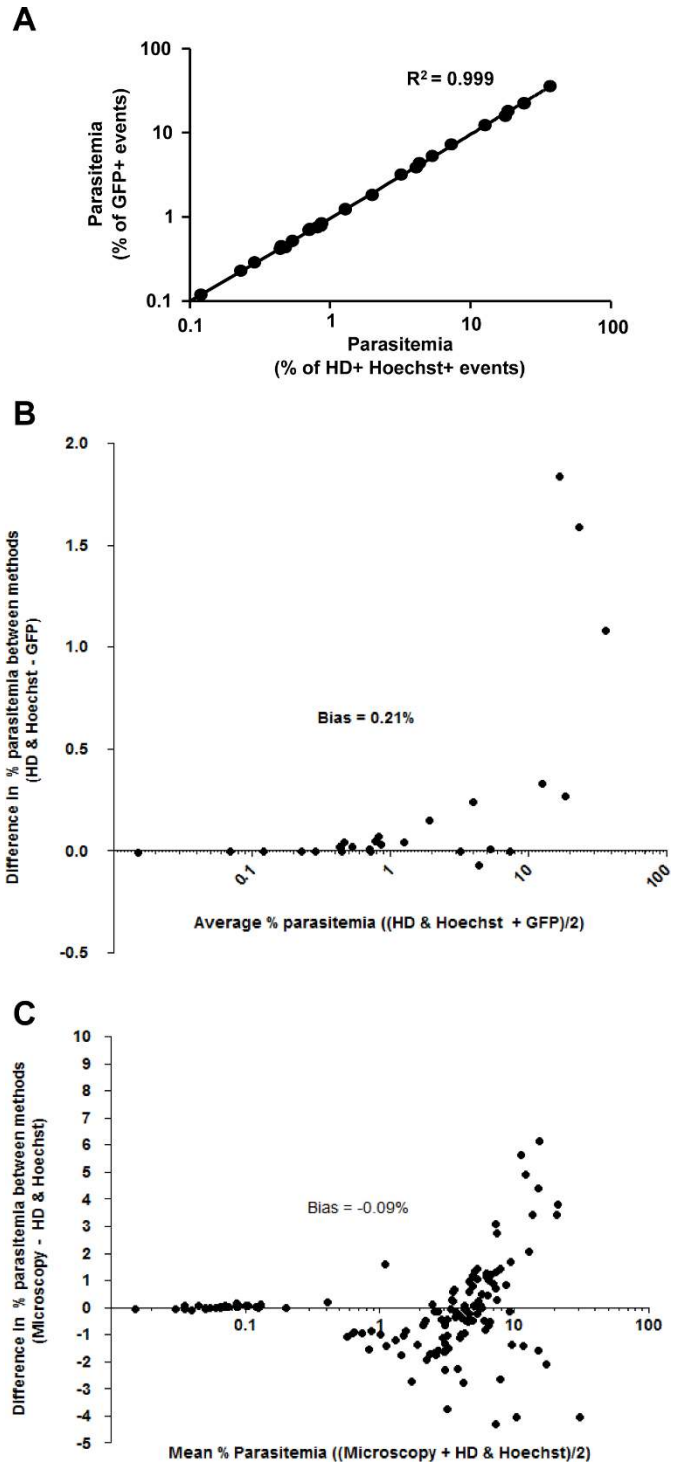

Figure 3 | Correlation analysis with Spearman test and agreement analysis with Bland-Altman statistical test. (A) Correlation between parasitemia of *P. berghei* ANKA GFP parasitemia monitored using GFP expression and with TCM. (A) Spearman rank correlation analysis of the parasitemia determined from GFP expression and the TCM in blood samples from 27 infected mice (slope = 1.044, R^2=0.999). (B) Bland-Altman agreement analysis between the parasitemia quantified with GFP expression and with TCM (bias=0.21%). (C) Bland-Altman agreement analysis between the parasitemia quantified with microscopy counting and TCM (bias=−0.09%).

Figure S3, parasitemia develop steadily until a majority of mice develop experimental cerebral malaria and die between days 10–12. In the two remaining mice, parasitemia increased rapidly until day 18 when they died of hyperparasitemia and anaemia. An advantage of the TCM was that the WBC percentage could also be monitored in parallel. A marked increased of nucleated cells (leukocytosis) appeared at the time of ECM and continued increasing in the mice which did not die of ECM (Figure S3B).

Monitoring *P. falciparum* growth *in vitro*. We next applied the TCM protocol to human parasites, staining and analyzing a synchronized *P. falciparum* 3D7 parasite culture. Parasites were also sorted to identify the different parasite maturation stages in the different clusters on the Ethidium/Hoechst dot plot. At the beginning at the culture, the majority of the parasites were detected in the left half of the double-positive gate. These were at an early stage of development and double rings could be distinguished from single rings on the basis of higher Hoechst staining (Figure 4A, left panel). After 24 hours of culture, the predominant forms of the parasite were in the right half and corresponded to late stages: trophozoites, early schizonts, and late schizonts, with increasing Hoechst staining (Figure 4A, right panel). No gametocytes were detected since our culture conditions did not favour their development.

