



Unambiguous determination of *Plasmodium vivax* reticulocyte invasion by flow cytometry



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ABSTRACT

The invasion of CD71+ reticulocytes by *Plasmodium vivax* is a crucial yet poorly characterised event. The application of flow cytometry to ex vivo invasion assays promises to facilitate the quantitative analysis of *P. vivax* reticulocyte invasion. However, current protocols suffer from a low level of sensitivity due to the absence of a particular design for *P. vivax* cell tropism. Importantly, merozoite invasion into contaminating red blood cells from the schizont inoculum (auto-invasion) may confound the analysis. Here we present a stable two-color flow cytometry assay for the accurate quantification of *P. vivax* merozoite invasion into intracellularly labelled CD71+ reticulocytes. Various enzymatic treatments, antibodies and invasion inhibitory molecules were used to successfully demonstrate the utility of this method. Fluorescent labelling of red blood cells did not affect the invasion and early intra-erythrocytic development of *P. vivax*. Importantly, this portable field assay allows for the economic usage of limited biological material (parasites and reticulocytes) and the intracellular labeling of the target cells reduces the need for highly purified schizont inoculums. This assay will facilitate the study of *P. vivax* merozoite biology and the testing of vaccine candidates against vivax malaria.

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1. Introduction

Plasmodium vivax is the most widespread, difficult to diagnose and treat cause of malaria. There are 70–130 million cases annually, mainly in Asia and the Americas, with 2.5 billion people currently at risk (Mendis et al., 2001; Gething et al., 2012). The continual emergence of drug-resistant vivax malaria (Rijken et al., 2011; Price et al., 2014) emphasizes the need for an effective vaccine against *P. vivax*. Currently much of the vivax malaria vaccine development efforts have focused on the intra-erythrocytic stage of the parasite (Vicentin et al., 2014; de Cassan et al., 2015; Moreno and Joyner, 2015).

However, the preclinical screening of vaccine candidates against *P. vivax* merozoite invasion of erythrocytes has been hampered by the absence of a successful continuous in vitro culture protocol. Much of the difficulty in developing a culture method for *P. vivax* is due to this species' highly restricted tropism for CD71+ reticulocytes (an early and short-lived stage of red blood cell (RBC) development) (Kitchen, 1938; Mons et al., 1988; Malleret et al., 2015). Therefore research into *P. vivax* invasion has relied largely on ex vivo (Russell et al., 2012) or in vivo studies of malaria-infected patients or non-human primates (Miller et al., 1976, 1978; Nichols et al., 1987; Barnwell et al., 1989). In both cases, fresh isolates are difficult to obtain, have low parasitemias (below 0.5%) and mixed stages which further complicate the precise investigation needed to study RBC invasion. Consequently, the preclinical development and testing of blood stage vaccines against *P. vivax* have focused on biochemical assays (Adams et al., 1992; Chitnis and Miller, 1994; VanBuskirk et al., 2004;

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Grimberg et al., 2007; Sampath et al., 2013) or in silico models (Batchelor et al., 2011) which do not provide final proof of the vaccine candidate effectiveness in blocking RBC invasion.

In 2011, Russell and colleagues established and validated a microscopy-based assay using concentrated reticulocytes (target cells) from cord blood and enriched mature parasites ex vivo from patient isolates (Russell et al., 2011). While this methodology allowed the initial investigation of different interventions, there were important limitations: (i) the concentrated parasite inoculum from a donor's blood contains contaminating RBCs, including reticulocytes (i.e. it is not possible to produce a 100% pure schizont preparation) and (ii) during parasite enrichment, schizonts can burst and release merozoites which are then free to invade the contaminating endogenous reticulocytes before the target cells are added. Microscopic observation will not differentiate between parasites that have invaded contaminating reticulocytes (autologous invasion) and those that have invaded target reticulocytes, thus confounding the precise measure of inhibitor efficacy against the merozoite invasion. Furthermore, microscopy is laborious, requires special training and lacks objectivity due to inter-reader variability.

Recently, a study promoted the idea of flow cytometry for observing *P. vivax* development with better accuracy, faster quantification and larger total cell counts (Malleret et al., 2011). This protocol was adapted for a drug susceptibility assays using a portable flow cytometer suitable for field laboratories (Russell et al., 2013). The application of flow cytometry and magnetic concentration of schizonts (Ribaut et al., 2008) in a clinical setting in Thailand helped to specify the tropism of *P. vivax* merozoites for CD71+ reticulocytes (Malleret et al., 2013, 2015). Although the flow cytometry method improved the objectivity of *P. vivax* invasion assays, it still suffers from the confounding background of autologous invasion.

In order to examine the invasion biology of *P. vivax* merozoites and the efficacy of vaccine candidates/therapies, we require an assay that can unambiguously detect the invasion of target cells with a high degree of sensitivity and specificity. Here we introduce a novel flow cytometry-based invasion inhibition assay adapted from the assay developed by Theron et al. (2010). This assay can distinguish newly invaded parasites from other endogenous confounding factors and reduces the need for highly purified parasite inoculums by using an intracellular dye (carboxyfluorescein succinimidyl ester (CFSE)). In this study we adapted the method to study *P. vivax* in field laboratories and enabled fast, efficient and unambiguous investigation of *P. vivax* merozoite invasion.

2. Materials and methods

2.1. Ethics statement

The clinical infected RBC (iRBC) and umbilical cord blood samples used in this study were collected under the following ethical guidelines and approved protocols; OXTREC 027-025 (University of Oxford, Centre for Clinical Vaccinology and Tropical Medicine, UK) and MUTM 2008-215 from the Ethics committee of Faculty of Tropical Medicine, Mahidol University, Thailand. The use of human samples was approved by the National University of Singapore Institutional Review Board (NUS-IRB reference no. 09-256), and written informed consent was obtained from all participants.

