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Stable Integration and Comparison of hGrx1-roGFP2 and sfroGFP2 Redox Probes in the Malaria Parasite *Plasmodium falciparum*

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Abstract

Studying redox metabolism in malaria parasites is of great interest for understanding parasite biology, parasite–host interactions, and mechanisms of drug action. Genetically encoded fluorescent redox sensors have recently been described as powerful tools for determining the glutathionedependent redox potential in living parasites. In the present study, we genomically integrated and expressed the ratiometric redox sensors hGrx1-roGFP2 (human glutaredoxin 1 fused to reduction–oxidation sensitive green fluorescent protein) and sfroGFP2 (superfolder roGFP2) in the cytosol of NF54-*attB* blood-stage *Plasmodium falciparum* parasites. Both sensors were evaluated *in Vitro* and in cell culture with regard to their fluorescence properties and reactivity. As genomic integration allows for the stable expression of redox sensors in parasites, we systematically compared single live-cell imaging with plate reader detection. For these comparisons, short-term effects of redox-active compounds were analyzed along with mid- and long-term effects of selected antimalarial agents. Of note, the single components of the redox probes themselves did not influence the redox balance of the parasites. Our analyses revealed comparable results for both the hGrx1-roGFP2 and sfroGFP2 probes, with sfroGFP2 exhibiting a

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ASSOCIATED CONTENT

Supporting Information

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Figure S1, fluorescence spectra of recombinant hGrx1-sfroGFP2 and sfroGFP2; Figure S2, equilibration of recombinant redox probes with GSH and GSSG; Figure S3, effects of antimalarial drugs on reduced sfroGFP2; Figure S4, stably transfected *P. falciparum* NF54-*attB* parasites expressing the hGrx1-roGFP2 or the sfroGFP2 redox probes; Table S1, primer sequences for cloning hGrx1 and roGFP2 into the expression vector pARL1a(+) and hGrx1-roGFP2 into the expression vector pDC2-CAM-*attP* for in-cell experiments; Table S2, primer sequences for cloning sfroGFP2 into the expression vector pQE30 for *in Vitro* experiments; Table S3, primer sequences for confirmation of genomic integration of sfroGFP2 in NF54-*attB*; Table S4, EC₅₀ values of antimalarial drugs determined by the SYBR Green assay (PDF)

more pronounced fluorescence intensity *in cellulo*. Accordingly, the sfroGFP2 probe was employed to monitor the fluorescence signals throughout the parasites' asexual life cycle. Through the use of stable genomic integration, we demonstrate a means of overcoming the limitations of transient transfection, allowing more detailed in-cell studies as well as high-throughput analyses using plate reader-based approaches.

Graphical Abstract

Keywords

Plasmodium falciparum; stable integration; NF54; genetically encoded redox probe; hGrx1-roGFP2; sfroGFP2

Although malaria mortality rates decreased by 29% between 2010 and 2015, malaria is still a serious health concern with around 445 000 deaths in 2016.¹ Artemisinin-based combination therapy is the treatment of choice for uncomplicated malaria caused by *Plasmodium falciparum* (*P. falciparum*).² However, increasing resistance to commonly used antimalarials like artemisinin (ART) and chloroquine (CQ)³ drives the search for new drug targets.

During their complex life cycle, malaria parasites have to adapt to different environments, have high proliferation rates, are under attack by the host immune system, and digest hemoglobin, thereby producing pro-oxidative heme.⁴ Antioxidant defense, redox balance, and efficient redox signaling are therefore of major importance for the parasite host cell unit, represent interesting drug targets, and are involved in mechanisms of drug action and resistance.^{3–5} Sources of oxidative and nitrosative challenges in protozoan parasites as well as techniques for monitoring redox changes under oxidative, pharmacological, or metabolic stress have recently been reviewed.⁶ Among these are cell-disruptive techniques as well as nondisruptive methods, which include the genetically encoded biosensor hGrx1-roGFP2 (human glutaredoxin 1 fused to reduction–oxidation sensitive green fluorescent protein) and molecular probes such as CM-H₂DCFDA and ThiolTracker Violet, as well as biochemical assays with Ellman's reagent and naphthalene dicarboxyaldehyde (NDA). The suitability of these different approaches in *Plasmodium* was systematically assessed by Mohring et al.⁷

The glutathione biosensor hGrx1-roGFP2 consists of human glutaredoxin 1 coupled to roGFP2. RoGFP2 equilibrates with the glutathione redox pair^{8,9} and does not react with reactive oxygen species under physiological conditions.¹⁰ RoGFP-based sensors have been

successfully applied in organisms like yeast,¹¹ *Arabidopsis thaliana*,⁹ and HeLa cells.¹² In *Plasmodium*, this genetically encoded probe has been episomally expressed and shown to be suitable for monitoring drug-induced redox changes in cytosol and organelles.^{13,14}

In the present work, we aimed to further optimize reliable and highly sensitive tools to study redox metabolism and mechanisms of drug action and resistance in *Plasmodium* and to make these methods available to the broader malaria community. As shown below, we therefore generated CQsensitive *P. falciparum* blood-stage parasites stably expressing the glutathione biosensor that has been genomically integrated using the *attB* × *attP* integration method.¹⁵ The serine integrase of mycobacteriophage Bxb1 (expressed by the pINT-*neo* plasmid) mediates homologous recombination between the genomic *attB* and the plasmid *attP* sites. These genetically and phenotypically homogeneous parasites showed enhanced biosensor signal intensity and stability compared to episomally transfected biosensor parasites. In our study, we describe and evaluate the superfolder roGFP2 (sfroGFP2) probe, a roGFP2 variant comprising the full set of sfGFP mutations (S30R, Y39N, N105T, Y145F, I171V, A206V) according to Pédélec et al.,¹⁶ as well as the cycle-3 mutations (F99S, M153T, V163A) that have also been described in that report. In addition, we introduced the F223R mutation, which is also present in the roTurbo probe.¹⁷

Following the successful integration of the hGrx1-roGFP2 and sfroGFP2 redox probes into the CQ-sensitive NF54-*attB* *P. falciparum* parasite line, we verified and compared the suitability of the sensors in cell culture. We furthermore assessed the effects of selected antimalarial agents on the probes using, in parallel, confocal laser scanning microscopy (CLSM) and a newly established microplate reader approach. We discuss the applications of the latter approach to high-throughput screens of *P. falciparum* redox state.

EXPERIMENTAL SECTION

Drugs and Chemicals.

All chemicals used were of the highest available purity and were obtained from Roth (Karlsruhe, Germany), Sigma-Aldrich (Steinheim, Germany), or Merck (Darmstadt, Germany). The following reagents were used: RPMI 1640 medium from Gibco (Paisley, United Kingdom); artemisinin (ART) from Roth (Karlsruhe, Germany); chloroquine (CQ), artesunate (ATS), amodiaquine (AQ), and atovaquone (ATQ) from Sigma-Aldrich (Steinheim, Germany); mefloquine (MQ) from Roche (Mannheim, Germany); artemether (ATM) from TCI Germany (Eschborn); quinine (QN) from Acros Organics (Geel, Belgium). Lumefantrine (LUM) was kindly provided by the Novartis Institute of Tropical Diseases to David Fidock (Columbia University, New York, USA), WR99210 by Jacobus Pharmaceuticals (Princeton, NJ, USA), and compound 1o by Reimar Krieg (Jena, Germany). Stock solutions of diamide (DIA), 1,4-dithiothreitol (DTT), and CQ were dissolved in sterile distilled H₂O, while ART, ATM, ATS, AQ, QN, MQ, ATQ, LUM, and compound 1o were dissolved in dimethyl sulfoxide (DMSO).