To further confirm that the increase of Ethidium and Hoechst staining was indeed associated with parasite maturation, we used a magnetic enrichment technique to separate the mature parasite forms containing hemozoin pigment from younger forms which do not contain hemozoin (Hz) [21]. Before magnetic sorting, it was evident that the unsynchronized *P. falciparum* 3D7 *in vitro* culture contained a mixture of different parasite development forms (Figure 4B, left panel). After magnetic enrichment, of 90% of the parasites were in the Ethidium high gate and microscopic examination confirmed that these had synthesized hemozoin (Figure 4B, right panel).

Assessment of parasitemia in human parasite field isolates. Moving onwards from the 3D7 laboratory strain, we next tested the TCM on two clones of *P. falciparum* parasite field isolates that have been propagated *in vitro*. As expected, CD45 staining was undetectable as white blood cells had been depleted using CF11. Freshly thawed cloned parasites were assayed just after thawing or after 24 hours of *in vitro* culture at 37°C. Initially, the preparation contained mainly ring forms (>92% for the two isolates tested), due to the fact that young forms of malaria parasites are more resistant to freeze/thaw than mature forms [22]. Three different clusters with increasing Hoechst and Ethidium fluorescence were observed, corresponding to single, double or triple rings in one iRBC caused by multiple infection by *P. falciparum* merozoites (upper left panel Figure 5A).

Total initial parasitemia determined by the TCM method was similar to that measured by microscopy (1.75% versus 1.9% for clone 1 and 1.7% versus 2.1% for clone 2). After 24 hours of *in vitro* maturation, the intensity of the Ethidium and Hoechst signals increased, reflecting the growing RNA and DNA content in IRBC parasite transition and maturation from rings to schizonts.

The TCM was next used with cryopreserved whole blood samples from two patients from Thailand infected by *P. falciparum* (CF11 depletion was not conducted on these samples) (Figure 5B). After thawing and staining, it was observed that the WBC proportion was low in these two samples. The calculated parasitemia values were 0.19% in patient #1 and 9.8% in patient #2. As expected for *P. falciparum* samples taken from patients, mainly rings forms were detected in peripheral blood as the mature forms are sequestered. Distinct clusters corresponding to double and triple rings were not observed as multiple invasions are unusual *in vivo* [23].

We also thawed and examined three different *Plasmodium vivax* isolates using the TCM (Figure 6A). The blood of patient #5 contained parasites distributed between two clusters on the Hoechst/Ethidium dot plot. These two populations were sorted, smeared on slides and stained with Giemsa, revealing that the left cluster corresponded to ring forms and the right cluster to mature forms

