Physiological jump in erythrocyte redox potential during *Plasmodium falciparum* development occurs independent of the sickle cell trait

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1. Introduction

The cellular redox state affects numerous biological pathways in health and disease and, therefore, has gained substantial attention [1]. However, most of our current understanding of cellular redox processes and their maintenance is based on common model organisms, in particular, *Escherichia coli* [2], plants [3], and opisthokonts such as yeast [4] and animals [1]. A non-opisthokont organism that faces several unique redox challenges and which also has a specialized export system for disulfide-containing proteins is the apicomplexan parasite *Plasmodium falciparum*. *P. falciparum* is responsible for the most virulent form of human malaria, killing an estimated 627,000 people in 2020 alone [5]. *P. falciparum* is transmitted to humans by the bite of an infected *Anopheles* mosquito. Once transmitted, the parasite infects hepatocytes before it changes its host cell specificity and establishes a 48 h replicative cycle within red blood cells [6]. The intraerythrocytic life cycle is defined by four morphological stages of development: the ring stage lasting the first 20 h post invasion (hpi), the trophozoite stage (24–36 hpi) being characterized by a large hemoglobin-containing digestive vacuole, the schizont stage (40–48 h post invasion) during which nuclear and cellular division occurs, and finally the merozoites, which are released from ruptured mature schizonts and which perpetuate the red blood cell cycle by re-infecting erythrocytes.

In the erythrocyte, the parasite is enveloped by a parasitophorous vacuolar membrane, separating the parasite from the host cell cytosol and serving as an intermediary compartment for parasite-encoded proteins allotted for export into the red blood cell [7–9]. Some of these exported proteins contain structural disulfide bonds [10]. Furthermore,
large amounts of hemoglobin are taken up by endocytosis at the parasi-tophorous vacuolar membrane and are degraded in a digestive vacuole to provide amino acids, maintain the colloid osmotic balance of the host-parasite unit, and create space for parasite growth [11-13].

During intraerythrocytic development, the parasite is believed to face high amounts of superoxide anion ($\text{O}_2^-$) and hydrogen peroxide ($\text{H}_2\text{O}_2$), which originate from the metabolic activity of the parasite and the role of the host cell as a carrier of molecular oxygen [14,15]. In particular, the decomposition of hemoglobin, spontaneously in the red blood cell and enzymatically in the parasite’s digestive vacuole, generates $\text{O}_2^-$ and ferric protoporphyrin IX [16]. While superoxide anions are thought to spontaneously disproporionate to $\text{H}_2\text{O}_2$ and $\text{O}_2$ at the acidic pH in the digestive vacuole, more than 95% of ferric protoporphyrin IX that is liberated during hemoglobin degradation is detoxified as hemozoin [16-18], thus preventing Fenton reactions in the presence of reducing agents. In general, $\text{O}_2^-$ and/or $\text{H}_2\text{O}_2$ can also be generated by flavoenzymes and at specific sites of electron transport chains [2,19-21]. However, it remains to be shown to which degree and during which developmental stage these processes play a role in the parasite’s cytosol, endoplasmic reticulum, mitochondrion or apicoplast.

To maintain the redox steady-state, the host-parasite unit relies on numerous enzymes. For example, kinetic models for uninfected erythrocytes suggest that human superoxide dismutase maintains a steady-state $\text{O}_2^-$ concentration of around 0.5 pM and that a combination of human glutathione peroxidase 1, catalase, and peroxiredoxin II keeps particular, the decomposition of hemoglobin, spontaneously in the red the role of the host cell as a carrier of molecular oxygen [14,15]. In general, $\text{O}_2^-$ and/or $\text{H}_2\text{O}_2$ can also be generated by flavoenzymes and at specific sites of electron transport chains [2,19-21]. However, it remains to be shown to which degree and during which developmental stage these processes play a role in the parasite’s cytosol, endoplasmic reticulum, mitochondrion or apicoplast.