Cloning the hGrx1-roGFP2 and sfroGFP2 Constructs.

For in-cell experiments using stable integration with the NF54-*attB* strain, hGrx1-roGFP2 and sfroGFP2 were cloned into the pDC2-CAM-*bsd-attP* expression vector with the CAM 5' promoter using *AvrII* and *XhoI* restriction sites. The starting plasmid pDC2-CAM-[sfroGFP2]-*bsd-attP* was a generous gift from the lab of Myles Akabas (Albert Einstein College of Medicine, NY, USA). This plasmid already contained the superfolder mutations (S30R, Y39N, N105T, Y145F, I171V, and A206V)¹⁶ as well as the cycle-3 mutations (F99S, M153T, V163A),¹⁶ the F223R mutation,¹⁷ and the established roGFP2-mutations (L68V, A72S, Q80R, the enhanced GFP-mutations F64L, S65T, and the cysteine mutations S147C and Q204C). Prior to cloning the hGrx1-roGFP2 construct into the pDC2-CAM-*bsd-attP* plasmid, the *XhoI* restriction site within the hGrx1-gene was eliminated by site-directed mutagenesis. All primers used are listed in the Supporting Information. For cloning hGrx1 and roGFP2 into the pARL1a(+) expression vector, *KpnI* and *XmaI* restriction sites were used (Table S1). For heterologous overexpression, sfroGFP2 was cloned into the pQE30 expression vector with *BamHI* and *HindIII* restriction sites (Table S2).

Heterologous Overexpression of Recombinant Redox Sensors.

For *in Vitro* characterization and comparison, the different redox sensors were heterologously overexpressed. Recombinant hGrx1-roGFP2 was produced according to Kasozi et al.¹³ Recombinant sfroGFP2 and hGrx1-sfroGFP2 were overexpressed as followed: *E. coli* M15 [pREP4] cells (kanamycin resistance, Kan^R) were transformed with sfroGFP2 in pQE30 (carbenicillin resistance, Cn^R). *E. coli* BL21 cells were transformed with hGrx1-sfroGFP2 in pET28 (kanamycin resistance, Kan^R). For both expressions, a preculture in lysogeny broth (LB) medium (containing 100 µg/mL carbenicillin (Cn) and/or 50 µg/mL kanamycin (Kan)) was inoculated with a colony and grown for 6–7 h at 37 °C with vigorous shaking. After incubation, the preculture was poured into 50 mL of LB medium with antibiotics and grown overnight at 37 °C with constant shaking. 500 mL of antibiotic-containing LB medium was inoculated with 10–15 mL of the overnight culture and grown at 37 °C. The culture was induced at an optical density at 600 nm (OD₆₀₀) of 0.6 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated overnight at room temperature (RT). The cells were harvested via centrifugation (8000 revolutions per minute (rpm), 15 min, 4 °C), resuspended (4 mL buffer/1 g pellet) in Hepes buffer (50 mM Hepes, 300 mM NaCl, pH 7.5), and mixed with protease inhibitors (150 nM pepstatin, 40 nM cystatin, 100 µM phenylmethylsulfonyl fluoride, PMSF) before storage at –20 °C. All proteins were purified via hexahistidyl affinity chromatography on Ni-NTA material, concentrated using 10 kDa or 30 kDa Vivaspins columns (Sartorius, Göttingen, Germany), and stored at –20 °C.

In Vitro Characterization of Recombinant Redox Sensors.

All drugs, as well as DIA, DTT, reduced glutathione (GSH), and oxidized glutathione (GSSG), were diluted with a standard reaction buffer (100 mM potassium phosphate, 1 mM ethylenediaminetetraacetic acid, EDTA, pH 7.0) and used immediately. Before the experiments, the reaction buffer was degassed for 1 h at RT. Purified proteins were reduced with 20 mM DTT for 30 min at 4 °C, desalted (Zeba Spin Desalting Columns, Thermo

Scientific, Waltham, USA), and diluted in reaction buffer to a final concentration of 1.25 μM . A 5-fold dilution (10 μL) of drugs or GSH/GSSG was mixed with 40 μL of 1.25 μM proteins in a 96-well microplate (black, half area, μClear , Greiner Bio-One, Frickenhausen, Germany). Fluorescence spectra of sfroGFP2 and hGrx1-sfroGFP2 were measured in a plate reader (Clariostar, BMG Labtech, Ortenberg, Germany) with excitation ranging from 350 to 490 nm; emission was recorded with a 1400-CLA-530–40 filter. Proteins were fully reduced with 10 mM DTT or oxidized with 1 mM diamide (Figure S1). For studying the equilibration with glutathione, sfroGFP2, sfroGFP2 plus *Pf*Grx1 (equimolar, corresponding to 1 μM), hGrx1-sfroGFP2, and hGrx1-roGFP2 were incubated with different concentrations of GSH (0.1, 2.5, and 5 mM) or GSSG (5, 10, 20, and 50 μM). The redox ratio was determined after 5 min and 4 h with the plate reader. For the incubation with GSH, 200 μM nicotinamide adenine dinucleotide phosphate (NADPH) and 1 U/mL human glutathione reductase were added to the buffer to avoid oxidation of the GSH over time. The emission after excitation at 405 and 480 nm was measured in a plate reader with optimal reading settings. Data from two independent experiments with two technical replicates were analyzed for each concentration (Figure S2). To exclude a direct interaction of the drugs with the new redox sensor sfroGFP2, recombinant protein was incubated with different drug concentrations and ratios (405/475 nm) were measured after 5 min and 4 and 24 h (Figure S3).

***P. falciparum* Cell Culture.**

The CQ-sensitive 3D7 and NF54-*attB* lines of *P. falciparum* were cultured as described.¹⁸ Parasites were propagated in red blood cells (RBCs) (A+) in RPMI 1640 medium supplemented with 0.5% Albumax, 9 mM glucose, 0.2 mM hypoxanthine, 2.1 mM L-glutamine, 25 mM Hepes, and 22 $\mu\text{g/mL}$ gentamycin at 3.3% hematocrit and 37 °C in a trigas consisting of 3% O₂, 3% CO₂, and 94% N₂. Synchronization of *P. falciparum* parasites was performed with 5% (w/v) sorbitol.¹⁹ *P. falciparum* trophozoites were enriched via magnet separation.²⁰ Cell lysates were obtained via saponin lysis.²¹ Parasitemia was counted using Giemsa-stained blood smears.

Transfection of *P. falciparum*.