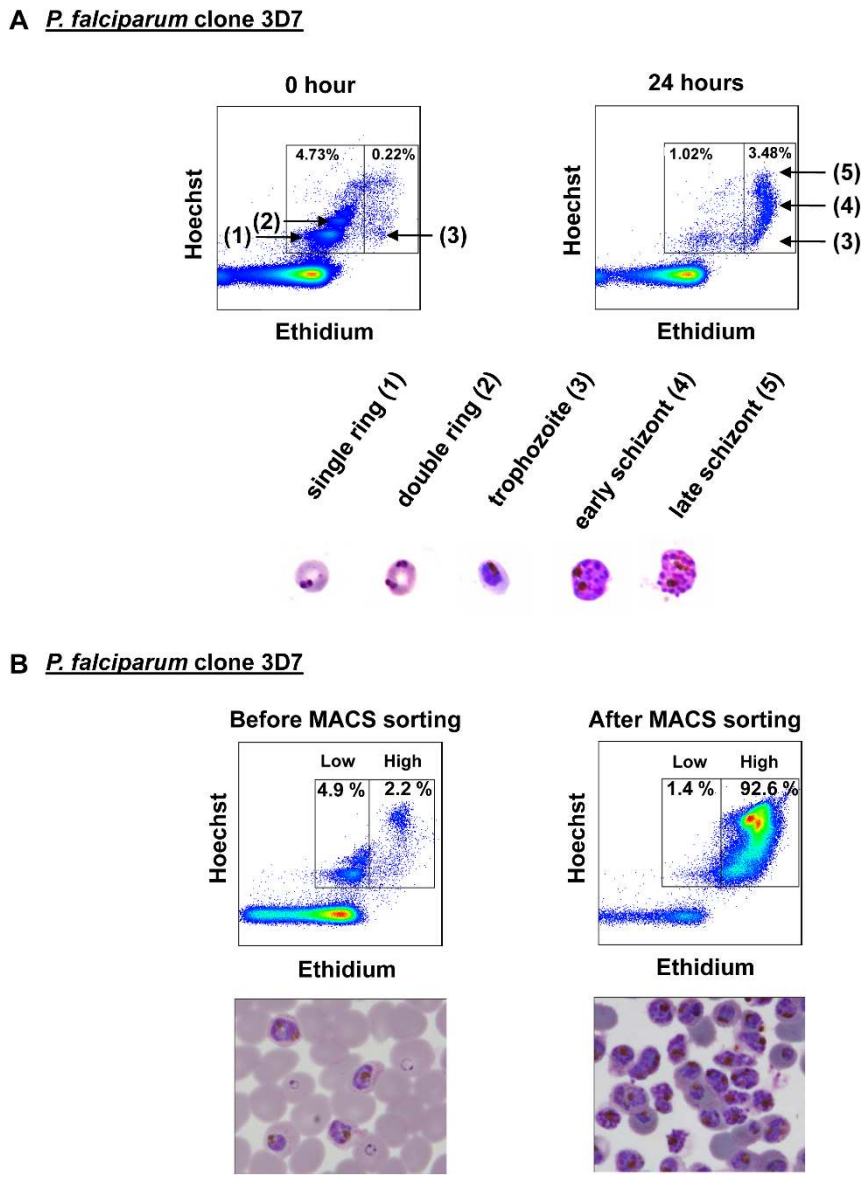

Figure 4 | Monitoring of *P. falciparum* parasitemia grown *in vitro* by the TCM. (A) Ethidium/Hoechst dot plot of parasites obtained from a synchronized culture of *P. falciparum* (3D7) after excluding debris and doublets. Cryopreserved parasites were thawed and stained by the TCM at two different times after thawing. The indicated cell populations were sorted by flow cytometry and stained with Giemsa; they corresponded to different parasite stages: (1) single ring, (2) double ring, (3) trophozoite, (4) early schizont and (5) late schizont. (B) Ethidium/Hoechst dot plot of parasites from an asynchronous culture of *P. falciparum* 3D7 before (left panel) and after (right panel) magnetic sorting using a MACS column. Predominantly, the stages containing the pigment (hemozoin) are in the Ethidium^{high} gate as shown in representative Giemsa-stained smears (right panel).

(Figure 6B). The blood of patient #3 contained almost exclusively ring forms while that of patient #3 and #5 contained predominantly mature stages. For each sample, the parasitemia determined by the TCM matched microscopy results to within 0.15% (data not shown). These results demonstrate that TCM can be applied successfully to assess parasitemia of human malaria parasites from whole blood.

Drug assay using TCM. The ability of the TCM not only to rapidly measure parasitemia but also to resolve rings and late stages suggests that it could be harnessed for the crucial application of performing drug assays to evaluate drug resistance and efficacy. As a proof of concept, an inhibition of maturation assay was performed using two different drugs as described in material and methods. Different doses of artesunate (AS) and chloroquine (CQ) were added to a synchronised culture of *P. falciparum* (3D7 cloned line) containing 95% of rings; 44 h later, the cultures were analyzed by the TCM (Figure 7A). Control cultures contained 81.91% ± 3.03% and 84.84 ± 1.19% of mature forms respectively. In cultures treated with the highest doses of the drugs tested, the percentage of inhibition for AS (19 ng/mL) and CQ (512 ng/mL) were 94.12% ± 2.79% and 89.81 ± 1.99% respectively. A dose response for CQ after 44 h of culture was calculated (Figure 7B). The geometric mean IC50 for CQ on *P. falciparum* 3D7 was 10 ng/mL and was similar to values obtained with flow cytometry methods using thiazole orange staining [24], by microscopy [25] or [^{3}H]-hypoxanthine incorporation [26].

An alternative method for 2 laser cytometers. We foresee that the principal limitation to widespread adoption of the TCM is that it requires the use of a UV laser for Hoechst detection. At present, this laser is only available in large, sophisticated instruments that are not suited for deployment in the field. We thus sought to adapt the TCM for 2 laser cytometers (with 488 nm and 633 nm lasers) that are now available in a portable format (Accuri Cytometers, USA). We replaced Hoechst dye with SYBR Green I, allowing detection with

