REVIEW

CYTOMETRY
Journal of Quantitative Cell Science PART A

ISAC
INTERNATIONAL SOCIETY FOR ADVANCEMENT OF CYTOMETRY

Live and Let Dye: Visualizing the Cellular Compartments of the Malaria Parasite *Plasmodium falciparum*

Marleen Linzke,[1] Sun Liu Rei Yan,[1] Attila Tárnok,[2] Henning Ulrich,[3] Matthew R. Groves,[4*] Carsten Wrenger[1*]

[1]Unit for Drug Discovery, Department of Parasitology, Institute of Biomedical Sciences, University of São Paulo, Avenida Professor Lineu Prestes 1374, São Paulo, São Paulo, 05508-000, Brazil

[2]Institute for Medical Informatics, Statistics and Epidemiology, Medical Faculty, University Leipzig, D-04107, Härtelstraße 16-18, Leipzig, Germany

[3]Department of Biochemistry, Institute of Chemistry, University of São Paulo, Avenida Professor Lineu Prestes 748, São Paulo, São Paulo, 05508-900, Brazil

[4]Structural Biology Unit, Department of Pharmacy, Faculty of Science and Engineering, University of Groningen, 9713AV, Antonius Deusinglaan 1, AV Groningen, The Netherlands

Received 30 January 2019; Revised 3 October 2019; Accepted 24 October 2019

Grant sponsor: Fundação de Amparo à Pesquisa do Estado de São Paulo, Grant number2012/50393-6, Grant number2012/50880-4, Grant number2014/23330-9, Grant number2015/26722-8, Grant number2017/03966-4, Grant number2017/26358-0; Grant sponsor: Rijksuniversiteit Groningen, Grant numberUbbo Emmius student fellowship

*Correspondence to: Matthew R. Groves, Structural Biology Unit, Department of Pharmacy, Faculty of Science and Engineering, University of Groningen 9713 Antonius Deusinglaan 1, AV Groningen, The Netherlands Email: m.r.groves@rug.nl Carsten Wrenger, Unit for Drug Discovery, Department of Parasitology, Institute of Biomedical

Abstract

Malaria remains one of the deadliest diseases worldwide and it is caused by the protozoan parasite *Plasmodium spp*. Parasite visualization is an important tool for the correct detection of malarial cases but also to understand its biology. Advances in visualization techniques promote new insights into the complex life cycle and biology of *Plasmodium* parasites. Live cell imaging by fluorescence microscopy or flow cytometry are the foundation of the visualization technique for malaria research. In this review, we present an overview of possibilities in live cell imaging of the malaria parasite. We discuss some of the state-of-the-art techniques to visualize organelles and processes of the parasite and discuss limitation and advantages of each technique.

Key terms

staining; dyes; GFP; aptamer; malaria

DETECTING *PLASMODIUM SPP.*

Malaria is an infectious disease caused by the protozoan parasite from the genus *Plasmodium*. The disease is common in sub- and tropical regions, threatening around 40% of the population worldwide. About 216 million people were infected by the parasite and an estimated 445,000 malaria deaths were reported in 2016 only (1).

Five species of the genus *Plasmodium* are known to cause human malaria. *P. falciparum* is the most severe one, being responsible for 90% of malaria deaths worldwide. Great efforts in vector control and malaria prevention, as well as an effective drug treatment, achieved the reduction of malaria infections and mortality in recent years. However, increasing resistance against antimalarial drugs endangers the current progress made in malaria control.

Fast diagnosis of malaria and the identification of the infecting species are one of the starting points to determine the correct drug treatment. A variety of malaria detection methods has been proposed, but diagnosis is still commonly performed by visual detection of the intraerythrocytic parasite by transmitted light microscopy of a peripheral blood smear stained with Giemsa stain (2). The staining approach consists of mixing an acidic (Eosin Y) and basic dye (Methylene blue and Azure B) to stain cytoplasm and chromatin, respectively. The technique can determine the parasitemia and plasmodial species in a low-cost and rapid manner and, although other methods have been successfully developed, it remains the preferred diagnostic method for malaria.

Visualization of *Plasmodium* greatly contributed to understanding its biology. With help of new probes, dyes and fluorescent proteins, organelles and dynamic processes and changes in the metabolism of the parasite can be specifically studied.

Sciences, University of São Paulo, Avenida Professor Lineu Prestes 1374, São Paulo, São Paulo 05508-000, Brazil Email: cwrenger@icb.usp.br

Published online 18 November 2019 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.23927

These tools combined with technical advances in microscopy and flow cytometry paved the way for new discoveries in malaria research.

In this review, we will shed light on some of the state-of-the-art techniques to visualize organelles and processes, focusing on the *Plasmodium* parasite. We focus on live cell imaging in combination with fluorescence microscopy or flow cytometry to provide an overview of the most common imaging techniques. Possible limitations and clear advantages of the techniques are also discussed.

THE TOOLS TO VISUALIZE *PLASMODIUM*

To dye or not to dye?

Dyes are colored organic substances that can specifically bind to their substrate, such as cells, organelles, or even molecules. Some basic requirements for dyes are important for biological applications: they should be sensitive enough to track biological processes at nanomolar or micromolar range; resolution has to be sufficient to verify dynamic cell signaling processes and the required instruments should be financially affordable.

In the context of parasitic diseases, some dyes may be able to appropriately stain targeted organelles of the studied parasite, but they can show an unexpected anti-parasitic effect as well. Methylene Blue, used in the Giemsa stain as a dye for nucleic acids, was the first fully synthetic dye used as an antimalarial drug (3). Following drug screenings have identified several synthetic dyes with *in vitro* and *in vivo* activity against asexual stages of the malaria parasite *P. falciparum* (4–6). For example, three mitochondrial dyes—MitoRed, dihexyloxacarbocyanine iodide (DiOC6), and rhodamine B—were highly active (IC_{50} - values <200 nM) and could be a starting point for the development of novel antimalarial lead compounds.

There are several well-characterized natural and commercially available dyes in different spectra, which target specific compartments of the parasite, such as the nucleus, mitochondrion, endoplasmic reticulum or the food vacuole or dynamic processes like oxidative stress or Ca^{2+} effluxes (Table 1).

Dyes interacting with nucleic acid are essential in malaria research and are often used for detection, growth analysis, drug screening, and invasion studies using flow cytometry or fluorescence microscopy. Studies take advantage of the fact that a normal circulating red blood cells (RBCs) lack DNA and thus, parasitized RBCs can be easily distinguished from non-infected RBCs by DNA stains. There exist a wide variety of dyes interacting with the DNA of the cell, either by intercalating with it, for example, ethidium bromide, propidium iodide, and acridine orange, or by binding to it like 4′,6-diamidino-2-phenylindole (DAPI) and the family of Hoechst dyes. However, several of them are not cell-permeable and thus not suited for live cell imaging.

Hoechst 33342 (bisbenzimide) and SYBR Green I are commonly used DNA dyes in malaria research. Hoechst 33342 binds to the minor groove of double-stranded DNA, with preference for adenine and thymine (AT) rich regions (7,8), which are most prevalent in the *Plasmodium* genome (over 80% in most species). It has been successfully used in fluorescence microscopy (9) and flow cytometry (10). However, it was shown that Hoechst 33342 also exhibited a strong *in vitro* antiplasmodial activity at a concentration range from 4.1 nM to 30.8 nM and low cytotoxicity to human HeLa cells (4), which can lead to difficulties in live cell imaging of the parasites. The intercalating dye SYBR Green I is another widely used nucleic acid dye that has been employed for more than a decade as a probe to measure the malaria parasite growth inhibition (10–15). Mostly used in flow cytometry analysis, it provides the chance of studying developmental stages of intraerythrocytic growth of *Plasmodium sp*., their quantification and further determine antimalarial drug candidates' sensitivity for a variety of *Plasmodium* strains. SYBR Green I strongly reacts with double-stranded DNA, but it also has the capacity of staining single stranded DNA and RNA, although to a lesser extent. Treatment with RNAse is recommended by the manufacturers to reduce the background, but sensitivity to RNA is low and can be neglected for live cell imaging (16).

Another nuclei acid dye for flow cytometry analysis is the commercially available Vybrant® DyeCycle™ (ThermoScientific, Waltham, Massachusetts, USA). The dye is structurally related to the family of Hoechst dyes and it comes in several spectra. The dye is normally used for studies of the cell cycle and as an indicator for viable cells. Due to its high sensitivity, it has been used for differentiation of the blood stages in *Plasmodium* (17).

Analysis of the viability of the parasite within the erythrocytes is important to study the effects of drugs on its growth. Analysis of the apoptotic mechanism in *Plasmodium* raised the theory that a number of parasites within the erythrocytes, especially *in vitro* culture, are actually dead (17). Although the parasites may be dead, the erythrocyte stays intact and thus, typical live/dead stains like propidium iodide cannot enter the cell (18). Also, most nucleic acid dyes do not distinguish between dead and live parasites. Stains depending on membrane potentials have been commonly used as indicators for the viability of the parasite. Dyes targeting the mitochondrion, such as Rhodamine 123 and MitoTracker™ can be used for visualization of the mitochondrion of *Plasmodium* (19–29), but they can be also employed as a marker for

viability (23–29). This is due to the unique membrane potential of the organelle with a negative charge inside of the membrane (30). Another dye which targets the mitochondrial membrane potential and that can be used as a viable stain is DiOC6 (3). First used as a marker for the endoplasmic reticulum (31,32), the dye stains membrane bilayers. In *Plasmodium*, the dye has been used as a viability dye in flow cytometry for drug screenings (33). Yet, for both dyes, antiplasmodial effects were also reported (4,5,21,34–36).

