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

Benoît Mallerêt1, Carla Claser1, Alice Soh Meoy Ong1, Rossarin Suwanarusk1, Kanlaya Sriprawat2, Shanshan Wu Howland1, Bruce Russell1, François Nosten2,3 & Laurent Rénia1

1Laboratory of Malaria Immunobiology, Singapore Immunology Network (SIgN), Agency for Science, Technology and Research (A*STAR), Biopolis, Singapore, 2Mahidol–Oxford University Tropical Medicine Research Programme, Shoklo Malaria Research Unit, Mae Sot, Thailand, 3Centre for Vaccinology and Tropical Medicine, Churchill Hospital, Oxford, UK.

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.

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 24.

**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 parasitemia.

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**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<sup>low</sup> Ethidium<sup>low</sup> (1) or Hoechst<sup>high</sup> Ethidium<sup>high</sup> (2) populations, smeared on glass slides and stained with Giemsa. Rings and trophozoites were found in the Hoechst<sup>low</sup>Ethidium<sup>low</sup> population whereas multi-rings, schizonts and gametocytes were found in the Hoechst<sup>high</sup>Ethidium<sup>high</sup> 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; R² = 0.999) (Figure 3A). Bland–Altman analysis<sup>19</sup> 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/6. The profile of parasitemia development was similar to profiles previously described and determined by microscopy<sup>20</sup>. As shown in

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**Figure 2 | Flow cytometry gating for P. berghei ANKA parasitemia determination.** The TCM method was performed with blood from (A) an uninfected C57BL/6 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) a mix of multi-rings, schizonts and gametocytes as shown in representative Giemsa-stained smears obtained after sorting by flow cytometry.
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 increase 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 of 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, 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).

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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 artemisinin (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, by microscopy or [3H]-hypoxanthine incorporation.

**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...
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 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.
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 \textit{ex-vivo} measurement or with \textit{in vitro} cultures of malaria parasites. It uses a small volume of blood (1 $\mu$L) 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 \textit{P. falciparum} and \textit{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 \textit{in vivo} or human parasite growth \textit{in vitro}\textsuperscript{27, 15, 28, 17}. None of these techniques were able to measure parasitemia either \textit{ex-vivo} or \textit{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\textsuperscript{12, 29–31, 15, 9, 32} or from WBC or erythroblasts containing...
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 34. 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 IC₅₀ value we measured was comparable with microscopy 35. 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/c (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. yoelli 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 (10⁷ parasitized erythrocytes per ml in Alsever’s solution). All mice were infected intraperitoneally (i.p.) with 10⁷ 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 Glycerol 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 sealed flasks using RPMI-HEPES medium at pH 7.4 supplemented with hypoxanthine 50 µg/mL, NaHCO₃ 25 mM, gentamicin 2.5 µg/mL, and Albumax II (Gibco, Singapore) 0.5% wt/vol in an atmosphere containing 5% CO₂, 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 whole blood was passed through the LD column as described elsewhere. 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 5B1, Miltenyi) or mouse IgG2a anti-human CD45 (clone 30F11.1, 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).
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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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