To maintain the redox steady-state, the host-parasite unit relies on numerous enzymes. For example, kinetic models for uninfected erythrocytes suggest that human superoxide dismutase maintains a steady-state $\text{O}_2^-$ concentration of around 0.5 pM and that a combination of human glutathione peroxidase 1, catalase, and peroxiredoxin II keeps the steady-state $\text{H}_2\text{O}_2$ concentration at around 50 pM [22]. Protein thiols in the erythrocyte are kept in a reduced state in the presence of approximately 1.8 mM glutathione with the help of human glutathione reductase and glutaredoxin 1 (bGrx1) [23-26]. While all these redox enzymes, together with hemoglobin, are taken up into the digestive vacuole, *P. falciparum* also harbors five endogenous peroxiredoxins, two superoxide dismutases, and the typical enzyme repertoire to maintain glutathione and protein thiols in a reduced state [15]. Furthermore, the parasite exports reduced glutathione (GSH) into the erythrocyte and induces a GSH efflux system in the erythrocyte plasma membrane via parasite-induced new permeation pathways [27].

Several antimalarial compounds, including artesiminin, chloroquine, ferroquine, and plasmiodione, were suggested to alter the redox metabolism either directly or indirectly and/or to induce downstream oxidative toxicity [14,28-33]. Furthermore, an imbalance in antioxidant defenses and oxidant production has been implicated in the malaria-protective function of the sickle cell trait and other hemoglobin polymorphisms [14,34,35]. The alleged pro-oxidative environment of sickle cell trait erythrocytes is thought to result from an inherent instability of the sickle cell hemoglobin S (HbS) [36,37], which carriers inherited in form of a mutated $\beta$-globin gene from one parent, while receiving a wild-type $\beta$-globin gene from the other parent. HbS, which differs from the wild-type HbA hemoglobin by a single amino acid substitution of valine for glutamate at position 6, is prone to autoxidation, resulting in elevated levels of methemoglobin, irreversibly oxidized hemechromes, ferryl hemoglobin, protein radicals, free ferryl heme and other oxidants including Fe$^{2+}$-Fe$^{3+}$ [38-40]. The pro-oxidative reactivity of HbS is thought to overwhelm the redox-balancing systems of the parasite and the red blood cell, which, in turn, disturbs physiological and pathophysiological processes of the parasite, including intraerythrocytic parasite development [41,42] and the ability to engage in disease-defining cytoadhesive interactions with the microvascular lining [35,43-46], while increasing immunogenicity and splenic clearance efficiency [34,35,47].

While this oxidative stress hypothesis provides a plausible explanation for the malaria-protective function of the sickle cell trait and related hemoglobinopathies, it has not yet been formally tested. Furthermore, it is unclear if and to what extent the infection with *P. falciparum* impacts on the redox potential of a wild-type red blood cell, not to mention that of a sickle trait erythrocyte. It is further unclear whether the erythrocyte cytosol and the parasitophorous vacuole are in a steady-state redox equilibrium with one another. Finally, time-resolved information regarding putative redox dynamics are not yet available for the erythrocyte/parasite system.

Traditionally, the redox potential in human erythrocytes was measured using invasive techniques that yielded values ranging from $-276$ mV to $-250$ mV for uninfected wild-type (HbAA) erythrocytes [48-50], and $-220$ mV for homozygous sickle cells (HbSS) [50]. Advances in genetically-encoded fluorescent redox reporters, such as the redox-sensitive green fluorescent protein 2 (roGFPP2), now allow one to quantify the redox potential in defined subcellular compartments in a non-invasive manner [51,52]. The fluorescence properties of roGFPP2 depend on whether cysteine residues 147 and 204 are in the reduced thiol or the oxidized sulfide state, which can be monitored ratiometrically by measuring the fluorescence intensities at an excitation wavelength of 405 nm and 488 nm [51]. In a cellular system, roGFPP2 is in a glutaredoxin-catalyzed steady-state equilibrium with highly concentrated glutathione and, thus, reports predominantly the glutathione redox potential [51,53-55]. In the case of *P. falciparum*, roGFPP2 constructs have been used to quantify the glutathione redox potential in the parasite cytosol (between $-304$ mV and $-314$ mV) [56-58], the apicoplast ($-267$ mV) [57] and the mitochondrion ($-329$ mV) [57], and to monitor $\text{H}_2\text{O}_2$ dynamics in the parasite cytosol upon antimalarial drug application [31,32,59].

Here, we targeted parasite-encoded roGFPP2 and the superfolder mutation FFrGFPP2 to the cytosol of *P. falciparum*-infected wild-type and sickle trait erythrocytes, allowing us to monitor the steady-state redox potential in the red blood cell compartment in a single live cell set-up over time. We observed a sudden physiological jump in the redox potential from a reducing to a more oxidizing environment during trophozoite maturation. Growing the parasite in sickle trait erythrocytes had no impact on the stage-specific steady-state redox potential, contrary to the predictions made by the oxidative stress hypothesis. We further determined the steady-state redox potential in the parasite enveloping parasitophorous vacuole and found it to be more oxidizing in comparison with its adjacent compartments.