Not only used as a viability indicator, MitoTracker™ can also be used for co-localization studies of specific targets within the parasite (20–22). For co-localization, the studied target is normally detected by tagging with GFP or by use of specific antibodies against it and its localization is compared to dyes staining the proper compartments of the parasite. Next to MitoTracker™ for staining of mitochondrion, other commercially dyes are available and have been used in *Plasmodium*. ER-Tracker™ and LysoTracker® are both commercially available in different spectra and highly selective and photostable. Both probes have been used for staining of the endoplasmic reticulum and the food vacuole, respectively (37).

Fluorescent proteins for *Plasmodium* studies

Fluorescent proteins allowed a new approach to shed light on the biochemical processes of the *Plasmodium* parasite. These proteins can be used for live cell imaging, providing clear advantages over immunofluorescence or electron microscopy, as these techniques require a good preservation of the *Plasmodium* samples (38). Fluorescent proteins may also present some limitations: their photophysical properties are not as good as of organic dyes; also, some of them are not very bright or photostable; their expression can vary considerably even within the same cell types or same temperature; moreover, their fluorescence signal can be lost during fixation of the cells, thereby antibodies are required to enhance the signal (39).

The fluorescent protein must be incorporated into the *Plasmodium* parasite by transfection and maintained episomal under drug pressure or integrated into the genome of the parasite. New sets of transfection vectors (40,41) and the improvements of the transfection process (42) improved the efficiency and success in generating transgenic parasite strains.

Undoubtedly, green fluorescent protein (GFP) (43) has opened an avenue to better understand cellular events in several diseases. First used in *P. falciparum* in 1997 (44), GFP facilitated the investigation of protein localization and interaction (45), trafficking between the different compartments of the parasite, visualization of a specific organelle or even the whole parasite during the life cycle (45–50). More recently, the formation of the parasite plasma membrane was elucidated in *Plasmodium berghei* by GFP-tagged localized protein (51), thereby providing data on membrane dynamics in live cell imaging.

However, conjugation of GFP can impair its function and thus lead to impairment in growth. There are reports that GFP can be toxic to cells due to aggregation and phototoxicity. It was also shown that GFP can induce apoptosis in living cells (52). Impairment of the biological function after conjugation with GFP was reported for the glycosylphosphatidylinositol (GPI)-anchored protein of *P. falciparum* (53,54). The protein forms a coat on the surface of developing merozoites and an inducible expression system solved this problem.

Autofluorescence of the erythrocytes can be challenging for studies with GFP. Red fluorescence proteins are favorable because of the reduction of autofluorescence in the red emission range. Also, the long wavelength used for excitation of RFP allows a better penetration of cells and tissues, and consequently intravital imaging for murine malaria parasites.

Malarial parasite proteins have been detected in nearly all compartments within the parasite and beyond (38), including cytosol (55–57), mitochondria (20,57), apicoplast (20,57), food vacuole (45), parasitophorous vacuole, and infected erythrocytes (58). While the destination of proteins can be predicted by their targeting sequence, many groups have used GFP-fusion proteins to study trafficking into the respective organelles within the parasite (45). For example, Waller and collaborators discovered the involvement of the ER in the trafficking of proteins to the apicoplast by selective truncation of the bipartite targeting sequence (46). The bipartite targeting sequence consists of a signal peptide and a plant-like transit peptide. The former leads the protein through the secretory pathway of the ER and the latter is responsible for the transport from the ER to the apicoplast. Recent reports suggest a direct connection between the ER and apicoplast since trafficking of stromal apicoplast proteins are not disrupted by treatment with AlF4-, an inhibitor for G protein-dependent vesicular fusion (59).

Since there is no specific stain available yet for the apicoplast, visualization depends mostly on fluorescent proteins. By targeting it to the apicoplast, the morphology of the organelle has been studied in the asexual stages in *P. falciparum* (60,61) and in the asexual and sexual stages for *P. berghei* (62). Appearing as a small round organelle, the apicoplast undergoes great morphological changes during the erythrocytic stages of the life cycle. During maturation within the erythrocyte, the apicoplast starts to enhance and branches extensively until it eventually divides into the newly formed merozoites. During the whole life cycle, the apicoplast was shown to associate with the mitochondrion most likely due to the metabolic dependency of both organelles and their shared metabolic pathway of biosynthesis of heme (63).

Fluorescence proteins are not only used for localization studies but they can also be used for the detection of subtle changes in a specific milieu within a cell. HyPer and roGFP are mutated versions of known fluorescent proteins, respectively, YFP and GFP, which can be used for the detection of change in redox milieu. For HyPer, the circularly permuted YFP (cpYFP) was fused to OxyR-RD, the H_2O_2-sensing regulatory domain of the transcription factor OxyR from *Escherichia coli* (64). Several versions have been created through single point mutations. There are some concerns whether HyPer is a sole H_2O_2 sensing probe or whether it also interacts with the glutathione system of the cell (65). Another drawback of HyPer is its sensitivity toward pH changes. This can be addressed by using the H_2O_2 insensitive

version of the probe, SypHer (66). In *P. falciparum*, HyPer-3 has been used to study drug-induced changes in H_2O_2 levels in a faster and more sensitive range than the roGFP probe (67). However, the control probe SypHer shows different behavior at a pH higher than 7, so it has to be used with caution.

The fluorescent protein roGFP was created by introduction of a redox-sensitive cysteine, which upon change in the oxidative state leads to a change in fluorescent properties (68). Further point mutation generated more sensitive versions of this redox sensor. While, the probe alone detects changes in the local thiol/disulfide equilibrium, it can also be coupled to redox-sensitive enzymes and signal peptides for detection of specific redox events and to be target to a specific organelle. In *Plasmodium*, the enzymes thiol peroxidase (Orp1) and human glutaredoxin 1 (hGrx1) have been coupled to the redox-sensitive GFP to detect changes in the H_2O_2 and glutathione milieu, respectively (67,69–71). Both probes have been targeted to sub-cellular compartments of the parasite as well by fusing the probe to the signal sequences of the acyl carrier protein (ACP, Apicoplast) and citrate synthase (CS, Mitochondrion) (71,72). The probes are sensitive to even low range changes in the redox milieu, but they might interfere with the redox state due to the episomal transfection *in vivo* and the correlated use of a selection drug to maintain the transfection (69). This can be circumvented by genomically integrating the redox sensor into the parasite, which has been successfully performed and characterized (73).

Hence, all probes discussed above are important indicators for their specific milieu but they do have clear limitations. Monitoring such a fast and delicate process such as redox metabolism requires experiment planning and the use of the most suitable probes in order to provide reliable experimental results for the raised research hypothesis.

FLOW CYTOMETRY: A TECHNIQUE FOR ANALYZING THE *Plasmodium* PARASITE

Flow cytometry enables measurements of optical and fluorescent characteristics of cells or particle populations. The fluid sample is injected into the flow cytometry analyzer where each single cell or particle passes through a light source. The obtained data of the scattered light provide valuable information about biochemical, biophysical, and molecular aspects of the studied sample. Flow cytometry is commonly used in immunophenotyping, measurements of apoptosis or membrane potentials, analysis of the cell cycle or cell proliferation and cell sorting (74).

Flow cytometry analysis in malaria research takes advantage of the fact that normal circulating red blood cells (RBCs) do not contain DNA and parasitized RBCs can be detected by nucleic acid stains. Exceptions are the immature forms of red blood cells (reticulocytes), which contain small amounts of RNA or remnants of DNA in erythrocytes named Howell–Jolly bodies. Although they are rare occurrences in healthy subjects, levels of reticulocytes can change upon infection with malaria and developing of anemia (75). Also, Howell–Jolly bodies that are cleared by the spleen in healthy patients can occur more often in sickle cell disease due to the ongoing decline of splenic function (76).

Flow cytometry has been used for detection of infection, analysis of parasite growth and inhibition of invasion as well as IC_{50} determination of possible antimalarial drugs.

Several nucleic acid stains have been already used for quantification of parasite growth, including ethidium bromide (77–79), Hoechst 33342 (79–81), SYBR Green I (11,81–83), SYTOX Green (84), among others, each with their own advantages and limitations.

Fixation of the parasite might be need for some nucleic stains or when used in combinations with antibodies. Fixation procedures can lead to aggregations of RBCs into clumps in solution (85) or autofluorescence of RBCs, which increase the background of the sample. However, effective fixation procedure has been widely reported and used which circumvent these problems (86–88).

Another additional step might be the treatment with RNases to reduce RNA contaminations and thus the background of the sample, which is recommended for some nucleic acid stains (89).