2.2. *Plasmodium vivax* mature schizont enrichment

Clinical isolates of *P. vivax* were collected from malaria patients receiving treatment from clinics operated 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 mL of whole blood were collected in lithium heparin tubes. These samples were processed for leukocyte-depletion using powdered cellulose (Sigma–Aldrich, Singapore) columns, then were either cultured directly or cryopreserved directly in Glycerolyte 57 (Baxter, USA) (Sriprawat et al., 2009). Parasites were thawed as previously described (Blomqvist, 2008) and were cultured to the schizont stage in 10 mL of McCoy 5A medium supplemented with 2.4 g/L of D-glucose, and 20% heat-inactivated human serum (matching blood type of donor), in a gas mixture of 5% CO₂, 5% O₂ and 90% N₂ at 37.5 °C.

The *P. vivax* peripheral blood samples received at the clinic were at different intra-erythrocytic stages with low parasitemias (Fig. 1D). Consequently, a maturation step was required to maximise the yield of mature schizonts (Fig. 1E, F). At 40 h post-cultivation, selection of the late developmental stages of *P. vivax* containing hemozoin pigment was performed using the magnetic-activated cell sorting (MACS) system (Miltenyi, Singapore). Approximately 1 mL of blood at 50% hematocrit in McCoy 5A medium was passed through a “large depletion” (LD) column as described previously (Ribaut et al., 2008). After washing twice with 2 mL of McCoy 5A medium, the developmental stages were collected from the magnetically-retained fraction. The mature intra-erythrocytic parasites were then diluted in McCoy 5A medium 1:2 and 0.2 µL added to make 0.1 µL of packed iRBCs per well.

2.3. Enrichment and intracellular staining of reticulocytes

Twenty mL of uninfected cord blood were collected in lithium heparin tubes from umbilical cords immediately after the delivery of the child for all samples. The blood group was determined using a TransClone ABO antisera kit (Bio-Rad, USA). After plasma removal, the packed RBCs were washed in McCoy 5A medium. Host white blood cells and platelets were depleted from the RBCs using two rounds of powdered cellulose (Sigma–Aldrich) column filtration (Sriprawat et al., 2009).

CD71+ reticulocyte enrichment was done using the MACS system (Miltenyi). Approximately 2 mL of blood at 50% haematocrit (Hct) in PBS were mixed with CD71 MicroBeads (Miltenyi) and passed through a large selection (LS) column to obtain a CD71 depleted (negative) fraction and a CD71-rich fraction (positive). A LS column favors purity over quantity when sorting for the cell of interest (positive fraction); in other words, some CD71+ reticulocytes can be lost in selection (negative fraction). The purity levels of these fractions were verified by flow cytometry using nucleic acid stain (Thiazole Orange, Sigma–Aldrich) and were corroborated with the new methylene blue stain (Sigma–Aldrich) (Fig. 1A–C).

The use of erythrocyte labelling with CFSE in *Plasmodium* studies has been reported (Koka et al., 2008; Theron et al., 2010; Gallo et al., 2012). Here we stained CD71+ reticulocytes with CFSE at 30 µM final concentration with 1% Hct in PBS for 30 min at room temperature, followed by 5 min incubation with 1 mL of warm FBS to stop the labelling reaction.

2.4. Invasion inhibition assay

The assay was set up in a 96-well flat-bottomed plate and each well contained either no inhibitor (positive control) or different inhibitors. In each well, 100 µL of complete McCoy 5A medium and 0.8 µL of CFSE labelled RBCs (~4 million erythrocytes) were added to bring the final target cell Hct to 0.8%. To this, 0.1 µL of magnetic positive fraction was added and, where required, candidate antibodies or mimetics were added before the complete set up of the assay. Maturation was obtained after incubation in an

