A Simple Monochromatic Flow Cytometric Assay for Assessment of Intraerythrocytic Development of Plasmodium falciparum

Kasem Kulkeaw (kasem.kuk@mahidol.edu)
Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University
https://orcid.org/0000-0002-6652-9244
Nutpakal Ketprasit
Chulalongkorn University
Anchalee Tungtrongchitr
Mahidol University
Duangdao Palasuwan
Chulalongkorn University

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Abstract

Background Gold standard microscopic examination of P. falciparum intraerythrocytic stage remains an important process for staging and enumerating parasitized erythrocytes in culture; however, microscopy is laborious, and its accuracy is dependent upon the skill of the examiner.

Methods In this study, we used ViSafe Green (VSG), which is a nucleic acid-binding fluorescent dye, to assess in vitro development of P. falciparum using flow cytometry.

Results Fluorescence intensity of VSG was found to depend on the developmental stage of parasites. Specifically, multiple-nuclei-containing schizonts were observed in the VSG high population, and growing trophozoites and ring-shaped forms were observed in the VSG intermediate and VSG low populations. The VSG-based assay was found to be comparable to the microscopic examination method, and capable of detecting as few as 0.001% of the parasitemia estimated by Giemsa staining. Moreover, when applying VSG for antimalarial drug test, we were able to observe the growth inhibitory effect of dihydroartemisinin, the front-line drug for malaria therapy.

Conclusions Taken together, the results of this study suggest the VSG-based flow cytometric assay to be a simple and reliable assay for assessing P. falciparum malaria development in vitro .

Background

Plasmodium falciparum remains a wide-spreading and highly virulent parasitic protozoan worldwide [1]. The mortality rate is highest in tropical and subtropical areas. Despite the development of effective antimalarial drugs, drug-resistant strains of malaria are reported annually [1] [2], which emphasizes the need for ongoing drug resistance surveillance, the study of the underlying mechanisms of drug resistance, and novel drug development. Culture of laboratory strains or field-isolates of *P. falciparum* has been widely used for these investigations. Microscopic examination is an effective method for assessing *in vitro* growth of malaria parasites in *P. falciparum* culture, as well as for drug sensitivity testing [3] [4] [5]. Nevertheless, the counting of malaria-infected erythrocytes under a microscope is tedious and time-consuming. This method requires a well-trained and experienced microscopist to enumerate and differentiate various stages of malaria parasites. Inter-rater variability among microscopists is, therefore, a drawback of the microscopic examination method.

Flow cytometry facilitates quantitative analysis of cells at high-speed, at high sensitivity, and in a semi-automatic manner. Given that erythrocytes lack nuclear DNA, detection of malarial DNA in erythrocytes is one of the most common assays. Several DNA-binding fluorescent dyes (fluorochromes) are available. Some fluorochromes, including hydroethidine [6], ethidium bromide [7], propidium iodide [8], SYBR Green I [9] [10], YOYO-1 [11], Hoechst 33258 [12], and Hoechst 33342 [13], are employed to stain the DNA of the malaria parasite in erythrocytes. To use hydroethidine and Hoechst 33342, cells need to be incubated at 37°C, which lengthens the processing time. The use of ethidium bromide has decreased due to its carcinogenic property. Propidium iodide was useful for evaluating invasion of merozoites into

erythrocytes in antimalarial drug test [8]. SYBR Green I facilitated quantification of parasitized erythrocytes at different stages of development [9]. However, propidium iodide, SYBR Green I, YOYO-1, and Hoechst require an extra step of cell membrane permeabilization for which aldehyde-based or ethanol-based fixation is often used. Since these methods alter cell structure, morphologic study of malaria parasite cannot be performed after their use.

Many nucleic acid-binding fluorochromes are commercially available and have been applied for visualizing DNA or RNA in agarose or polyacrylamide gel. ViSafe Green (VSG) is a stable, sensitive and environmentally safe nucleic acid-binding fluorescent dye. The VSG can be activated by a 250-300 nm wavelength and emit spectra similar to that of ethidium bromide[14]. Thus, the VSG is an alternative to ethidium bromide. Given the availability of new nucleic acid-binding fluorochromes, we set forth to develop a simple and fixation-free method that uses VSG to enumerate malaria-infected erythrocytes and assess intraerythrocytic development in culture. In addition, we demonstrate its utility for antimalarial drug susceptibility assay.

Methods

Parasite and culture

5. *falciparum* strain K1 was used in this study. Parasites were maintained as described previously [15]. Briefly, malaria culture medium (MCM) was prepared that consisted of Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich Corporation, St. Louis, MO, USA) supplemented with 5.96 g/L hydroxethylypiperazine-ethanesulphonic (HEPES), 2 g/L sodium bicarbonate ($NaHCO_3$), and 10% heat-inactivated human AB serum. The parasites were cultured in a T-25 flask containing 5% human O+ erythrocytes in MCM in a 5% CO_2 environment at 37°C. To assess the developmental stages of the studied parasites, a thin blood smear was prepared on a glass slide. Cells were visualized by staining with Giemsa dye prior to observation under light microscope [16].

Synchronization of *P. falciparum*-infected erythrocytes

Parasites were maintained in a synchronicity manner as described previously [17]. Briefly, parasites were allowed to grow to the ring stage, and they could not be older than 10 to 12 hours after merozoite invasion. The parasite culture was spun down at 2,000 revolutions per minute (rpm) for 5 minutes. After removal of the supernatant, an equal volume of sterile 5% D-sorbitol in distilled water was mixed with the packed erythrocytes and the mixture was incubated at 37°C for 10 minutes. After incubation, the cell suspension was spun down at 2,000 rpm for 5 minutes and then washed three times with RPMI 1640. The parasitemia and synchronicity were evaluated by counting the infected cells per 1,000 erythrocytes on a Giemsa-stained thin blood smear under a microscope. Ninety percent synchronicity was accepted for this experiment. The synchronized parasites were adjusted to 1% parasitemia with fresh human O+ erythrocytes and cultured in MCM as described above.

Preparation of *P. falciparum* gametocyte

Gametocytes were prepared as described previously [18]. Briefly, the parasites were allowed to grow to the ring stage at 3-5% parasitemia in MCM and then adjusted to 1% ring-stage parasitemia with fresh human O+ erythrocytes. To induce gametocyte formation, MCM was replaced with a gametocyte-inducing medium, which is MCM consisting of 0.37 mM hypoxanthine (Sigma-Aldrich, MO) and 10% human AB serum without heat inactivation. A 75% volume of the gametocyte-inducing medium was replaced daily. To assess sexual development of *P. falciparum*, a thin blood smear was prepared on a glass slide and stained with Giemsa dye prior to observation under a light microscope. Gametocytes were identified as described in a published method [19].