For stable transfection, 5 mL of NF54-*attB* culture (ring-stage 8–10 h, 5–8% parasitemia) was centrifuged at 1500 rpm for 3 min and the supernatant was discarded. For each transfection, 50 μg of purified pDC2-plasmid bearing the genes coding for the redox sensors and 50 μg of purified pINT DNA was mixed and placed on ice. The parasite pellet was resuspended with an equal volume of sterile cytomix (120 mM KCl, 0.15 mM CaCl₂, 2 mM EGTA, 5 mM MgCl₂, 10 mM K₂HPO₄/KH₂PO₄, 25 mM Hepes, pH 7.6) and spun down at 1500 rpm for 3 min. Excess cytomix was discarded, and the volume remaining was brought up with extra cytomix to approximately 450 μL ; then, it was added to the DNA on ice. The parasites were electroporated (310 V, 950 μF , capacitance ∞ ; using a Bio-Rad Gene Pulser).²² The time constant was between 10 and 15 ms. After electroporation, the parasites were immediately resuspended with 1 mL of complete media and transferred to a 15 mL conical tube containing 3.5 mL of complete media and RBCs (4% hematocrit). The conical tube was spun down, resuspended in new complete media (5 mL), and plated out. After 24 h, the selection drugs blasticidin [2.5 $\mu\text{g/mL}$] and genitcin (G418) [125 $\mu\text{g/mL}$] were added for 6

days to the cells to select for transfectants. Culture medium was changed every day for the first 6 days and thereafter every other day. On day 6 post-electroporation, 50 μL of fresh RBCs was added to the cells. From day 7 on, only blasticidin was used for selection. Between days 10 and 13, the parasites were diluted 1:2 weekly until the appearance of parasites (usually 3–4 weeks).²³ As detailed elsewhere,²⁴ blood PCR reactions (KAPA Biosystems) were used to assess for successful integration in bulk cultures and in cloned recombinant parasites. Parasites were cloned by limiting dilution and subjected to blood PCR with primer pair cg6 5' F + bsd R (Table S3).²⁵ Representative NF54^[sfroGFP2] parasite clones are shown (Figure 1), revealing the expected 1.6 kb PCR product indicative of successful *attB* $\times$ *attP*-based integration. After confirmation of the stable integration, the selection drug blasticidin was omitted for the maintenance of the culture.

Transfection of *P. falciparum* 3D7 parasites was carried out as described above for NF54-*attB* with the following slight modifications. Electroporation was performed using 150 μg of purified plasmid. To select for transfectants, 2 nM WR99210 was added to the culture 24 h post-transfection. The culture medium was changed every day for the first 5 days under constant drug pressure, and drug concentration was increased to 5 nM after the appearance of transfectants (usually after 3–4 weeks). After 1 week, 50 μL of fresh RBCs were added to the parasites, and thereafter, the culture was maintained by diluting 1:2 weekly with fresh media and RBCs until the growth of the parasites.

Time Course Experiment sfroGFP2.

Under the experimental conditions chosen, the NF54^[sfroGFP2] strain usually has an asexual intraerythrocytic developmental cycle of 48 h. For the time course experiment, a parasite culture with 8% parasitemia was synchronized using a Percoll (GE Healthcare Lifescience, Little Chalfont, UK) gradient with minor changes, as described by Ljungström et al.²⁶ By this procedure, schizonts can be isolated. After allowing 3 h for parasite reinvasion, the Percoll separation was repeated to isolate the newly invaded red blood cells at the ring-stage, followed by treatment with 5% (w/v) sorbitol,²⁰ which eliminates remaining late stages. The first samples were taken directly after sorbitol treatment ($t = 0$) and then every 5 h postinvasion (5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h). Giemsa-stained smears were prepared and samples for Western blot analysis were taken. For the blots, 15 μL of infected red blood cells (iRBCs) were resuspended in 400 μL of saponin lysis buffer (0.02% saponin, 10 mM NaH_2PO_4 , 10 mM Na_2HPO_4 , 145 mM NaCl, 3 mM KCl, pH 7.2) and incubated for 3 min at RT with inverting. After centrifugation (13 400 rpm, 3 min), the pellets were resuspended in 20 μL of M-PER buffer (Thermo Scientific, Waltham, USA), followed by 5 min of incubation at RT and further centrifugation (13 400 rpm, 3 min). Fifteen μL of the supernatants were mixed with 5 μL of 4 $\times$ sample buffer + DTT. After heating for 5 min at 95 $^\circ\text{C}$, the samples were stored at $-20\text{ }^\circ\text{C}$ until Western blotting. 3.25 μg of recombinant protein and proteins from the parasite lysate were separated onto 12% sodium dodecyl sulfate (SDS) gels and transferred to a nitrocellulose membrane (GE Healthcare Lifescience, Little Chalfont, UK). Membranes were probed with α -GFP (1:1000; Roche) followed by secondary α -mouse IgG antibodies (1:10 000; Dianova, Hamburg, Germany). All antibodies for Western blotting were diluted in 3% bovine serum albumin (BSA) in Tris-buffered saline with 0.05% Tween 20. In addition to the Western blots, fluorescence measurements at the

confocal microscope were performed at 20 to 50 h time points. For these experiments, the parasite culture was washed once with prewarmed (37 °C) Ringer's solution (122.5 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl₂, 0.8 mM MgCl₂, 11 mM D-glucose, 25 mM Hepes, 1 mM NaH₂PO₄, pH 7.4) and resuspended in Ringer's solution. For additional information, see section "Confocal Live-Cell Imaging and Image Processing".

Determination of Thiol and Glutathione Concentrations in Parasite Lysate.

60 mL of transfected *P. falciparum* 3D7[pARL1a(+)], 3D7[hGrx1], 3D7[roGFP2], and 3D7[hGrx1-roGFP2] trophozoites (30–34 h post-invasion) at 8–10% parasitemia and 5% hematocrit were saponin-lysed²⁷ and harvested.²¹ Total thiols in fresh parasite lysate were measured spectrophotometrically on the basis of their reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB).²⁸ Briefly, the lysate was mixed with 150 mM potassium phosphate buffer, pH 8.0, and 400 μ M DTNB, and directly measured at 412 nm at RT, accounting for control absorption values. Free nonprotein thiols were measured analogously in the supernatant of a parasite lysate previously precipitated with 3 volumes of 5% sulfosalicylic acid (SSA). Total glutathione was measured with the glutathione reductase-coupled DTNB-GSH-recycling assay.²⁹ Briefly, 10 μ L of SSA supernatant was mixed with 490 μ L of buffer containing 142 mM NaH₂PO₄, 6 mM EDTA, and 0.3 mg/mL NADPH, pH 7.5. After adding 0.6 mM DTNB, the mixture was incubated for 10 min at RT. Human glutathione reductase (0.5 U/ml) was added, and the DTNB increase was detected immediately for 30 s at 25 °C. GSH was quantified with a NDA assay.³⁰ One volume of SSA supernatant was added to ten volumes of freshly prepared NDA derivatization mix (1 mL of 10 mM NDA in DMSO, 7 mL of 50 mM Tris, pH 10, and 1 mL of 0.5 M NaOH). Fluorescence was measured at ex 485 nm/em 520 nm together with a glutathione standard curve in 96-well plates in a microplate reader (Clariostar, BMG Labtech, Ortenberg, Germany). Data include three biological replicates. All 3D7 samples were tested on the same day to exclude systematic errors. Graphs were plotted using GraphPad Prism 5 software (San Diego, CA, USA). To calculate the significance of changes in thiol levels after drug treatment, all groups were compared with the control by the Dunnett's Multiple Comparison Test (GraphPad Prism, San Diego, CA, USA).

In Vitro *P. falciparum* Drug Susceptibility Assays.