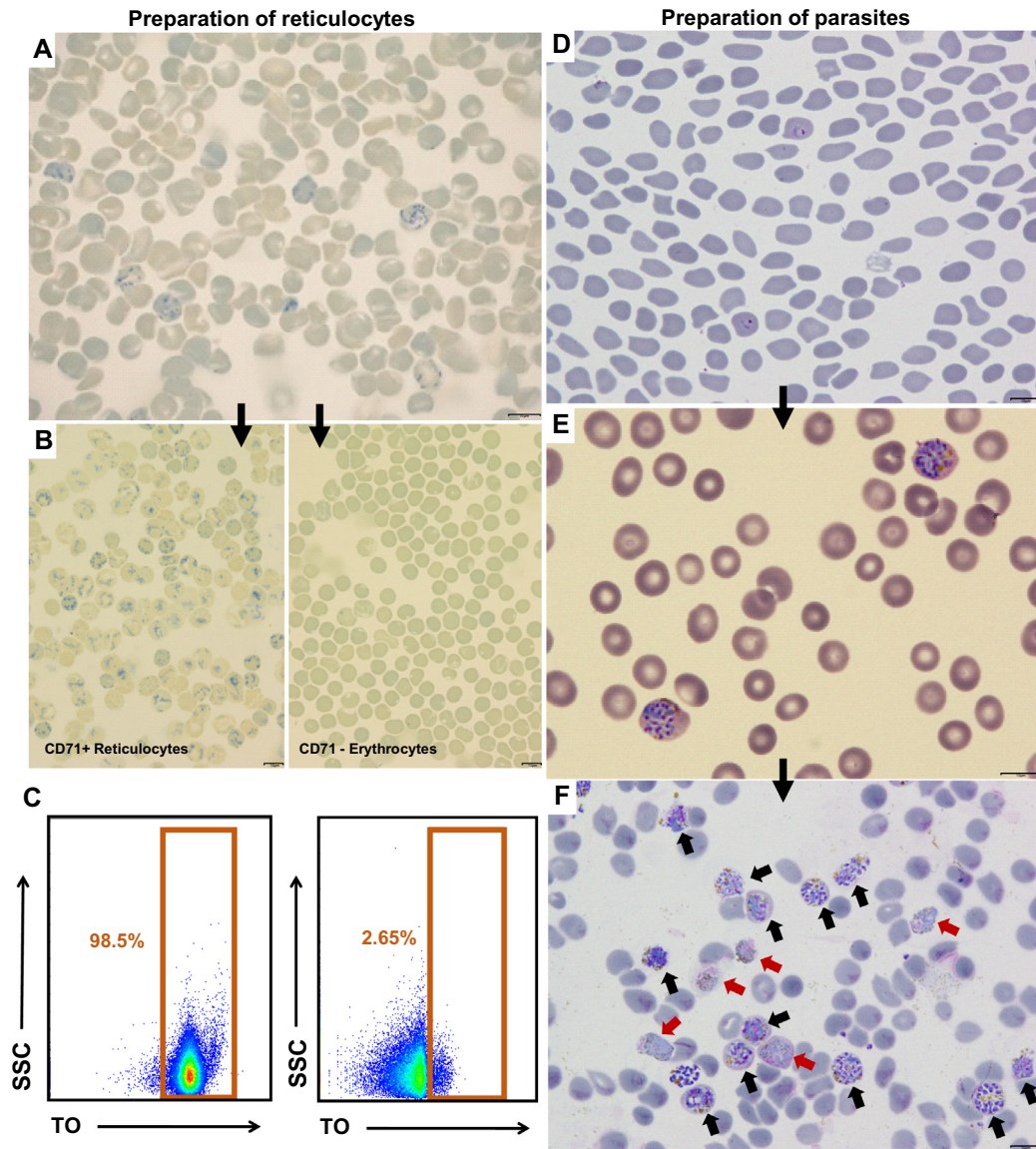


Fig. 1. Preparation procedure for target cells and *Plasmodium vivax* parasites. (A, B) New methylene blue stained reticulocytes from cord blood before and after immunomagnetic selection. (C) Flow cytometry of CD71 positive and negative fractions labelled with Thiazole orange after immunomagnetic sorting (Thiazole orange signal for X-axis and side scatter signal, SSC for Y-axis). (D, E) Giemsa stain of *P. vivax* from infected patients maturing from early intra-erythrocytic phase to late stage schizonts. (F) After magnetic sorting enrichment, two late stage parasites can be seen: schizonts (black arrows) and gametocytes (red arrows). Scale bars = 10 μ m.

atmosphere of 5% O₂ at 37.5 °C for an average of 24 h, subsequently assessed by flow cytometry and microscopy. The murine anti-Fy6 (2C3) murine antibody (1 mg/mL) (Wasniowska et al., 2002), the anti-Fy6 single domain antibody (SdAb) (1 mg/mL) from a camelid VHH library (Smolarek et al., 2010) and the small molecule inhibitor against Merozoite Surface Protein (MSP)-1 (NIC: 2-butyl-5-chloro-3-(4-nitro-benzyl)-3H-imidazole-4-carbaldehyde, 50 mM) were obtained as previously described (Wasniowska et al., 2002; Smolarek et al., 2010; Chandramohanadas et al., 2014). The rabbit azide-free monoclonal antibody (mAb) anti-FyB antibody (0.31 mg/mL) was commercially purchased (Abcam, Singapore). Antibodies were tested for inhibitory potential by using anti-Fy6 (2C3 epitope on DARC, Duffy antigen/chemokine receptor) antibody as a positive control. Both murine and camelid anti-Fy6 antibodies were used at 20 μ g/mL, NIC at 25 μ M and anti-FyB antibody at 18.6 μ g/mL, respectively, in the final assay volume of 100 μ L.

Invasion inhibition efficiency was calculated based on the positive control (CD71+ reticulocytes only) after 24 h: (Inhibition

efficiency = 100 - ((CFSE + Rings % with tested condition)/(CFSE + Rings % with positive control) \times 100)).

2.5. Microscopy

After 24 h, thin film smears were made for all samples and stained with Giemsa (Sigma–Aldrich) for examination by light microscopy. All fluorescent microscopy was done live with samples in humidifying chambers, using Hoechst 33342 (Sigma–Aldrich) to stain the nuclear material. Cells were imaged using a 100 \times oil immersion objective (Olympus IX73).

2.6. Determination of parasitemia by flow cytometry

At 0 h and 24 h of the assay, 20 μ L of culture from each well were mixed with 80 μ L of PBS, then stained with 1 μ L of Hoechst 33342 (Sigma–Aldrich) in a small curved bottom tube (Micronic, Netherlands). After 20 min incubation at room temperature and

protected from direct light, 300 μL of PBS were added to stop the staining reaction for immediate flow cytometry analysis (optimal signal during the first 2 h post-staining). The absence of fixation and/or permeabilisation steps reduces the risk of RBC agglutination that can affect flow cytometry acquisition and analysis. At each time point, the samples were acquired on an Accuri C6 (Becton Dickinson, Singapore) using the near UV laser (427 nm) to detect Hoechst 33342 and the blue laser for FITC (530 nm). The gating strategy was based on methods by Malleret et al. (2011) and analysed using FlowJo (Tree Star, USA). When determining invasion efficiency, we applied the same gating strategy to a CFSE+ target cell-only control (no schizonts added) which was observed for any non-specific background events. These events were used to set a baseline for measuring 'true' parasite invasion efficiency into CFSE+ target cells (Fig. 4D).

2.7. Enzyme treatment

Erythrocytes were pre-treated with either trypsin (1 mg/mL), chymotrypsin (1 mg/mL) or neuraminidase (100 mU/mL) for 1 h at 37 °C. The enzyme treatment was stopped by washing three times with warm PBS.

2.8. DNA extraction

Before any procedures commenced, dried blood spots of cord blood and parasite-infected patient samples were made on Whatman filter paper and stored under sterile conditions. Alternatively, 200 μL of cultured parasites or human donor blood samples were stored at -20 °C until DNA extraction using a QIAamp DNA mini kit (Qiagen, Santa Clarita, CA, USA).