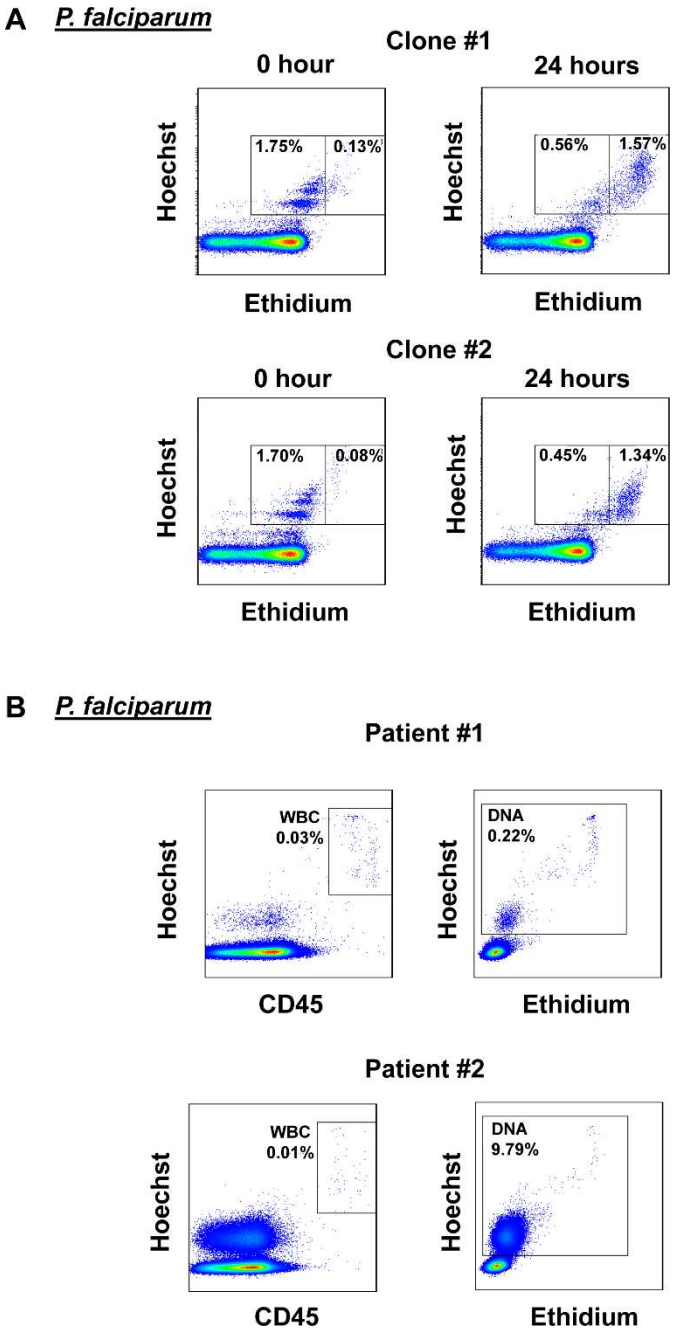

Figure 5 | Determination of parasitemia in *P. falciparum* field isolates samples. Cryopreserved parasites were thawed and stained by the TCM at two different times after thawing. (A) Ethidium/Hoechst dot plot of parasites from synchronized culture of two clones derived from a *P. falciparum* field isolate before and after 24 hours of culture. (B) Dot plots representing CD45/Hoechst and Ethidium/Hoechst staining of cells in whole blood from two patients from Thailand infected with *P. falciparum*. Note that the blue laser voltage was lower in this particular experiment.