Parasite growth inhibition studies and calculations of the half maximal effective concentration (EC₅₀) of antimalarial drugs or compounds against *P. falciparum* were performed using SYBR Green I-based fluorescence assays according to Ekland et al.³¹ with modifications. Serial double dilutions (50 μ L) of the compounds in complete medium were performed in 96-well half area microtiter plates (black, half area, μ Clear, Greiner Bio-One, Frickenhausen, Germany). Synchronized ring-stage parasites (50 μ L) were added to each well (0.15% parasitemia, 1.25% final hematocrit) and incubated for 48 h at 37 °C. Then, 20 μ L of 5 $\times$ SYBR Green (10 000 $\times$ stock solution) in lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 0.16% w/v saponin, and 1.6% v/v Triton X-100) was added to each well for 24 h at RT in the dark. Fluorescence was measured in a Clariostar plate reader at ex 494 nm/em 530 nm. Curve-fitting the percentage growth inhibition against log drug concentration with a variable slope sigmoidal function led to the determination of EC₅₀ values (Table S4).

Effects of Redox-Active Compounds and Antimalarial Drugs on Redox Homeostasis.

The effects of antimalarial drugs on *P. falciparum* were investigated in mid- (4 h) and long-term (24 h) incubation experiments. For 4 h experiments, trophozoites (26–30 h postinvasion) of NF54^[hGrx1-roGFP2] and NF54^[sfroGFP2] (6–8% parasitemia) were magnetically enriched (Miltenyi Biotec, Bergisch Gladbach, Germany), counted using the improved Neubauer hemocytometer (Brand GmbH, Wertheim, Germany), and returned to cell culture (at 2.0×10^5 trophozoites/ μL) for at least 1 h to recover. Parasites were treated with antimalarial drugs at $100 \times \text{EC}_{50}$ for 4 h under cell culture conditions. Subsequently, free thiol groups were blocked with 2 mM N-ethylmaleimide (NEM) for 15 min at 37 °C. For 24 h experiments, a 5 mL culture (6–8% parasitemia) of ring-stage parasites (6–10 h post-invasion) was treated with antimalarial drugs at $10 \times \text{EC}_{50}$. Prior to enrichment, cysteines were blocked with 2 mM NEM. After incubation, cells were washed and resuspended in Ringer's solution for all incubation periods. All experiments included nontreated parasites as control, as well as fully oxidized and fully reduced parasites obtained by 2 min of incubation with 1 mM DIA and 10 mM DTT, respectively, prior to blocking with NEM. Each experiment was conducted three times using freshly prepared parasites.

Confocal Live-Cell Imaging and Image Processing.

Magnetically enriched *P. falciparum*-infected erythrocytes (trophozoite-stage) were washed with prewarmed (37 °C) Ringer's solution and seeded onto poly-L-lysine-coated μ -slides VI (for time course experiments) or flat μ -slides 18 well for end point experiments (Ibidi, Martinsried, Germany). We used a Leica confocal system TCS SP5 inverted microscope equipped with the objective HCX PL APO 63.0 $\times$ 1.30 GLYC 37 °C UV connected to a 37 °C temperature chamber. The argon laser power was set to 20%, and scanning was performed at 400 Hz frequency and at a 512×512 pixel resolution. The smart gain and smart offset were 950 V and -0.9% , respectively. With a sequential scan, we excited the probes at 405 nm and at 488 nm and detected emissions at 500–550 nm. Laser intensity for both lines NF54^[hGrx1-roGFP2] and NF54^[sfroGFP2] was adjusted to match the full dynamic range of the probes to the dynamic range of the detector. According to Morgan et al.,¹¹ the dynamic range of the setting is calculated by dividing the highest ratio of the DIA time course (R_{ox}) by the lowest ratio of the DTT time course (R_{red}). For time series, images were acquired every 5 s over a time course of 3 min after 15 s of basal measurements. Autofluorescence images were simultaneously taken at ex 405 nm/em 430–450 nm and individually defined together with the background for every image, but no disturbing fluorescence signal was detected. The Leica LAS AF Lite software for fluorescence analysis was used. The 405/488 nm ratios were calculated. Graphs were plotted using GraphPad Prism 5 software (San Diego, CA, USA). We chose only parasites showing fluorescent signals at both 405 and 488 nm excitation and an intact host cell. In order to investigate the short-term effects of DIA and DTT (dynamic range measurements) on the parasites, $50 \mu\text{L}$ of cells (1.0×10^6 trophozoites) were exposed to 1 mM DIA and 10 mM DTT, and the fluorescence signals were monitored in a time course of 3 min. All experiments included nontreated parasites as controls and end point experiments with both fully oxidized and fully reduced parasites (2 min incubation prior to measurements). Each incubation time and drug concentration treatment was carried out three times. For each time course, at least three parasites were assessed resulting in at least nine experimental values per data point. End

point experiments comprised at least ten parasites resulting in at least 30 experimental values per incubation. The contrast of the confocal images shown in this publication has been slightly enhanced (all by the same degree) to facilitate visualization for the naked eye.

Plate Reader Analyses.

For 4 and 24 h experiments, cells were washed and resuspended after incubation in prewarmed Ringer's solution to a concentration of 2.0×10^5 iRBCs/ μL . Ten μL of cells (1×10^6 iRBCs) were transferred to each well of a 384-well plate (black, flat bottom, Greiner bio-one, Frickenhausen, Germany) and measured with a Clariostar plate reader (BMG labtech, Ortenberg, Germany) with excitation wavelengths at 405 and 475 nm (emission 510 nm). To investigate the short-term effects of DIA and DTT (dynamic range measurements) on the parasites, 10 μL of cells (1.0×10^6 trophozoites) were exposed to 1 mM DIA and 10 mM DTT, and the fluorescence signals were monitored in a time course of 3 min. For end point measurements, 10 μL of cells (1.0×10^6 trophozoites) were transferred to a 384-well plate. The gain of the 405 and 475 nm excitation wavelength was adjusted to match the full dynamic range of the redox probes. 475 nm instead of 488 nm was chosen due to the available filters of the used plate reader as well as to ensure that the excitation wavelength did not adversely influence the emission. The 405/475 nm ratio was calculated. Graphs were plotted using GraphPad Prism 6 software (San Diego, CA, USA). One-Way ANOVA tests with 95% confidence intervals with the Dunnett's Multiple Comparison Test were applied for statistical analysis of significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Calculations of Degree of Oxidation and Glutathione Redox Potential.

The degree of oxidation (OxD) was determined according to the following equation (eq 1)⁸

$$\text{OxD}_{\text{roGFP2}} = \frac{R - R_{\text{red}}}{\frac{I_{488\text{ox}}}{I_{488\text{red}}}(R_{\text{ox}} - R) + (R - R_{\text{red}})} \quad (1)$$

where R is the ratio of excitation at 405/488 nm; R_{red} is the ratio of fully reduced roGFP2 with 10 mM DTT; R_{ox} is the ratio of fully oxidized roGFP2 with 1 mM DIA; $I_{488\text{ox}}$ is the fluorescence intensity at 488 nm for fully oxidized roGFP2; $I_{488\text{red}}$ is the fluorescence intensity at 488 nm for fully reduced roGFP2.

Also, the glutathione redox potential was calculated according to Schwarzländer et al.⁸ (eq 2)

$$E'_{\text{roGFP2}} = E'_{0(\text{roGFP2})}^{\text{pH}} - \frac{2.303RT}{zF} \log_{10} \frac{(1 - \text{OxD}_{\text{roGFP2}})}{\text{OxD}_{\text{roGFP2}}} \quad (2)$$

where R is the gas constant ($8.315 \text{ J K}^{-1} \text{ mol}^{-1}$); T is the absolute temperature (310.45 K); z is the number of transferred electrons (2); F is the Faraday constant (96485 C mol^{-1}).