SYBR Green I is one of the widely used probes in flow cytometry for malaria research due to its high sensitivity and easy to use staining protocol. Bei and collaborators (82) established a flow-cytometer-based method to establish *P. falciparum* virulence by determining both fold increase in parasitemia after each asexual growth and the ratio of observed multiply infected erythrocytes to those predicted by Poisson distribution. The authors showed that SYBR Green I flow cytometry method provided a very high linear correlation to the parasitemia measured by the traditional light microscopy method. For better accuracy in parasitemia measurements, the parasites must be synchronized at ring-stage and the erythrocytes should not harbor more than three parasites each, as the fluorescence emitted by these multiply infected erythrocytes may not be distinguishable when compared to early trophozoite-parasite stage.

High-throughput protocol approach for SYBR Green I is also reported in the literature (11,90). The protocol combines flow cytometry and the SYBR Green I dye, which allows sufficient resolution between the peaks of non-infected RBCs, infected RBCs and white blood cells. Two standard antimalarial drugs (pyrimethamine and chloroquine) were used to validate the results and the determination of and the 50% effective dose (ED_{50}) of both drugs were calculated. A disadvantage of this method is that it requires a longer incubation period—about 30 min—compared to the gold-standard staining protocol.

Other methods using SYBR Green I have been developed such as *ex vivo* methods suitable for both *P. vivax* and *P. falciparum* based on flow cytometry for drug screening, with a sensitivity very close to the traditional microscopy results (91,92). SYBR Green I and hydroethidine (HE) are also reported in the literature for the quantification of red blood cells with a flow-cytometry-based *ex vivo* assay (92). SYBR Green I in combination with dihydroethidium and Hoechst 33342 was also explored as a potential way to quantify and

Table 1. List of reported dyes in the literature for imaging use in *Plasmodium* species

	DYE NAME	$\lambda_{EX}/\lambda_{EM}$ (NM)	REPORTED LOCALIZATION/ FUNCTION	REPORTED TECHNIQUES	*PLASMODIUM* SPECIES	REFERENCES
Nucleic acid stain	DAPI	358/461	Nucleic acid stain	• Cytotoxicity assay • Fluorescence microscopy • Flow cytometry • Immunofluorescence	*falciparum*	(116,117)
	Hoechst Hoechst 33342 Hoechst 33258	 350/461 352/461	Nucleic acid stain	• Flow cytometry • Fluorescence microscopy	*falciparum* *chabaudi* *berghei*	(45,118,119)
	Ethidium bromide	210/605	Nucleic acid stain	• Flow cytometry	*falciparum*	(77–79,120)
	SYBR green I	497/520	Nucleic acid stain	• Cytotoxicity assay • Flow cytometry	*berghei* *falciparum* *yoelli* *knowlesi*	(11,81–83,121)
	PicoGreen	485/530	Nucleic acid stain	• Flow cytometry • Cytotoxicity assay	*falciparum* *vivax*	(122,123)
	SYTO SYTO 16 SYTO 21 SYTO 59 SYTO 61 SYTOX Green	 488/518 494/517 622/645 620/647 504/523	Nucleic acid stain	• Flow cytometry • Fluorescence microscopy • Cell microarray chip • Cytotoxicity assay	*yoelii* *falciparum*	(84,120,124–126)
	YOYO-1	491/509	Nucleic acid stain	• Flow cytometry • Cytotoxicity assay	*falciparum* *yoelii* *berghei* *vinckei* *chabaudi*	(33,127,128)
	Thiazole Orange	512/533	Nucleic acid stain	• Flow cytometry	*falciparum*	(100)
Nucleic acid stain (cont.)	Propidium Iodide	493/636	Nucleic acid stain	• Flow cytometry	*berghei* *falciparum*	(87,129)
	Hydroethidine	518/606	Superoxide indicator Nucleic acid stain	• Flow cytometry	*falciparum*	(130–132)
	Styryl dye 132A 107E 107F	 500/610 500/610 500/610	RNA dyes	• Fluorescence microscopy	*falciparum*	(133)
	Vybrant® DyeCycle™ Violet	369/437	Nucleic acid stain	• Flow cytometry	*falciparum*	(17)
	Acridine Orange	500/526	Nucleic acid stain Digestive vacuole	• Flow cytometry • Fluorescence microscopy	*falciparum*	(134,135)
	Rhodamine 123	505/529	Mitochondrion		*falciparum*	(36)

(Continues)

Table 1. Continued

	DYE NAME	$\lambda_{EX}/\lambda_{EM}$ (NM)	REPORTED LOCALIZATION/ FUNCTION	REPORTED TECHNIQUES	*PLASMODIUM* SPECIES	REFERENCES
				• Fluorescence microscopy • Flow cytometry		
	JC-1	515/529	Mitochondrion	• Flow cytometry • Fluorescence microscopy	*chabaudi* *falciparum*	(136)
Mitochondrial membrane potential	MitoTracker® family		Mitochondrion	• Flow cytometry • High-throughput screening • Fluorescence microscopy	*falciparum* *berghei*	(116,137–139)
	Mitotracker Red CMXRos	579/599				
	MitoTracker Red CM-H2XRos	579/599				
	MitoTracker Green FM	490/516				
	MitoTracker Deep Red	644/665				
	MitoTracker Red FM	581/644				
	MitoTracker Red 580	580/644				
	Mitofluor Green	490/516	Mitochondrion	• Fluorescence microscopy	*chabaudi* *falciparum*	(140)
	DiOC6(3)	482/504	Membrane structure	• Fluorescence microscopy • Spectrofluorimetry	*falciparum*	(33) (45,141)
Lipid and membrane structure	ER-Tracker™		Endoplasmic Reticulum	• Fluorescence microscopy	*falciparum* *vivax*	
	ER-Tracker Green	504/511				
	ER-Tracker Red	587/615				
	ER-Tracker Blue-White DPX	374/430–640				
	BODIPY		Membrane structure	• Fluorescence microscopy • Immunofluorescence	*falciparum* *vivax* *chabaudi*	(141–144)
	BODIPY-TR-Ceramide	589/617	Golgi apparatus			
	BODIPY FL C5 ceramide	503/512	Neutral lipids			
	BODIPY 493/503	480/515	Food vacuole			
	BODIPY 505/515	488/510				
	Nile Red	550/640	Lipid bodies	• Fluorescence microscopy • Immunofluorescence	*falciparum*	(141)
	Lysotracker Red	577/590	Food vacuole	• Fluorescence microscopy	*falciparum*	(145)
Acidic compartment	Lysosensor™		Food vacuole	• Fluorescence microscopy	*falciparum*	(45,141)
	LysoSensor Blue-DND192	373/422				
	Lysosensor Green DND189	443/505				
Undefined compartment	Rhodacyanine derivative SSJ-127		Mitochondrion Apicoplast	• Fluorescence microscopy • Fluorescence microscopy	*berghei* *falciparum*	(146) (147)

(Continues)

Table 1. Continued

	DYE NAME	$\lambda_{EX}/\lambda_{EM}$ (NM)	REPORTED LOCALIZATION/ FUNCTION	REPORTED TECHNIQUES	*PLASMODIUM* SPECIES	REFERENCES
	C120 conjugated to betalamic acid, BtC		Intraparasitic compartment		*berghei* *chabaudi*	
	BCECF-AM	500 (high pH) 450 (low pH) / 531	pH indicator	• Fluorescence microscopy	*falciparum*	(144,148)
Dynamic process	Fluo-4 AM	494/506 (bound)	Ca^{2+} indicator	• Fluorescence microscopy • Spectrofluorimetry	*falciparum* *berghei* *yoelii*	(33,149,150)
	Fura-2 AM	363/512 (free) 335/505 (bound)	Cytosolic Ca^{2+} indicator	• Fluorescence microscopy • Spectrofluorimetry	*falciparum* *berghei*	(149)
	Fura-Red AM	472/657 (free) 436/637 (bound)	Cytosolic Ca^{2+} indicator	• Fluorescence microscopy	*falciparum*	(149,150)
	Mag-fura-2	369/511 (free) 329/508 (bound)	Ca^{2+} indicator	• Fluorescence microscopy • Spectrofluorometry	*falciparum*	(151)
	Rhod-2 AM	552/581 (bound)	Mitochondrial Ca^{2+} indicator	• Fluorescence microscopy	*chabaudi* *falciparum*	(140)
	ThiolTracker™ Violet	405/525	Thiol quantification indicator	• Fluorescence microscopy • Spectrofluorimetry	*falciparum*	(69)
	CM-H2DCFDA	492–495/517–527	General oxidative stress indicator	• Fluorescence microscopy • Flow cytometry • Spectrofluorimetry	*falciparum*	(120)
	APF	490/515	Indicator of $^1O_2$	• Fluorescence microscopy	*falciparum*	(152)
	MitoSOX	510/580	Mitochondrial superoxide indicator	• Fluorescence microscopy	*falciparum*	(153)
	DAR-4 M AM	560/575	Nitrogen species indicator	• Fluorescence microscopy • Spectrofluorimetry	*falciparum*	(153)
	Dihydroethidium	518/606	Superoxide indicator	• Flow cytometry	*falciparum* *vivax*	(91)
	DiBAC4(3)	493/516	Membrane potential indicator	• Spectrofluorimetry	*falciparum*	(154)
	BODIPY 581/591-PC	581/595	Oxidative stress indicator	• Fluorescence microscopy • Flow cytometry	*falciparum*	(155)

stage through flow cytometry a number of malaria parasites in red blood cells in whole blood and *in vitro* parasites (13).

