Original Research *Feature article*

Cell trace far-red is a suitable erythrocyte dye for multi-color *Plasmodium falciparum* invasion phenotyping assays

Laty G Thiam[1,2], Yaw Aniweh[1,2], Evelyn B Quansah[1,2], Jacob K Donkor[2], Theresa M Gwira[1,2], Kwadwo A Kusi[1,2,3], Makhtar Niang[4] and Gordon A Awandare[1,2]

[1]West African Centre for Cell Biology of Infectious Pathogens, College of Basic and Applied Sciences, University of Ghana, P. O. Box LG54, Legon, Accra; [2]Department of Biochemistry, Cell and Molecular Biology, College of Basic and Applied Sciences, University of Ghana, P. O. Box LG54, Legon, Accra; [3]Department of Immunology, Noguchi Memorial Institute for Medical Research, University of Ghana, P. O. Box LG 581, Legon, Accra; [4]Unité d'Immunologie, Institut Pasteur de Dakar, Dakar 220, Senegal
Corresponding authors: Gordon A Awandare. Email: gawandare@ug.edu.gh; Yaw Aniweh. Email: aniweh@gmail.com

Impact statement

In recent years, flow cytometry has become a cornerstone in investigating *P. falciparum* phenotypic diversity using multiple dyes to discriminate between donor and acceptor erythrocytes. To broaden the applicability of such assays, we optimized the staining conditions of a newly developed cytoplasmic dye, cell trace far-red (CTFR), and assessed its suitability for use in *P. falciparum* invasion phenotyping assays. We showed that CTFR has a very narrow emission peak excited by red lasers. Furthermore, CTFR labeling of target erythrocytes, achieved even at low concentrations, is stable over time and did not impair parasite development. *P. falciparum* erythrocyte invasion phenotyping assays revealed that CTFR is suitable for use in combination with several DNA dyes in multiplex assays. This will allow for high throughput phenotyping of parasites as well as facilitate the evaluation of preference of erythrocytes by merozoites. Altogether, these make screening for potential invasion-blocking interventions possible.

Abstract

Plasmodium falciparum erythrocyte invasion phenotyping assays are a very useful tool for assessing parasite diversity and virulence, and for characterizing the formation of ligand–receptor interactions. However, such assays need to be highly sensitive and reproducible, and the selection of labeling dyes for differentiating donor and acceptor erythrocytes is a critical factor. We investigated the suitability of cell trace far-red (CTFR) as a dye for *P. falciparum* invasion phenotyping assays. Using the dyes carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) and dichloro dimethyl acridin one succinimidyl ester (DDAO-SE) as comparators, we used a dye-dilution approach to assess the limitations and specific staining procedures for the applicability of CTFR in *P. falciparum* invasion phenotyping assays. Our data show that CTFR effectively labels acceptor erythrocytes and provides a stable fluorescent intensity at relatively low concentrations. CTFR also yielded a higher fluorescence intensity relative to DDAO-SE and with a more stable fluorescence intensity over time. Furthermore, CTFR did not affect merozoites invasion of erythrocytes and was not toxic to the parasite's intraerythrocytic development. Additionally, CTFR offers flexibility in the choice of combinations with several other DNA dyes, which broaden its usage for *P. falciparum* erythrocyte invasion assays, considering a wider range of flow cytometers with various laser settings.

Keywords: Cell trace far-red, flow cytometry, *Plasmodium falciparum*, erythrocyte invasion

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Introduction

Erythrocyte invasion is critical for *Plasmodium falciparum* establishment, multiplication, and transmission. The process of merozoite invasion is complex and well regulated, involving an array of interactions between parasite ligands and erythrocyte receptors.[1] In response to immune pressure or natural genetic variation, *P. falciparum* alters the usage of these ligand–receptor interactions to successfully invade the host cells.[2,3] Collectively, these interactions define distinct invasion pathways, hence the parasite invasion phenotype. Invasion phenotyping assays have traditionally been monitored using microscopy,[4,5] which is time-consuming and requires technical expertise. As an alternative, early flow cytometry-based approaches made

use of DNA labeling dyes such as SYBR Green I to assess the parasite invasion efficiency.[6] Nevertheless, this approach does not distinguish acceptor erythrocytes from culture or patient-derived uninfected erythrocytes. Screening large numbers of *P. falciparum* clinical isolates is necessary for vaccine candidate prioritization, therefore, requiring high throughput assay system with different levels of discrimination. Recent progress in flow cytometry applications has entrenched its wider usage in *P. falciparum* erythrocyte invasion phenotyping assays. So far, the commonly used flow cytometry-based assay employs a dual-dye combination approach requiring an amine-reactive cytoplasmic dye to be used in combination with fluorescent DNA dye to label the cytoplasm of acceptor erythrocytes and the parasite's DNA, respectively.[7] Advantages and disadvantages of the most commonly used dye-combinations have earlier been assessed, and as a result, an adaptable flow cytometry-based assay has been proposed.[7] Given their ability to passively diffuse through the lipid bilayer of different cell types, amine-reactive dyes have been preferred over lipophilic dyes that bind to the plasma membrane.[8] Furthermore, lipophilic dyes may alter the nature of ligand–receptor interactions during merozoites invasion. Consequently, the most commonly used cytoplasmic dyes are carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) and 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) succinimidyl ester (DDAO-SE). First used in tracking lymphocyte migration,[9] CFDA-SE has since been used in a wide range of applications[10–12] and has been shown to be non-toxic to *P. falciparum*.[13] Despite being extremely bright and readily affordable, CFDA-SE is limited by its poor spectral properties, preventing its usage in multi-color assays involving dyes that emit through the green channel.[14] DDAO-SE, which is a red laser-excitable dye, has been found to be an excellent alternative for CFDA-SE in *P. falciparum* erythrocyte invasion phenotyping assays.[7] However, despite its numerous properties, DDAO-SE has recently been discontinued (Thermo Fisher Scientific, United Kingdom), hence the need for an alternative dye that might help free up the green channel for multi-color assays. CTFR, a newly developed amine-reactive dye (Thermo Fisher Scientific, United Kingdom), is excited by the red channel at 640 nm and can be used in red-laser equipped flow cytometers. CTFR has been proposed as a candidate for monitoring early events of bacterial invasion,[15] but also as an alternative to dyes with limited spectral properties such as CFDA-SE in cell proliferation assays.[14] However, the use of CTFR in flow cytometry-based *P. falciparum* assays, and particularly the effect of the dye on the parasites' invasion or subsequent intraerythrocytic development are yet to be reported. In this study, we used a dye-dilution approach to assess the applicability and limitations of erythrocyte staining with CTFR in *P. falciparum* erythrocyte invasion phenotyping assays. Using both DDAO-SE and CFDA-SE as comparators, the suitability of CTFR was evaluated by assessing the strengths and weaknesses of the dye with regard to its spectral properties, the persistence of staining as well as toxicity to *P. falciparum* invasion and development.