To adjust the midpoint redox potential for pH, the following equation (eq 3) was used.

$$E'_{0(\text{roGFP2})}^{\text{pH}} = E'_{0(\text{roGFP2})} - \frac{2.303RT}{zF}(\text{pH} - 7) \quad (3)$$

According to Dooley et al.,³² the consensus midpoint potential of roGFP2 is −280 mV. The pH of the *P. falciparum* cytosol was determined to be 7.16 using pHluorin.¹⁴ This value was used for our calculations.

RESULTS

Comparison of the Recombinant Redox Sensors *in Vitro*.

Recombinant sfroGFP2, hGrx1-sfroGFP2, and hGrx1roGFP2 were heterologously overexpressed and purified for comparative studies. hGrx1-sfroGFP2 and sfroGFP2 exhibited spectra under reducing and oxidizing conditions (Figure S1) that were comparable to the hGrx1-roGFP2 probe characterized by Meyer and Dick.¹⁰ Thus, we could exclude adverse effects of the mutations, introduced to create sfroGFP2, on the spectral properties of the probe. To verify the ability of the sfroGFP2 probe to equilibrate with GSH and GSSG, the recombinant protein was incubated with reduced and oxidized glutathione in the absence or presence of an equimolar concentration (1 μM) of Pf Grx1 for 5 min and 4 h (Figure S2). For direct comparison, hGrx1-sfroGFP2 and hGrx1roGFP2 were tested in parallel. Incubation with GSH (0.1, 2.5, and 5 mM) indicated constant and comparable probe reduction over time. GSSG (5, 10, 20, and 50 μM) had a clear time-dependent oxidative effect on all probes. This effect was more pronounced when Grx was present in the incubation mix and even more so when Grx was directly linked to the probe (Figure S2).

Direct Interaction of Antimalarial Drugs with Recombinant sfroGFP2 *in Vitro*.

Prior to the drug treatments described below, we studied the *in Vitro* interactions of the drugs with the redox sensor sfroGFP2 as described¹³ to exclude a direct influence of the drugs on the probe. The tested drugs included ART, ATM, ATS, and the quinoline drugs CQ, MQ, AQ, and QN, as well as LUM and ATQ. All compounds were tested at concentrations of 10 nM, 100 nM, 1 μM , 10 μM , 100 μM , 1 mM, and 10 mM in standard reaction buffer in a plate reader. Compound 1o has been previously described in Krieg et al.³³ None of the drugs significantly affected the fluorescence ratio of sfroGFP2 up to a concentration of 10 μM . Only after long incubation times and very high, pharmacologically irrelevant concentrations, effects were seen. Figure S3 shows the drug concentrations and the effects on the redox sensor as determined after a 5 min, 4 h, and 24 h incubation at 25 °C. These findings are in agreement with the previously published data on recombinant hGrx1-roGFP2.^{13,14}

Expression of the hGrx1-roGFP2 and sfroGFP2 Sensors in *P. falciparum* NF54-attB Parasites.

Within the framework of this study, we aimed to create a recombinant parasite line stably expressing the redox sensors, which can be used reliably in the malaria community. To reach this goal and additionally introduce an optimized probe, hGrx1-roGFP2 and sfroGFP2 redox

sensors were integrated into the NF54-*attB* strain under the control of the CAM promoter using the pDC2-CAM-[X]-*bsd-attP* plasmid for stable integration. Parasites appeared in cell culture 3 weeks after transfection, and integration was verified with PCR (Figure 1). 100% of the genetically identical parasites exhibited fluorescence signals.

Total Thiols and Glutathione Levels in *P. falciparum* 3D7 Parasites.

As discussed in Mohring et al.,⁷ we had previously observed a slightly altered redox status in parasites transfected with the hGrx1-roGFP2 probe in comparison with nontransfected parasites. We therefore aimed to systematically study the potential influence of the different parts of the hGrx1-roGFP2 probe on the cellular thiol status. We determined total thiols (determined by DTNB end point, including low molecular weight free thiols and protein bound thiols), free thiols (representing low molecular weight thiols mainly consisting of glutathione; measured by DTNB in supernatant after precipitation of the cell lysate with sulfosalicylic acid), total glutathione (comprising GSH and GSSG but not protein bound glutathione; obtained after protein precipitation; determined in the DTNB recycling assay), and GSH levels (determined in the NDA assay after protein precipitation) of transiently transfected 3D7^[pARL1a(+)], 3D7^[hGrx1], 3D7^[roGFP2], and 3D7^[hGrx1-roGFP2] trophozoites as described by Mohring et al.⁷ As shown in Figure 2, no significant differences in thiol status (absolute values and ratios between different parameters (not shown)) were detected between the four parasite lines: (1) the line containing the empty transfection vector pARL1a(+); (2) the line expressing only the hGrx1-part of the sensor; (3) the line expressing only the roGFP2-part; and (4) the line expressing the full-length redox sensor hGrx1-roGFP2. These findings indicate that there is no major influence of the redox probe hGrx1-roGFP2 on the glutathione metabolism in the parasite. Therefore, as hypothesized before, previous changes observed with transiently transfected parasites⁷ were most likely due to stress conferred by the selecting agent.

Dynamic Range and Glutathione Redox Potential As Determined by the Stably Integrated Redox Probes hGrx1-roGFP2 and sfroGFP2 in Living Parasites.

For the direct comparison of the two redox sensors *in cellulo*, a set of three independent experiments with the same microscope settings (laser: 405 nm 10%, 488 nm 6%) were performed with NF54^[hGrx1-roGFP2] and NF54^[sfroGFP2] magnetically enriched trophozoites. To calibrate 100% probe reduction and 100% probe oxidation, cells were incubated with 10 mM DTT and 1 mM DIA, respectively (Figure S4). Parasites expressing the sfroGFP2 probe showed higher fluorescence levels (I_{488ox} : 49.77 vs 26.84; I_{488red} : 113.71 vs 81.86), whereas the dynamic range of both probes was comparable (hGrx1-roGFP2: 4.23; sfroGFP2: 4.56). While the OxD under basal conditions was slightly higher in NF54^[hGrx1-roGFP2] (20.8%) compared to NF54^[sfroGFP2] (15.9%), both probes indicated a similar cytosolic glutathione redox potential of -304 and -303 mV, respectively (Table 1). Furthermore, both stably transfected parasite lines showed 100% expression of the sensors and higher fluorescence intensities than the previously reported transiently transfected cells.

Transfer of the Method to the Plate Reader.

With the stably integrated redox probes, it became possible to determine the cytosolic glutathione redox potential in malaria parasites with a plate reader. This has great advantages

for future experiments. To establish this method, we systematically compared CLSM and plate reader detection of the fluorescence signals. NF54[hGrx1-roGFP2] and NF54[sfroGFP2] magnetically enriched trophozoites were exposed to 1 mM DIA and monitored for 3 min using either CLSM (Figure 3A) or a plate reader (Figure 3B). Reduction capacity after DIA treatment was investigated via subsequent treatment with 10 mM DTT. As shown in Table 1, the basal ratio of individual trophozoites ranged between 0.38 and 0.85 for NF54[hGrx1-roGFP2] and between 0.47 and 0.95 for NF54[sfroGFP2]. For comparability and clarity of Figure 3, the obtained ratio values of the time courses were all related to the first basal ratio value, which was set to 100. The data indicate that the sensors in both parasite strains were sensitive to dynamic oxidation and reduction processes. Furthermore, the obtained profiles were comparable for CLSM and plate reader measurements. The higher DIA sensitivity observed for the sfroGFP2 probe showed rather high standard deviations and was not observed in all experiments.