Invasion studies to determine the effect of antibodies or different RBC populations have to carefully distinguish between the donor RBCs and the targeted, treated RBCs. One possibility is to purify the schizont stage via magnetic beads and thus, reduce the contamination of the donor RBCs (93,94). Additionally, several two-color flow cytometry approaches have been developed that utilized dyes of markers to distinguish the target RBCs by fluorescence (15,81,95). The group of Rayner reported a method for staining the targeted RBC population with the intracellular dyes carboxylfluorescein diacetate succinimidyl ester (CFDA-SE) or 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) succinimidyl ester (DDAO-SE) and the nucleic acid dyes Hoechst 33342 or SYBR Green I, respectively. Their study demonstrated that the used dyes are not interfering with the invasion process of *Plasmodium* like reported for surface labeling of the target RBC population (77). Further development of this technique was recently published by Vimonpatranon et al. (15), as an *in vitro* culture system that combines biotin/streptavidin-labeling of different red blood cells populations and fluorescent DNA dyes such as Hoechst 33342 or SYBR Green I. Several concentrations of biotin have been tested which can be easily distinguished by flow cytometry. This combination allowed the article authors to determine the invasion efficacy of malaria parasites in heterogeneous red blood cell populations.

Parasitemia is mostly determined in flow cytometry by staining DNA. Transgenic parasites expressing GFP can be detected and sorted by its fluorescence signal, which can be beneficial during the other stages of the parasite life cycle (96,97).

Another approach in flow cytometry that appears to have an important impact in the early diagnosis of malaria is the in vivo photoacoustic flow cytometry (PAFC). With PAFC, a focused linear laser beam irradiates directly circulating cells in the peripheral vessels through the skin. By using an ultrasound transducer, laser-induced photoacoustic waves can be detected. The fluorescent light can be detected by a photodetector. The main advantage in using PAFC is the high sensitivity: it has a sensitivity of twofold to threefold higher than a conventional *in vitro* flow cytometry approach. (98).

Imaging flow cytometry (IFC) is a high-throughput technique that combines fluorescence microscopy and flow cytometry. This combination allows the measurement of both fluorescence intensities and the spatial distribution of such fluorescence in large sets of cell populations by using light microscopy (99). As the malaria parasite presents different asexual stages of development which includes rings, trophozoites and schizonts, IFC can be a valuable tool for distinguishing such different stages. Dekel and collaborators (100) developed methods of IFC that allowed the detailed morphological quantification of different malarial parasite asexual stages by using both Hoechst and Thiazole Orange dyes.

A recent work from Chia and colleagues (101) demonstrated a proof-of-concept of the high-throughput capacity by using IFC in malarial drug screening. A chemical library of 4,440 compounds at 10 μM was screened, where 245 hits showed effective parasite's digestive-vacuole disruption and about 0.6% of the hits demonstrated a potency at a concentration lower than 1 μM. The cutoff for digestive-vacuole disruption was arbitrarily determined as about a twofold increase from the cells that received phosphate buffered saline (PBS) as negative control. Fluo-4 is a fluorescent Ca^{2+} binding dye that was successfully used by the authors to check the disruption of the vacuole.

For extracellular vesicles (EVs) secreted into the intercellular compartment by *Plasmodium*, recently Ofir-Birin and colleagues (18) monitored the uptake of malaria-derived vesicles by host monocytes. This was performed by using IFC in combination with the use of different specific stains for the cargo compartments, such as Thiazole Orange for RNA-cargo, Ghost Dye UV for protein cargo, and DiI, DiD or DiO for lipid cargo.

As flow cytometer analyzers become financially less expensive and proper training for using them is provided, they become more available in endemic areas, where *ex vivo* fast detection of parasitemia can be performed. The use of flow cytometry allows a rapid screening for drug resistance and therefore the development of new or improved dyes is valuable for malaria research.

DNA AND RNA IN SHAPES: APTAMERS

The recent grow in aptamer research gave way for the development of aptamers not only for therapeutical use but also for biosensing and imaging purposes. The short chained DNA or RNA nucleotides are considered rivals for antibodies due to higher conformational stability, in particular at higher temperature and pH, specificity to targets and convenience of chemical synthesis (102).

Modification of the synthesis process *Systematic Evolution of Ligands by EXponential enrichment* (SELEX) opened the field for more complex targets like whole cells or microorganism (for more information, see review (103)).

By tagging aptamers to radioactive and fluorescent molecules or even nanoparticles, aptamers can be easily used in *in vitro* and *in vivo* imaging of the target. Additionally, aptamers specific to fluorophore or their quenchers can interact with their target and thereby regulate the fluorescence (104,105). Coupling the aptamers with fluorescein isothiocyanate (FITC) or gold particles (AuNPS) have been proven to be useful for visualization of the selected target (102,106).

While aptamers play an increasing role in cancer research (107–109), aptamers against parasites are rare. The few publications studying aptamers and parasites are shown in the literature and most of them are focusing on *Plasmodium*, *Leishmania*, and *Trypanosoma* (110). As an example of aptamers development, A RNA aptamer against live *Trypanosoma cruzi* trypomastigotes was developed which binds with a high affinity against trypomastigotes from different strains but not to epimastigotes (111). *T. cruzi*-binding aptamers have also been used to develop an assay for detection of the parasites at low levels of concentration (112).

In *Plasmodium*, most publications are focusing on the use of aptamers as possible inhibitors for the parasite's growth and few are addressing the topic of detection the parasite. One example is an aptamer against the plasmodial biomarker lactate dehydrogenase (pLDH) of *P. vivax* and *P. falciparum* (112,113). The protein shows high expression level in both the sexual and asexual stages of parasites. The pL1 aptamer was developed as an aptamer-based assay for detection (102). Aggregation of the conjugated AuNPs depends on the level of pLDH and shows a color shift from red to blue in the assay. Using this methodology, parasites were detectable at low concentrations in human blood samples. SELEX was also applied for the selection of RNA aptamers against a conserved protein in *Plasmodium* (*Pf*EMP1), but the focus of the group lies more on therapeutic use of the aptamer then detection of the parasite (114).

Another study reports an improvement of cell-SELEX by combining inertial microfluidics and SELEX to create a platform named I-SELEX which has broad application in aptamer discovery (115). The platform has been tested on *Plasmodium* infected RBC and one of the selected aptamers have been showed to be specific for *Pf*EMP1. Further advances in this highly growing field will facilitate the selection of new aptamers and will lead to new possibilities for their usage in the future.

CONCLUSIONS

Whether to visualize a sub-cellular compartment, verify the localization of a target or even detecting dynamic and changeable processes, there is a variety of imaging probes to choose from. After careful consideration, the optimal probe for each research question can be found in dyes of a wide range of spectra, fluorescent proteins, immunohistochemistry or even aptamers. Therefore, it is important to consider the advantages and disadvantages of each probe. Fluorescent dyes are easy to utilize, fast and usable in fixed and living cells and can detect subcellular compartments. However, some dyes display antimalarial activity, thus they should be used with caution. Dyes are often susceptible to photo bleaching and consequently cannot be used for imaging over long time scales. For this type of experiments, fluorescence proteins are more favorable. Fluorescence proteins are genetically encoded and stably expressed in the cell, either by episomal transfection or by genomic integration. However, conjugation of a protein to fluorescence protein might impair its function or lead to phototoxicity inside the cell. Also, transfection requires usage of a selection drug which might interfere with the process under examination. Both approaches allow imaging of cellular processes over longer periods of time.

Recent advances in flow cytometry, especially the reducing cost and handiness of the analysers, give rise for this technique for the detection of malaria in endemic areas. Flow cytometry prevails over other detection methods like light microscopy by its accuracy and sensitivity. The analysis is fast and can provide information on drug resistant parasite strains.

Additionally, to the established methods, the new field of aptamer research opened new approaches in the imaging of the *Plasmodium* parasite. Aptamers can be easily modified and through the intense selection process are specific for their target. The first successful attempts for detection of malaria parasites have been reported and more will surely follow in the future.

Even with declining mortality, understanding the metabolic and cellular processes of *Plasmodium* parasites is as important as ever. Technical advances in imaging techniques and stronger cooperation between scientific fields will inevitably lead to new discoveries and opportunities in the field of imaging.