ViSafe Green staining and flow cytometric analysis

Given that no previous study used VSG (Vivantis Technologies, Salangor, Malaysia) for nucleic stain in viable cells, fluorescent dye concentration was initially optimized. Briefly, cells were suspended in diluted concentrations of VSG (10x, 5x, 2.5x, 2x, 1x, 0.5x, and 0.25x) in PBS and kept in the dark at room temperature for 20 minutes. Cells were then subjected to flow cytometric analysis and cell sorting using a FACS Aria II Instrument (BD Biosciences, San Jose, CA, USA) without cell washing. A type of VSG-activating laser and a suitable fluorescence detector of an emitted fluorescent signal were determined. Given that FACS Aria II is equipped with 488-, 561- and 445-nm laser, all three lasers were used for VSG activation. Fluorescence detector of FITC (500-560 nm), PE (543-627 nm), PE-Texas Red (593-639 nm), PerCP-Cy5-5 (655-735 nm), PE-Cy7 (720-840 nm), APC (640-680 nm), A700 (685-775 nm), APC-Cy7 (720-840), BV421 (400-500 nm), BV510 (500-560 nm) and BV605 (590-630 nm) were used for detection of the emitted fluorescent signals. The flow cytometric data were analyzed using FlowJo version 10 software (Tree Star, Inc). To increase the accuracy of flow cytometric analysis, non-single cells were excluded by gating according to forward scatter (FSC) and side scatter (SSC) characteristics of cells. Briefly, cells were first gated using forward scatter area (FSC-A) parameter at the X-axis, and using forward scatter height (FSC-H) parameter at the Y-axis. Cells having the characteristic of FSC-A equal to FSC-H were gated. Then, side scatter width (SSC-W) and side scatter height (SSC-H) were set at the X-axis and Y-axis, respectively, in order to exclude cells having SSC-W^{high}, which are not single cells. Cells were then further gated according to forward scatter width (FSC-W) and forward scatter height (FSC-H). Cells were sorted into PBS containing 1% fetal bovine serum (FBS) for morphologic analysis.

Giemsa staining and microscopy

Cells were affixed to glass slides using a CytoSpinTM4 Cytocentrifuge (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 450 rpm for 7 minutes, and then rapidly air-dried. Cells were fixed with absolute methanol and stained using a 1:18 diluted Giemsa solution at room temperature for 30 minutes. After one wash with running tap water, slides were air-dried and covered with glass coverslips with one drop of mounting solution. Cell morphology was assessed using an Olympus BX53 at magnification of 100X of

objective lens. For Giemsa-stained thin film of the culture, a minimum of 100 fields was examined at 100X magnification with oil immersion [20].

Fluorescence microscopic imaging

To ensure that VSG is able to pass through the cell membrane and bind to the parasite's nucleic acid, 50-100 mL of VSG-stained cells were dropped onto a glass slide and covered with a thin glass. The VSG-stained cells were observed under a laser-scanning confocal microscope (*ECLIPSE Ti-Clsi4 Laser Unit,* Nikon Corporation, Tokyo, Japan). Differential interference contrast and 488-nm argon-ion laser was used for microscopic imaging.

Reliability and sensitivity

To test the reliability of the VSG-based flow cytometric assay, we compared the parasitemia estimated from microscopic examination of the Giemsa-stained blood smear (the standard method) with the percentage of VSG+ cells obtained from flow cytometry. Various concentrations of parasitemia were prepared by diluting the parasitized erythrocytes in a 5% uninfected erythrocyte suspension. Spearman's rank correlation coefficient was used to assess the strength of association between the standard microscopic assay and VSG-based flow cytometry. For sensitivity testing, culture of *P. falciparum* was diluted to 0.001% parasitemia, which is the limit of detection in routine microscopic diagnosis [20], and then stained with VSG as describe above and analyzed by flow cytometry.

Lethal induction of *P.falciparum* using dihydroartemisnin

Dihydroartemisinin (DHA) (Sigma-Aldrich), which is a primary drug for falciparum malaria treatment, was used in this study to induce lethal form of the parasites.
DHA was prepared at a concentration of 700 nM in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) as described in previous study [21]. In short, 2 mg of DHA was resuspended in 2 mL of DMSO and used as stock solution. The solution was then diluted 5-fold in DMSO to achieve a drug concentration of 200 µg/mL (700 mM). Synchronized ring stages of *P. falciparum* K1 strain were diluted with 5% hematocrit O cell and MCM to obtain 1% parasitemia and 2% hematocrit. Twenty µL of DHA solution or DMSO was mixed with 2 mL of MCM to obtain a concentration of 700 nM. The parasites were exposed to DHA or DMSO (as control) in a 5% CO_2 atmosphere at 37°C for 24 hours. Later, cells were washed once with MCM, stained with Giemsa dye, and enumerated under a microscope as described above.

Statistical analysis

Data analysis and graph generation were performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Results are expressed as mean ± standard deviation (SD) and coefficient of variation (CV). Spearman's rank correlation coefficient was used to measure the strength of association between standard microscopy and VSG-based flow cytometry. Statistically

significant differences were identified using non-parametric Student's t-test. A *p*-value less than 0.05 was regarded as being statistically significant.

Results

Cell permeability of VSG dye

To ensure that VSG is cell-permeable and that it binds to nucleic acid, the non-synchronized culture of *P. falciparum* (Fig. 1A) was incubated with VSG dye without fixation and subjected to laser-scanning confocal microscope imaging in which emitted fluorescent signal of VSG was displayed as green. At lower magnification, cells having green color were observed (Fig. 1B, upper panels). Higher magnification images revealed green color inside erythrocytes (Fig. 1B, yellow and blue arrows), which suggests cell membrane permeability of VSG. Moreover, the intensity of green color was shown to vary, with intensity roughly grouped into low or high intensity (Fig. 1B, yellow and blue arrows, respectively). Two green dots were also observed in a single erythrocyte as same as those found in the Geimsa-stained thin blood smear, which suggests multiple infection of *P. falciparum*. These findings indicate that VSG was able to permeate the *P. falciparum*-infected erythrocytes.

Optimization of VSG stain for flow cytometer

Given that VSG has never been used for flow cytometry, we first had to identify a type of VSG-activating laser and a suitable fluorescence detector. The concentration of VSG was then optimized. In flow cytometry analysis, non-single cells were excluded by gating according to forward scatter (FSC) and side scatter (SSC) characteristics of cells. Briefly, cells were first gated using FSC-A parameter at the X-axis, and using FSC-H parameter at the Y-axis (Fig. 2A, upper panel). Cells having the characteristic of FSC-A equal to FSC-H were gated. Then, SSC-W and SSC-H were set at the X-axis and Y-axis, respectively (Fig. 2A, middle panel), in order to exclude cells having SSC-W^{high}, which are not single cells. Cells were then further gated according to FSC-W and FSC-H (Fig. 2A, lower panel). These initial gating steps aimed to obtain single cells, which increases the accuracy of flow cytometric analysis. Using a FACS Aria II, only 488-nm laser could activate VSG and effectuate emission of a fluorescent signal, whereas 561-nm and 445-nm laser did not (Fig. 2B). When we used a detector of FITC fluorochrome (500-560 nm), VSG+ cells (green-colored lines) could be separated from the unstained cells (magenta-colored lines). By contrast, when we used detector of PE (543-627 nm) and PE-Texas Red (593-639 nm), VSG+ cells (green-colored lines) overlapped with the unstained cells (magenta-colored lines), limiting analysis of parasitized cells. Therefore, we decided to use the 488-nm laser for VSG activation, and the FITC detector to read the emitted fluorescent signal.

