Field based flow cytometry for \textit{ex vivo} characterization of \textit{Plasmodium vivax} and \textit{P. falciparum} antimalarial sensitivity

Running Title: \textit{Ex vivo} flow cytometry antimalarial sensitivity assay

Russell B\textsuperscript{1,2*}, Malleret B\textsuperscript{3*}, Suwanarusk R\textsuperscript{3*}, Anthony C\textsuperscript{2}, Kanlaya S\textsuperscript{4}, Lau YL\textsuperscript{2}, Woodrow CJ\textsuperscript{5,6}, Nosten F\textsuperscript{4,5,6}, Renia L\textsuperscript{3}

1. Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore (NUS), National University Health System (NUHS), Singapore
2. Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia
3. Laboratory of Malaria Immunobiology, Singapore Immunology Network, Biopolis, Agency for Science Technology and Research (A*STAR), Singapore.
4. Shoklo Malaria Research Unit (SMRU), Mae Sot, Tak Province, Thailand
5. Mahidol-Oxford-University Research Unit, Bangkok, Thailand,
6. Centre for Tropical Medicine, University of Oxford, Churchill Hospital, Oxford, United Kingdom.

* Equal contribution authors

#Correspondence to: Bruce Russell; Vivax Malaria Laboratory, Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, 5 Science Drive 2, Blk MD4, Level 5, Singapore 117545.
Email: micbmr@nus.edu.sg, Tel. +65 6516 3517, Fax. +65 6776 6872

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Abstract

Ex vivo antimalarial sensitivity testing in human malaria parasites has largely depended on microscopic determination of schizont maturation. While this microscopic method is sensitive, it suffers from poor precision and is laborious. The recent development of portable, low cost cytometers has allowed us to develop and validate a simple, field optimised protocol using SYBR Green and dihydroethidium for the accurate and objective determination of antimalarial drug sensitivity in freshly isolated \textit{Plasmodium vivax} and \textit{Plasmodium falciparum}

Key Words: \textit{Plasmodium vivax}, \textit{Plasmodium falciparum}, artesunate, chloroquine, flow cytometry, ex vivo susceptibility assay
Microscopic examination of ex vivo matured malaria parasites, remains the gold standard method used to determine the intrinsic sensitivity of fresh Plasmodium vivax and Plasmodium falciparum isolates to antimalarial drugs (2, 6, 20, 21, 24, 25, 26, 31). The modified WHO microtest assay is sensitive, relatively simple, inexpensive and continues to be applied to a range of studies (1, 4, 8, 9, 12, 22, 27, 29, 30), especially those seeking novel antimalarial therapeutics against drug resistant malaria (15, 17, 19, 23). However, the microscopic examination of Giemsa stained thick films central to this method is tedious, time-consuming and requires skilled microscopists. Moreover, large inter and intra-observer variations of parasite staging are frequently observed (25). Attempts to find an alternative ex vivo method suitable for both P. vivax and P. falciparum have been largely unsuccessful due to the high background of noise present in clinical isolates (caused by a number of factors including leukocytes, red blood cell autofluorescence, gametocytes and contaminating protein signatures in host plasma) compared with the low target signal of the maturing parasite (clinical isolates frequently have parasitemias <0.01%) (3, 5, 13). Perhaps the most objective and direct method to determine schizont maturation is through the use of flow cytometry (7, 10, 18, 32). However, the high expense and fragility of most flow cytometers significantly limits their use in field laboratories. Fortunately, the recent development of relatively cheap, portable 2 laser flow cytometers (such as the Accuri C6, Becton Dickinson) for the first time allows flow cytometric evaluation of ex vivo susceptibility assays in malaria endemic areas (16). Capitalising on this new capacity, we have developed a precise, accurate, fast and simple flow cytometry (FC) method to conduct ex vivo drug sensitivity assays of P. vivax and P. falciparum under field conditions using only 2 colours.

Forty eight isolates of P. vivax and 15 isolates of P. falciparum with parasitemias of between 0.05% and 0.5%, predominantly at the early ring stage (>80% of the total stages present) were collected from patients attending clinics at the Thai-Myanmar border (Collected under the following approved ethics protocol FMT-019-10 (Mahidol University, Faculty of
The isolates were transported to the SMRU field laboratory within 6 hours of collection and were depleted of WBCs by cellulose medium fiber (Sigma Catalogue No. C6288) filtration as previously described (28); and cultured in the presence of between 8-514 ng/ml of chloroquine diphosphate (CQ), or 0.3-19 ng/ml sodium artesunate (AS) using the protocol described in Russell et al. 2008 (24). At harvest (~42 hours post culture) the 200 µl of blood media mixture of each well was mixed and 20 µl from each well was dispensed into a small curved bottom tube (Micronic) and stained with 2 µl of Dihydroethidium (Sigma) and 5 µl of Sybr Green (made up with 63 µl of PBS) (Sigma) and incubated for 20 min at RT. During the staining time, thick films (3 µl packed RBCs) were made from each of the wells for Giemsa staining and microscopic examination. The fluorescent staining reaction was stopped after 20 min with the addition of 400 µl of PBS and was stored on an ice brick until FC analysis. The analysis of the FC was conducted using an Accuri C6 (Becton Dickinson), and the gating strategy as per the method by Malleret et al. 2011 (16). However, two special modifications were made to this protocol. Firstly only 60,000 events rather then 300,000 events were counted (reducing the count time per well from ~1.2 min to ~15 s). Secondly, no CD45 staining was necessary as >98% of the WBCs were removed from the isolates by cellulose. Slide counts for the microscopy were conducted as described by Russell et al. 2008 (24). The proportion of events in the target gate (for cytometry) or the mature schizonts (for microscopy) at each of the treatments were normalised to the drug free control. The proportion of schizont maturation at each corresponding drug concentration was then entered into the online ICEstimator (http://www.antimalarial-icestimator.net/MethodIntro.htm) and the IC_{50} calculated by non-linear regression analysis (11, 14).