ACKNOWLEDGMENTS

The authors would like to acknowledge the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grants 2017/26358-0 to SY, 2014/23330-9 to M.L., 2012/50880-4, 2012/50393-6 to H.U. and 2017/03966-4, 2015/26722-8 to C.W.) and the Brazilian National Council for Scientific and Technological Development (CNPq) for financial support. The work on aptamer selection distinguishing between *P. falciparum*-infected and uninfected erythrocytes work was supported by a FAPESP-BMBF network grant. ML is a recipient of an *Ubbo Emmius* student fellowship and M.L. and S.L.R.Y. are part of the USP/RUG PhD double degree program. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

LITERATURE CITED

1. WHO. World Malaria Report 2016. 2017:2017.
2. Barcia JJ. The giemsa stain: Its history and applications. Int J Surg Pathol 2007;15: 292–296.
3. Schirmer RH, Adler H, Pickhardt M, Mandelkow E. Lest we forget you—methylene blue…. Neurobiol Aging 2011;32:2325.e7–2325.e16.
4. Joanny F, Held J, Mordmüller B. In vitro activity of fluorescent dyes against asexual blood stages of *Plasmodium falciparum*. Antimicrob Agents Chemother 2012; 56:5982–5985.
5. Gebru T, Mordmüller B, Heldb J. Effect of fluorescent dyes on in vitro-differentiated, late-stage *Plasmodium falciparum* gametocytes. Antimicrob Agents Chemother 2014;58:7398–7404.
6. Vennerstrom JL, Makler MT, Angerhofer CK, Williams JA. Antimalarial dyes revisited: Xanthenes, azines, oxazines, and thiazines. Antimicrob Agents Chemother 1995;39:2671–2677.
7. Crissman HA, Hirons GT. Staining of DNA in live and fixed cells. Methods Cell Biol 1994;41:195–209.
8. Portugal J, Waring MJ. Assignment of DNA binding sites for 4′,6-diamidine-2-phenylindole and bisbenzimide (Hoechst 33258). A comparative footprinting study. Biochim Biophys Acta 1988;949:158–168.
9. Sturm A, Graewe S, Franke-Fayard B, Retzlaff S, Bolte S, Roppenser B, Aepfelbacher M, Janse C, Heussler V. Alteration of the parasite plasma membrane and the parasitophorous vacuole membrane during exo-erythrocytic development of malaria parasites. Protist 2009;160:51–63.
10. Chiba P, Pferschy S, Vossen MG, Noedl H. The SYBR green I malaria drug sensitivity assay: Performance in low parasitemia samples. Am J Trop Med Hyg 2010; 82:398–401.
11. Izumiyama S, Omura M, Takasaki T, Ohmae H, Asahi H. Plasmodium falciparum: Development and validation of a measure of intraerythrocytic growth using SYBR green I in a flow cytometer. Exp Parasitol 2009;121:144–150.
12. Johnson JD, Dennull RA, Gerena L, Lopez-Sanchez M, Roncal NE, Waters NC. Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening. Antimicrob Agents Chemother 2007;51:1926–1933.
13. Malleret B, Claser C, Ong ASM, Suwanarusk R, Sriprawat K, Howland SW, Russell B, Nosten F, Rénia L. A rapid and robust tri-color flow cytometry assay for monitoring malaria parasite development. Sci Rep 2011;1:118.
14. Cheruiyot AC, Auschwitz JM, Lee PJ, Yeda RA, Okello CO, Leed SE, Talwar M, Murthy T, Gaona HW, Hickman MR, et al. Assessment of the worldwide antimalarial resistance network standardized procedure for *in vitro* malaria drug sensitivity testing using SYBR green assay for field samples with various initial parasitemia levels. Antimicrob Agents Chemother 2016;60:2417–2424.