2.9. PCR analysis and sequencing studies

Two PCRs were done for this project and the first PCR was done to determine if blood donors were Duffy negative. Prior to the PCR, phenotyping of Duffy negative donors was carried out on a flow cytometer as previously described (Malleret et al., 2013).

Primers Fynull F & Fynull R (Supplementary Fig. S2A) were designed to detect the C/T single nucleotide polymorphism (SNP) inside the GATA box motif (Tournamille et al., 1995). The second PCR was done to amplify a 586 bp product which includes the MSP1-19 region, which was then sequenced to detect SNPs in the predicted NIC binding site.

Each PCR contained 2.5–7 ng of DNA, 50 pmol of each primer, 2 nmol dNTPs, 1.0 U Phusion HF polymerase and buffer (Thermo Scientific, Singapore) in a total volume of 25 μL . Primer sequences are listed in Supplementary Fig. S2A.

The same PCR cycle conditions were used for both PCRs and the amplification profile used was 98 °C for 5 min followed by 30 cycles of denaturation at 98 °C for 15 s; annealing at 63 °C for 15 s; elongation at 72 °C for 30 s; followed by a final 10 min incubation at 72 °C. Amplified products were subjected to electrophoresis in 1% agarose gel in 0.5% Tris-Borate-EDTA (TBE) to verify the product quality, before PCR purification using a Qiagen kit and then sent for sequencing in the forward direction on an automated sequencer (AITBiotech, Singapore).

2.10. Statistical analysis

Parametric comparisons of invasion/ inhibition efficiencies were paired to be tested by either a Paired t test or an one-way ANOVA with Tukey post hoc test. All statistical analyses used Prism 5 for Windows (Version 5.01), Mackiev software. $P < 0.05$ was considered significant.

3. Results

3.1. Human reticulocyte enrichment

Concentration of immature RBCs from cord blood (Fig. 1A) is a non-invasive and efficient process. In the past, repeated powdered cellulose filtration was necessary to deplete leukocytes (Russell et al., 2011), however the introduction of non-woven fabric filter now allows for faster processing of blood (Tao et al., 2011). After leukocyte depletion and CD71 magnetic sorting, a mean of 88.58% CD71+ reticulocytes were obtained (95% confidence interval (CI): 84.91–92.25%) (Fig. 1B). The CFSE-labelled erythrocyte population was clearly distinguished from non-labelled erythrocytes under microscopy and flow cytometry (Fig. 2A, B).

3.2. Plasmodium vivax maturation and enrichment

The mean volume of the *P. vivax* late stage parasites (LSPs) after maturation and concentration from a 500 μL fresh isolate sample was 1.03 μL (95% CI: 0.802 μL –1.27 μL). The mean purity of LSPs was 36.3% (95% CI: 24.28–48.33%). Cryopreserved parasites also had similar volumes (1.35 μL , 95% CI: 0.959 μL –1.74 μL) and LSP concentrations (30.89%, 95% CI: 19.36–42.41%) compared with fresh isolates. It is important to note that the magnetically concentrated LSPs comprised of schizonts and gametocytes (Fig. 1F). The proportion of gametocytes in the LSP concentrate varied considerably between *P. vivax* isolates (a range of 0–48% of LSPs were gametocytes).

3.3. Ex vivo invasion assay

After preparing target reticulocytes and LSPs, those were incubated together for 24–26 h. Flow cytometry acquisition at 0 h and 24 h was done using the laser configuration described in Section 2.6, with additional microscopy observation at both of these time points. Therefore, to a standard volume of 100 μL of McCoy medium, 0.1 μL of packed iRBCs and 0.8 μL of CFSE-labelled target reticulocytes were added (Fig. 2A). However, due to the variable purity of schizonts after magnetic sorting (MACS), the ratio between the target cells and parasite inoculum (1:8) was challenging to maintain. Nevertheless, there was no significant association between the proportion of parasite population added at 0 h and CFSE+ parasitemia at 24 h (Pearson's correlation coefficient; $R^2 = 0.1121$, $P = 0.4176$).

At 0 h, a flow cytometry dot plot of CFSE/Hoechst shows three defined populations and events were gated accordingly: LSPs, contaminating erythrocytes and the target CFSE+ reticulocytes (Fig. 2B). After 24 h, two additional populations appeared; early stage parasites that either invaded the contaminating erythrocytes (CFSE negative) or target reticulocytes (CFSE positive) (Fig. 2B). CFSE labelling did not affect RBC morphology nor parasite invasion. Successful experiments were defined by positive controls of each experiment which showed a clear cluster of CFSE+ iRBC populations higher than 0.75% at 24 h. For microscopic observations, only rings and early trophozoites were counted. Dead rings and unhealthy early stages were also counted as they could be indications of new invasion. Overall, the mean parasitemia from flow cytometry acquisition at 24 h was 3.95% (95% CI: 1.23–6.63%) and the mean parasitemia from microscopic examination was 3.63% (95% CI: 0.09–7.18%). Flow cytometry measurement was comparable to that of microscopy (paired t test; $P = 0.736$), the gold standard method for parasitemia observation, consistent with previous publications (Malleret et al., 2011; Russell et al., 2013).

We demonstrated that *P. vivax* invasion is dependent on the proportion of CD71+ reticulocyte in the target cell population by