Sensor Expression Throughout the Asexual *P. falciparum* Life Cycle.

Tightly synchronized (in a window of about 3–4 h) NF54[sfroGFP2] parasites were monitored throughout a complete asexual life cycle in RBCs to determine at which stages the probe was expressed and fluorescence measurements could be performed. Twenty hours postinvasion, parasites develop from the ring to the trophozoite stage and fluorescence levels become sufficiently strong to reliably measure the redox ratio (Figure 4). On the basis of our data, the best time frame for measurements was from approximately 25 to 35 h post-invasion, i.e., in mature trophozoites (Figure 4). Forty hours post-invasion, most parasites were still late trophozoites, with some schizonts observed. Forty five hours post-invasion, the first schizonts ruptured, merozoites started to reinvade, and small rings became visible (Figure 4). After 50 h, rings became larger and only a few late schizonts and merozoites were still present. Although merozoites showed some fluorescence, measurements were challenging, since the signals were weak, and a proper adjustment to the correct z-level was difficult. Also, ring-stage parasite fluorescence signals were too weak for confocal redox measurements (Figure 4, 20 h).

In parallel with the images, samples for Western blot analysis were taken every 5 h. Although fluorescence levels were weak in ring-stages investigated with CLSM, we detected an α -GFP signal at every time point of the a sexual blood stage cycle (Figure 5). While bands of the late stages (35, 40, and 45 h) showed strong Western blot signals, the band became weaker at 50 h, when mainly rings and merozoites with weak fluorescence signals are present.

Mid- and Long-Term Drug Effects on the Oxidation Levels of NF54[hGrx1-roGFP2] and NF54[sfroGFP2] Transfected Parasites.

In order to test the impact of clinically employed antimalarial drugs and redox-active agents on the redox probes hGrx1-roGFP2 and sfroGFP2 in *P. falciparum*, mid- and long-term experiments were carried out with NF54[hGrx1-roGFP2] and NF54[sfroGFP2] transgenic parasites with both the CLSM and plate reader. To measure the completely oxidized and reduced state of the probes, magnetically enriched trophozoites were incubated for 2 min with 1 mM DIA or 10 mM DTT, respectively, and blocked thereafter with 2 mM NEM. For

mid-term experiments (4 h exposures), 26–30 h trophozoites were incubated with 100× EC₅₀ of ATM, ATQ, ATS, MQ, LUM, or compound **1o** and subsequently blocked with 2 mM NEM. Compound **1o** is a recently described antiparasmodial arylmethyldamino steroid, which might at least partially act on the basis of a redox mechanism as described in Krieg et al.³³ EC₅₀ values were confirmed to be in the expected nanomolar range and are shown in Table S4. None of the tested drugs showed significant effects on the 405/488 nm and 405/475 nm ratios in either CLSM or plate reader, respectively, after 4 h of incubation.

As shown in Figure 6, 1 mM DIA treatment led to a significant increase of the fluorescence ratio of both NF54^[hGrx1-roGFP2] (Figure 6A,B) and NF54^[sfroGFP2] (Figure 6C,D) transfectants with both the CLSM and plate reader.

For long-term drug exposure experiments (24 h exposure), ring-stage parasites were incubated with 10× EC₅₀ of the drugs. Prior to magnet enrichment, cysteines were blocked with 2 mM NEM. MQ and compound **1o** showed evidence of oxidation of both probes, which was most pronounced via plate reader detection although data did not reach statistical significance (hGrx1-roGFP2: MQ $p = 0.6223$, compound **1o** $p = 0.5568$; sfroGFP2: MQ $p = 0.2852$, compound **1o** $p = 0.3455$). NF54^[hGrx1-roGFP2] showed a 1.5-fold increase of fluorescence ratio with the CLSM and a 2.6-fold increase at the plate reader (Figure 7A,B). Interestingly, NF54^[sfroGFP2] was oxidized more than NF54^[hGrx1-roGFP2] with a 1.8-fold and a significant 2.8-fold ratio change at the CLSM and plate reader, respectively (Figure 7C,D). ATM, ATQ, ATS, and LUM showed no effect on the increase of fluorescence ratio for NF54^[hGrx1-roGFP2] and NF54^[sfroGFP2] using both CLSM and the plate reader (Figure 7A–D). One mM DIA treatment led to an increase of the fluorescence ratio of both NF54^[hGrx1-roGFP2] (Figure 7A,B) and NF54^[sfroGFP2] (Figure 7C,D) parasites with both the CLSM and plate reader.

DISCUSSION

In order to further improve the currently available tools for studying redox homeostasis in malaria parasites, we produced and evaluated the sfroGFP2 redox probe, which was likely to have improved fluorescence properties. To generate the probe, we introduced into roGFP2 the full set of sfGFP mutations (S30R, Y39N, N105T, Y145F, I171V, A206V), as well as the cycle-3 mutations (F99S, M153T, V163A) both according to Pédélecq et al.¹⁶ In addition, we inserted the F223R mutation, which has also been reported for the roTurbo probe.¹⁷ In summary, the two differences between the roTurbo probe described by Dooley et al.¹⁷ and our sfroGFP2 are the presence of the Y145F mutation in our protein and the A206V mutation that is an A206K mutation in Dooley et al.¹⁷ Notably, Hoseki et al.³⁴ (describing ER-targeted roGFP-iL) also introduced the Y145F mutation, which however, caused unwanted spectral changes in the reduced probe. We therefore analyzed the spectral properties of our sfroGFP2 (in direct comparison with recombinant hGrx1-sfroGFP2 (Figure S1)). In neither of the probes did we observe the problematic spectral changes described by Hoseki et al.³⁴ Rather, the oxidized as well as the reduced spectra were comparable to the normal (hGrx1)-roGFP2 probes described, e.g., by Meyer and Dick.¹⁰ Furthermore, the ability of the sfroGFP2 probe to equilibrate with GSH and GSSG in the presence or absence of Grx was verified (Figure S2). For direct comparison, hGrx1-sfroGFP2 and hGrx1-roGFP2

were tested in parallel. Incubation with GSH indicated comparable reduction for all probes. Oxidation of sfroGFP2 by GSSG was time dependent and more pronounced in the presence of Grx. The most efficient oxidation was monitored when Grx was directly linked to the probes (Figure S2). It should, however, be noted that the cytosolic Grx concentration might be higher than 1 μM thus allowing for more rapid equilibration and that we monitored comparable oxidative signals in our in-cell measurements with sfroGFP2 and hGrx1-roGFP2 (Figures 6 and 7). However, a stable integration of the hGrx1-sfroGFP2 probe into NF54-*attB* (which is currently underway in our lab) would allow for higher fluorescence intensity combined with rapid equilibration also in subcellular compartments lacking Grx.