Materials and methods

Ethics statement

The use of human blood for this study was approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research, University of Ghana and the Ghana Health Services Ethical Review Committee. Blood samples were obtained from healthy donors after they have endorsed a written informed consent form.

Labeling dyes

The properties of the different dyes used in this study are summarized in Table 1. Except for DDAO-SE, a generous gift from Dr Amy K. Bei, all dyes were purchased from Thermo Fisher Scientific, United Kingdom. CTFR (cat# C34564) and CFDA-SE (cat# V122883) were obtained in 15 and 50 μg lyophilized vials, respectively. Stock concentrations of 1 and 5 mM were respectively made by the addition of 20 and 180 μL dimethyl sulfoxide (DMSO), according to the manufacturer's recommendations. DDAO-SE was also received in a 50 μg lyophilized vial and was reconstituted into 19.8 μL DMSO to obtain 5 mM initial concentration. Nucleic acid dyes, SYBR Green I (cat# 668120), and Hoechst 33342 (cat# 62249) were purchased in solutions of 10,000× and 20 mM concentrations, respectively. For all dyes, working concentrations were made by the addition of adequate volumes of either RPMI 1640 or 1× phosphate buffer saline (PBS).

Staining with DDAO-SE and CFDA-SE

DDAO-SE and CFDA-SE staining procedures were adapted from Theron *et al*.[7] Briefly, freshly washed O^+ erythrocytes were suspended at 4% hematocrit with either 10 μM DDAO-SE or 20 μM CFDA-SE in RPMI 1640. The suspension was protected from any source of light and incubated at 37°C in a shaking incubator (150 r/min) for 2 h. The cells were subsequently pelleted by centrifugation at 2000 r/min and incubated for 10 min at room temperature in complete parasite medium (CPM), RPMI 1640 medium supplemented with 0.5% Albumax II (Gibco, Life Technologies, New Zealand), 2 mg/mL sodium bicarbonate and 50 μg/mL Gentamycin (Sigma, United Kingdom), and 2% normal human serum (NHS) to quench the excess of the dyes. The pellets were finally washed three times at 2000 r/min for 3 min with RPMI 1640, suspended at 25% hematocrit in CPM (without NHS), and stored at 4°C until use.

Staining with CTFR

CTFR was serially diluted with either RPMI 1640 or PBS to obtain working concentrations of 1, 2, and 5 μM (Table 1). Erythrocytes were suspended with the CTFR staining solution and incubated at 37°C in a shaking incubator (150 r/min). Cells were subsequently pelleted by centrifugation at 2000 r/min for 3 min, suspended in CPM containing 2% NHS, and incubated at room temperature for 10 min. The suspension was then centrifuged for 3 min at 2000 r/min and the pellet washed thrice with RPMI 1640 or

Table 1. Dye properties and their associated flow cytometer settings.

Staining parameters					Flow cytometer settings				
Dyes (form)	Diluent	Stock concentration	Working concentration	Staining properties	Excitation (nm)	Emission (nm)	Laser line (nm)	Filter sets	PMT (V)[a]
CFDA-SE (lyophilized)	DMSO	5 mM	20 μM	Cytoplasmic	492	517	Blue (488)	530/30 (505LP)	452
DDAO-SE (lyophilized)	DMSO	5 mM	10 μM	Cytoplasmic	648	657	Red (640)	670/30	505
CT far-red (lyophilized)	DMSO	1 mM	1, 2, or 5 μM	Cytoplasmic	633	661	Red (640)	670/30	505
Hoechst 33342 (solution)	–	20 mM	1 μM	Nuclear	361	497	UV (355)	450/30 (410LP)	300
SYBR Green I (solution)	–	10,000×	1×	Nuclear	NA	NA	Blue (488)	530/30 (505LP)	350

[a]These settings should be optimized when using a different flow cytometer model.
CFDA-SE : carboxyfluorescein diacetate succinimidyl ester ; DDAO-SE : dichloro dimethyl acridin one succinimidyl ester.

PBS. The resulting pellets were finally suspended in CPM (without NHS) and stored at 4°C until use. To optimize the staining conditions, parameters such as the hematocrit and the incubation time were varied in the preliminary experiments.

Assessment of dye stability

To assess the stability of CTFR-derived fluorescent intensity, the mean fluorescent intensities (MFIs) of erythrocytes labeled with 20 μM of CFDA-SE, 10 μM 0f DDAO-SE, or with different concentrations of CTFR in either PBS or RPMI 1640 were compared to those of unlabeled erythrocytes over time. Both labeled and unlabeled erythrocytes were kept at 4°C in separate tubes after processing. For each measured time point (days 1, 5, 10, and 15 post staining), equal volumes of labeled and unlabeled erythrocytes were mixed together and incubated at 37°C for 24 h. The resulting MFI of each erythrocyte population was assessed by flow cytometry and the stain index of labeled erythrocytes was assessed accordingly.