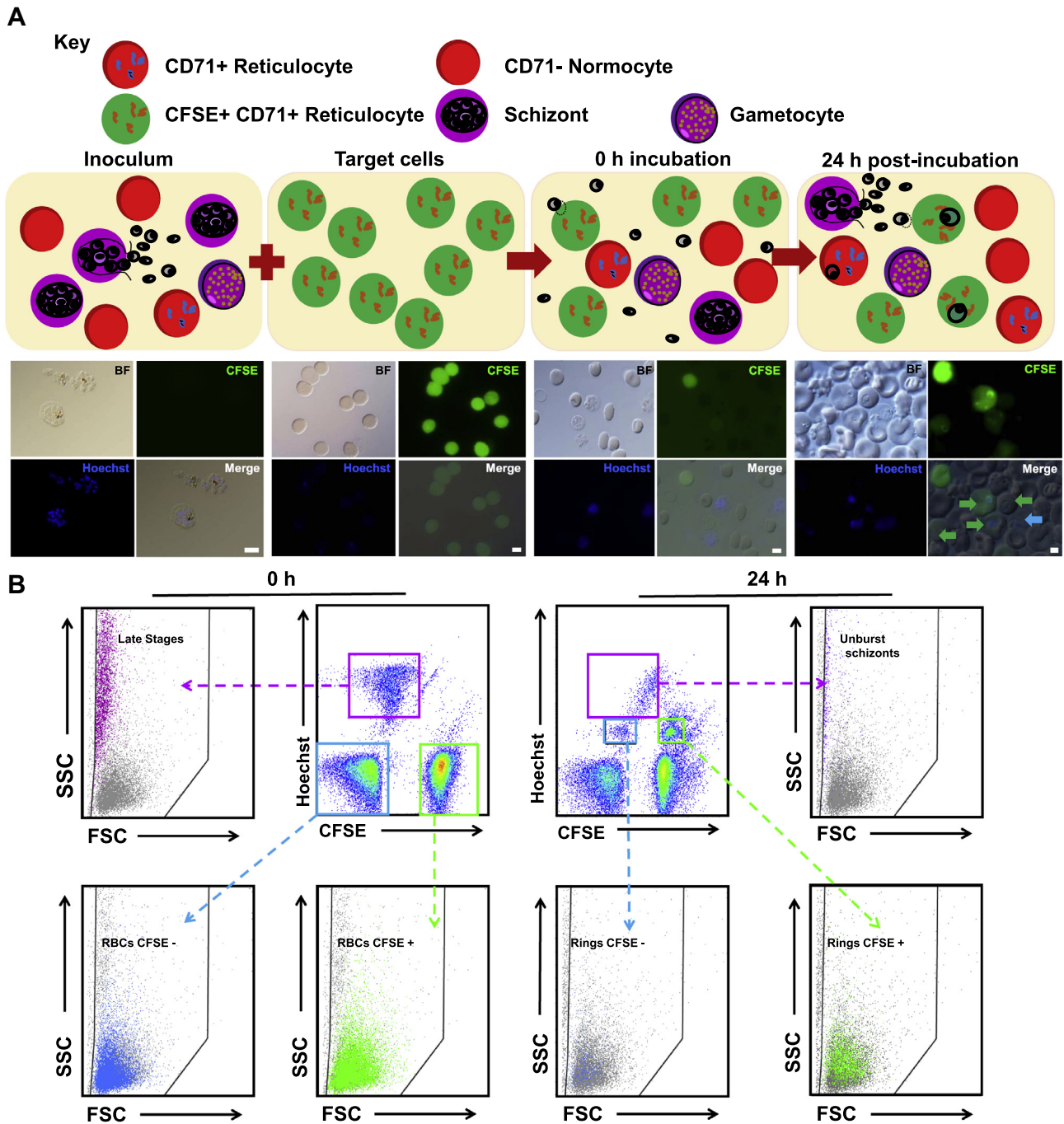


Fig. 2. Schematic outline of the invasion assay using a diagram, micrographs and flow cytometry plots. (A) Diagram and corresponding micrographs showing different cell populations present when parasite inoculum and target cells are added at 0 h and cultured for 24 h. Parasite DNA is visualised using Hoechst stain. Target cells stained with carboxyfluorescein succinimidyl ester are in fluorescent green. Green arrows indicate CFSE+ target cells that have been invaded by *Plasmodium vivax* at 24 h whereas the blue arrow shows *P. vivax* invasion into a contaminating endogenous CFSE- red blood cell (RBC) population. Scale bar = 5 μ m. (B) Flow cytometry gating strategy of the *P. vivax* invasion assay, Hoechst/CFSE dot plot at 0 h and 24 h with forward scatter/side scatter (FSC/SSC) back-gating for each cell population at 0 h: RBCs CFSE- (blue gate), RBCs CFSE+ (green gate), parasite late stages (purple gate) and at 24 h: rings CFSE- (blue gate), rings CFSE+ (green gate) and unburst schizonts (purple gate).

an additional LD column step of erythrocytes from the LS column negative fraction. The mean percentage of CD71+ reticulocytes in the negative fraction was 5.50% (95% CI: 1.49–9.50%) and after the LD column step the mean value decreased further to 1.87% (95% CI: -6.39–10.13%). As expected, we saw a positive correlation between invasion efficiency and the CD71+ reticulocyte percentage. The mean value of invasion efficiency was 36.95% \pm 8.66 for

the negative fraction from LS column which was significantly higher than the control (anti-Fy6) (one-way ANOVA; $P < 0.01$) and 17.05% \pm 5.11 from the negative fraction of the LD column (Supplementary Fig. S1).

Mean parasitemia for targeted cells at 24 h was 3.60% (95% CI: 1.15–6.04%) and for contaminant RBCs was 0.56% (95% CI: 0.18–0.94%) (Fig. 4A). Significant differences in parasitemias between