After 42 hours of culture in the drug free controls, the schizont ‘target gate’ on the cytometer plot corresponding to the cluster of events with the highest levels of DNA (SYBR Green, y axis) and RNA (Dihydroethidium, x axis) can be clearly discerned for both species.
(Figure 1) with the number of events in the FC plot ‘target gate’ corresponding to the presence or absence of schizonts in the thick films (Figure 1.).

The culture success rate for both species was good, with 95.8% (46/50) and 86.7% (13/15) of *P. vivax* and *P. falciparum* samples respectively reaching at least 60% schizonts in the drug free control after at least 42 hours culture. Of the 46 successful *P. vivax* cultures we were unable to model the IC$_{50}$ data for one of the CQ assays.

The geometric mean IC$_{50}$ of *P. vivax* CQ and AS determined by microscopy and FC was 17.93 ng/ml (95%CI 16.2-19.84, n=45) versus 17.20 ng/ml (95%CI 15.52-19.07, n=46) and 0.57 ng/ml (95%CI 0.45-0.72, n=45) versus 0.66 ng/ml (95%CI 0.51-0.86, n=45) respectively (Figure 2C). For *P. falciparum* the geometric mean IC$_{50}$ of CQ and AS determined by microscopy and FC was 45.82 ng/ml (95%CI 24.22-84.2, n=13) versus 46.22 ng/ml (95%CI 24.22-88.2, n=13) and 3.47 ng/ml (95%CI 2.38-5.1, n=13) versus 3.97 ng/ml (95%CI 2.78-5.67, n=13) (Figure 2 A). Paired t-test analysis showed that the only comparison where there was a significant difference was the sensitivity of *P. vivax* to AS (Fig1C) (p<0.01).

Bland-Altman analysis indicated good agreement between the methodologies (independent of drug type used) (Figure2 B & D). There was a slight bias towards higher IC$_{50}$ values with the flow cytometry method for both *P. falciparum* (-0.03 log$_{10}$ units) and *P. vivax* (-0.025 log$_{10}$ units)

In summary, the antimalarial sensitivity data for the new FC assay matched those of the traditional microscopy very closely. In the one case where there was a significant difference between the IC$_{50}$ analysis of FC and Microscopy the actual mean difference in AS IC$_{50}$ for *P. vivax* was less than 0.1 ng/ml which is unlikely to be of biological significance. This 0.1 ng/ml disparity should also be put in the context of inter reader variability between microscopists, which in the experience of the authors can be an order of magnitude greater. It should also be noted that the time to acquire data from the FC method is only ~2 min per drug
(8 wells) compared to 18 min by microscopy. In conclusion, our data support the use of this simple FC protocol as a precise and more objective alternative to the microscopic determination of antimalarial drug sensitivity in fresh isolates of *P. vivax* and *P. falciparum*. Further studies involving a wider range of drugs are now planned.

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References


**Figure 1.** Representative flow cytometry plot outputs from chloroquine (CQ) and artesunate (AS) sensitivity assays conducted on (A) *P. falciparum* and (B) *P. vivax*. The target gate representing schizont development events is indicated on each of the cytometry plots.
Underneath each of cytometry plots we show the corresponding micrographs of Giemsa stained thick films collected from the same culture wells.

Figure 2. The *ex vivo* sensitivity of (A) *Plasmodium falciparum* and (C) *Plasmodium vivax* to chloroquine (CQ) (open circles) and artemisinin (AS) (solid circles) compared using microscopy and flow cytometry (Accuri C6). Solid horizontal lines and associated numerals indicate the geometric mean IC$_{50}$ ng/ml. A paired t-test shows that there was a significant difference (p<0.01) between the AS IC50 as calculated by microscopy and FC. Bland-Altman comparisons of IC$_{50}$ values for (B) *Plasmodium falciparum* and (D) *Plasmodium vivax* (AS and CQ combined) determined by microscopy and flow cytometry. The upper and lower 95% limits of agreement are denoted by the dotted lines.