In vitro culturing and invasion assay set up

P. falciparum laboratory-adapted strains, 3D7 and Dd2, were maintained in culture at 4% hematocrit in complete parasite media and incubated under an atmosphere of 2% O_2, 5.5% CO_2, and 92.5% N_2. The parasites' growth patterns in labeled versus unlabeled erythrocytes were assessed throughout two successive asexual replication cycles. The parasitemias in culture aliquots, taken at 24-h intervals were assessed daily using flow cytometry after Hoechst 33342 staining. For the invasion assays, CTFR-labeled erythrocytes were suspended at 50% hematocrit in different tubes for enzyme treatments with either 250 mU/mL of neuraminidase (*Vibrio cholerae*; Sigma, United Kingdom), or 1 mg/mL of trypsin or chymotrypsin (Sigma, United Kingdom). Invasion assays were performed as described earlier[16] for both parasite strains. Briefly, schizont-infected erythrocytes were mixed with equal volumes of either enzyme-treated or untreated CTFR-labeled erythrocytes at 2% hematocrit in CPM. All assays were performed in triplicates in 96-well plates and incubated in blood gas atmosphere of 2% O_2, 5.5% CO_2, and 92.5% N_2. Parasites were left to grow at 37°C for 18 to 24 h, after which the plates were centrifuged at 1500 r/min for 3 min and the supernatant was discarded. Parasite DNA was then labeled by adding 100 μL of either 1× SYBR Green I or 1 μM Hoechst 33342 and incubated at 37°C in a shaking incubator for an hour. The plates were once more centrifuged, and the cells were washed with 1× PBS after removal of the supernatant. The cells were finally suspended into 1× PBS and the invasion efficiency was determined by flow cytometry. The percentage of erythrocytes positive for CTFR and either SYBR Green I of Hoechst 33342 was recorded as invasion rate.

Microscopy imaging

To ascertain the normal parasite development in labeled erythrocytes, *P. falciparum* culture aliquots, collected at different time-points for two complete asexual replication cycles, were smeared on glass microscope slides, fixed with 100% methanol and stained with 10% Giemsa. The parasites morphologies were assessed using a Cole Palmer light microscope (Vernon Hills, Illinois 60061, USA) coupled to a MoticamBTW8 camera (Motic China Group Co., Ltd). For the confocal imaging, schizont-infected erythrocytes were co-incubated with CTFR-labeled erythrocytes for 48 h in CPM at 37°C. Small aliquots of culture, taken at different time-points, were fixed with 4% paraformaldehyde, stained with SYBR Green I, and then smeared on glass microscope slides. Microscope slides were viewed under oil immersion (40×) on an LSM 800 confocal laser scanning microscope and images were acquired using ZEN 2.6 Blue Software (Carl Zeiss, Germany). All images were processed using Fiji ImageJ 1.52i (National Institutes of Health, USA).

Flow cytometry data acquisition

Labeled erythrocytes were analyzed using a BD LSR Fortessa™ X-20 (BD Biosciences, Belgium). CFDA-stained erythrocytes were excited with a blue laser (488 nm) and detected with a 530/30 (505LP) filter set, while both DDAO and CTFR-stained cells were analyzed with a red laser (640 nm) and detected with 670/30 filter set. SYBR Green I was detected with the blue laser associated to a 530/30 (505LP) filter set and Hoechst 33342 was excited by a UV laser (355 nm) associated to a 450/50 (410LP) filter set (Table 1). No compensation was necessary since the dyes were used separately. Data were acquired with the BD FACSDiva v.8.0 software (BD Biosciences, Oxford,

United Kingdom). Total erythrocytes were gated using 125 and 203 FSC and SSC voltages, respectively, with a threshold of 5000 on the FSC-A. Single erythrocytes were further gated using the forward scatter high (FSC-H) versus the forward scatter area (FSC-A). Data were acquired in triplicates and 100,000 events were recorded for each replicate using a low sample flow rate (12 μL/min).

Data analysis

Flow cytometry data were acquired with the FACS Diva software (BD Biosciences) and exported as FCS files for analysis with FlowJo v10.5.0 (FlowJo, LLC, Ashland OR). A similar gating strategy was applied to all data sets to identify acceptor erythrocytes and to distinguish positive and negative populations. The data were displayed on a logarithmic scale and total erythrocytes population was gated in FSC-A vs. SSC-A plot as the events falling between 0 and 10^5 on both sides. Single cells were gated using the FSC-H vs. FCS-A, from which acceptor erythrocytes were gated based on the fluorescence intensity of the corresponding dye. Data were primarily expressed as median fluorescent intensity (MFI) from which the stain index (SI) was calculated for each dye. SI is a normalized sensitivity metric, used for quantitating the brightness of immunofluorescent reagents through estimation of signal detection over the background. Here, the SI was calculated using the following formula

$$SI\,nonparametric = \frac{3.29 \times (50th\,positive - 50th\,negative)}{95th\,negative - 5th\,negative}$$

To acquire data from invasion phenotyping assays, parasitized erythrocytes were further gated from DDAO-SE or CTFR-labeled cells based on either SYBR Green I or Hoechst 33342 fluorescent intensity. Each condition was tested three times and the data were presented as the mean plus or minus the standard error from the different replicates. Additional calculations and graphs were generated using GraphPad Prism v.7.01 (GraphPad Software, Inc.).

Results

Gating strategy for the selection of CTFR-labeled erythrocytes

Although CTFR has a well-defined emission peak (661 nm; Table 1), its fluorescent signal can be detected with a wide range of filter sets. Here we used filters designed to acquire wavelength derived from Alexa Fluor 647 fluorochrome to detect CTFR-associated signal. Total erythrocytes were primarily gated using the FSC-A and SSC-A parameters (Figure 1(a)) and single erythrocytes (Figure 1(b)) were used to further gate CTFR positive population (Figure 1 (c) and (d)).