2. Materials and methods

2.1. Ethical clearance

The study was approved by the ethical review boards of Heidelberg University, Germany, and Mannheim University, Germany. Written informed consent was given by all blood donors.

2.2. Blood collection and hemoglobin phenotype determination

Blood was collected by venipuncture in diagnostic grade sodium isoctirate tubes and centrifuged (800 × g, 10 min, RT). The plasma and buffy coat were discarded, and the red blood cells (RBC) were washed and stored at 4 ºC in RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine and 25 mM HEPES. The HbAS phenotype was confirmed by HPLC testing in the Central Laboratory of Heidelberg University Hospital. Erythrocytes were used after blood donation within 10 days or less. The experimental data were obtained using blood from five different HbAA donors and two different HbAS donors.

2.3. *P. falciparum* culture

The *P. falciparum* FCR3 strain was used throughout this study. The parental strain and transfected parasites were cultured as described [60]. The parasites were propagated in wild-type (HbAA) A- and HbAS RBCs at a hematocrit of 5.5% in RPMI medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 0.2 mM hypoxanthine, 5% (v/v) human serum (AB + for HbAS RBCs), 5% (v/v) albumax and 20 μg ml$^{-1}$ gentamycin, and in an atmosphere of 5% O$_2$, 3% CO$_2$, 92% N$_2$, and 95% humidity at 37 ºC. Parasitemia was assessed using Giemsa-stained thin
blood smears. Parasite cultures were synchronized within a window of 4 h, using a combination of 5% sorbitol [61] and 50 units ml⁻¹ heparin (Sigma) [62].

2.4. Constructs and cloning

A fast folding variant of roGFP2 (FFroGFP2) was rationally designed to incorporate the mutations F99S, M153T, V163A [63] and five of the six superfolder mutations [64], namely, S30R, Y39N, N105T, I171V, and A206V, into roGFP2. The Y145F mutation, deemed to interfere with the hydrogen bonding dynamics of the chromophore and shown to abolish the ratiometric properties of roGFP-variants [65] was left out. FFroGFP2 is thus similar to rTurbo which omits M153T and includes A206K and F223R instead [66]. FFroGFP2 was ordered as a synthetic construct from GeneArt, Life Technologies (Fig. S1). Targeting of roGFP2 variants to the iRBC cytosol was achieved by fusing them with the first 80 N-terminal amino acids of a STEVOR protein containing the PEXEL motif [67]. To this end, the coding sequences of roGFP2, FFroGFP2 or roGFP2+C147S (generated by PCR mutagenesis) [68] were cloned in frame with the coding sequence of STEVOR+C147S into pARL1a_STEVOR-C147S [67], following digestion with the restriction endonucleases BsrHI and XmaI (FFroGFP2) or NcoI and XmaI (roGFP2 and roGFP2+C147S). Primers used for amplification and cloning are listed in Table S1. All vectors were confirmed by DNA sequencing and restriction analysis.

2.5. Transfection of P. falciparum

Transient transfection of P. falciparum was performed as described previously [69]. 200 μl of 1-2% sorbitol synchronized pelleted culture of early ring stage parasites was mixed with 370 μl of cytosol (120 mM KCl, 0.15 mM CaCl₂, 5 mM MgCl₂, 10 mM KH₂PO₄/K₂HPO₄, 25 mM HEPES, 2 mM EGTA, pH 7.6) and 30 μl of plasmid DNA in 10 mM Tris-HCl at pH 8.5. Electroporation was performed at 0.31kV/0.95μF/high capacitance in a 0.2 cm electroporation cuvette, before cells were returned to culture. 24 h after electroporation, transfected parasites were selected by continuous exposure to 5 nM of WR99210. A detectable parasitemia appeared after 4-5 weeks.