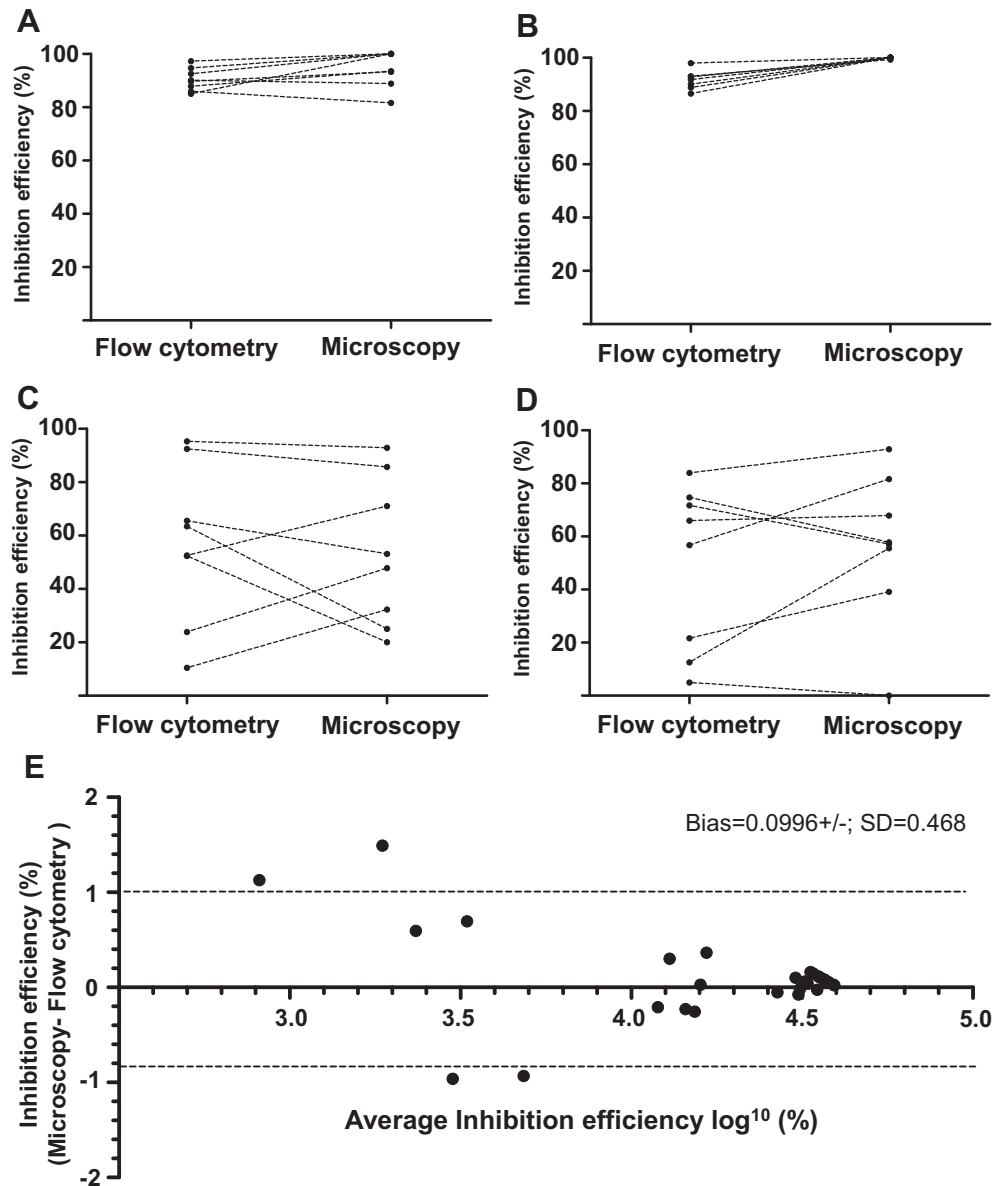


Fig. 3. Agreement between microscopy and flow cytometry in determining inhibition efficiency. (A–D) Inhibition efficiency (%) of merozoites from enriched *Plasmodium vivax* schizonts in the presence of different inhibitors. (A) Murine anti-Fy6 (2C3) antibody ($n = 8$ isolates). (B) Camelid anti-Fy6 antibody ($n = 7$ isolates). (C) Anti-FyB antibody ($n = 8$ isolates). (D) NIC (2-butyl-5-chloro-3-(4-nitro-benzyl)-3H-imidazole-4-carbaldehyde) ($n = 8$ isolates). (E) Bland-Altman comparisons of inhibition efficiencies for all tested *P. vivax* inhibitor candidates ($n = 31$ isolates) were determined by microscopy and flow cytometry. The upper and lower 95% limits of agreement are denoted by the dotted lines.

the two erythrocyte populations indicated that the contaminant reticulocytes are not preferentially invaded when the target cell-parasite inoculum ratio is followed (paired t test; $P = 0.014$).

3.4. Invasion inhibition assays

Murine anti-Fy6 (2C3) antibody exhibited a high reduction in parasite invasion in a previous study (Russell et al., 2011), thus it was used as a control inhibitor throughout this study (mean 87.65%; $SD \pm 9.17$). Camelid anti-Fy6 (2C3) antibody showed similar inhibition efficiency (mean 91.56%; $SD \pm 3.65$) (Fig. 4B) whereas the inhibition efficiency of anti-FyB antibody (mean 53.06%; $SD \pm 30.14$) and NIC (mean 48.59%; $SD \pm 29.11$) was significantly lower (one-way ANOVA, $P < 0.05$ (anti-FyB), $P < 0.01$ (NIC)) (Fig. 4B).

Inhibition efficiency of conditions for microscopy and flow cytometry were paired and displayed for each tested inhibitor

(Fig. 3A–D). These matched results were combined and analyzed to test the reliability of flow cytometry compared with the microscopy method. Bland-Altman analysis indicated good agreement between different intervention methodologies, with a slight bias towards higher inhibition efficiency with the microscopy method (0.0996 \log_{10} units) (Fig. 3E).