CTFR labeling requires minimal dye concentration and short incubation time

The first step for assessing the applicability of CTFR in *P. falciparum* phenotyping assays was to determine the optimal concentration to effectively distinguish the staining intensity over the autofluorescence background. Dye concentrations of 1, 2, and 5 μM were used and the resulting stain index (SI) was compared to that of CFDA-SE and DDAO-SE. Prior to data acquisition, labeled erythrocytes were pre-incubated with mock-labeled erythrocytes (negative control) at 37°C for at least 24 h. As expected, fluorescent intensity shows a dose-dependency with dye concentration, with 5 μM CTFR yielding a higher fluorescent intensity as compared to 1 and 2 μM, which nevertheless yielded distinct fluorescent peaks relative to the negative control (Figure 2(a) to (d)). Additionally, staining erythrocytes at 4% hematocrit yielded a better resolution between positive and negative populations (Figure 2(a) and (c)) relative to 8% hematocrit (Figure 2(b) and (d)). To prepare working concentrations from CTFR stock, we assessed the effectiveness of two different diluents, RPMI 1640 and PBS, to uniformly stain erythrocytes. Overall, similar staining patterns were observed when erythrocytes were stained in either labeling solution at the same dye or cell concentration (Figure 2). However, the data show that staining with RPMI at lower hematocrit gives a better resolution with minimal overlap between different concentrations of dye (Figure 2).

Furthermore, we assessed the minimal incubation time needed to allow the dye to penetrate the cells and develop a detectable positive signal. Strong and relatively similar fluorescent intensities were observed after 20, 40, and 60 min of incubation and a slightly higher MFI observed after 2 h of incubation (Figure SI 1).

CTFR labels erythrocytes with a stable fluorescent intensity

Conducting *P. falciparum* invasion phenotyping assays requires the co-incubation of the acceptor (labeled) with donor erythrocytes (unlabeled), and hence the stability of CTFR within stained cells was of interest. Passive dye-transfer from labeled to unlabeled cells has been reported in cell proliferation studies involving both membrane and cytoplasmic dyes.[17–20] Here, the stability of CTFR stain index was monitored for a period of two weeks and compared to that of DDAO-SE or CFDA-SE-stained cells. Overall, all dyes showed a decrease of SI, which seemed to be progressive over time and irrespective to cell concentration or staining solution in the case of CTFR (Figure 3).

However, staining with 1 μM CTFR in RPMI at 4% hematocrit yielded a more stable SI relative to the rest of the conditions (Figure 3, Figure SI 2). Given the relatively stable background fluorescence observed in unlabeled cells, which overall does not seem to increase over time as expected in case of dye transfer (Supplemental Table 1), our data suggest that the observed variations might be due to other erythrocyte intrinsic properties than to passive dye transfer between cells. This, therefore, indicates that