2.6. Spinning disc confocal microscopy

Synchronized parasites (100 μl) were seeded either into a 96-well plate with optical glass bottom (Thermo Fisher) or 18 well ibidi slides coated with a 0.01% poly-L-lysine solution (4 μg cm⁻²). The parasites were allowed to settle for 5–10 min, washed once with 100 μl Ringer solution (122.5 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl₂, 0.8 mM MgCl₂, 11 mM D-glucose, 10 mM HEPES, 1 mM NaH₂PO₄, pH 7.4) before adding another 100 μl of Ringer solution for imaging. Parasites were imaged within 1 h after seeding into the imaging wells. Imaging was performed with a PerkinElmer UltraView spinning disc confocal unit on a Nikon TiE inverted microscope (objective: Nikon Apo TIRF 60x NA 1.49 oil immersion; illumination sources: 405 nm and 488 nm diode lasers; fluorescence filter center wavelength 525 nm bandwidth 50 nm; EM-CCD camera: Hamamatsu C9100-23B). The system was embedded in an environmental box for temperature-, CO₂- and humidity control and equipped with a hardware-based Nikon perfect focus system (infrared LED, 880 nm, 0.1 mW, Inline CMOS sensor). Imaging was performed at 37 °C in ambient air. Images (16bit) were acquired with the PerkinElmer Velocity software (v6.2) with a frame rate of 10 s for 240 s. After 60 s, Diamond (1 mM final concentration) and after 180 s DTT (5 mM final concentration) were added to the wells, using gel-loading pipette tips.

2.7. Image analysis

Images were analyzed, using a FIJI-based [70] custom-made macro (https://github.com/mhaag-ux/FLIroGFP2). In some cases, images were analyzed manually, using FIJI. Briefly, mean fluorescence intensities were quantified in a region of interest (ROI) within the iRBC cytosol, the parasite cytosol or the PV. In addition, the background fluorescence intensity was determined and subtracted. The specific fluorescence intensities derived from the two excitation wavelengths were subsequently used to calculate the 405/488 ratio.

2.8. Calculation of the degree of oxidation of roGFP2 and redox potentials

Oxidation of roGFP2 was calculated as previously described [71]. For the calculation of a ratiometric readout the following equation was used:

\[ \text{OD}_{\text{roGFP}} = \frac{R_m - R_o}{R_o - R_m} \text{ red} \]

Where R is the 405/488 ratio (m: at the beginning of the experiment; red: fully reduced after the addition of DTT; ox: fully oxidized after the addition of Diamide) and I the fluorescence intensity of the fully oxidized (ox) and reduced (red) cell. A non-ratiometric readout was calculated as following:

\[ \text{OD}_{\text{roGFP}} = \frac{\text{I}_{\text{reduced}} - \text{I}_{\text{baseline}}}{\text{I}_{\text{reduced}} - \text{I}_{\text{oxidized}}} \]

Where \text{I}_{\text{baseline}} is the intensity of the fluorescent signal of roGFP at the beginning of the measurement, \text{I}_{\text{oxidized}} is the intensity after full oxidation of the protein and \text{I}_{\text{reduced}} is the intensity after full reduction. The redox potential of roGFP2 is calculated as follows:

\[ E_{\text{roGFP}} = E_{\text{roGFP}}^o - \frac{RT}{nF} \ln \left( \frac{1 - \text{OD}_{\text{roGFP}}}{\text{OD}_{\text{roGFP}}} \right) \]

Where R is the gas constant (8.315 J mol⁻¹ K⁻¹), T is the experimental temperature (37 °C = 310.15K), n is the number of transferred electrons (2), F is the Faraday constant (96,480 J mol⁻¹), \text{E}_{\text{roGFP}}^o is the midpoint potential of roGFP2 at 30 °C and pH 7.0 (~280 mV) [72,73]. In cellular compartments where roGFP2 equilibrates with the redox state of glutathione the following applies:

\[ E_{\text{GSH}} = E_{\text{GSSG}}^o - \frac{RT}{nF} \ln \left( \frac{\text{GSH}^-}{\text{GSSG}^-} \right) \]

Because 2 protons and 2 electrons are involved in reduction/oxidation of both GSH and roGFP2, ΔE/ΔpH is ~61.5 mV per 1 pH unit at 37 °C [74].