15. Vimonpatranon S, Chotivanich K, Sukapirom K, Lertjuthaporn S, Khowawisetsut L, Pattanapanyasat K. Enumeration of the invasion efficiency of *Plasmodium falciparum* in vitro in four different red blood cell populations using a three-color flow cytometry-based method. Cytometry 2019;95A: 737–745.
16. Suzuki T, Fujikura K, Higashiyama T, Takata K. DNA staining for fluorescence and laser confocal microscopy. J Histochem Cytochem 1997;45:49–53.
17. Philipp S, Oberg H-H, Janssen O, Leippe M, Gelhaus C. Isolation of erythrocytes infected with viable early stages of *Plasmodium falciparum* by flow cytometry. Cytometry 2012;81A:1048–1054.
18. Ofir-Birin Y, Abou karam P, Rudik A, Giladi T, Porat Z, Regev-Rudzki N. Monitoring extracellular vesicle cargo active uptake by imaging flow cytometry. Front Immunol 2018;9:1011.
19. Tanabe K. Staining of *Plasmodium yoelii*-infected mouse erythrocytes with the fluorescent dye rhodamine 123. J Protozool 1983;30:707–710.
20. Sato S, Rangachari K, Wilson RJM. Targeting GFP to the malarial mitochondrion. Mol Biochem Parasitol 2003;130:155–158.
21. Roberts CW, Roberts F, Henriquez FL, Akiyoshi D, Samuel BU, Richards TA, Milhous W, Kyle D, McIntosh L, Hill GC, et al. Evidence for mitochondrial-derived alternative oxidase in the apicomplexan parasite *Cryptosporidium parvum*: A potential anti-microbial agent target. Int J Parasitol 2004;34:297–308.
22. Pornthanakasem W, Kongkasuriyachai D, Uthaipibull C, Yuthavong Y, Leartsakulpanich U. Plasmodium serine hydroxymethyltransferase: Indispensability and display of distinct localization. Malar J 2012;11:387.
23. Abrahem A, Certad G, Pan XQ, Georges E. Pleiotropic resistance to diverse antimalarials in actinomycin D-resistant *Plasmodium falciparum*. Biochem Pharmacol 2000;59:1123–1132.
24. Katao M, Izumo A, Tanabe K. Vital staining of *Plasmodium falciparum* with cationic fluorescent rhodamine dyes. J Parasitol 1987;73:1058–1059.
25. Peatey CL, Chavchich M, Chen N, Gresty KJ, Gray KA, Gatton ML, Waters NC, Cheng Q. Mitochondrial membrane potential in a small subset of artemisinin-induced dormant *Plasmodium falciparum* parasites in vitro. J Infect Dis 2015;212: 426–434.
26. Totino PRR, Daniel-Ribeiro CT, Corte-Real S, de Fátima Ferreira-da-Cruz M. *Plasmodium falciparum*: Erythrocytic stages die by autophagic-like cell death under drug pressure. Exp Parasitol 2008;118:478–486.
27. Tiendrebeogo RW, Adu B, Singh SK, Dodoo D, Dziegiel MH, Mordmüller B, Nébié I, Sirima SB, Christiansen M, Theisen M, et al. High-throughput tri-colour flow cytometry technique to assess Plasmodium falciparum parasitaemia in bioassays. Malar J. 2014;13:412
28. Amaratunga C, Neal AT, Fairhurst RM. Flow cytometry-based analysis of artemisinin-resistant *Plasmodium falciparum* in the ring-stage survival assay. Antimicrob Agents Chemother 2014;58:4938–4940.
29. Painter HJ, Morrisey JM, Vaidya AB. Mitochondrial electron transport inhibition and viability of intraerythrocytic *Plasmodium falciparum*. Antimicrob Agents Chemother 2010;54:5281–5287.
30. McBride HM, Neuspiel M, Wasiak S. Mitochondria: More than just a powerhouse. Curr Biol 2006;16:R551–R560.
31. Quader H, Schnepf E. Endoplasmic reticulum and cytoplasmic streaming: Fluorescence microscopical observations in adaxial epidermis cells of onion bulb scales. Protoplasma 1986;131:250–252.
32. Terasaki M, Song J, Wong JR, Weiss MJ, Chen LB. Localization of endoplasmic reticulum in living and glutaraldehyde- fixed cells with fluorescent dyes. Cell 1984; 38:101–108.
33. Silva GNS, Schuck DC, Cruz LN, Moraes MS, Nakabashi M, Gosmann G, Garcia CRS, Gnoatto SCB. Investigation of antimalarial activity, cytotoxicity and action mechanism of piperazine derivatives of betulinic acid. Trop Med Int Health 2015;20:29–39.
34. Tanabe K. Inhibitory effect of rhodamine 123 on the growth of the rodent malaria parasite, *Plasmodium yoelii*. J Protozool 1984;31:310–313.
35. Izumo A, Tanabe K. Inhibition of in vitro growth of *Plasmodium falciparum* by a brief exposure to the cationic rhodamine dyes. Ann Trop Med Parasitol 1986;80: 299–305.
36. Divo AA, Geary TG, Jensen JB, Ginsburg H. The mitochondrion of *Plasmodium falciparum* visualized by rhodamine 123 fluorescence. J Protozool 1985;32: 442–446.
37. Yeoman JA, Hanssen E, Maier AG, Klonis N, Maco B, Baum J, Turnbull L, Whitchurch CB, Dixon MWA, Tilley L. Tracking glideosome-associated protein 50 reveals the development and organization of the inner membrane complex of *Plasmodium falciparum*. Eukaryot Cell 2011;10:556–564.
38. Tilley L, McFadden G, Cowman A, Klonis N. Illuminating *Plasmodium falciparum*-infected red blood cells. Trends Parasitol 2007;23:268–277.
39. Swenson ES, Price JG, Brazelton T, Krause DS. Limitations of green fluorescent protein as a cell lineage marker. Stem Cells 2007;25:2593–2600.
40. Tonkin CJ, van Dooren GG, Spurck TP, Struck NS, Good RT, Handman E, Cowman AF, McFadden GI. Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. Mol Biochem Parasitol 2004;137:13–21.
41. Waterkeyn JG, Crabb BS, Cowman AF. Transfection of the human malaria parasite *Plasmodium falciparum*. Int J Parasitol 1999;29:945–955.
42. Hasenkamp S, Russell KT, Horrocks P. Comparison of the absolute and relative efficiencies of electroporation-based transfection protocols for *Plasmodium falciparum*. Malar J 2012;11:210.
43. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science 1994;263:802–805.
44. VanWye JD, Haldar K. Expression of green fluorescent protein in *Plasmodium falciparum*. Mol Biochem Parasitol 1997;87:225–229.
45. Müller IB, Knöckel J, Eschbach M-LL, Bergmann B, Walter RD, Wrenger C. Secretion of an acid phosphatase provides a possible mechanism to acquire host nutrients by *Plasmodium falciparum*. Cell Microbiol 2010;12:677–691.
46. Waller RF, Reed MB, Cowman AF, McFadden GI. Protein trafficking to the plastid of *Plasmodium falciparum* is via the secretory pathway. EMBO J 2000;19:1794–1802.
47. Lopez-Estraño C, Bhattacharjee S, Harrison T, Haldar K. Cooperative domains define a unique host cell-targeting signal in *Plasmodium falciparum*-infected erythrocytes. Proc Natl Acad Sci U S A 2003;100:12402–12407.
48. Stanway RR, Mueller N, Zobiak B, Graewe S, Froehlke U, Zessin PJM, Aepfelbacher M, Heussler VT. Organelle segregation into Plasmodium liver stage merozoites. Cell Microbiol 2011;13:1768–1782.
49. Angrisano F, Delves MJ, Sturm A, Mollard V, McFadden GI, Sinden RE, Baum J. A GFP-Actin reporter line to explore microfilament dynamics across the malaria parasite lifecycle. Mol Biochem Parasitol 2012;182:93–96.
50. Heiny SR, Pautz S, Recker M, Przyborski JM. Protein traffic to the *Plasmodium falciparum* apicoplast: Evidence for a sorting branch point at the Golgi. Traffic 2014;15:1290–1304.
51. Burda P-C, Schaffner M, Kaiser G, Roques M, Zuber B, Heussler VT. A Plasmodium plasma membrane reporter reveals membrane dynamics by live-cell microscopy. Sci Rep 2017;7:9740.
52. Liu H-S, Jan M-S, Chou C-K, Chen P-H, Ke N-J. Is green fluorescent protein toxic to the living cells? Biochem Biophys Res Commun 1999;260:712–717.
53. Proellocks NI, Kovacevic S, Ferguson DJP, Kats LM, Morahan BJ, Black CG, Waller KL, Coppel RL. Plasmodium falciparum Pf34, a novel GPI-anchored rhoptry protein found in detergent-resistant microdomains. Int J Parasitol 2007;37: 1233–1241.
54. Meissner M, Krejany E, Gilson PR, de Koning-Ward TF, Soldati D, Crabb BS. Tetracycline analogue-regulated transgene expression in *Plasmodium falciparum* blood stages using toxoplasma gondii transactivators. Proc Natl Acad Sci U S A 2005; 102:2980–2985.
55. Müller IB, Wu F, Bergmann B, Knöckel J, Walter RD, Gehring H, Wrenger C. Poisoning pyridoxal 5-phosphate-dependent enzymes: A new strategy to target the malaria parasite *Plasmodium falciparum* Lewin A, editor. PLoS One 2009;4:e4406.
56. Ma J, Rahlfs S, Jortzik E, Heiner Schirmer R, Przyborski JM, Becker K. Subcellular localization of adenylate kinases in *Plasmodium falciparum*. FEBS Lett 2012;586: 3037–3043.
57. Knöckel J, Bergmann B, Müller IB, Rathaur S, Walter RD, Wrenger C. Filling the gap of intracellular dephosphorylation in the *Plasmodium falciparum* vitamin B1 biosynthesis. Mol Biochem Parasitol 2008;157:241–243.
58. Petersen W, Külzer S, Engels S, Zhang Q, Ingmundson A, Rug M, Maier AG, Przyborski JM. J-dot targeting of an exported HSP40 in *Plasmodium falciparum*-infected erythrocytes. Int J Parasitol 2016;46:519–525.
59. Chaudhari R, Dey V, Narayan A, Sharma S, Patankar S. Membrane and luminal proteins reach the apicoplast by different trafficking pathways in the malaria parasite *Plasmodium falciparum*. PeerJ 2017;5:e3128–e3128.
60. Van Dooren GG, Marti M, Tonkin CJ, Stimmler LM, Cowman AF, McFadden GI. Development of the endoplasmic reticulum, mitochondrion and apicoplast during the asexual life cycle of *Plasmodium falciparum*. Mol Microbiol 2005;57:405–419.
61. Okamoto N, Spurck TP, Goodman CD, McFadden GI. Apicoplast and mitochondrion in gametocytogenesis of *Plasmodium falciparum*. Eukaryot Cell 2009;8: 128–132.
62. Stanway RR, Witt T, Zobiak B, Aepfelbacher M, Heussler VT. GFP-targeting allows visualization of the apicoplast throughout the life cycle of live malaria parasites. Biol Cell 2009;101:415–435.
63. Ke H, Sigala PA, Miura K, Morrisey JM, Mather MW, Crowley JR, Henderson JP, Goldberg DE, Long CA, Vaidya AB. The heme biosynthesis pathway is essential for *Plasmodium falciparum* development in mosquito stage but not in blood stages. J Biol Chem 2014;289:34827–34837.
64. Van Dyk TK, Wei Y, Hanafey MK, Dolan M, Reeve MJG, Rafalski JA, Rothman-Denes LB, LaRossa RA. A genomic approach to gene fusion technology. Proc Natl Acad Sci 2001;98:2555–2560.
65. Lukyanov KA, Belousov VV. Genetically encoded fluorescent redox sensors. Biochim Biophys Acta - Gen Subj 1840;2014:745–756.
66. Poburko D, Santo-Domingo J, Demaurex N. Dynamic regulation of the mitochondrial proton gradient during cytosolic calcium elevations. J Biol Chem 2011;286: 11672–11684.
67. Rahbari M, Rahlfs S, Jortzik E, Bogeski I, Becker K. H2O2 dynamics in the malaria parasite *Plasmodium falciparum*. PLoS One 2017;12:e0174837.
68. Hanson GT, Aggeler R, Oglesbee D, Cannon M, Capaldi RA, Tsien RY, Remington SJ. Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators. J Biol Chem 2004;279:13044–13053.
69. Mohring F, Jortzik E, Becker K. Comparison of methods probing the intracellular redox milieu in *Plasmodium falciparum*. Mol Biochem Parasitol 2016;206:75–83.
70. Kasozi D, Mohring F, Rahlfs S, Meyer AJ, Becker K. Real-time imaging of the intracellular glutathione redox potential in the malaria parasite *Plasmodium falciparum*. PLoS Pathog 2013;9:1–18.
71. Mohring F, Rahbari M, Zechmann B, Rahlfs S, Przyborski JM, Meyer AJ, Becker K. Determination of glutathione redox potential and pH value in subcellular compartments of malaria parasites. Free Radic Biol Med 2017;104:104–117.
72. Rahbari M, Rahlfs S, Przyborski JM, Schuh AK, Hunt NH, Fidock DA, Grau GE, Becker K. Hydrogen peroxide dynamics in subcellular compartments of malaria parasites using genetically encoded redox probes. Sci Rep 2017;7:10449.