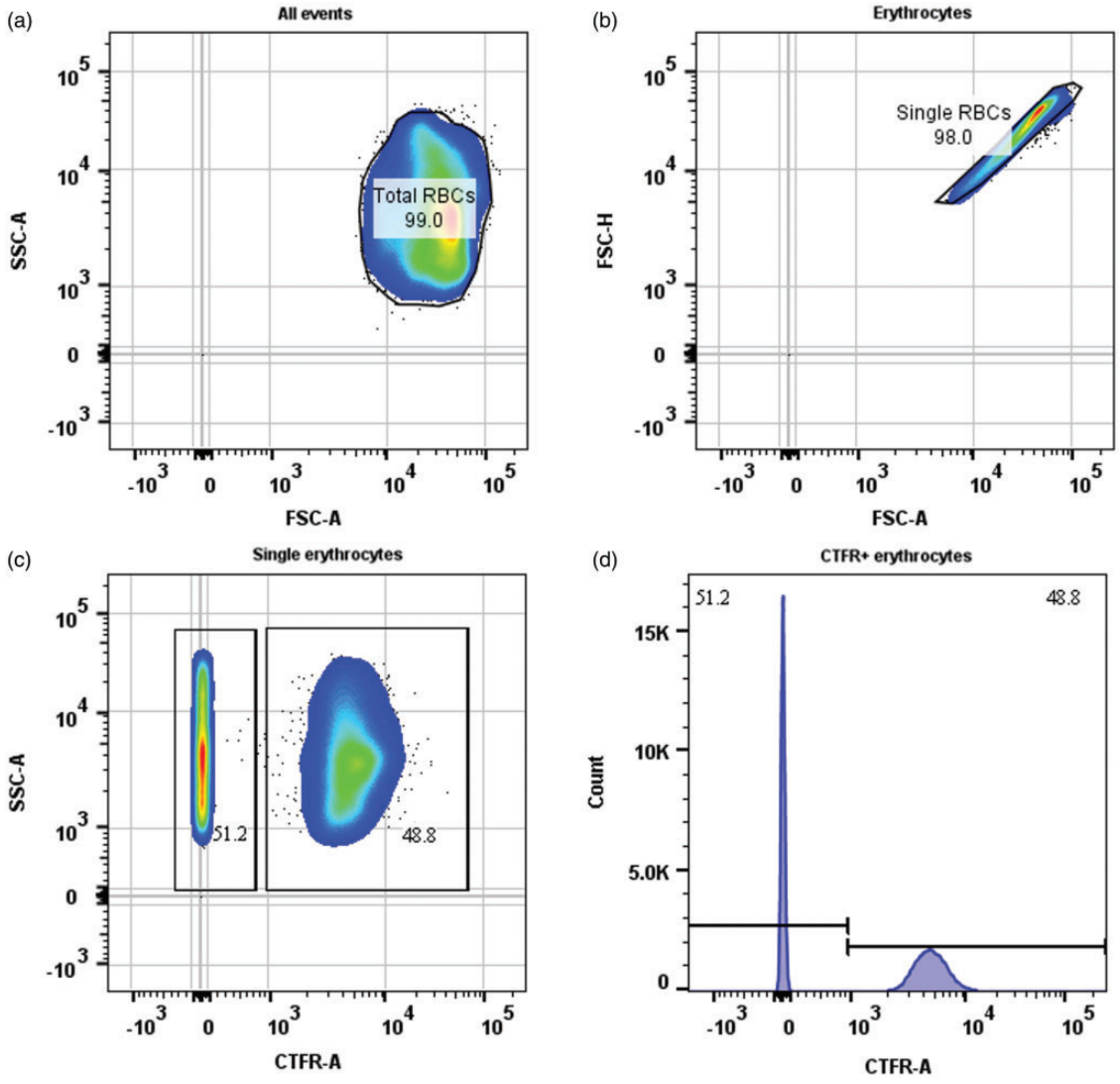

Figure 1. Gating strategy of data generated by CTFR staining. (a): Pseudocolor plot of all events gated in the forward scatter area (FSC-A) by side scatter area (SSC-A). (b): Selection of single erythrocytes using forward scatter hight (FSC-H) by the FSC-A. (c–d): Dot plot and histogram plot showing CTFR fluorescent intensity by the SSC-A and number of erythrocytes count, respectively. PMT voltages were adjusted so that CTFR-stained erythrocytes register between 10^3 and 10^5 on the logarithmic scale. Black dots represent outliers.

CTFR could be appropriate for labeling of acceptor erythrocytes for invasion phenotyping assays.

CTFR labeling does not impair erythrocyte invasion or normal parasite growth

Amine-reactive dyes, such as DDAO-SE and CFDA-SE, have been preferred over membrane dyes because they do not impair parasite invasion.[7] To test whether CTFR affects parasite invasion or early intraerythrocytic development, erythrocytes from tightly synchronized 3D7 and Dd2 cultures were co-incubated with erythrocytes labeled with CFDA-SE, DDAO-SE, or CTFR for two complete asexual cycles. Culture aliquots collected at different time points were analyzed by both flow cytometry and microscopy to assess the invasion rate and parasite morphology, respectively, in both labeled and unlabeled erythrocytes. Overall, there was no significant difference in the parasites' invasion rates into CTFR-labeled and unlabeled erythrocytes (Figure 4(a)). There was also no significant difference in the parasites' growth rates in CTFR-labeled erythrocytes relative to those in CFDA-SE- or DDAO-SE-labeled erythrocytes (Figure 4(a)). Across all assays, the ratio of invasion into labeled/unlabeled erythrocytes was not significantly different from 1 (Supplementary Figure 3 (a) and (b)), therefore suggesting that none of the dyes tested here, particularly CTFR, significantly affects parasite invasion relative to unlabeled erythrocytes. Besides, our data also showed that parasites were able to develop normally within both erythrocyte subpopulations, as they underwent complete intraerythrocytic development with no apparent morphological changes (Figure 4(b), Supplementary Figure 3(c) to (e)). This result mimics early reports regarding the nontoxic effect of CTFR when used to study other cell types,[14,15] but also confirms previous reports regarding the use of amine-reactive dyes in invasion phenotyping assays.[7]

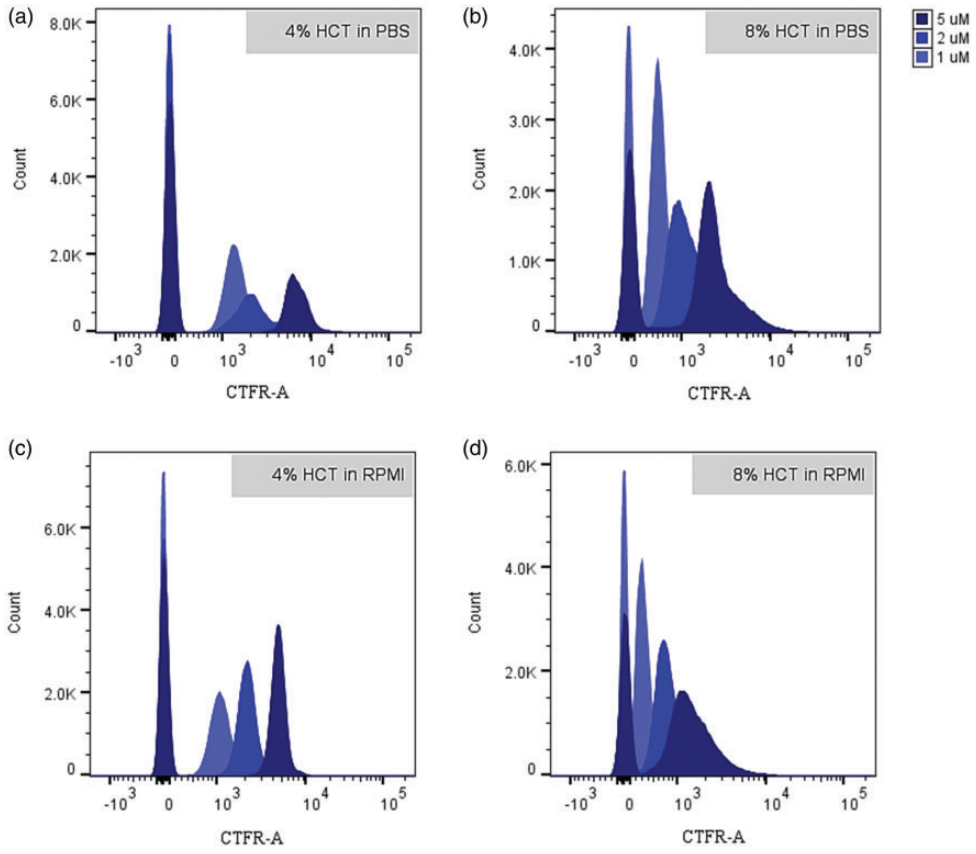

Figure 2. Labeling conditions of acceptor erythrocytes with CTFR. Erythrocytes were labeled using different concentrations of CTFR diluted in different solvents – 1× PBS (a–b) or RPMI 1640 (c–d) – at different hematocrits – 4% (a and c) or 8% (b and d) – and incubated at 37°C for 2 h in a shaking incubator. Labeled erythrocytes were further mixed with unlabeled erythrocytes and analyzed by flow cytometry.