2.9. Statistical analysis

Graphs and statistical analyses were done using GraphPad Prism version 9, GraphPad Software, San Diego, California USA, www.graphpad.com. Statistical significance was determined using the Kruskal-Wallis test with Dunn’s multiple comparison, the Mann-Whitney test or the F-test, where indicated. A p-value of <0.05 was considered as significant. Box plots show the median (black line), the mean (black cross), and the 25 and 75% quartile range. The error bars above and below the box indicate the 90th and 10th percentiles.
3. Results

3.1. The parasite cytosol is a stable reducing environment

To determine the steady-state redox potential in defined subcellular compartments of erythrocytes that are infected with *P. falciparum*, we generated parasite lines with genetically encoded fusion constructs between roGFP2 or the improved FFroGFP2 variant and appropriate protein sorting and targeting motifs. FFroGFP2 differs from roGFP2 in that it carries so-called superfolder mutations, resulting in improved stability and, hence, higher fluorescence intensities that facilitate redox measurements in the secretory pathway [65].

As a control for our experimental set-up, we initially quantified the steady-state redox potential in the parasite cytosol, using the previously published hGrx1_roGFP2 construct (Fig. 1A) [56]. Bright fluorescence signals at both the 405 nm and 488 nm excitation wavelength were observed in rings (8–16 h post invasion (hpi)), trophozoites (20–32 hpi), and schizonts (36–40 hpi) (Fig. 1B). The fluorescence intensities were stable over a period of at least 240 s (Fig. 1C), suggesting that our single-cell measurements were not compromised by technical difficulties, such as focus drifts or photobleaching. To calibrate the redox measurements, we used the oxidizing agent 3-(dimethylcarbamoylimino)-1,1-dimethylurea (Diamide) [75] and the reducing agent dithiothreitol (DTT) [76]. We first exposed the cells to 1 mM Diamide followed by the addition of 5 mM DTT, while constantly monitoring fluorescence intensities at the two excitation wavelengths in single living cells (Fig. 1C). As expected, the fluorescence intensity at 405 nm excitation increased upon the addition of Diamide, whereas that at 488 nm excitation decreased. The opposite effect was observed following the addition of DTT. Accordingly, the ratio of the two fluorescence intensities increased following treatment with Diamide and decreased in the presence of DTT. In total, we recorded data from 22 rings, 27 trophozoites and 20 schizonts and found that the parasite cytosol provides a constant reducing environment throughout the intraerythrocytic cycle, as indicated by a mean roGFP2 oxidation of 24 ± 20% in rings, 35 ± 23% in trophozoites and 29 ± 23% in schizonts (Fig. 1D). Using the Nernst equation for glutathione at pH 7.2 and 37 °C [74, 77, 78], these values were converted to steady-state redox potentials of −313 ± 21, −304 ± 17, and −306 ± 18 mV, respectively (Table 1), consistent with previous reports on strain 3D7 [56–58].
In summary, we established and validated an experimental setup for single-cell roGFP2 measurements revealing a stable reducing environment in the parasite cytosol throughout the asexual intraerythrocytic development of *P. falciparum*.

### 3.2. Sudden jump in erythrocyte redox potential during parasite development

We next set out to quantify the unknown steady-state redox potential in the cytosol of the *P. falciparum*-infected erythrocyte. To this end, a *P. falciparum* line was established, expressing FFroGFP2 fused N-terminally with the first 80 amino acids of STEVOR [67]. This domain contains all trafficking and sorting elements for entering the secretory pathway and passing the parasite plasma membrane, the parasitophorous vacular lumen, and the parasite-enveloping parasitophorous vacular membrane to reach the cytosol of the infected erythrocyte (Fig. 2A) [8,9,67].

Highly synchronized parasite cultures were used and four time points between 14 and 40 h post invasion (hpi) were analyzed: late rings (14–18 hpi), early trophozoites (20–24 hpi), late trophozoites (28–32 hpi), and schizonts (35–40 hpi). Stable fluorescence signals at both excitation wavelengths were detectable in the cytosol of infected red blood cells (iRBC) at all time points (Fig. 2B). In addition, fluorescence was detected in various subcellular compartments along the protein export pathway, including the ER and the parasitophorous vacuole, and the digestive vacuole. We further noted that the overall signal strengths were generally weaker as compared with measurements for hGrx1_roGFP2 that was localized in the parasite cytosol. Nevertheless, ratiometric redox imaging was possible. The cytosol of iRBC containing rings that were younger than 14 hpi was not or only weakly fluorescent and, therefore, was excluded from the analysis. The weak fluorescence might be explained by a premature protein export pathway and/or insufficient export of the redox sensor into the red blood cell compartment.