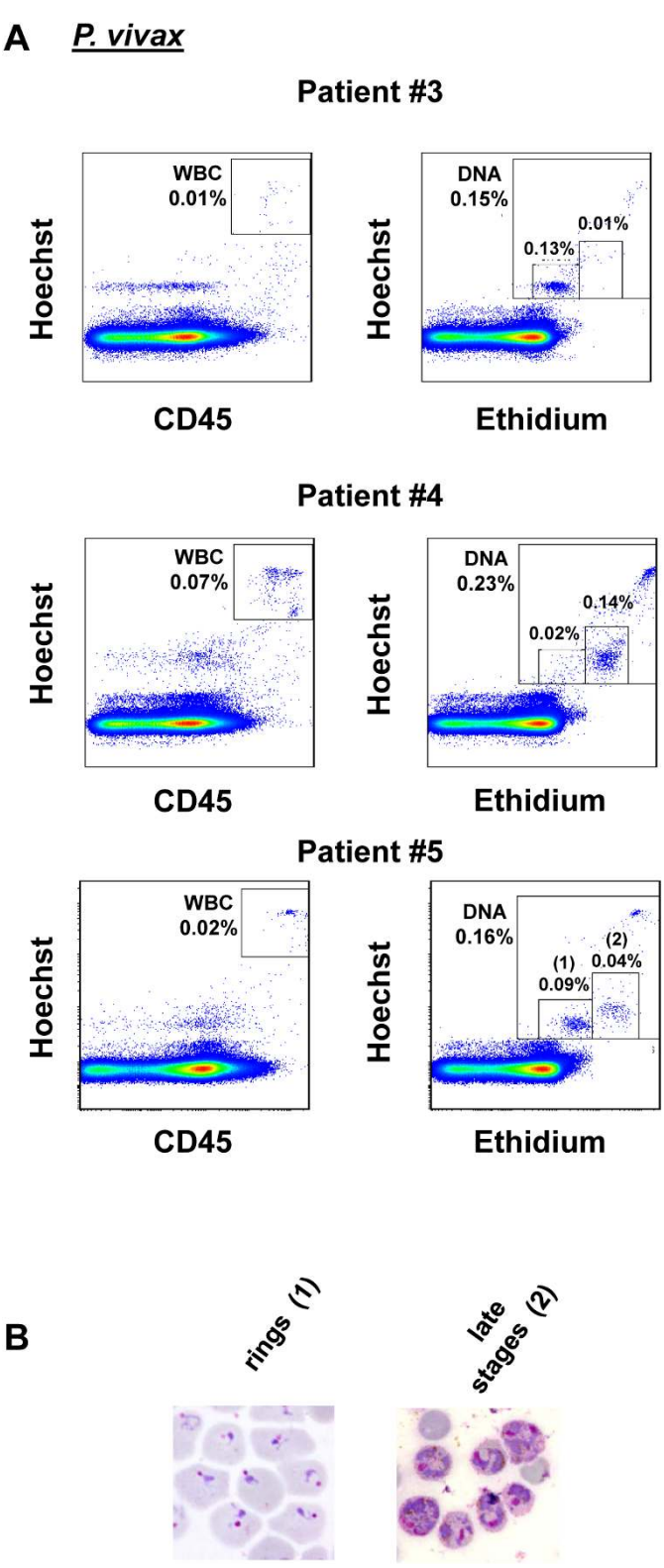

Figure 6 | Determination of parasitemia in *P. vivax* field isolates samples. Cryopreserved whole blood from three different patients from Thailand were thawed and stained by the TCM after thawing. (A) Dot plots representing CD45/Hoechst and Ethidium/Hoechst staining of cells in whole blood from patients from Thailand infected with *P. vivax*. (B) Representative images of Giemsa-stained blood smears of the indicated parasite populations sorted by flow cytometry; they corresponded to rings (1) and late stages (2) i.e. trophozoites and schizonts.

a 488 nm laser. We validated this new protocol using whole blood from a *P. berghei* ANKA-infected mouse (7 days post-infection). The parasitemia values calculated by the two methods were comparable: 5.02% with SYBR Green (Figure 8A) and 5.37% with Hoechst (Figure 8B). A more extensive comparison between the dyes was performed using 10 mice, and again the calculated parasitemia were comparable (Figure S4). These data show that SYBR Green I can substitute Hoechst staining with the limitation that the parasite staging is less well defined.