73. Schuh AK, Rahbari M, Heimsch K, Mohring F, Gabryszewski S, Weder S, Buchholz K, Rahlfs S, Fidock DA, Becker K. Stable integration and comparison of hGrx1-roGFP2 and sfroGFP2 redox probes in the malaria parasite *Plasmodium falciparum*. ACS Infect Dis 2018;4:1601–1612.
74. Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: Basic principles and applications. Crit Rev Biotechnol 2017;37:163–176.
75. Leowattana W, Krudsood S, Tangpukdee N, Brittenham G, Looareesuwan S. Defective erythropoietin production and reticulocyte response in acute *Plasmodium falciparum* malaria-associated anemia. Southeast Asian J Trop Med Public Health 2008;39:581–588.
76. Ware RE, Dertinger S, Howard TA, Zimmerman SA. Quantitative analysis of Howell-jolly bodies in children with sickle cell disease. Exp Hematol. 2007;35: 179–183.
77. Gomez-Escobar N, Amambua-Ngwa A, Walther M, Okebe J, Ebonyi A, Conway DJ. Erythrocyte invasion and merozoite ligand gene expression in severe and mild *Plasmodium falciparum* malaria. J Infect Dis 2010;201:444–452.
78. Baum J, Maier AG, Good RT, Simpson KM, Cowman AF. Invasion by *P. falciparum* merozoites suggests a hierarchy of molecular interactions. PLoS Pathog 2005;1:e37.
79. Grimberg BT, Jaworska MM, Hough LB, Zimmerman PA, Phillips JG. Addressing the malaria drug resistance challenge using flow cytometry to discover new antimalarials. Bioorg Med Chem Lett 2009;19:5452–5457.
80. Grimberg BT, Erickson JJ, Sramkoski RM, Jacobberger JW, Zimmerman PA. Monitoring *Plasmodium falciparum* growth and development by UV flow cytometry using an optimized Hoechst-thiazole orange staining strategy. Cytometry A 2008; 73:546–554.
81. Theron M, Hesketh RL, Subramanian S, Rayner JC. An adaptable two-color flow cytometric assay to quantitate the invasion of erythrocytes by Plasmodium falciparum parasites. Cytometry A. 2010;77:1067–1074
82. Bei AK, Desimone TM, Badiane AS, Ahouidi AD, Dieye T, Ndiaye D, Sarr O, Ndir O, Mboup S, Duraisingh MT. A flow cytometry-based assay for measuring invasion of red blood cells by *Plasmodium falciparum*. Am J Hematol 2010;85:234–237.
83. Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob Agents Chemother 2004;48:1803–1806.
84. Chandramohanadas R, Davis PH, Beiting DP, Harbut MB, Darling C, Velmourougane G, Lee MY, Greer PA, Roos DS, Greenbaum DC. Apicomplexan parasites co-opt host calpains to facilitate their escape from infected cells. Science 2009;324:794–797.
85. Morel FMM. Quantitation of human red blood cell fixation by glutaraldehyde. J Cell Biol 1971;48:91–100.
86. Bengtsson D, Sowa KM, Salanti A, Jensen AT, Joergensen L, Turner L, Theander TG, Arnot DE. A method for visualizing surface-exposed and internal PfEMP1 adhesion antigens in *Plasmodium falciparum* infected erythrocytes. Malar J 2008;7:101.
87. Pattanapanyasat K, Webster HK, Udomsangpetch R, Wanachiwanawin W, Yongvanitchit K. Flow cytometric two-color staining technique for simultaneous determination of human erythrocyte membrane antigen and intracellular malarial DNA. Cytometry 1992;13:182–187.
88. Bengtsson DC, Sowa KMP, Arnot DE. Dual fluorescence labeling of surface-exposed and internal proteins in erythrocytes infected with the malaria parasite *Plasmodium falciparum*. Nat Protoc 2008;3:1990–1996.
89. Grimberg BT. XXX. XXX 2011;367:1–16.
90. Somsak V, Srichairatanakool S, Yuthavong Y, Kamchonwongpaisan S, Uthaipibull C. Flow cytometric enumeration of Plasmodium berghei-infected red blood cells stained with SYBR green I. Acta Trop 2012;122:113–118.
91. Russell B, Malleret B, Suwanarusk R, Anthony C, Kanlaya S, Lau YL, Woodrow CJ, Nosten F, Renia L. Field-based flow cytometry for ex vivo characterization of Plasmodium vivax and *P. falciparum* antimalarial sensitivity. Antimicrob Agents Chemother 2013;57:5170–5174.
92. Wirjanata G, Handayuni I, Prayoga P, Apriyanti D, Chalfein F, Sebayang BF, Kho S, Noviyanti R, Kenangalem E, Campo B, et al. Quantification of Plasmodium ex vivo drug susceptibility by flow cytometry. Malar J 2015;14:417.
93. Bates AH, Mu J, Jiang H, Fairhurst RM, Su X. Use of magnetically purified *Plasmodium falciparum* parasites improves the accuracy of erythrocyte invasion assays. Exp Parasitol 2010;126:278–280.
94. Dent AE, Bergmann-Leitner ES, Wilson DW, Tisch DJ, Kimmel R, Vulule J, Sumba PO, Beeson JG, Angov E, Moormann AM, et al. Antibody-mediated growth inhibition of *Plasmodium falciparum*: Relationship to age and protection from parasitemia in Kenyan children and adults. PLoS One 2008;3:e3557.
95. Clark MA, Goheen MM, Spidale NA, Kasthuri RS, Fulford A, Cerami C. RBC barcoding allows for the study of erythrocyte population dynamics and P. falciparum merozoite invasion. PLoS One. 2014;9:e101041.
96. Vorobjev IA, Buchholz K, Prabhat P, Ketman K, Egan ES, Marti M, Duraisingh MT, Barteneva NS. Optimization of flow cytometric detection and cell sorting of transgenic Plasmodium parasites using interchangeable optical filters. Malar J 2012;11:312.
97. Kenthirapalan S, Waters AP, Matuschewski K, Kooij TWA. Flow cytometry-assisted rapid isolation of recombinant Plasmodium berghei parasites exemplified by functional analysis of aquaglyceroporin. Int J Parasitol 2012;42: 1185–1192.
98. Cai C, Carey KA, Nedosekin DA, Menyaev YA, Sarimollaoglu M, Galanzha EI, Stumhofer JS, Zharov VP. In vivo photoacoustic flow cytometry for early malaria diagnosis. Cytometry 2016;89A:531–542.
99. Blasi T, Hennig H, Summers HD, Theis FJ, Cerveira J, Patterson JO, Davies D, Filby A, Carpenter AE, Rees P. Label-free cell cycle analysis for high-throughput imaging flow cytometry. Nat Commun 2016;7:10256.
100. Dekel E, Rivkin A, Heidenreich M, Nadav Y, Ofir-Birin Y, Porat Z, Regev-Rudzki N. Identification and classification of the malaria parasite blood developmental stages, using imaging flow cytometry. Methods 2017;112:157–166.
101. Chia WN, Lee YQ, KS-W T. Imaging flow cytometry for the screening of compounds that disrupt the *Plasmodium falciparum* digestive vacuole. Methods 2017; 112:211–220.
102. Jeon W, Lee S, Dh M, Ban C. A colorimetric aptasensor for the diagnosis of malaria based on cationic polymers and gold nanoparticles. Anal Biochem 2013; 439:11–16.
103. Ulrich H, Wrenger C. Disease-specific biomarker discovery by aptamers. Cytometry 2009;75A:727–733.
104. Dolgosheina EV, Jeng SCY, Panchapakesan SSS, Cojocaru R, Chen PSK, Wilson PD, Hawkins N, Wiggins PA, Unrau PJ. RNA mango aptamer-fluorophore: A bright, high-affinity complex for RNA labeling and tracking. ACS Chem Biol 2014;9:2412–2420.
105. Kato T, Shimada I, Kimura R, Hyuga M. Light-up fluorophore–DNA aptamer pair for label-free turn-on aptamer sensors. Chem Commun 2016;52:4041–4044.
106. Kim EY, Kim JW, Kim WK, Han BS, Park SG, Chung BH, Lee SC, Bae K-H. Selection of aptamers for mature white adipocytes by cell SELEX using flow cytometry. PLoS One 2014;9:e97747.
107. Zhou Z, Liu M, Jiang J. The potential of aptamers for cancer research. Anal Biochem 2018;549:91–95.
108. Hori S, Herrera A, Rossi J, Zhou J. Current advances in aptamers for cancer diagnosis and therapy. Cancers (Basel) 2018;10:9.
109. de Franciscis V. Challenging cancer targets for aptamer delivery. Biochimie 2018; 145:45–52.
110. Tonelli RR, Colli W, Alves MJM. Selection of binding targets in parasites using phage-display and aptamer libraries in vivo and in vitro. Front Immunol 2012; 3:419.
111. Ulrich H, Magdesian MH, Alves MJM, Colli W. *In Vitro* selection of RNA aptamers that bind to cell adhesion receptors of *Trypanosoma cruzi* and inhibit cell invasion. J Biol Chem 2002;277:20756–20762.
112. Frith K-A, Fogel R, Goldring JPD, Krause RGE, Khati M, Hoppe H, Cromhout ME, Jiwaji M, Limson JL. Towards development of aptamers that specifically bind to lactate dehydrogenase of *Plasmodium falciparum* through epitopic targeting. Malar J 2018;17:191.
113. Lee S, Song K-M, Jeon W, Jo H, Shim Y-B, Ban C. A highly sensitive aptasensor towards Plasmodium lactate dehydrogenase for the diagnosis of malaria. Biosens Bioelectron 2012;35:291–296.
114. Barfod A, Persson T, Lindh J. In vitro selection of RNA aptamers against a conserved region of the *Plasmodium falciparum* erythrocyte membrane protein 1. Parasitol Res 2009;105:1557–1566.
115. Birch CM, Hou HW, Han J, Niles JC. Identification of malaria parasite-infected red blood cell surface aptamers by inertial microfluidic SELEX (I-SELEX). Sci Rep 2015;5:11347.
116. Sienkiewicz N, Daher W, Dive D, Wrenger C, Viscogliosi E, Wintjens R, Jouin H, Capron M, Müller S, Khalife J. Identification of a mitochondrial superoxide dismutase with an unusual targeting sequence in *Plasmodium falciparum*. Mol Biochem Parasitol 2004;137:121–132.
117. Baniecki ML, Wirth DF, Clardy J. High-throughput *Plasmodium falciparum* growth assay for malaria drug discovery. Antimicrob Agents Chemother 2007;51: 716–723.
118. van Vianen PH, van Engen A, Thaithong S, van der Keur M, Tanke HJ, van der Kaay HJ, Mons B, Janse CJ. Flow cytometric screening of blood samples for malaria parasites. Cytometry 1993;14:276–280.
119. Howard RJ, Battye FL, Mitchell GF. Plasmodium-infected blood cells analyzed and sorted by flow fluorimetry with the deoxyribonucleic acid binding dye 33258 Hoechst. J Histochem Cytochem 1979;27:803–813.
120. Fu Y, Tilley L, Kenny S, Klonis N. Dual labeling with a far red probe permits analysis of growth and oxidative stress in *P. falciparum* -infected erythrocytes. Cytometry 2010;9999A;77: 253–263.
121. Meissner KA, Kronenberger T, Maltarollo VG, Trossini GHG, Wrenger C. Targeting the *Plasmodium falciparum* plasmepsin V by ligand-based virtual screening. Chem Biol Drug Des 2019;93:300–312.
122. Quashie NB, de Koning HP, Ranford-Cartwright LC. An improved and highly sensitive microfluorimetric method for assessing susceptibility of *Plasmodium falciparum* to antimalarial drugs in vitro. Malar J 2006;5:95.
123. Kosaisavee V, Suwanarusk R, Nosten F, Kyle DE, Barrends M, Jones J, Price R, Russell B, Lek-Uthai U. Plasmodium vivax: Isotopic, PicoGreen, and microscopic assays for measuring chloroquine sensitivity in fresh and cryopreserved isolates. Exp Parasitol 2006;114:34–39.
124. Jiménez-Díaz MB, Mulet T, Gómez V, Viera S, Alvarez A, Garuti H, Vázquez Y, Fernández A, Ibáñez J, Jiménez M, et al. Quantitative measurement of *Plasmodium* -infected erythrocytes in murine models of malaria by flow cytometry using bidimensional assessment of SYTO-16 fluorescence. Cytometry 2009;75A:225–235.
125. Evers A, Heppner S, Leippe M, Gelhaus C. An efficient fluorimetric method to measure the viability of intraerythrocytic *Plasmodium falciparum*. Biol Chem 2008; 389:1523–1525.
126. Yatsushiro S, Yamamura S, Yamaguchi Y, Shinohara Y, Tamiya E, Horii T, Baba Y, Kataoka M. Rapid and highly sensitive detection of malaria-infected erythrocytes using a cell microarray chip. PLoS One 2010;5:e13179.