The fluorescence intensities of trophozoite-infected erythrocytes without the addition of redox agents were again stable over a period of at least 240 s while fluorescence intensities responded upon the addition of 1 mM Diamide and 5 mM DTT (Fig. 2C). Quantification of the fluorescence signals revealed a reducing environment in the cytosol of infected erythrocytes for late rings and early trophozoites, as indicated by FFroGFP2 oxidation states of 31 ± 17% (n = 9) and 47 ± 19% (n = 22), respectively (Fig. 2D). Unexpectedly, the FFroGFP2 oxidation state in the erythrocyte jumped to 72 ± 15% (n = 14) in late trophozoite-infected cells and remained high until the end of schizogony (78 ± 27%; n = 18). Based on the assumption that the redox probe predominately reflects a hGrx1-catalyzed steady-state equilibrium with glutathione, we used again the Nernst equation to calculate the corresponding steady-state redox potentials, revealing a jump in the erythrocyte redox potential from −304 ± 11 to −278 ± 12 mV (Table 1). Thus, the steady-state redox potential in the erythrocyte cytosol appears to change from a highly reducing to a more oxidizing milieu as the parasite matures from early to late trophozoites.

Using STEVOR<sup>1–80</sup>_roGFP2 instead of STEVOR<sup>1–80</sup>_FFroGFP2 as a redox probe confirmed these results (Fig. 3). Again, a change in the steady-state redox potential in the cytosol of infected erythrocytes was observed during trophozoite maturation, with ring- and early trophozoite-stage iRBC providing a reducing environment (24 ± 17% roGFP2 oxidation in late ring iRBC and 36 ± 22% in early trophozoite iRBC), whereas late trophozoite and schizont iRBC provided a more oxidizing milieu (roGFP2 oxidation 81 ± 20% and 71 ± 14%, respectively) (Fig. 3E). Using the Nernst equation to calculate the according steady-state redox potentials revealed again a jump in the erythrocyte redox potential from −309 ± 12 to −272 ± 14 mV (Table 1). Although the results obtained using STEVOR<sup>1–80</sup>_FFroGFP2 were quantitatively and qualitatively comparable to those made using STEVOR<sup>1–80</sup>_FFroGFP2, a previously described non-ratiometric data analysis strategy [51,53,79], based on the 488 nm excited fluorescence signal only, had to be applied because of a non-quantifiable fluorescence signal in the erythrocyte cytosol at 405 nm excitation (Fig. 3D). We explain this lack of a detectable excitation at 405 nm by a lower export efficiency and a lower mean fluorescence intensity of roGFP2 compared with FFroGFP2.

To further support the non-ratiometric data analysis strategy, we investigated parasites expressing the redox-inactive STEVOR<sup>1–80</sup>_roGFP2<sup>(C147S)</sup> in the erythrocyte cytoplasm. A 488 nm excited fluorescence signal was detected in the erythrocyte cytoplasm, which, however, was unresponsive to the addition of Diamide or DTT (Fig. 3D), contrary to the STEVOR<sup>1–80</sup>_roGFP2 signal that responded to the two redox agents, thus, allowing for quantitative data analysis.

In summary, ratiometric STEVOR<sup>1–80</sup>_FFroGFP2 and single wavelength STEVOR<sup>1–80</sup>_FFroGFP2 measurements both revealed a highly reducing erythrocyte steady-state redox potential during ring-stage development. Between 24 and 28 hpi, a jump in the erythrocyte redox potential around −30 mV occurs during trophozoite-stage development and is maintained in schizont stage iRBC.

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### Table 1

Summary of steady-state redox potentials in subcellular compartments of the erythrocyte-*P. falciparum* host-parasite unit calculated at pH 7.2.