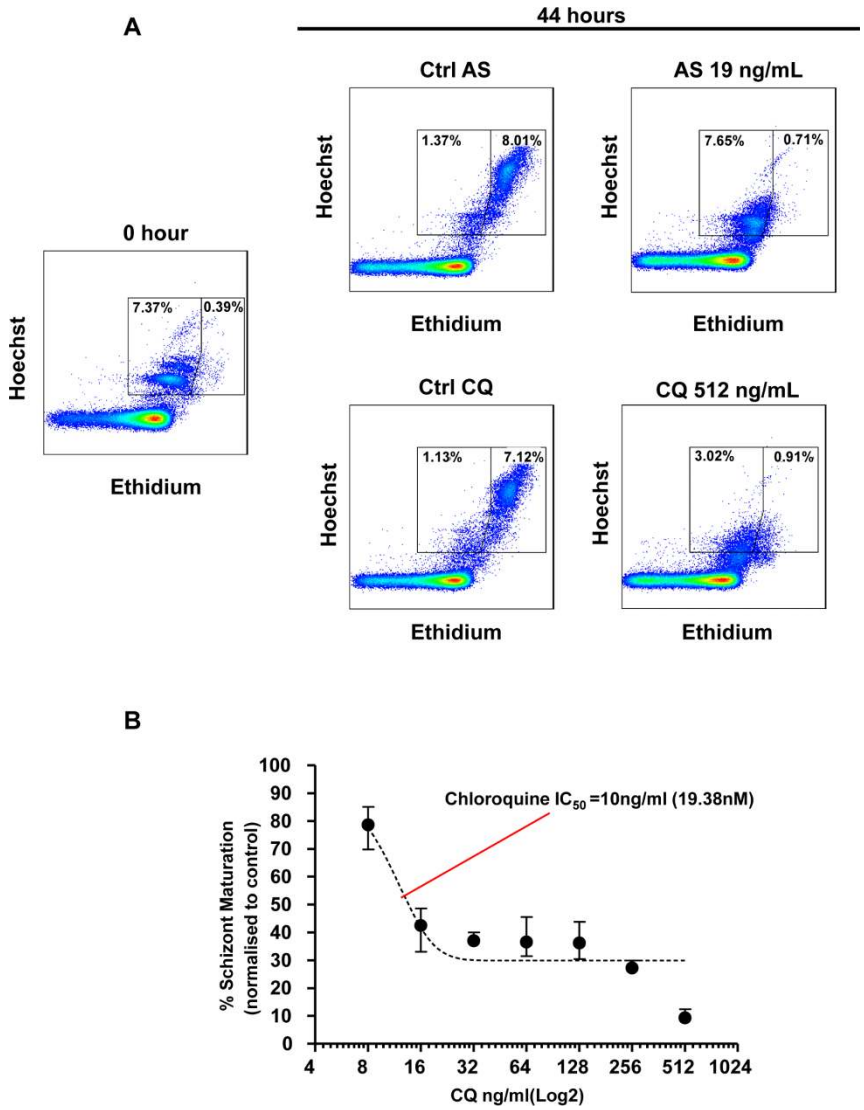

Figure 7 | Inhibition assay of *P. falciparum* schizont maturation after chloroquine or artesunate treatment. (A) Ethidium/Hoechst dot plots of control and drug-treated synchronized *P. falciparum* clone 3D7 cultures before and after 44 hours of treatment with high dose artesunate (19 ng/ml) or chloroquine (512 ng/ml). (B) Dose response curve of *P. falciparum* 3D7 after 44 hours of chloroquine treatment, error bars represent the error on triplicate cultures.

Discussion

Here, we have described a tri-color flow cytometry assay, designed for the accurate and fast quantification and staging of human and rodent malaria parasites. The technique can be performed with whole blood for *ex-vivo* measurement or with *in vitro* cultures of malaria parasites. It uses a small volume of blood (1 μL) and is performed in a single step at room temperature, without washing or fixation, and is compatible with high-throughput platforms. The time required to complete staining and flow cytometry acquisition does not exceed half an hour. The lower limit of parasite detection allowing accurate counting for the assay is a parasitemia of 0.02%, making TCM less sensitive than techniques such as real-time PCR but more sensitive than Giemsa-stained blood smear (accurate counting when parasitemia $\geq$ 0.05%). As such, it is not suited for diagnosis because it cannot be used for the detection of very low parasitemias; additionally we recommend the inclusion of an uninfected blood control for samples close to this threshold. However for monitoring parasitemia above 0.02%, the TCM is both faster than non-flow cytometry methods and more accurate than other flow cytometry methods due to the use of CD45 to exclude nucleated erythroblasts and leukocytes. The simplicity of the method, the rapidity of sample processing and data acquisition and its relative low reagent cost compares favorably with microscopy (Supplementary Table 2).

Moreover, the low inter-operator variability (once cytometer settings have been fixed) is such that we routinely train undergraduate students to perform the assays with minimal supervision.

In addition, TCM was adapted for use with portable two laser flow cytometers and allowed parasitemia determination in rodent malaria samples (Figure 8) but also more recently in *P. falciparum* and *P. vivax* samples processed in field conditions (data not shown). However, it has to be emphasized that the resolution of the different parasite stages with SYBR Green was not as sharp as that obtained with the Hoeschst dye.

Previous flow cytometry methods were designed for monitoring rodent parasite development *in vivo* or human parasite growth *in vitro* [27, 15, 28, 17]. None of these techniques were able to measure parasitemia either *ex-vivo* or *in vivo* for all malaria parasites, making the TCM the first such generally applicable assay.

Previous single or double-color methods were not sensitive enough to discriminate between different parasite maturation stages [12, 29–31, 13, 9, 32] or from WBC or erythroblasts containing