In order to provide a robust and reliable *Plasmodium* strain for comparable results in the malaria community, we expressed and characterized the hGrx1-roGFP2 and the optimized sfroGFP2 probes in *P. falciparum* NF54-*attB* blood stage parasites. In contrast to episomally transfected parasites,^{7,14} 100% of the cloned, isogenic parasites showed stable fluorescence. We note that, as described by Mohring et al.,⁷ slight differences in the redox milieu of malaria parasites had been observed after episomal transfection with the hGrx1roGFP2 probe. These changes were hypothesized to be caused by WR99210, the drug used for selection. To further substantiate this hypothesis, we determined total thiols, free thiols, total glutathione, and GSH levels of transiently transfected 3D7[pARL1a(+)], 3D7[hGrx1], 3D7[roGFP2], and 3D7[hGrx1-roGFP2] parasites (Figure 2). Indeed, no significant differences were detected between parasites carrying different parts of the sensor or the transfection vector pARL1a(+) alone, indicating that there is no direct influence of the redox probe on glutathione metabolism in the parasite. This further substantiates the use of stably expressed redox probes in future analyses.

On the basis of these results, we were able to study redox changes in living parasite populations in liquid culture using a plate reader. The plate reader detection is a rapid, easy, and frequently available method to compare many treatments at one time point. This method also allows dynamic measurements for longer time periods, which is not possible with CLSM due to photobleaching and cell disruption.¹⁴ Finally, the analysis of a whole cell population, rather than of several single cells, results in a robust readout. Nevertheless, we recommend, when using a plate reader, to briefly validate the quality of the parasite culture by microscopy in order to detect morphological changes, e.g., due to drug effects. Plate reader measurements had already been established for roGFP-based probes in other cells like HeLa cells³⁵ and yeast.¹¹ Also in *P. falciparum*, both methods, fluorescence microscopy and plate reader measurements with the stably expressed sensors, proved to be reliable tools with comparable sensitivities toward oxidation/reduction in dynamic live cell imaging. With -304 mV for NF54[hGrx1-roGFP2] and -303 mV for NF54[sfroGFP2], both strains exhibited nearly the same glutathione redox potential, which is comparable to the results measured in 3D7 parasites by Kasozi et al.¹³ (-314 mV) and Mohring et al.¹⁴ (-309 mV). As discussed in other publications,^{32,10} slightly diverging values for the midpoint potential of roGFP2 have been described, depending on the methods used and the lab, in which the analyses were carried out. It has therefore been suggested to use a consensus E'_0 of -280 mV,^{32,10} which was also used for our calculations. From the OxDs of our untreated cells, which have been determined to be 16% for sfroGFP2 and 21% for hGrx1-roGFP2, it can be assumed that

both probes have a comparable midpoint potential and are mainly reduced in the cytosol of *Plasmodium* (as has been reported for other cells) but that they still can be used to monitor oxidative effects *in cellulo*. Notably, the sfroGFP2 sensor showed higher fluorescence intensity than the hGrx1-roGFP2 probe in the cells (please see Table 1, Figure S4, and abstract graphic), suggesting it to be (ideally in fusion with Grx) a valuable candidate for future studies in small organisms and subcellular compartments.

Furthermore, probe fluorescence and protein levels throughout the asexual life cycle of *P. falciparum* were monitored, and trophozoite stages 25–40 h post-infection were found to be the best time point for redox analysis with the genetically encoded roGFP2 sensors. Because of the increased fluorescence levels of the sfroGFP2, we could, for the first time, detect fluorescence signals in merozoites. However, reliable measurements remained challenging because a proper adjustment to the correct z-level was difficult to achieve (Figure 4).

Finally, on NF54-*attB* parasites stably transfected with hGrx1-roGFP2 or sfroGFP2, we assessed the effects of antimalarial drugs after mid-term (4 h) and long-term (24 h) exposures (Figures 6 and 7). Prior to cell culture experiments, we recombinantly produced and purified sfroGFP2 to exclude direct effects of the compounds on the redox ratio of the probe. None of the drugs showed interactions at pharmacologically relevant concentrations even after 24 h of incubation (Figure S3). Also, for the hGrx1-roGFP2 probe, major direct interactions with antimalarial drugs had been excluded in previous studies.^{13,14} It should be noted that within parasites the observed moderate interactions are likely to be even less pronounced due to the presence of multiple membranes that need to be passed, compound degradation, and/or drug binding to cellular proteins.

Incubations with ATS did not cause an increase in the cytosolic fluorescence ratio of the probes. Notably, Kasozi et al.¹³ observed strain-specific oxidizing effects of ATS on *P. falciparum* 3D7 and Dd2 parasites episomally transfected with hGrx1-roGFP2. The recently described compound **1o**, an antiplasmodial arylmethylamino steroid, led to a strong increase of the redox ratio after 24 h of incubation, which points to a redox-based mechanism of action of the drug and is in accordance with the data reported by Krieg et al.,³³ with transiently transfected 3D7 parasites.

The synthetic drug MQ showed an oxidizing effect after 24 h. This change in redox ratio was more prominent in the plate reader, in which a whole cell population can be analyzed simultaneously. In this context, it should be taken into account that the absolute cell number (which can be compromised by antimalarials) needs to be high enough in the plate reader to obtain reliable results. Therefore, we usually use at least 1 million RBCs with 60–80% parasitemia per well (after magnetic enrichment) and recommend performing CLSM in parallel to monitor viability and morphology of the parasites. An increase in the redox ratio of the hGrx1-roGFP2 probe after 4 and 24 h of incubation with MQ has been previously described by Kasozi et al.¹³ for the *P. falciparum* 3D7 strain. Since the mechanism of action of MQ, a compound structurally related to QN and CQ, is not yet fully understood, it would be worth studying time-, concentration-, strain-, and compartment-dependent redox changes induced by the drug.

CONCLUSIONS

Our results suggest that the redox biosensors hGrx1-roGFP2 and sfroGFP2 are both sensitive and suitable tools to study redox homeostasis in the cytosol of *P. falciparum*. In contrast to the previously used episomal transfection, the stable integration facilitates rapid and reliable analyses since all parasites express the sensor. This also enhances the feasibility of more complex in-cell studies allowing for plate reader analyses that can be leveraged with fluorescence microscopy studies. Furthermore, problems with episomally transfected parasite lines, in which some parasites may develop resistance against selection drugs thus losing their plasmid and the expression of the fluorescent probe, were overcome by stable integration. The robust fluorescence levels of sfroGFP2 will likely be of substantial benefit for researchers working with small organisms or in subcellular compartments.