| Organelle       | Construct          | RBC     | Stage          | Redox potential | Reference |
|-----------------|--------------------|---------|----------------|-----------------|-----------|
| apicoplast      | APC_roGFP2_hGrx1   | HbAA    | ring           | −267 mV         | [57]      |
|                 | roGFP2_hGrx1       | HbAA    | trophozoite    | −329 mV         | [57]      |
| parasite cytosol| roGFP2_hGrx1       | HbAA    | early trophozoite | −314 mV       | [56]      |
|                 |                    |         | late trophozoite | −309 mV         | [57]      |
|                 | hGrx1_roGFP2       |         |                | −304 mV         | [58]      |
| PV              | Stevor<sup>1–80</sup>_FFroGFP2 | HbAA | ring           | −313 ± 21 mV    | this study |
|                 |                    |         | schizont       | −306 ± 18 mV    | this study |
| iRBC cytosol    | Stevor<sup>1–80</sup>_FFroGFP2 | HbAA | ring           | −304 ± 11 mV    | this study |
|                 |                    |         | early trophozoite | −295 ± 15 mV    | this study |
|                 |                    |         | late trophozoite | −278 ± 12 mV    | this study |
|                 |                    |         | schizont       | −282 ± 10 mV    | this study |
|                 | Stevor<sup>1–80</sup>_roGFP2 | HbAA | ring           | −309 ± 12 mV    | this study |
|                 |                    |         | early trophozoite | −303 ± 19 mV    | this study |
|                 |                    |         | late trophozoite | −272 ± 14 mV    | this study |
|                 |                    |         | schizont       | −279 ± 10 mV    | this study |
|                 | Stevor<sup>1–80</sup>_roGFP2 | HbAS | ring           | −306 ± 7 mV     | this study |
|                 |                    |         | early trophozoite | −306 ± 14 mV    | this study |
|                 |                    |         | late trophozoite | −270 ± 26 mV    | this study |
|                 |                    |         | schizont       | −276 ± 15 mV    | this study |

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In summary, we established and validated an experimental setup for single-cell roGFP2 measurements revealing a stable reducing environment in the parasite cytosol throughout the asexual intraerythrocytic development of *P. falciparum*. 

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**Table 1**

Summary of steady-state redox potentials in subcellular compartments of the erythrocyte-*P. falciparum* host-parasite unit calculated at pH 7.2.
3.3. More oxidizing conditions in the parasitophorous vacuole

We observed that several iRBC containing STEVOR1-80_FFroGFP2 accumulated the protein not only in the erythrocyte cytosol but also in the parasitophorous vacuolar lumen on its route to the host cell cytosol (Fig. 4A and B). This provided us with the opportunity to explore the unknown steady-state redox potential of the parasitophorous vacuole in selected trophozoites with clearly discernible fluorescence signals. Fluorescence signals in the parasitophorous vacuole were detectable at both excitation wavelengths and did not change over time when no redox agents were added (Fig. 4C). While fluorescence signals slightly changed upon the addition of Diamide, the change upon the addition of

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Fig. 2. The FFroGFP2 probe reveals an increase in erythrocyte steady-state redox potential during parasite maturation. A Cartoon highlighting the mode of action and localization (green) of Stevor1-80_FFroGFP2 in the RBC cytosol and PV. B Representative fluorescence microscopy images of different parasite stages expressing Stevor1-80_FFroGFP2, which were excited at 405 nm and 488 nm scale bar 4 μm. C Time series of erythrocyte fluorescence intensities excited at 405 nm and 488 nm in the trophozoite control without the addition of redox agents, rings, early and late trophozoites, and schizonts (left). Arrows mark the addition of Diamide and DTT. Shown are the mean ± SEM and the number of individual cells examined per parasite stage (n). Ratios of fluorescence intensities over time (right). Shown is the mean ± SEM. D Oxidation of Stevor1-80_FFroGFP2 in the RBC cytosol as a function of parasite maturation. A box plot analysis of single cell determinations is shown. The number of individual cells examined (n) is indicated. p-values were calculated using the Kruskal-Wallis test with Dunn’s Multiple comparison. Abbreviations and details on box plot analysis are given in the legend of Fig. 1.
DTT was more drastic (Fig. 4D). Quantification of data from 12 early and 12 late trophozoites revealed an FFroGFP2 oxidation state of 63 ± 14% and 55 ± 11%, respectively (Fig. 3E). Based on the assumption that the redox state of the probe in this compartment also reflects predominantly the glutathione redox state at the same pH, we calculated steady-state redox potentials of 284 ± 10 and 289 ± 6 mV for early and late trophozoites, respectively (Table 1). This finding suggests that the vacuole surrounding the parasites is a redox-protected compartment that maintains a redox gradient towards both the erythrocyte and the parasite cytosol in early trophozoites and towards the parasite cytosol in late trophozoites.