To determine the optimal concentration of VSG, *P. falciparum*-infected erythrocytes were incubated with 0.25x, 0.5x, 1x, 2.5x, 5x, and 10x dilution of VSG. The optimal VSG concentration was determined based on its ability to fractionate *P. falciparum*-infected erythrocytes from non-infected cells. As shown in Fig.

2C, 10x and 5x VSG were the concentrations that yielded the highest fluorescence intensity in VSG5+ cells. Moreover, different intensity of fluorescence was observed in the 10x and 5x VSG-stained samples (Fig. 2C, histogram), which is a finding that is consistent with confocal microscopic data. We excluded the 100x, 50x, and 25x VSG concentrations due to an upward shift in the dots on the flow cytometric profile (data not shown), which suggested an increase in non-specific staining (high background). Microscopic observation of sorted VSG+ cells showed that 5x diluted VSG yielded all stages of intraerythrocytic development of *P. falciparum* (Fig. 2D). In agreement with Fig. 2D, Giemsa staining of pre-sorted sample showed 10.4% parasitemia that consisted of 9.8% ring form, 0.1% trophozoites and 0.5% schizonts, implying accuracy of VSG at 5x dilution. Therefore, 5x diluted VSG was used for other experiments in this study.

Validation of the VSG staining method

To test that each stages of intraerythrocytic development of *P. falciparum* could be fractionated based on intensity of VSG, a non-synchronized culture of malaria parasites was prepared. As a standard method, Giemsa staining of thin blood film showed 14% parasitemia that consisted of 13% ring form, 0% trophozoites and 1.1% schizonts (Fig. 3A). The VSG+ cells were separated according to intensity into low, intermediate, or high (hereafter referred to as VSG^{low}, $VSG^{intermediate}$, and VSG^{high}, respectively) (Fig. 3B), and their morphologies were examined. Schizonts were observed only in VSG^{high} fraction, and ring forms and growing trophozoites were observed only in $VSG^{intermediate}$ and VSG^{low} fraction (Fig. 3C). Moreover, we could observe different morphology of the *P. falciparum* parasites in $VSG^{intermediate}$ and VSG^{low} fraction. The cytoplasm of *P. falciparum* in the $VSG^{intermediate}$ was thicker than that in the VSG^{low} fraction (Fig. 3D). These findings were in agreement with microscopically examined Giemsa-stained thin blood film that revealed ring form, trophozoites, and schizonts in the culture, which suggests that our protocol was optimal. Thus, fluorescence intensity of VSG depends on the stage of *in vitro* malaria development.

To test whether the VSG-based flow cytometric analysis could distinguish gametocytes from schizonts, we cultured *P. falciparum* strain K1 in gametocyte-inducing culture medium and performed VSG-based flow cytometric analysis. Cells in VSG^{low}, $VSG^{intermediate}$, and VSG^{high} fraction were sorted and stained with Giemsa dye. In the VSG^{high} fraction, we could observe parasitized erythrocytes having granular distribution of hemozoin, resembling stage-IB gametocyte. Some elongated and D shaped within erythrocytes, which are key characteristics of stage-II gametocytes. Early schizonts having 2 and 6 divided nucleus and mature schizonts consisted of 14 merozoites were also observed in the VSG^{high} fraction, whereas ring forms and trophozoites were observed in the VSG^{low} and $VSG^{intermediate}$ fraction, respectively (Fig. S1). Thus, VSG-based flow cytometric assay is not able to distinguish gametocytes from schizonts.

Given the ability of VSG to differentiate intraerythrocytic stages, we explored whether change in cell granularity are related to the developmental stages of *P. falciparum*. VSG^{low}, $VSG^{intermediate}$, and VSG^{high}

cells were gated and analyzed for SSC-A (which is an indicator of cell granularity). As shown in Fig. 3E, the median of SSC-A increased about 2 times when VSG^{low} and $VSG^{intermediate}$ cells developed into VSG^{high} cells. These results suggest that change in cell granularity is related to intraerythrocytic development of *P. falciparum,* and that this change can be assessed using VSG-based flow cytometry.

Linearity and sensitivity of the VSG-based flow cytometric assay

To evaluate the optimized protocol relative to its ability to enumerate parasitized erythrocytes, we tested whether it could detect malaria-infected erythrocytes in a dose-dependent manner. Various concentrations of malaria-infected erythrocytes were prepared. Two-fold dilutions of infected cells were prepared using non-infected erythrocytes as diluent. That analysis revealed that VSG-based flow cytometry could detect malaria-infected erythrocytes in a dose-dependent manner (Fig. 4A). The relative values correlated well between the two assays (r^2=0.75-0.97; p<0.05). The same results were observed from three independent experiments (CV = 11.2%), indicating reproducibility of linearity measurement.

To assess the sensitivity of VSG-based flow cytometry, parasitized erythrocytes were diluted to 0.001%, which is the limit of detection in routine microscopic diagnosis [20]. As shown in Fig. 4B, two independent cultures were analyzed for each cytometer run. There were 11% and 9% parasitemia enumerated using Giemsa-based microscopy. The parasites were diluted to 0.001% using non-infected erythrocytes as diluent. The diluted samples having 0.001% parasitemia were then subjected to flow cytometry analysis. VSG-based flow cytometry was capable of detecting 0.3% and 1.1% of VSG+ cells, which is 300-1,000 times higher than the detection rate (0.001% parasitemia) by Giemsa-based microscopy. Next, we examined the reproducibility of the developed assay for enumeration of low parasitemia. We prepared three independent settings of malaria culture and diluted them to 0.01% parasitemia, a minimum value that correlated well with standard microscopic examination (Fig. 4A). All three independent runs of VSG-based flow cytometer were able to detect 0.9±0.2% of VSG+ cells (CV = 22%, Fig. 4C), implying reproducibility comparable to Giemsa-based microscopy (CV = 21.8%) in detection of low parasitemia.