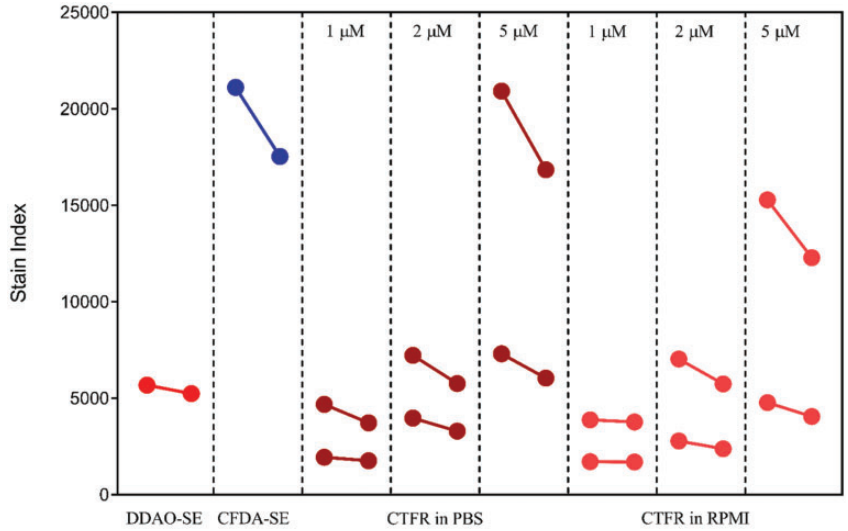

Figure 3. Graph showing the variation of CTFR stain index at two-time points, day 0 and day 15 post-staining. To monitor the stability of CTFR, labeled erythrocytes were co-incubated with unlabeled erythrocytes for at least 24 h prior to data acquisition by flow cytometry. CTFR SI was monitored for 15 days and compared to that of CFDA-SE and DDAO-SE.

A CTFR-based dual-dye flow cytometry assay for P. falciparum invasion phenotyping assays

Appropriate discrimination between infected and uninfected erythrocytes is key for developing a highly sensitive flow cytometry-based *P. falciparum* invasion assay. Based on the previously described approach,[7] we assessed the sensitivity of SYBR Green I and Hoechst 33342, two nucleic acid dyes in differentiating infected and uninfected erythrocytes and showed that both were able to similarly detect infected erythrocytes, with Hoechst 33342 yielding a