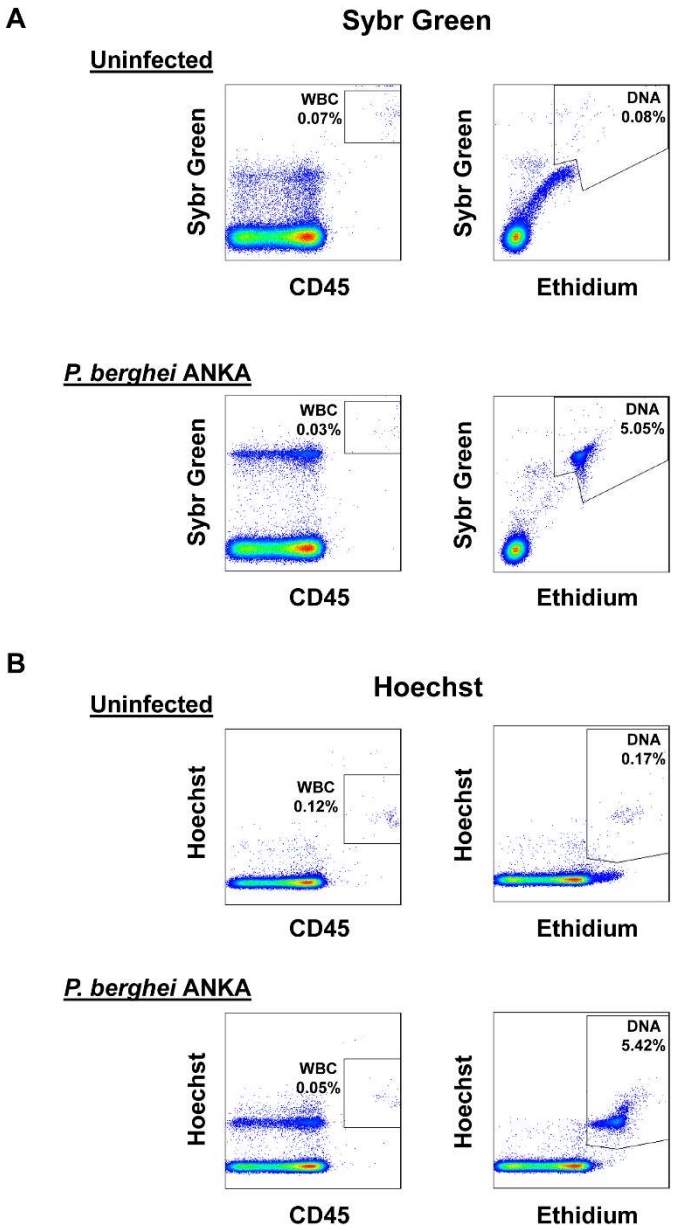

Figure 8 | Detection of *P. berghei* ANKA parasitemia using SYBR Green and a 2 laser flow cytometer. The TCM method using SYBR Green (A) or Hoechst (B) was performed side by side with blood from an uninfected mouse or a mouse infected 7 days previously with *P. berghei* ANKA. The same flow cytometry gating strategy described in Figure 1 was used. Two parameters dot plots representing Ethidium/SYBR Green or Ethidium/Hoechst are compared. Gates containing WBC or cells containing DNA are indicated.

DNA or RNA. The present method allows uninfected normocytes and uninfected reticulocytes to be separated from IRBC and measures the percentage of WBC (Figures 1 and 2). In addition, the different parasite maturation stages can be resolved for *P. falciparum* and *P. vivax* (Figures 5 and 6).

Antimalarial drug discovery has been hampered by the absence of high throughput techniques able to distinguish the parasite maturation stages. So far, only one assay using SYBR Green alone on *P. falciparum* has been amenable to high-throughput screening [33]. In the present study, we applied the TCM to an *in vitro* maturation assay for assessing the effects of CQ and artesunate against *P. falciparum* (3D7). We confirmed that ring stage parasites failed to mature in the presence of CQ [34, 35]. Furthermore, the CQ IC_{50} value we measured was comparable with microscopy [25]. Importantly the precision of the TCM is not subject to inter and intra-reader variability or the fatigue factor commonly associated with microscopic examination of thick films.

In conclusion, the TCM is a fast, accurate and versatile technique for parasitemia determination. We are confident that this method will be further optimised for use in low cost 2 laser flow cytometers, thus facilitating malaria research in field laboratories.

Methods

Ethics Statement. All mouse experiments and procedures were conducted according to the rules of the Agri-Food and Veterinary Authority (AVA) and the National Advisory Committee for Laboratory Animal Research (NACLAR), under BRC Institutional Animal Care and Use Committee (IACUC) approval (IACUC number 080321). The clinical IRBC samples examined in this study were collected under the following ethical guidelines in the approved protocols; OXTREC 027-025 (University of Oxford, Centre for Clinical Vaccinology and Tropical Medicine, UK) and MUTM 2008-215 from Ethic committee of Faculty of Tropical Medicine, Mahidol University.

Mice. C57BL/6J and BALB/cJ (7–8 week old) were obtained from Biomedical Resource Centre (BRC, Biopolis, Singapore) and bred and kept under specific pathogen-free conditions.

Rodent parasites. A GFP-transfected clone derived from *P. berghei* ANKA clone 15cy1 [36] and the clone lined 1.1 of *P. yoelii* 17X (Py17X) [37] were used. Infected red blood cells (IRBC) stabilates used to initiate infections were free from other infectious agents and were prepared through *in vivo* passage in C57BL/6J mice and stored in liquid nitrogen (10^7 parasitized erythrocytes per ml in Alseveer's solution). All mice were infected intraperitoneally (i.p.) with 10^6 iRBC.

Human parasites. Clinical isolates of *P. vivax* and *P. falciparum* were collected from malaria patients receiving treatment from clinics run by the Shoklo Malaria Research Unit on the North Western border of Thailand. All patients were briefed on the project and provided informed consent prior to collection of blood by venipuncture. Five milliliters of whole blood was collected in lithium heparin collection tubes. This sample was either cryopreserved directly in Glycerolyte 57 (Baxter) or leukocyte depleted using CF11 cellulose columns [38] prior to cryopreservation.