To examine variability of VSG-based flow cytometric assay in enumeration and identification of *P. falciparum*-infected erythrocytes among different sets of parasite culture, we prepared parasite culture on different date and compared enumerated values of parasitized cells (mean±SD) obtained from the Giemsa-based microscopes with those obtained from VSG-based flow cytometric assays (Table 1). There were two types of culture: ring-form and trophozoite predominant culture. In both types of culture, CV of VSG-based flow cytometric assay in enumeration of parasitemia is relatively lower than that of microscope, implying smaller variability of VSG-based flow cytometric assay. When analyzing variability of assays according to developmental stages, high CV values were obtained from both Giemsa-based microscopy and VSG-based flow cytometric assays, likely due to low parasitemia of each developmental

stages. Collectively, VSG-based flow cytometry is reliable, sensitive and reproducible for enumeration of parasitemia.

Application of VSG-based flow cytometry for synchronicity assessment and drug sensitivity testing

Synchronization of *P. falciparum* development is a common method used in a routine culture and aims to obtain a predominant intraerythrocytic stage of parasites. To explore whether VSG-based flow cytometry is capable of assessing synchronicity of *P. falciparum* development in a routine culture, synchronized and non-synchronized cultures of *P. falciparum* were prepared (Fig. 5A), stained with VSG and subjected to flow cytometry analysis. Given an ability of flow cytometry to detect cell size and granularity using respective FSC and SSC, we hypothesized that synchronized parasites have same size and granularity, a status of homogeneity. Thus, we selected a quantile contour plot, which is an effective way to visualize distinct populations regardless of the numbers of cells displayed [22], to assess cell homogeneity. In Fig. 5B, only VSG+ cells were displayed based on their size (as indicated by FSC-A on the X-axis) and granularity (as indicated by SSC-A on the Y-axis). To enhance the visualization of distinct cell population having various cell size and granularity, histograms of FSC-A and SSC-A are also shown at the top and left side of the contour plots, respectively. There were at least three distinct populations observed in the non-synchronized culture (Fig. 5B, upper panel), as follows: (1) cells having small size with various granularity; (2) cells having a relatively large size with high granularity; and, (3) cells having a relatively larger size with low granularity. In contrast, only one major and one minor population were observed in the synchronized culture. They had a similar size, but different level of granularity (Fig. 5B, lower panel). To confirm heterogeneity in the non-synchronized culture, we statistically analyzed the CV, which is a measure of relative *variability*, of FSC-A and SSC-A. Despite statistical non-significance ($p>0.05$), the non-synchronized culture tended to have a higher degree of CV of FSC-A and SSC-A (Fig. 5C), which confirms the relatively high heterogeneity of VSG+ cells. Thus, VSG-based flow cytometry is an effective alternative method for assessing synchronicity of *P. falciparum* development in erythrocytes.

To demonstrate the use of VSG for assessment of growth inhibitory effect of antimalarial drug, malaria-infected erythrocytes were incubated with DHA following a standard assay. The DHA- and DMSO-treated cells were stained with VSG and analyzed by using flow cytometry. In both the presence and absence of the drug, there were VSG+ cells exhibiting $VSG^{intermediate}$ and VSG^{low} (Fig. 5D), which are likely resemble to trophozoite and ring form, respectively. Our results revealed that the number of VSG+ cells decreased following the DHA treatment (Fig. 5D, right panel) compared to the DMSO-treated control (Fig. 5D, left panel). The majority of the DHA-treated VSG+ cells appear as VSG^{low}, implying that ring form was predominant. In contrast, both $VSG^{intermediate}$ and VSG^{low} cells were observed in the DMSO-treated control (Fig. 5D, left panel), implying that both ring from and trophozoite were presence in the culture. According to the VSG-based flow cytometric data, the DHA likely inhibited growth of parasite. Therefore, the VSG-based flow cytometric assay can be used as an alternative assay for assessment of *P. falciparum* growth in the presence of antimalarial drug *in vitro*.

Discussion

Many flow cytometric assays have been developed to detect the malaria parasite; however, these assays are complicated, time consuming, and/or insufficiently sensitive. In this study, we used VSG to detect and purify *P. falciparum*-infected erythrocytes using flow cytometry. VSG could enter and bind to the nucleic acid of ring form, trophozoite, schizont and early stage of gametocytes of *P. falciparum* growing in culture. Twenty-minute incubation at room temperature without fixation makes this method faster and simpler to perform than other malaria detection assays.

The degree of parasitemia detected by VSG-based flow cytometry was different from the degree of parasitemia detected by the standard microscopic method. This difference is likely due to the method of cell analysis. It is recommended that 10,000 cells be counted under the microscope in order to accurately quantify the number of malaria parasite-infected cells. In contrast, hundreds of thousands of cells could be analyzed by flow cytometry. Thus, different percentages of infected cells could be expected from these two different methods. Although the percentages of infected cells are different between methods, the VSG-based flow cytometric assay was found to be reliable for detecting the malaria parasite in a dose-dependent manner, and it was able to detect parasitized cells as low as 0.001%, which is the detection threshold for the standard microscopic method [20].

The fixation-free flow cytometric method profiled in this report also provides morphologic information. To our knowledge, this is the first study to report high specificity of the method by showing the morphology of fluorochrome-binding cells. VSG+ cells were infected by *P. falciparum*, and all 4 major stages of malaria parasite could uptake VSG. Moreover, the intensity of VSG was found to be commensurate with the amount of DNA, and the VSG intensity of schizonts was higher than that of ring-shaped forms.

VSG is commercially available for nucleic acid detection in agarose gel electrophoresis and is tough to be cell impermeable; however, we report data that shows that VSG could enter *P. falciparum*-infected erythrocytes. It needs to be further investigated whether the permeability of VSG is due to increased membrane transport for nucleosides, amino acids, and carbohydrates, as described in previous studies [23] [24].

Compared to other DNA-binding fluorochromes, VSG is more suitable for analyzing malaria parasites for the following reasons. First, VSG can rapidly enter cells and bind to nucleic acid at ambient temperature. As such, there is no need for cell permeabilization, which shortens the pre-flow cytometry process. Second, the fluorescence emission spectrum of VSG is similar to spectra of FITC, the most widely used fluorochrome. This factor facilitates application of VSG with other flow cytometry. Third and last, although Hoechst is frequently used as nuclear DNA stains, these fluorochrome probes cannot be used when the cytometer being used does not have a UV laser. Thus, VSG can be used as an alternative choice in the aforementioned setting. VSG is an attractive alternative in flow cytometric assay due to its speed and ease of use compared to that of other DNA-binding fluorochrome probes, including hydroethidine [6],

ethidium bromide [7], propidium iodide [8], SYBR Green [9] [10], YOYO-1 [11], Hoechst 33258 [12], and Hoechst 33342 [13]. In comparison with Coriphosphine O [25], a fluorochrome activated by 488-nm laser, use of VSG is simpler; no requirement for incubation at 37°C and cell washing prior to flow cytometric analysis. Moreover, cost of VSG is cheaper than that of Coriphosphine O. Although merit of the VSG is comparable to that of SYBR Green I regarding cost and ease of use (without cell fixation); however, the resolution of the different *Plasmodium* stages stained with SYBR Green was not sharp [26], limiting parasite stage identification. Moreover, additional step of cell fixation using paraformaldehyde (PFA) or 1% glutaraldehyde is reportedly required in the previous study [9]. Importantly, VSG-based flow cytometric assay was able to detect parasitemia lower than Coriphosphine O and SYBR Green I did.