In the future, it would be of particular interest to establish flow cytometric measurements and other potentially high-throughput compatible techniques on roGFP2 transfected parasites, in order to enable detailed analyses of a whole cell population. Furthermore, the usage of a stably integrated hGrx1-sfroGFP2 sensor will allow one to analyze glutathione-dependent redox changes with high spatiotemporal resolution even in Grx-free subcellular compartments. Finally, it would be desirable to systematically study and directly compare all GFPbased glutathione redox sensors *in Vitro* and *in cellulo* including redox titrations and determination of midpoint potentials and dynamic changes as well as spectral characteristics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ART	artemisinin
ATS	artesunate
AQ	amodiaquine
ATM	artemether
ATQ	atovaquone
BSA	bovine serum albumin

CLSM	confocal laser scanning microscopy
Cn	carbenicillin
CQ	chloroquine
DIA	diamide/diazenedicarboxamide
DMSO	dimethyl sulfoxide
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DTT	1,4-dithiothreitol
EC₅₀	half maximal effective concentration
EDTA	ethylenediaminetetraacetic acid
GFP	green fluorescent protein
Grx	glutaredoxin
GSH	reduced glutathione
GSSG	oxidized glutathione
hGrx1-roGFP2	human glutaredoxin 1 fused to reduction– oxidation sensitive green fluorescent protein
IPTG	isopropyl- β -D-thiogalactopyranoside
iRBCs	infected red blood cells
Kan	kanamycin
LB	Luria–Bertani medium
LUM	lumefantrine
MQ	mefloquine
NADPH	nicotinamide adenine dinucleotide phosphate
NDA	naphthalene dicarboxaldehyde
NEM	N-ethylmaleimide
OD₆₀₀	optical density at 600 nm
OxD	degree of oxidation
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
P. falciparum	Plasmodium falciparum

PMSF	phenylmethylsulfonyl fluoride
QN	quinine
RBCs	red blood cells
roGFP	reduction–oxidation sensitive green fluorescent protein
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
sfroGFP2	super folder reduction–oxidation sensitive green fluorescent protein
SSA	sulfosalicylic acid

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Figure 1.

Schematic representation of redox sensor integration into the *P. falciparum* genome and representative PCR showing evidence of integration. (A) NF54-*attB* parasites were cotransfected with an *attP* plasmid (*bsd* selection marker) encoding the hGrx1-roGFP2 (shown) or sfroGFP2 gene along with the integrase-encoding plasmid pINT (*neo* selection marker). Integrase-mediated *attB* × *attP*-based genomic recombination facilitated the insertion of hGrx1-roGFP2 (shown) or sfroGFP2 into the genomic *attB* locus. (B) Integration was verified using cg6 5' F and bsd R primers, as marked above, resulting in the expected 1.6 kb PCR fragment. Results are shown for five separate sfroGFP2 parasite clones. The specificity of integration was confirmed using sequence analysis of the recombinant bands

Figure 2.

Determination of total thiols, free thiols, total glutathione, and GSH levels in *P. falciparum* 3D7-transfected parasites. Transiently transfected 3D7[pARL1a(+)], 3D7[hGrx1], 3D7[roGFP2], and 3D7[hGrx1-roGFP2] trophozoites were saponin-lysed, and their thiol and glutathione levels were determined. Data from three independent experiments revealed no significant differences between the four transfected parasite lines. Means and standard errors of the mean (SEM) are shown.

Figure 3.

Real-time imaging of the dynamic range of hGrx1-roGFP2 and sfroGFP2 in NF54-*attB* parasites using CLSM and plate reader. After 15 s of baseline monitoring, NF54-*attB* parasites transfected with hGrx1-roGFP2 or sfroGFP2 were exposed to 1 mM DIA and monitored for 3 min before adding 10 mM DTT. Ratiometric readings employed either the CLSM (A) or a plate reader (B). The fluorescence ratios of 405/488 nm and 405/475 nm measured using CLSM or the plate reader, respectively, were plotted across time. CLSM data (mean $\pm$ SEM) were composed of values from 9 trophozoites analyzed in each of three independent experiments. Plate reader data show mean values $\pm$ SEM of three independent experiments. For further experimental details, please see the Experimental Section.

Figure 4. SfroGFP2 signals throughout the asexual blood stage cycle of *P. falciparum* NF54-attB. 405 nm, 488 nm, and bright field images were taken with a confocal microscope. The white magnification bar represents 10 μm . Giemsa-stained images were photographed with a Zeiss Axiophot with a 100/1.3 oil Plan-NEOFLUAR objective. The black magnification bar represents 20 μm .

Figure 5.

Western blot time course from 5 to 50 h post-invasion (for NF54^[sroGFP2] parasites). For each lane, 15 μ L of infected RBCs with an initial parasitemia of 2.5% were collected and saponin-lysed. Samples were taken every 5 h after invasion until 50 h. For technical reasons, the figure is composed of bands from two separate SDS gels/membranes; however, all samples were prepared simultaneously, and the two gels were run and blotted at the same time and detected with identical settings. The 26 kDa band (theoretical MW) of sroGFP2 is clearly visible at every time point. Late trophozoite stages (35–45 h) show strong signals, whereas the signal at 50 h (where merozoites and rings are present) is weaker. The protein ladder is labeled with M; positive control recombinantly expressed sroGFP2 was used and is labeled with C.

4h

Figure 6.

Mid-term effects of antimalarial agents and redox-active compounds on the redox ratio of *P. falciparum* NF54^[hGrx1-roGFP2] and NF54^[sfroGFP2] parasites. A 4 h incubation of NF54^[hGrx1-roGFP2] and NF54^[sfroGFP2] transgenic parasites with 100× EC₅₀ drug concentrations did not affect the fluorescence ratio of the redox sensors with either the CLSM (A, C) or the plate reader (B, D). One mM DIA led to a significant increase in fluorescence ratio for both probes and detection methods. CLSM data were composed of 10–20 trophozoites analyzed per experiment for each incubation. Mean values and standard error of the mean (±SEM) are shown for three independent experiments. A one-way ANOVA test with 95% confidence intervals with the Dunnett's Multiple Comparison Test was applied for statistical analysis of significance (*, $p < 0.05$).

Figure 7.

Long-term effects of antimalarial agents and redox-active compounds on the redox ratio of *P. falciparum* NF54^[hGrx1-roGFP2] and NF54^[sfroGFP2] transfected parasites. 24 h of incubation of NF54^[hGrx1-roGFP2] and NF54^[sfroGFP2] transgenic parasites with 10× EC₅₀ drug concentration showed increased fluorescence ratios of the redox sensors for MQ and compound 1o with the CLSM (A, C) and the plate reader (B, D). DIA led to an increase in fluorescence ratio for both probes and detection methods. CLSM data were collected from 10–20 trophozoites analyzed per experiment. Mean values and standard errors of the mean (±SEM) are shown for three independent experiments. A one-way ANOVA test with 95% confidence intervals with the Dunnett's Multiple Comparison Test was applied for statistical analysis of significance (*, $p < 0.05$; ****, $p < 0.0001$).

Table 1.Comparison of E_{GSH} in the Cytosol of NF54^[hGrx1-roGFP2] and NF54^[sfroGFP2]^a

	NF54 ^[hGrx1-roGFP2]	NF54 ^[sfroGFP2]
R_{basal} mean	0.65 ± 0.11	0.70 ± 0.12
R_{basal} range	0.38–0.85	0.47–0.95
R_{ox} mean	2.19 ± 0.11	2.51 ± 0.004
R_{ox} range	1.76–2.79	1.95–3.33
R_{red} mean	0.52 ± 0.01	0.55 ± 0.12
R_{red} range	0.40–0.62	0.34–0.76
dynamic range ($R_{\text{ox}}/R_{\text{red}}$)	4.23	4.56
$I_{488\text{ox}}$	26.84	49.77
$I_{488\text{red}}$	81.86	113.71
$I_{488\text{ox}}/I_{488\text{red}}$	0.33	0.44
OxD	20.8%	15.9%
glutathione redox potential	–304 mV	–303 mV

^aR = ratio (405 nm/488 nm) I = fluorescence intensity; OxD = degree of oxidation. Three replicates with 11 parasites each were used for calculation (for details, please see Experimental Section).