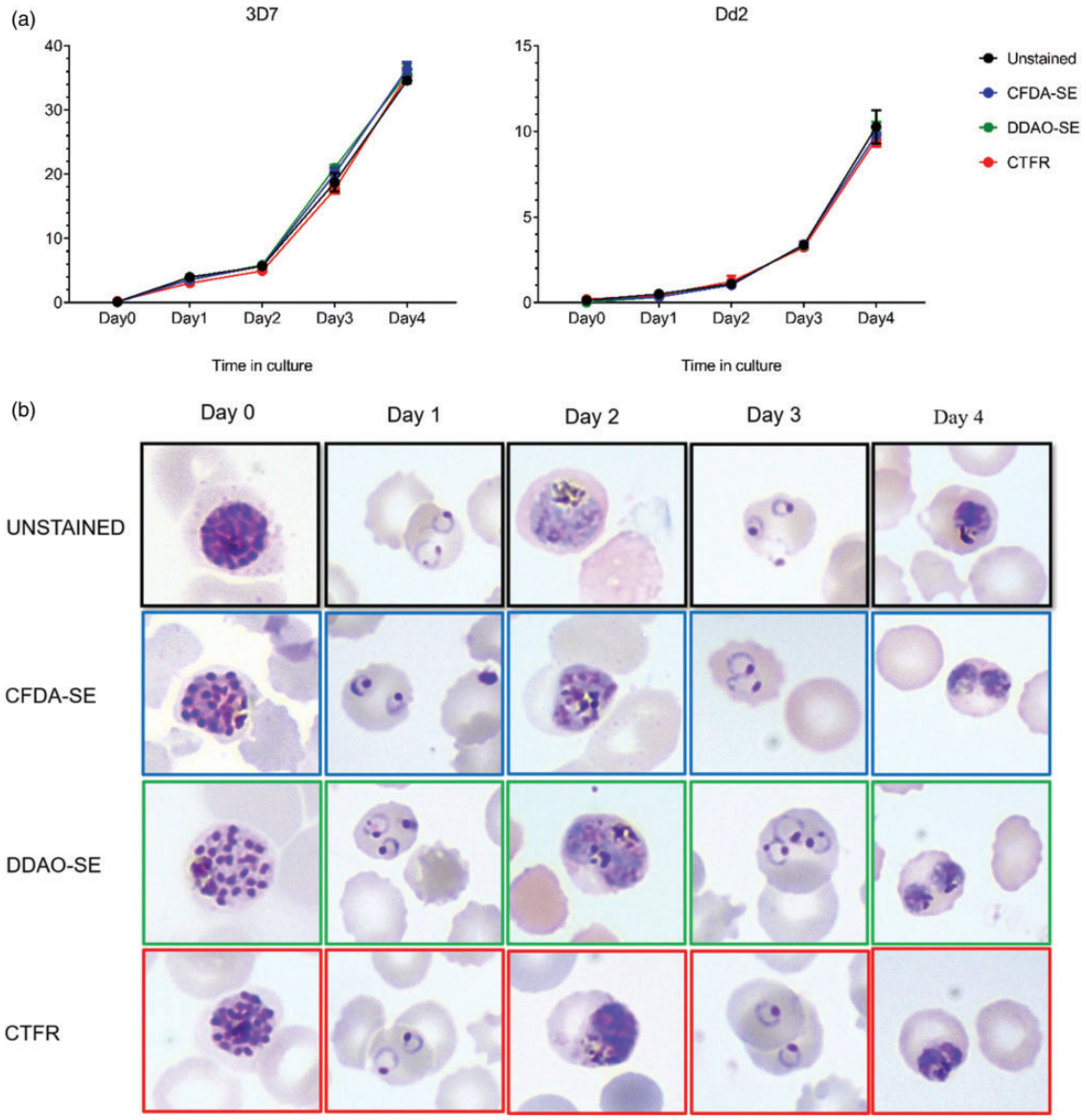

Figure 4. CTFR labeling does not impair *P. falciparum* erythrocyte invasion and normal parasite growth. Erythrocytes labeled with either CFDA-SE (20 μM), DDAO-SE (10 μM), or CTFR (2 μM) were individually incubated with equal volumes of schizont-infected erythrocytes from 3D7 and Dd2 parasite cultures under normal *P. falciparum* culturing conditions. (a) Graphs showing the parasite growth patterns for two successive asexual replication cycles in labeled erythrocytes relative to the unlabeled control erythrocytes. (b) Giemsa-stained parasites showing the parasite morphology in unstained (black squares), CFDA-SE-stained (blue squares), DDAO-SE-stained (green squares), and CTFR-stained erythrocytes (red squares). Culture aliquots harvested at regular time intervals were fixed with 100% methanol and stained with 10% Giemsa on glass microscope slides. Images were taken with a Cole Palmer light microscope (Vernon Hills, Illinois 60061, USA) coupled with a MoticamBTW8 camera (Motic China Group Co., Ltd).

relatively lower level of background staining with uninfected erythrocytes (Supplementary Figure 4). To further confirm the suitability of CTFR for *P. falciparum* invasion phenotyping assays, we used it to assess the invasion phenotypes of two *P. falciparum* laboratory strains, 3D7 and Dd2, with known invasion phenotypes.[21–23] Schizont-infected erythrocytes were co-incubated with either DDAO-SE- or CTFR-labeled and enzyme-treated erythrocytes for 24 h, and infected cells were then labeled post-incubation with either SYBR Green I or Hoechst 33342 to determine the parasitemia. Mock-treated erythrocytes were used as positive controls to estimate the invasion efficiency, wherein the observed parasitemia of a given treatment group was expressed as a percentage of the former one. The effect of enzyme treatment was assessed for each isolate using DDAO-SE or CTFR-labeled erythrocytes. As expected, both isolates retained similar invasion phenotypes following staining with either SYBR Green I or Hoechst 33342, irrespective of the erythrocyte dye (Figure 5). *P. falciparum* 3D7 invaded neuraminidase-treated erythrocytes with higher invasion efficiency (>50%) relative to that of Dd2 (< 50%), therefore, confirming previously published data regarding the degree of sialic acid dependency of these isolates (Figure 5). We also assessed invasion rates into trypsin- and chymotrypsin-treated erythrocytes by both *P. falciparum* isolates. Both isolates showed a relatively high invasion of chymotrypsin-treated erythrocytes (> 50%), while 3D7