Recently, effective, accurate detections and quantifications of *P. falciparum*-infected erythrocytes using an automated Sysmex Haematology Analyzer XN-30 were published [27, 28]. This automatic machine employs a 405-nm laser to detect cells, which need to be partially lysed to increase permeability of florescent dye before cell analysis. Thus, this method limits morphological observation of parasitized cells post cell analysis. Importantly, specific florescent dye can be used with this cell analyzer. In comparison with this report, VSG is commercially available and activated using a 488-nm excitation laser, which is one of common lasers equipped with several models of cell analyzers; FACSCalibur, FACS Aria (BD Bioscineces), ZE5 Cell Analyzer (Bio-Rad), CellSimple™ Cell Analyzer (Cell Signaling Technology) and Guava® easyCyte™ Systems (Luminex), allowing broad application of VSG. However, VSG-based flow cytometry is not able to distinguish early gametocyte stage in the culture containing schizonts.

Antimalarial drug susceptibility assay is very useful for identifying pharmacologically active compounds, to monitor drug resistance, and to investigate the mechanism underlying drug resistance. Effect of antimalarial drugs is mainly characterized by the inhibition of parasite growth or maturation and multiplication. These parameters are often measured by uptake of radioisotope [H^3] hypoxanthine into nucleic acid [29], enzymatic assay of *P. falciparum*-specific lactate dehydrogenase [30], or detection of *P. falciparum*-specific antigen histidine-rich protein 2 in the culture [31]. We demonstrate the utility of VSG for assessing the pharmacologic effect of antimalarial drugs on organism development.

Conclusion

Given a relative ease of use of fluorescent dyes, VSG-based flow cytometry could be an alternative assay for enumeration of parasitemia, assessment of intraerythrocytic development and synchronicity, and antimalarial drug effect.

Abbreviations

CV: Coefficient of variation; DHA: Dihydroartemisinin; DIC: Differential interference contrast; DMSO: Dimethyl sulfoxide; FBS: Fetal bovine serum; FSC-A: Forward scatter area; FSC-H: Forward scatter height;

FSC-W: Forward scatter width; HEPES: hydroxethylypiperazine-ethanesulphonic; MCM: malaria culture medium; NS: Non-synchronized; RPM: revolutions per minute; RPMI: Roswell Park Memorial Institute; S: Synchronized; SD: standard deviation; SSC-H: Side scatter height; SSC-W: Side scatter width; VSG: ViSafe Green.

Declarations

Ethics approval and consent to participate

Ethical approval were obtained from the Ethical Review Committee for Research Involving Human Subjects in Research, Chulalongkorn University, in accordance with the International Conference on Harmonization-Good Clinical Practice (COA no. 128/2012). Written informed consent was obtained from each study participant before giving blood for malaria culture. The protocol of malaria culture was approved by the Siriraj Safety Risk Management Taskforce, Faculty of Medicine Siriraj Hospital, Mahidol University (COA no. SI 2018-011).

Consent for publication

All authors reviewed and consented the final manuscript for publication.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding authors on reasonable request.

Competing interest

All authors declare no competing interests, no personal or professional conflicts of interest, and no financial support from the companies that produce and/or distribute the drugs, devices, or materials described in this report.

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Authors' contributions

KK, AT, and DP conceived and designed the study; KK, NK, and DP performed the experiments, analyzed and interpreted the data, and wrote the manuscript.

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Table 1

Table 1 Comparison of a standard optical microscope and VSG-based flow cytometer for enumeration and identification of *P. falciparum*-infected erythrocytes

Ring-form predominant culture	Enumerated value of parasitized erythrocyte					
	Microscope			VSG-based flow cytometer		
	Mean	SD	CV (%)	Mean	SD	CV (%)
Ring-forms	11.5	2.3	20.4	10.4	3.7	35.4
Trophozoites	0.1	0.1	141.4	1.7	1.6	91.5
Schizonts	0.8	0.4	53.0	0.5	0.3	52.1
All stages	12.3	2.7	21.8	12.5	1.8	14.1

Trophozoite predominant culture	Enumerated value of Parasitized erythrocyte					
	Microscope			VSG-based flow cytometer		
	Mean	SD	CV (%)	Mean	SD	CV (%)
Ring-forms	1.1	1.5	141.4	2.7	0.2	8.0
Trophozoites	8.5	0.3	3.3	4.4	0.4	9.6
Schizonts	0.4	0.5	141.4	0.1	0.04	60.6
All stages	9.9	1.7	17.1	7.1	0.7	9.5

Figures

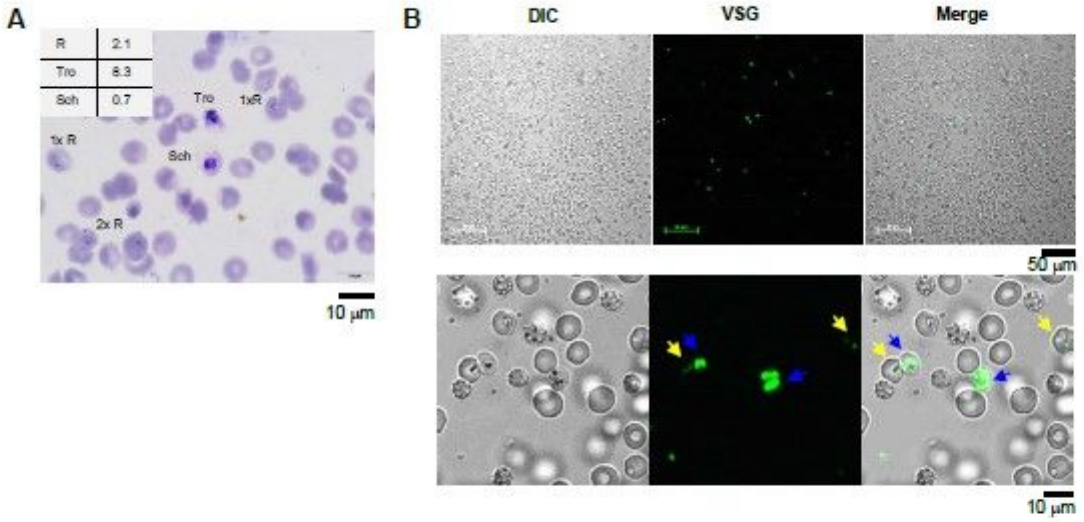


Figure 1

Cell permeability of VSG dye. (A) Geimsa-stained thin blood smear of the non-synchronized culture revealed mixed developmental stages of falciparum malaria parasites. Scale bars: 10 m. (B) Laser scanning confocal microscopic images of P. falciparum-infected erythrocytes uptaking VSG. Differential interference contrast images are shown (far left panel). VSG was activated by a 488-nm argon-ion laser, and are displayed as green in the middle panel. Differential interference contrast and fluorescent images are merged in the far-right panel. Yellow and blue arrows indicate low and high intensity of VSG,

respectively. Scale bars: 50 and 10 μm at upper and lower panels, respectively. Abbreviations: R, ring form; Tro, trophozoite; Sch, schizoint; DIC, differential interference contrast; VSG, ViSafe Green