3.5. Other applications

Trypsin, Chymotrypsin and Neuraminidase are three enzymes commonly used to examine the invasion of erythrocytes by parasites (Barnwell et al., 1989). As expected, chymotrypsin treatment abrogated invasion (mean 83.00%; $SD \pm 15.05$) whereas trypsinised cells showed partial disruption to parasite invasion (mean 59.11%; $SD \pm 26.50$) (Fig. 4C). Both treatments showed no significant difference in invasion inhibition efficiency compared with the control. Neuraminidase treatment of RBCs often resulted in increased RBC

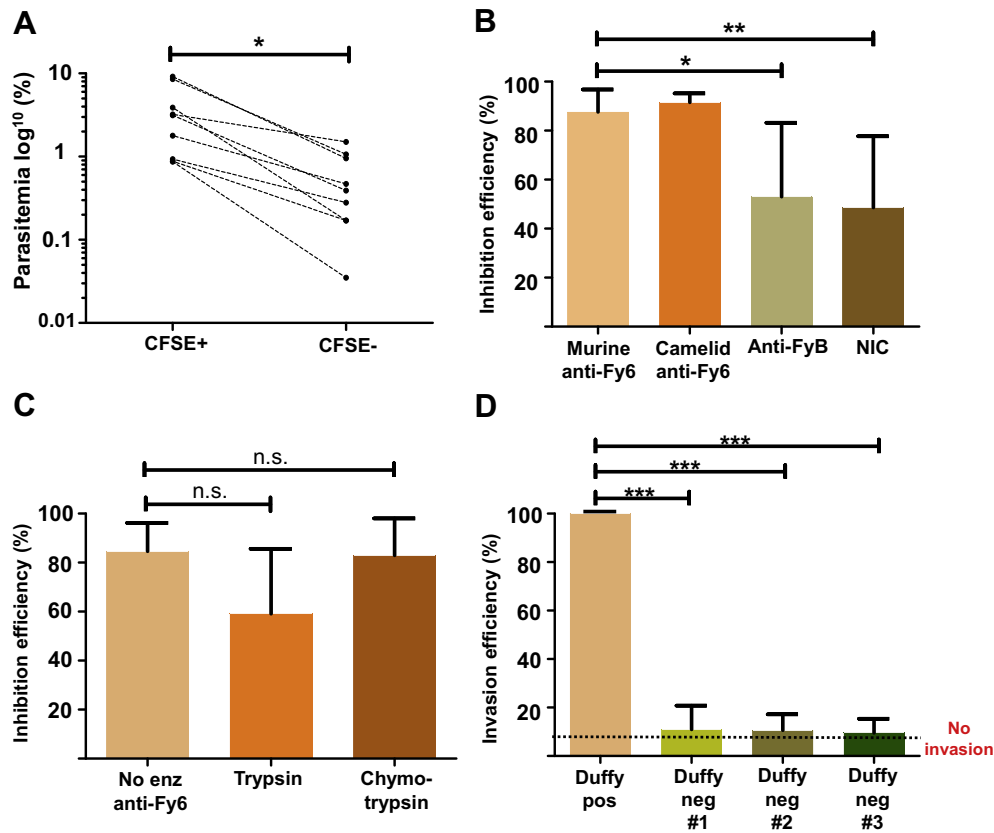


Fig. 4. Application of invasion assay. (A) Comparison of parasitemia between carboxyfluorescein succinimidyl ester (CFSE)+ target red blood cells and CFSE– contaminant RBCs at 24 h. Two dots joined by line represent one experiment ($n = 9$ isolates). (B) The mean inhibition efficiency of different antibodies and sugar mimetic against either DARC epitopes (anti-Fy6 or anti-FyB antibodies) or parasite protein (NIC) are shown with respective S.D.s.: murine anti-Fy6 (2c3) (mean 87.65%; S.D. \pm 9.17), camelid anti-Fy6 (2C3) (mean 91.56%; S.D. \pm 3.65), anti-FyB antibody (mean 53.06%; S.D. \pm 30.14) and NIC (mean 48.59%; S.D. \pm 29.11), ($n = 9$ isolates for murine anti-Fy6 antibody and NIC; $n = 7$ isolates for camelid anti-Fy6 antibody). (C) Inhibition efficiency of trypsinised (mean 59.11%; S.D. \pm 26.50) and chymotrypsinised-erythrocytes (mean 83.00%; S.D. \pm 15.05) using murine anti-Fy6 antibody (20 μ g/mL; mean 84.59%; S.D. \pm 11.59) as a control ($n = 5$ isolates for all). (D) The mean invasion efficiency of Duffy negative reticulocytes; Donor #1 (mean 10.97%; S.D. \pm 9.80), Donor #2 (mean 10.66%; S.D. \pm 6.57), Donor #3 (mean 9.52%; S.D. \pm 5.90), ($n = 3$ isolates for Donor 1; $n = 4$ isolates for Duffy positive control and Duffy negative Donors 2 and 3) Dotted line reflects CFSE+ Hoechst+ events acquired by a flow cytometer at 24 h in a CFSE+ target red blood cells only control well. Asterisks indicate statistical significance determined with a one-way ANOVA with Tukey post hoc test ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s. not significant).

fragility and thus made it difficult to determine the true effect of the enzyme.

Plasmodium vivax did not invade reticulocytes from three Duffy negative African donors (Fig. 4D). The invasion efficiency into each reticulocyte sample was: Donor #1 (mean 10.97%; S.D. \pm 9.80), Donor #2 (mean 10.66%; S.D. \pm 6.57), Donor #3 (mean 9.52%; S.D. \pm 5.90). They were all significantly lower than Duffy positive reticulocytes (one-way ANOVA, $P < 0.001$).

4. Discussion

Accurate analysis of *P. vivax* invasion assays is essential for the assessment of neutralising antibodies and thus discovery and pre-clinical development of new vaccine targets and chemical inhibitors of invasion. The use of CFSE labeling of target cells in combination with flow cytometry allows for the unambiguous and quantitative determination of *P. vivax* invasion. CFSE labelling not only differentiates between target and autologous invasions; it also excludes other confounding events such as slow growing rings or dead parasites from the 0 h parasite inoculum (Fig. 2B). Moreover, the CFSE processing of target reticulocytes does not appear to affect the receptiveness of the labeled reticulocytes compared with the health of parasite CFSE– contaminating RBCs (Fig. 4A). These results are in line with past studies showing that CFSE

labelling does not affect *Plasmodium falciparum* merozoite invasion of normocytes (Theron et al., 2010).