127. Jiménez-Díaz MB, Rullas J, Mulet T, Fernández L, Bravo C, Gargallo-Viola D, Angulo-Barturen I. Improvement of detection specificity ofPlasmodium-infected murine erythrocytes by flow cytometry using autofluorescence and YOYO-1. Cytometry 2005;67A:27–36.
128. Li Q, Gerena L, Xie L, Zhang J, Kyle D, Milhous W. Development and validation of flow cytometric measurement for parasitemia in cultures Of*p. falciparum* vitally stained with YOYO-1. Cytometry 2007;71A:297–307.
129. Gerena Y, Gonzalez-Pons M, Serrano AE. Cytofluorometric detection of rodent malaria parasites using red-excited fluorescent dyes. Cytometry 2011;79A:965–972.
130. Sobolewski P, Gramaglia I, Frangos JA, Intaglietta M, van der Heyde H. Plasmodium berghei resists killing by reactive oxygen species. Infect Immun 2005;73:6704–6710.
131. Engelbrecht D, Coetzer TL. The walking dead: Is hydroethidine a suitable viability dye for intra-erythrocytic *Plasmodium falciparum*? Parasitol Int 2012;61:731–734.
132. Jouin H, Daher W, Khalife J, Ricard I, Puijalon OM, Capron M, Dive D. Double staining Of*plasmodium falciparum* nucleic acids with hydroethidine and thiazole orange for cell cycle stage analysis by flow cytometry. Cytometry 2004;57A:34–38.
133. Cervantes S, Prudhomme J, Carter D, Gopi KG, Li Q, Chang Y-T, Le Roch KG. High-content live cell imaging with RNA probes: Advancements in high-throughput antimalarial drug discovery. BMC Cell Biol 2009;10:45.
134. Bhakdi SC, Sratongno P, Chimma P, Rungruang T, Chuncharunee A, Neumann HPH, Malasit P, Pattanapanyasat K. Re-evaluating acridine orange for rapid flow cytometric enumeration of parasitemia in malaria-infected rodents. Cytometry 2007;71A:662–667.
135. Bray PG, Saliba KJ, Davies JD, Spiller DG, White MRH, Kirk K, Ward SA. Distribution of acridine orange fluorescence in *Plasmodium falciparum*-infected erythrocytes and its implications for the evaluation of digestive vacuole pH. Mol Biochem Parasitol 2002;119:301–304.
136. Pasini EM, van den Ierssel D, Vial HJ, Kocken CH. A novel live-dead staining methodology to study malaria parasite viability. Malar J 2013;12:190.
137. Duffy S, Avery VM. Identification of inhibitors of *Plasmodium falciparum* gametocyte development. Malar J 2013;12:408.
138. Chang ZW, Malleret B, Russell B, Rénia L, Claser C. Ex vivo maturation assay for testing antimalarial sensitivity of rodent malaria parasites. Antimicrob Agents Chemother 2016;60:6859–6866.
139. Ha YR, Hwang B-G, Hong Y, Yang H-W, Lee SJ. Effect of farnesyltransferase inhibitor R115777 on mitochondria of *Plasmodium falciparum*. Korean J Parasitol 2015;53:421–430.
140. Gazarini ML, Garcia CRS. The malaria parasite mitochondrion senses cytosolic Ca2+ fluctuations. Biochem Biophys Res Commun 2004;321:138–144.
141. Hartwig CL, Rosenthal AS, D'Angelo J, Griffin CE, Posner GH, Cooper RA. Accumulation of artemisinin trioxane derivatives within neutral lipids of *Plasmodium falciparum* malaria parasites is endoperoxide-dependent. Biochem Pharmacol 2009; 77:322–336.
142. Behari R, Haldar K. Plasmodium falciparum: Protein localization along a novel, lipid-rich tubovesicular membrane network in infected erythrocytes. Exp Parasitol 1994;79:250–259.
143. Adisa A, Rug M, Klonis N, Foley M, Cowman AF, Tilley L. The signal sequence of exported protein-1 directs the green fluorescent protein to the parasitophorous vacuole of transfected malaria parasites. J Biol Chem 2003;278: 6532–6542.
144. Bracho C, Dunia I, Romano M, Benedetti EL, Perez HA. Plasmodium vivax and Plasmodium chabaudi: Intraerythrocytic traffic of antigenically homologous proteins involves a brefeldin A-sensitive secretory pathway. Eur J Cell Biol 2001;80: 164–170.
145. Bohórquez EB, Chua M, Meshnick SR. Quinine localizes to a non-acidic compartment within the food vacuole of the malaria parasite *Plasmodium falciparum*. Malar J 2012;11:350.
146. Ikegami-Kawai M, Arai C, Ogawa Y, Yanoshita R, Ihara M. Selective accumulation of a novel antimalarial rhodacyanine derivative, SSJ-127, in an organelle of Plasmodium berghei. Bioorg Med Chem 2010;18:7804–7808.
147. Gonçalves LCP, Tonelli RR, Bagnaresi P, Mortara RA, Ferreira AG, Bastos EL. A nature-inspired betalainic probe for live-cell imaging of Plasmodium-infected erythrocytes. PLoS One 2013;8:e53874.
148. Wissing F, Sanchez CP, Rohrbach P, Ricken S, Lanzer M. Illumination of the malaria parasite *Plasmodium falciparum* alters intracellular pH. Implications for live cell imaging. J Biol Chem 2002;277:37747–37755.
149. Rohrbach P, Friedrich O, Hentschel J, Plattner H, Fink RHA, Lanzer M. Quantitative calcium measurements in subcellular compartments of *Plasmodium falciparum*-infected erythrocytes. J Biol Chem 2005;280:27960–27969.
150. Glushakova S, Lizunov V, Blank PS, Melikov K, Humphrey G, Zimmerberg J. Cytoplasmic free Ca2+ is essential for multiple steps in malaria parasite egress from infected erythrocytes. Malar J 2013;12:41.
151. Gazarini ML, Thomas AP, Pozzan T, Garcia CRS. Calcium signaling in a low calcium environment: How the intracellular malaria parasite solves the problem. J Cell Biol 2003;161:103–110.
152. Butzloff S, Groves MR, Wrenger C, Müller IB. Cytometric quantification of singlet oxygen in the human malaria parasite *Plasmodium falciparum*. Cytometry 2012; 81A:698–703.
153. Ostera G, Tokumasu F, Oliveira F, Sa J, Furuya T, Teixeira C, Dvorak J. Plasmodium falciparum: Food vacuole localization of nitric oxide-derived species in intraerythrocytic stages of the malaria parasite. Exp Parasitol 2008;120:29–38.
154. Allen RJW, Kirk K. The membrane potential of the intraerythrocytic malaria parasite *Plasmodium falciparum*. J Biol Chem 2004;279:11264–11272.
155. Fu Y, Klonis N, Suarna C, Maghzal GJ, Stocker R, Tilley L. A phosphatidylcholine-BODIPY 581/591 conjugate allows mapping of oxidative stress in *P. falciparum* -infected erythrocytes. Cytometry 2009;75A:390–404.