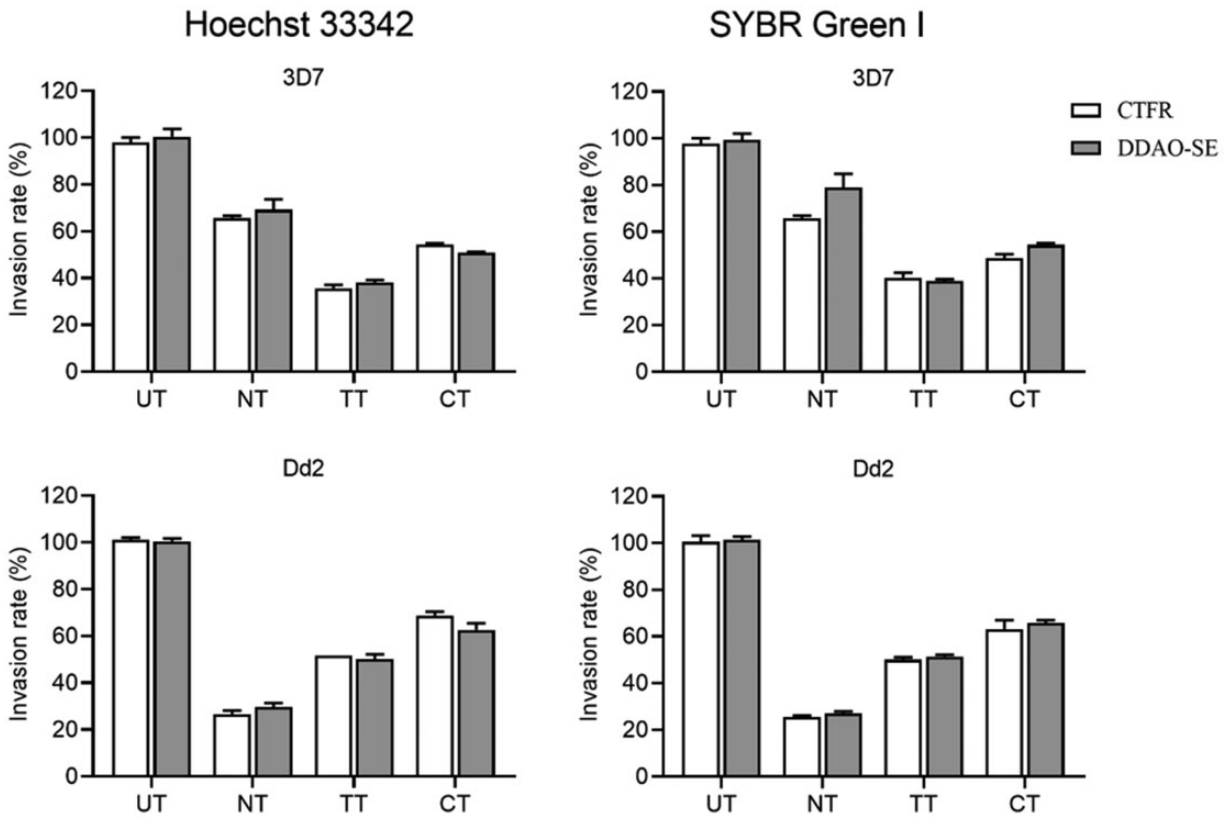

Figure 5. Invasion phenotypes of two *P. falciparum* laboratory strains: 3D7 and Dd2 into enzyme-treated erythrocytes (define abbreviations here). Schizont-infected unlabeled erythrocytes were co-incubated in a 1:1 ratio with either DDAO-SE or CTFR-labeled erythrocytes, at 37°C for 18–24 h. Following incubation, parasites were stained with either Hoechst 33342 or SYBR Green I and final parasitemias in the target populations were determined by gating on the fluorescently labeled erythrocyte population using flow cytometry. Invasion efficiencies were determined as a percentage of the final parasitemia of a mock-treated and labeled positive control erythrocyte population. The data are presented as the mean ± standard error of the mean of two independent experiments conducted in triplicates. UT: untreated; NT: neuraminidase treatment; TT: trypsin treatment; CT: chymotrypsin treatment.

invaded trypsin-treated cells with less efficiency (< 50%) relative to Dd2, which showed invasion rates of above 50% (Figure 5).

Discussion

The pathogenic phase associated with *P. falciparum* infection is initiated following erythrocyte invasion by the merozoite form of the parasite. Erythrocyte invasion is a very complex process and its molecular mechanisms need further understanding, particularly the specific ligand–receptor interactions involved. The development of new flow cytometry-based approaches offers higher sensitivity, specificity, and speed compared to other routinely used technologies such as microscopy.[6] Furthermore, flow-cytometry based approaches have enabled the easy investigation of *P. falciparum* invasion phenotypes in the field of malaria research. One of the advantages of flow cytometry is its ability to provide a platform for high throughput data generation. This study describes a flow cytometry-based assay to assess the suitability of CTFR, a newly developed red excited cytoplasmic dye, for *P. falciparum* erythrocyte invasion phenotyping assays. CTFR can passively diffuse through the plasma membrane to covalently bind to cell cytoplasm and to develop a stable fluorescence.[14] Besides, CTFR has a very narrow emission band 633–661 nm, which allows its usage in multiplex experiments with minimal risk of spectral overlap.[14] To our knowledge, this is the first study that assessed the applicability of CTFR for labeling erythrocytes for merozoites invasion assays. For parasite quantification, previously described DNA dyes, namely SYBR Green I and Hoechst 33342,[7] were used to distinguish between infected and uninfected erythrocytes. To minimize the effect on cell integrity or metabolism, if any, a good cytoplasmic dye should be able to brightly label the acceptor cells with relatively low concentrations. Consequently, we have shown that, even at low concentrations (1 μM), CTFR is able to yield high fluorescence upon excitation with the red laser and this is consistent with a previous report on the labeling of other cell types.[14] Routine *P. falciparum* phenotyping assays involve co-incubation of labeled acceptor erythrocytes with unlabeled donor erythrocytes from the starting culture and therefore necessitate a clear distinction between these two populations of erythrocytes. Confounders, such as dye-transfer between co-incubated positive and negative cells, have previously been shown to affect the sensitivity of cell proliferation assays using cytoplasmic dyes.[17,18] However, such variations were not observed in our study which shows that the dye was well retained in erythrocytes which is in agreement with the dye being carried in mammalians cells for several generations,[24,25] and therefore suggesting its stable

binding to the free amino groups of intracellular macromolecules. Moreover, the preference of cytoplasmic dyes over membrane dyes is determined by their ability to not impair erythrocyte invasion. We showed that CTFR has the ability to efficiently stain erythrocytes without impairing the parasite invasion or development. Furthermore, we also showed that combination of CTFR with either SYBR Green I or Hoechst 33342 reveals some common features of the CTFR with DDAO-SE in terms of spectral properties as previously reported.[7] Besides, our study also reports similar rates of merozoites invasion between CTFR and DDAO-SE. This further reiterates the ability of CTFR to be used as an alternative for DDAO-SE in flow cytometry-based *P. falciparum* invasion assays. Our assay is optimized for utilization with instruments that are not equipped to simultaneously excite CFDA-SE and Hoechst 33342, but also for experiments where there is the need to free-up the green channel (e.g. experiments requiring SYBR Green or GFP detection). In conclusion, we have optimized a sensitive CTFR-based dual-dye flow cytometry assay for assessing the invasion phenotypic diversity of *P. falciparum* and providing highly reliable and reproducible data comparable to existing assays. Also, given the ability of CTFR to stain acceptor erythrocytes at relatively low concentrations, combined to its narrow emission peak, it would be a dye of choice to consider for more complex experimental designs, such as the recently developed erythrocyte preference assay[26] that requires dyes able to provide scalable assay design.

Authors' contributions: LGT, YA and GAA conceived the study; LGT, EBQ and JKD performed the experiments LGT, GAA and YA analyzed the data and drafted the manuscript; YA, GAA, MN, KA and TMG supervised the study. All authors critically reviewed and edited the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID IDs

Laty G Thiam https://orcid.org/0000-0001-7086-4726
Gordon A Awandare https://orcid.org/0000-0002-8793-3641

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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