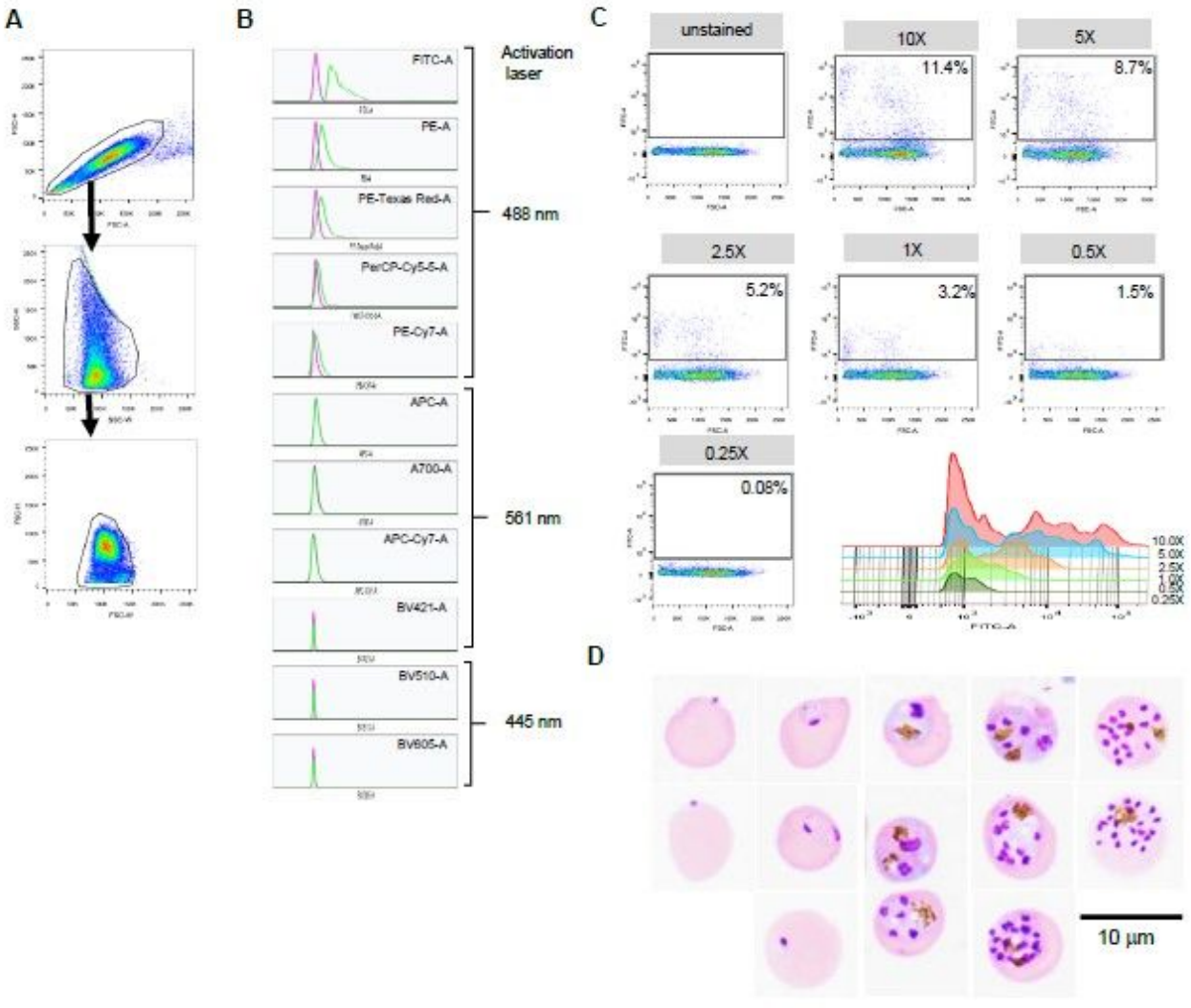


Figure 2

Optimization of VSG staining of P. falciparum-infected erythrocytes. (A) Gate setting for flow cytometric analysis. Non-single cells were excluded by gating according to FSC-H, FSC-W, SSC-H, and SSC-W. (B) Histograms show fluorescence intensity of VSG+ cells (green) excited by 488-nm, 561-nm, and 445-nm lasers. To read the emitted fluorescent signal, detectors of FITC (500-560 nm), PE (543-627 nm), PE-Texas Red (593-639 nm), PerCP-Cy5-5 (655-735 nm) and PE-Cy7 (720-840 nm) were used for 488-nm activating laser, detectors of APC (640-680 nm), A700 (685-775 nm) and APC-Cy7 (720-840) were used for 561-nm activating laser and detectors of BV421 (400-500 nm), BV510 (500-560 nm) and BV605 (590-630 nm) were used for 445-nm activating laser. Histogram of sample not stained with VSG was set as VSG negative (shown in magenta). (C) Representative flow cytometric profiles of samples stained with VSG at 10x, 5x, 2.5x, 1x, 0.5x, and 0.25x dilution relative to the 10,000x concentration of the commercial version. Overlaid histogram of VSG+ cells obtained from staining with different concentrations of VSG is shown on the left side of flow cytometric images. (D) Representative images of Giemsa-stained erythrocytes in

VSG+ fraction acquired using an objective lens at 100X. Scale bars: 10 □m. Abbreviations: FSC-A, forward scatter area; FSC-H, forward scatter height; FSC-W, forward scatter width; SSC-W, side scatter width; SSC-H, side scatter height; DIC, differential interference contrast; VSG, ViSafe Green

