Flow Cytometry-Based Analysis of Artemisinin-Resistant *Plasmodium falciparum* in the Ring-Stage Survival Assay

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The ring-stage survival assay (RSA) is a powerful tool for phenotyping artemisinin-resistant *Plasmodium falciparum* but requires experienced microscopists to count viable parasites among 10,000 erythrocytes in Giemsa-stained thin blood smears. Here we describe a rapid flow cytometric assay that accurately counts viable parasites among 250,000 erythrocytes in suspension. This method performs as well as light microscopy and can be used to standardize the collection of RSA data between research groups in laboratory and field settings.

Since the World Health Organization recommended artemisinin (ART)-based combination therapies (ACTs) for uncomplicated *Plasmodium falciparum* malaria in 2006 (1), 79 of 88 countries where *P. falciparum* malaria is endemic have adopted ACTs as first-line treatments (2). Increased availability of ACTs in these countries is considered a major factor in reducing malaria-attributable morbidity and mortality in recent years (2, 3). The efficacy of all ACTs is highly dependent on the potency of the short-acting ART component and the effectiveness of a long-acting partner drug (e.g., piperaquine, lumefantrine) in clearing any parasites that remain after ART exposure (4). Recent studies in Southeast Asia have defined clinical ART resistance as a slow parasite clearance rate (i.e., a long parasite clearance half-life) following ACT (5–8). One clinical impact of this slow-clearance phenotype is that greater numbers of parasites surviving ART treatment are exposed to partner drug monotherapy. Early efforts to monitor this threatening situation, in which emerging ART resistance promotes the development of partner drug resistance and thus ACT failure, were hampered by the lack of genotypic and phenotypic markers of ART resistance.

In exploring the genetic basis of ART resistance, Miotto et al. used whole-genome sequence data to define distinct *P. falciparum* subpopulations in Cambodia, one (KH1) that is ART sensitive and three (KH2, KH3, and KH4) that are ART resistant (9). These four subpopulations were subsequently found to associate with the wild-type, C580Y, R539T, and Y493H alleles, respectively, of the “K13-propeller” domain in a putative kelch protein (UniProt database accession number Q8IDQ2.PLAF7, encoded by PF3D7_1343700) (10). While K13-propeller polymorphism is a useful marker for surveying the spread of ART resistance in the field, the 0- to 3-h ring-stage survival assay (RSA0-3 h) is a useful tool for investigating the mechanism of ART resistance in the laboratory (11). In this assay, parasites are synchronized at the 0- to 3-h ring stage of development, exposed to 700 nM dihydroartemisinin (DHA) for 6 h *in vitro*, and then cultured for 66 h; the percent survival of these parasites at 72 h is calculated relative to...
dimethyl sulfoxide (DMSO)-exposed parasites (11). Survival of ART-sensitive parasites is typically <1%, while survival of ART-resistant parasites ranges from 1 to 100% (unpublished). Calculation of percent survival values requires two experienced microscopists to manually count the number of viable parasites among 10,000 erythrocytes in Giemsa-stained thin blood smears and a third microscopist to reconcile results that differ by more than 20%.

To reduce the inherent variability of microscopy data, we developed a two-color flow cytometric method to accurately quantify viable parasites in the RSA0-3 h (see the detailed protocol at http://www.wwarn.org/toolkit/procedures/ring-stage-survival-assays-rsa-evaluate-susceptibility-p-falciparum). This method (RSA-2FACS, where FACS is fluorescence-activated cell sorting) uses SYBR green I (SYBR) to indiscriminately stain DNA in both viable and pyknotic parasites and MitoTracker deep red FM (MTDR) to selectively stain mitochondria in viable parasites. Since MTDR staining relies on the unique membrane polarity of functional mitochondria, this reagent labels viable but not pyknotic parasites. We selected MTDR because this far-red dye does not emit fluorescence in the FL1 channel, where SYBR is detected. MTDR clearly distinguishes populations of pyknotic and viable parasites (Fig. 1A and B). Some previously reported flow cytometric methods have relied on a single DNA dye (e.g., SYTO 61) to distinguish viable from pyknotic parasites in other ART susceptibility assays (12, 13). While the simplicity of this single-laser, single-channel approach is attractive, we found that the indiscriminate staining of DNA in both viable and pyknotic parasites (Fig. 1C) made it impossible to accurately identify viable parasites using SYBR alone. More recently, the combination of SYBR and MTDR was used to quantify viable parasites in erythrocytes following exposure to chloroquine and azithromycin in conventional drug assays (14).

To compare the performance of RSA-2FACS with that of microscopy, we subjected four Cambodian P. falciparum isolates (5) which differ in KH subpopulation assignment (9) and K13-propeller allele (10) to the RSA0-3 h in three independent experiments. In each experiment, we simultaneously prepared Giemsa-stained thin blood smears from DHA- and DMSO-exposed cultures and processed samples as technical triplicates for flow cytometry at 72 h as follows. We washed 20 μl of packed cells three times with 500 μl of Hanks balanced salt solution (HBSS) plus 2% fetal bovine serum (FBS). To 2.5 μl of packed cells, we added 50 μl of HBSS plus 2% FBS containing 0.2 μM SYBR (Invitrogen, USA) and 0.3 μM MTDR (Invitrogen). After incubating the cells for 30 min at 37°C in the dark, we washed the samples three times with HBSS plus 2% FBS before analyzing them on an Accuri C6 flow cytometer (Becton Dickinson, USA) equipped with 488-nm and 640-nm lasers. For each sample, 250,000 ungated events were collected and

FIG 2 Flow cytometry scatterplots of DHA (A)- and DMSO (B)-exposed parasites. In each plot, the percentage of viable parasites in 250,000 events is shown in the upper right quadrant. (C) In a mode-normalized histogram overlay of these upper quadrants, populations of viable and pyknotic parasites are clearly separated in both samples. Percent survival was calculated by multiplying the ratio of viable parasites in DHA- and DMSO-exposed samples (e.g., 0.11%/2.17% multiplied by 100). (D) Percent survival values for four Cambodian parasite isolates that differ in KH subpopulation (KH1, KH2, KH3, and KH4) and K13-propeller allele (wild type, C580Y, R539T, and Y493H) are shown. For each isolate, percent survival values calculated from microscopy or flow cytometry data were not significantly different. Data from three independent experiments per isolate are shown.
processed in FlowJo v10 (Tree Star, Inc., USA) using debris and doublet exclusion gates.

Figure 2A and B show representative scatterplots for DHA- and DMSO-exposed samples. After quadrant gates were applied based on distinct histogram peaks, viable and pyknotic parasites were clearly detected in the upper right and upper left quadrants, respectively. These data can also be visualized in a mode-normalized histogram overlay (Fig. 2C) of these upper quadrants. Viable parasite survival in the RSA0-3 h. Percent survival was calculated by multiplying the ratio of viable parasites in the DHA- and DMSO-exposed samples by 100. Figure 2D shows that percent survival values calculated from flow cytometry or microscopy data do not differ significantly in the <1% to 30% range (although the numbers of viable parasites estimated by flow cytometry tend to be slightly higher than those estimated by microscopy). These data indicate that the RSA-2FACS accurately quantifies viable parasites and thus reliably detects ART-resistant parasites in the RSA0-3 h. With the growing availability of portable, durable, and inexpensive two-laser flow cytometers, automation of the RSA-2FACS should facilitate more-comprehensive assessments of P. falciparum resistance to ART and perhaps other antimalarial drugs in both field and laboratory settings.

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We declare that we have no conflicts of interest.

REFERENCES