P. falciparum (3D7 clone of the NF54 strain) samples were obtained from cultures performed using sealable flasks using RPMI-HEPES medium at pH 7.4 supplemented with hypoxanthine 50 μg/mL, $NaHCO_3$ 25 mM, gentamicin 2.5 μg/mL, and Albumax II (Gibco, Singapore) 0.5% wt/vol in an atmosphere containing 5% CO_2, as previously described [39].

The selection of the late developmental stages of *P. falciparum* containing the hemozoin pigment was performed using the MACS system (Miltenyi, Singapore). One to two milliliters of blood at 50% hematocrit in PBS were passed through the LD column as described elsewhere [40]. After washing twice with 2 ml of PBS, the late developmental stages were collected from the magnetically-retained fraction.

Determination of parasitemia by flow cytometry. One microliter of whole blood was added to a tube containing 100 μl of PBS. Dihydroethidium (Sigma, Singapore), Hoechst 33342 (Sigma) and anti-CD45 coupled to allophycocyanine (APC) were added together to the blood sample. In preliminary experiments, we determined that the optimal doses for dihydroethidium and Hoechst 33342 were 5 μg/ml and 8 μM respectively using *P. berghei*-infected red blood cells (Figure S1). We also determined that the staining was stable over a 24 hours period (Figure S2). Rat IgG2a anti-mouse CD45 (clone 30F11.1, Miltenyi) or mouse IgG2a anti-human CD45 (clone 5B1, Miltenyi) monoclonal antibodies were used at a 1:50 dilution. In one set of experiments, Hoechst was substituted by SYBR Green I (Sigma, Singapore) at 0.25x dilution.

The diluted whole blood samples were incubated for 20 minutes at room temperature in the dark. After the incubation, 400 μl of cold PBS was added. The samples were acquired on an LSR II flow cytometer (Becton Dickinson, Singapore) using the UV laser (305 nm) to detect Hoechst 33342, the blue laser (488 nm) for GFP and Ethidium, and the red laser (633nm) for APC. In experiments using SYBR Green, samples were acquired with the Accuri C6 flow cytometer (Accuri cytometers Inc., Ann Arbor, MI) or LSR II flow cytometer (Becton Dickinson, Singapore). For samples with parasitemia less than 1%, 500,000 events were recorded, otherwise 100,000 events were recorded. FlowJo (Tree Star) was used for all flow cytometry analyses. In experiments using blood from infected mice, a negative control sample from a non-infected mouse was tested each day in parallel to define the threshold of positivity for the parasitemia.

Parasite sorting by flow cytometry. Human and rodent parasites were sorted using the TCM staining protocol from 25 μL infected blood samples. Cell sorting was performed using a FACSAria II (Becton Dickinson).

Microscopy. Microscopic enumeration of IRBC was performed using thin blood smears stained with Giemsa. A minimum of 4,000 red blood cells were counted (20 fields at 100x magnification).

Drug assay. Chloroquine diphosphate (molecular weight [MW], 519.9; Sigma-Aldrich) and artesunate (AS) (base MW, 282.3 Holly Pharmaceuticals Co Ltd) were used. A stock solution of each drug was prepared in methanol in glass vials coated with a 0.2% (vol/vol) AquaSil water solution (Pierce) to prevent the drug from binding to the glass surface. One or two dilutions of each drug were subsequently made in methanol to obtain the drug at the desired testing concentration. Twenty microliters of the final drug solution were added to each well of a 96-well microtiter plate (Nunc, Singapore), and two-fold serial dilutions were made in methanol. The predosed plates were then dried in an incubator at 37°C overnight, further covered with Plate Sealer (Linbro), and stored at 4°C. *P. falciparum*-3D7-infected red blood cells synchronized to rings were prepared from cultures. Fifty microliters of uninfected-blood-medium mixture (2% hematocrit) was added to each well of the predosed drug plates. Plates were next incubated in an atmosphere containing 5% CO_2 at 37°C, until $\geq$ 50% of the ring stage parasites had matured to schizonts (24 to 36 h). The parasitemia for each well is quantified using TCM protocol as described above.

Statistical analysis. The nonparametric Spearman rank correlation test was used to investigate the relationship between the parasitemia determined by detection of GFP fluorescence and by the new technique. We also used the method of Bland-Altman for assessing agreement between two methods of clinical measurement [19]. The drug assay method used was based on a modified World Health Organization microtest [41] for determination of *P. falciparum* drug sensitivity. Dose-response data were analyzed by nonlinear regression analysis (Table Curve software, Jandel Scientific) to obtain the 50% inhibitory concentrations (IC50s) (geometric means). The experiment was performed in triplicate.

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Author Contributions

BM designed and performed research, analysed data and wrote the paper. CC, ASMO, RS performed research. KS, FN obtained ethics authorization, clinical management as well as preparation and collection of fresh isolates. SWH analysed the data and wrote the paper. BR, LR designed research, analysed data and wrote the paper.

Additional information

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