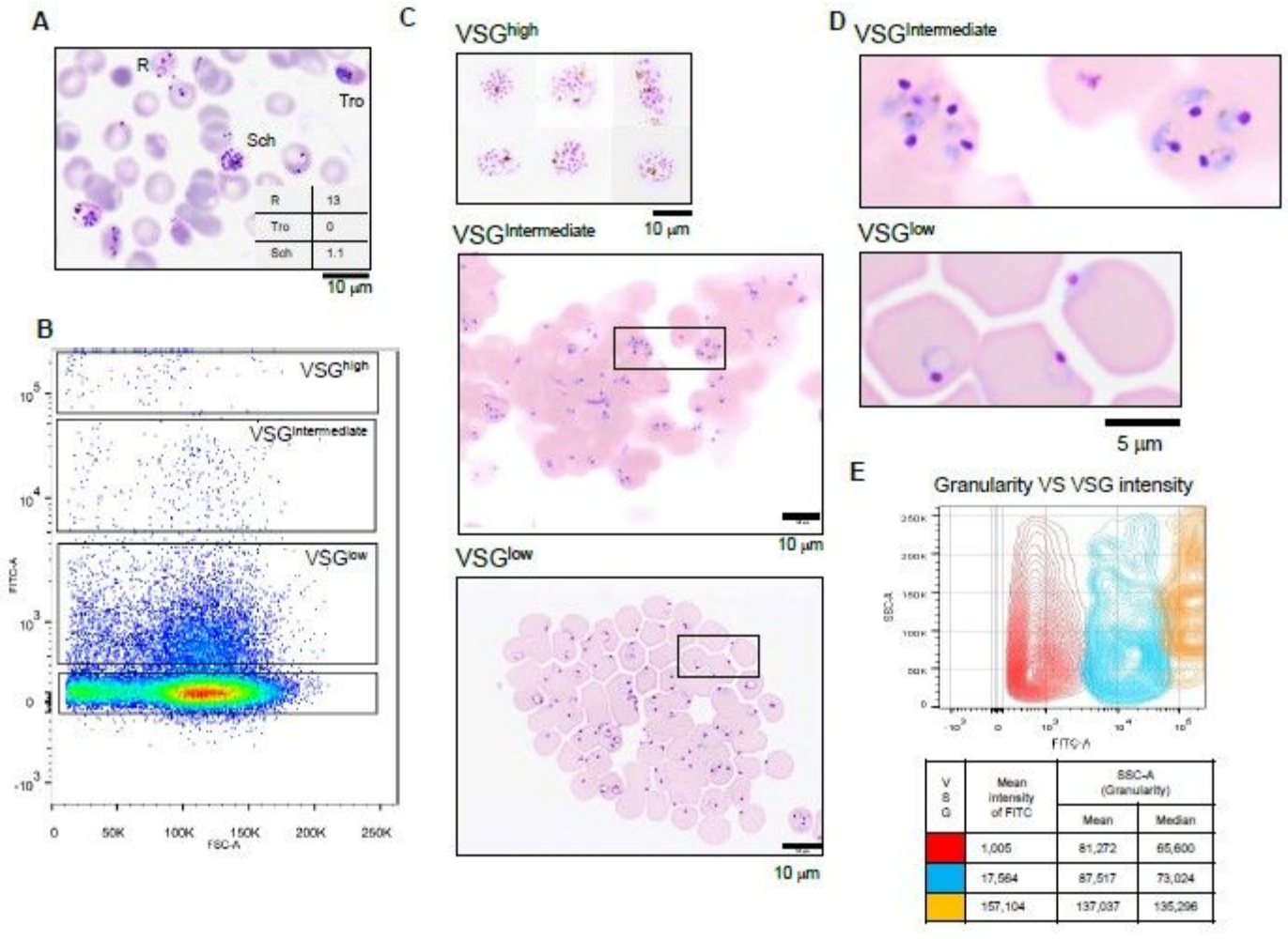
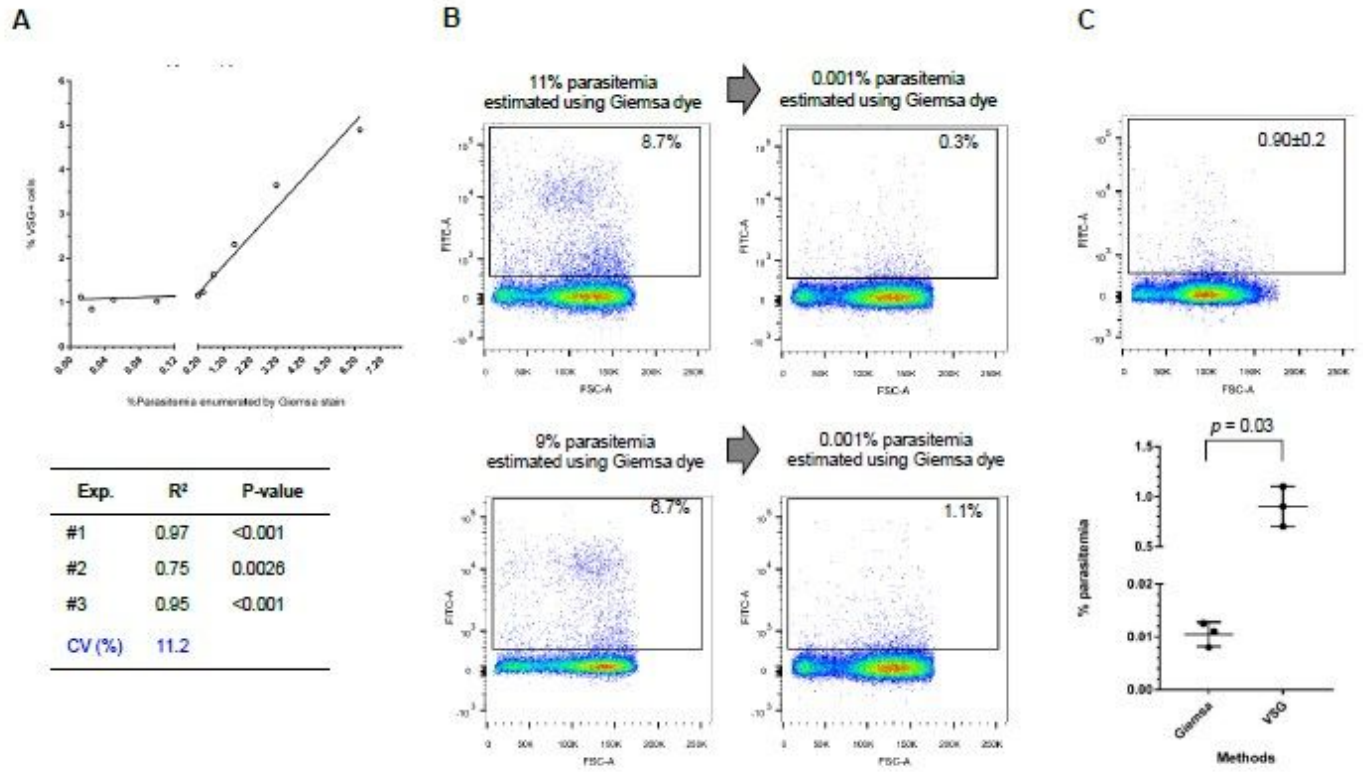