To demonstrate the utility of this assay, we used a range of antibodies and inhibitors known to abrogate *P. vivax* merozoite invasion (Fig. 4B). All invasion inhibition efficiencies were calculated using the parasitemia from the treated well versus the intervention-free positive control. Importantly, the data from different experiments was not normalised using the respective LSP population at 0 h for two reasons. First, the LSP population includes gametocytes and late trophozoites in addition to schizonts that do not egress within the incubation time. The presence of gametocytes in peripheral parasitemia is more frequent in *P. vivax* infection than *P. falciparum* (McKenzie et al., 2006). Under flow cytometry, it is difficult to differentiate gametocytes from schizonts, and factors that drive differentiation into gametocytes are still unknown. Second, there is no significant association between the proportion of LSP that was added at 0 h and that of newly iRBCs at 24 h. A positive control for each isolate will elucidate the approximate proportion of merozoites which successfully invaded RBCs. Thus, CFSE+ parasitemia from the positive control can be used to normalise the results from the same isolates to compare the effects of different interventions or conditions.

Although the emergence of Duffy antigen/chemokine receptor (DARC)-independent *P. vivax* isolates have been reported (Menard et al., 2010, 2013), merozoite invasion of *P. vivax* strains from Thailand into erythrocytes is DARC-dependent (Russell

Table 1
Comparison of different methodologies to study parasite development and invasion. The Inhibition Assay includes antibody or therapeutic inhibitors. Tropism studies refer to observation of various *Plasmodium* spp. cell tropism and effects of mutants or red blood cells with different phenotypes on *Plasmodium vivax* invasion.

Methodology	Advantage	Disadvantage	Inhibition assay	Tropism studies
Microscopy	Morphology observation Qualitative and quantitative evidence Low cost	Time consuming Special training required Inter-reader variability Donor and target cell populations are not distinguished	✓ (With limitation)	X
Flow Cytometry (FC)	Objective Fast More cells counted with less samples Reduced inter-operator variability	Morphology cannot be observed Debris can be counted as events Dead or slow-growing parasites cannot be excluded Donor and target cell populations are not distinguished	✓ (With limitation)	X
FC with target cell labelling	Objective Fast More cells counted with less samples Reduced inter-operator variability Donor and target cell populations differentiated Accurate quantification of parasite invasion into target cell Optimised for portable flow cytometer (usable in field settings)	Morphology cannot be observed Debris can be counted as events May be not be suitable for some invasion inhibitory compounds with fluorescent properties	✓	✓

X, non-quantifiable.

et al., 2011). Therefore the use of murine and camelid monoclonal antibodies against the 2C3 epitope on DARC (anti-Fy6) and anti-FyB monoclonal antibody against a Duffy antigen could be used to validate the assay.

The inhibition efficiency of anti-FyB antibodies varied within different reticulocyte samples. This variation may be associated with the Fy phenotype of cord blood donors which needs to be assessed. The FyB allele frequency in the south-eastern Asia region is reported to be below 10% (Howes et al., 2011), thus we did not expect anti-FyB antibody to inhibit *P. vivax* invasion. However, although inhibition efficiency compared with anti-Fy6 antibody was significantly less, mean inhibition efficiency of anti-FyB was still over 50% (Fig. 4B). The possible reason for this is that FyB (Asp) differs by one amino acid residue from FyA (Gly) and this may cause partial binding of antibody which may affect parasite invasion.

To demonstrate a wider utility of the assay we used NIC, a small molecule shown to inhibit merozoite invasion through its interaction with MSP-1 (Chandramohanadas et al., 2014). NIC-mediated invasion inhibition was highly variable depending on the parasite isolate (Fig. 4B). However, DNA sequencing results showed complete conservation at the putative binding region of NIC on all *P. vivax* MSP-1 genes of parasite isolates (Supplementary Fig. S2C) (Chandramohanadas et al., 2014). This suggests that MSP-1 involvement in invasion may be redundant in some *P. vivax* isolates, however this clearly deserves further investigation.

While this assay is particularly useful in observing antibodies or inhibitors that have a specific target(s) in the merozoite invasion process, it may not have a greater advantage for parasitocidal or growth-inhibitory drugs compared with microscopy. In fact, some inhibitory compounds may have fluorescent properties that could interfere with the sensitivity of the new assay (Table 1). On the other hand, target cell labelling allowed investigations using RBCs with various phenotypes (from either genetic deficiencies or modifications). For example, we confirmed the in vivo study that shows the DARC-dependence of *P. vivax* (Miller et al., 1978) under ex vivo conditions by using Duffy negative RBCs (Supplementary Fig. S2A, B) from three African donors instead of introducing different inhibitors (Fig. 4D). Similarly, our assay can be utilised to study the effects of enzymatic treatment on RBCs (Fig. 4C) and the host cell tropism of different *Plasmodium* spp. by introducing various target cells with different phenotypes (data not shown).

The comparability between microscopy and flow cytometry in detecting parasitemia and drug sensitivity was demonstrated previously (Malleret et al., 2011; Russell et al., 2013). Here, we present the agreement between the two methods in determining inhibition efficiency, thus supporting flow cytometry as a sensitive means to verify potential inhibitors. In addition to the ability to distinguish debris, 50,000 events/cells were acquired by flow cytometry compared with 1000 cells counted using a microscope which indicates that the overall sensitivity and specificity (due to CFSE differentiation) is increased by this novel assay whilst using less parasites and reticulocytes (Table 1). Also, cytoplasmic labelling reduced the requirement for high purity and yield for LSP concentration (Fig. 2B) and negated the need for an extra trypsin treatment prior to parasite concentration with Percoll (isotonic) which may affect invasion (Carvalho et al., 2010; Russell et al., 2011).

Flow cytometry has lower inter-operator variability and can obtain the results quickly, which is desirable for high throughput screening. We hope that this field applicable flow cytometry invasion assay, based on the successful Theron et al. (2010) *P. falciparum* invasion assay, will help facilitate the investigation of *P. vivax* merozoite invasion biology and accelerate the screening of new vaccine candidates against vivax malaria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijpara.2015.08.003>.

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