Figure 3

Intensity of VSG depends on the stage of intraerythrocytic development of P. falciparum. (A) Geimsa-stained thin blood smear of the non-synchronized culture revealed mixed developmental stages of falciparum malaria parasites. (B) Flow cytometric profile of P. falciparum-infected erythrocytes. The non-synchronized culture of malaria parasites was stained with 5x VSG. Single cells were separated based on fluorescence intensity into high, intermediate, or low (hereafter referred to as VSGhigh, VSGintermediate, and VSGlow, respectively). (C) Morphology of Giemsa-stained VSGhigh, VSGintermediate, and VSGlow cells. (D) Higher magnification of P. falciparum-infected cells in the boxed area of VSGintermediate, and VSGlow cells in Fig. 3C. (E) Cell granularity of VSGhigh, VSGintermediate, and VSGlow cells was assessed based on SSC-A, respectively. Scale bars: 10 µm for Fig. 3A and 3C and 5 µm for Fig. 3D. Abbreviations: FSC-A, forward scatter area; VSG, ViSafe Green



Exp.	R^2	P-value
#1	0.97	<0.001
#2	0.75	0.0026
#3	0.95	<0.001
CV (%)	11.2	

Figure 4

Linearity and sensitivity of the VSG-based flow cytometric assay. (A) Representative graph of the Spearman's rank correlation coefficients. Percentage of P. falciparum-infected erythrocytes was obtained from VSG-based flow cytometry (Y-axis) and from Giemsa staining of thin blood film (X-axis). Three independent analyses were performed that revealed a range of infected erythrocytes of 0.01-6.4%, as shown in the table. (B) Sensitivity of VSG-based flow cytometry. Culture of P. falciparum was diluted to 0.001% parasitemia, which is the limit of detection in routine microscopic diagnosis [20], and analyzed by flow cytometry. Representative flow cytometric profile and data are shown as mean±SD. Graph shows comparison of parasitemia detected by standard microscope and VSG+ cells detected by flow cytometry. (C) Reproducibility of the VSG-based flow cytometric assay for low parasitemia culture. Three independent settings of P. falciparum culture was diluted to 0.01% parasitemia and analyzed using flow cytometry. Abbreviations: VSG, ViSafe Green; FSC-A, forward scatter-area

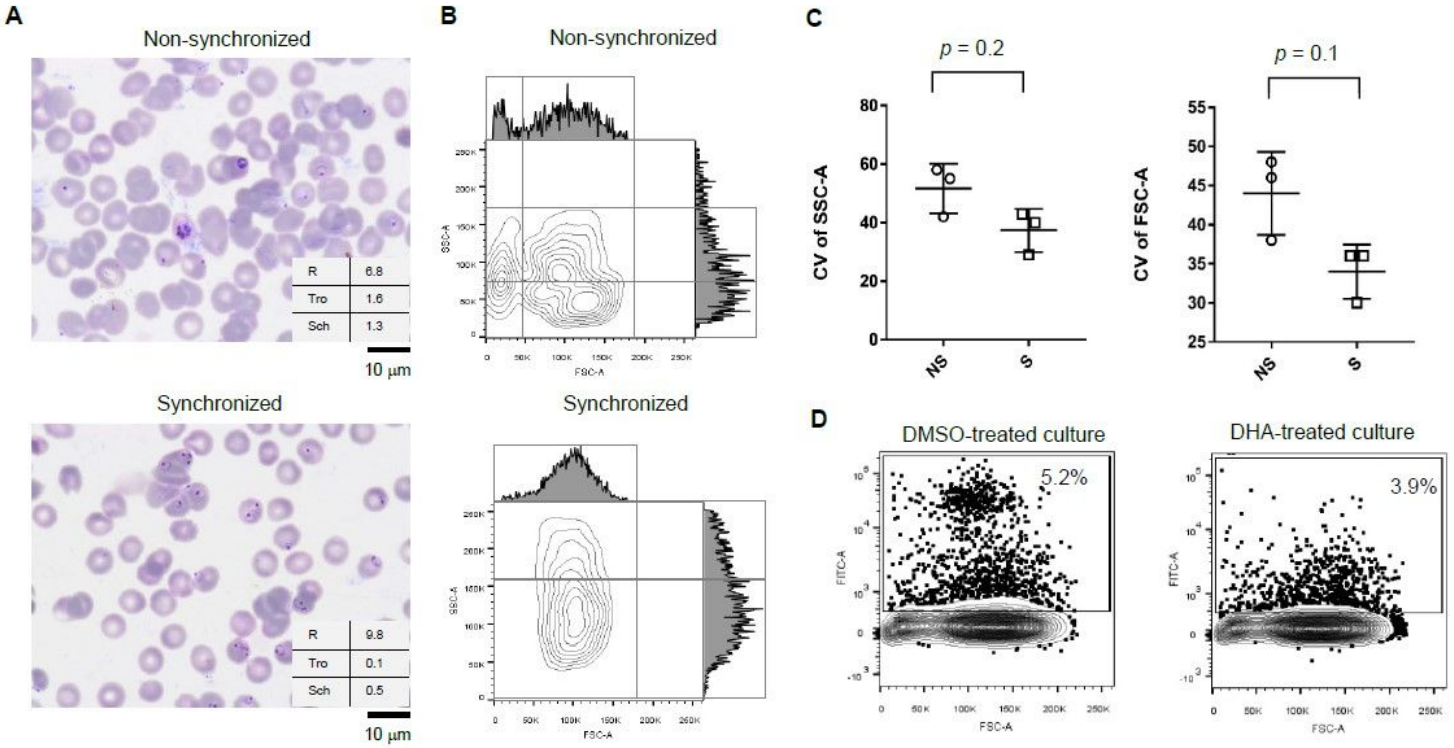


Figure 5

Application of VSG-based flow cytometry in synchrony assessment and anti-malarial drug assay. (A) Microscopic images of P. falciparum-infected erythrocytes derived from non-synchronized and synchronized cultures and stained with Giemsa dye. (B) Flow cytometric profiles of VSG+ cells are displayed according to FSC-A (X-axis) and SSC-A (Y-axis) in contour plots. Histograms of FSC-A and SSC-A are shown at the top and left side of the contour plots, respectively. (C) Graph of coefficient of variation of FSC-A and SSC-A. Dots represent three independent experiments. (D) P. falciparum-infected erythrocytes were treated with anti-malarial drug dihydroartemisinin (DHA) and subjected to VSG-based flow cytometric analysis. Mixed stages and synchronized ring forms were prepared. Abbreviations: FSC-A, forward scatter-area; SSC-A, side scatter-area; NS, non-synchronized culture; S, synchronized culture; CV, coefficient of variation

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigS1.pdf