3.4. No evidence for the oxidative stress hypothesis in malaria

We finally investigated the steady-state redox potential in the cytosol of infected HbAS erythrocytes to test the oxidative stress hypothesis. The oxidative stress hypothesis in malaria explains the lower risk that carriers of the sickle cell trait have in developing severe malaria by a redox imbalance. The redox imbalance is thought to result from the instability of hemoglobin S and its tendency to autoxidize and initiate downstream radical reactions, which, in turn, are thought to interfere with physiological and pathophysiological functions of the parasite [34, 35]. To this end, we infected HbAS erythrocytes with the P. falciparum line expressing STEVOR1-80_roGFP2 and determined the steady-state redox potential in highly synchronized parasite cultures, as explained above (Fig. 5A). We detected a weak but still quantifiable fluorescence signal in STEVOR1-80_roGFP2 parasites excited at 488 nm, responding to the two redox agents, thus, allowing for quantitative data analysis (Fig. 5B). In this regard, parasitized HbAS cells expressing STEVOR1-80_roGFP2 behaved similar to their HbAA counterparts. Again, a jump in the red blood cell steady-state redox potential was observed during trophozoite maturation, with the erythrocyte cytosol providing a reducing environment in late rings and early trophozoites and a more oxidizing environment in late trophozoites and schizonts.

Fig. 3. The roGFP2 probe confirms an increase in erythrocyte steady-state redox potential during parasite maturation. A Cartoon highlighting the mode of action and localization (green) of Stevor1-80_roGFP2 in the RBC cytosol. B Representative fluorescence microscopy images of different parasite stages expressing Stevor1-80_roGFP2, which were excited at 405 nm and 488 nm. Scale bar, 4 μm. C Images of a trophozoite expressing the redox-inactive Stevor1-80_roGFP2(C147S). Scale bar, 4 μm. D Time series of erythrocyte fluorescence intensities excited at 405 nm and 488 nm of control trophozoites, rings, early and late trophozoites and schizonts. Arrows mark the addition of Diamide and DTT. Shown are the mean ± SEM and the number of individual cells examined per parasite stage (n). E Oxidation of Stevor1-80_roGFP2 in the RBC cytosol as a function of parasite maturation. A box plot analysis of single cell determinations is shown. The number of individual cells examined (n) is indicated. The 25–75 percentile ranges of the corresponding results from Stevor1-80_FFrGFP2 expressing cells (Fig. 2D) are highlighted in red for visual comparison. p-values were calculated using the Kruskal-Wallis test with Dunn’s Multiple comparison. Abbreviations and details on box plot analysis are given in the legend of Fig. 1.
(roGFP2 oxidation: 27 ± 11% in late rings, 30 ± 18% in early trophozoites, 74 ± 21% in late trophozoites and 73 ± 21% in schizonts). In general, the data obtained using HbAA and HbAS erythrocytes were statistically not different from one another (F = 1.23; p > 0.99) (Fig. 5 C and D).

4. Discussion

The redox potential of human erythrocytes has so far only been determined using invasive methods, yielding values for the glutathione redox potential between 276 and 250 mV [48–50]. Our non-invasive measurements with genetically encoded roGFP2 variants revealed much more reducing values of 304 and 309 mV for ring-stage iRBC at pH 7.2 [80, 81]. Taking into account that the redox potential is lowered by 11.8 mV when the pH is increased from 7.2 to 7.4 [74, 77], our values are in good agreement with previous measurements in unperturbed transgenic mouse erythrocytes, yielding a redox potential of ~319 ± 8 mV [82]. Furthermore, similar cytosolic redox potentials around 320 mV were determined in a variety of cell types and organisms [51, 83]. We therefore hypothesize that the steady-state redox potentials in ring-stage iRBC and uninfected human erythrocytes are highly similar.

When glutaredoxins are present, the redox potential of unfused roGFP2 variants was shown to reflect predominantly the steady-state redox potential of glutathione in Arabidopsis thaliana [54], HeLa cells [52], Saccharomyces cerevisiae [55, 84], and P. falciparum [58]. Since erythrocytes contain about 1 μM hGrx1 [23], which roughly equals the concentration of ScGrx2 in yeast [21], we posit that our measured redox potentials in the erythrocyte also corresponds to the steady-state glutathione redox potential. Based on the assumption that the roGFP2 and glutathione redox couples are close to equilibrium at steady-state conditions, we can use the Nernst equation and a glutathione concentration of 1.8 mM glutathione to estimate an average GSH:GSSG ratio of 3.3 × 10^4 and a GSSG concentration in the erythrocyte cytosol of around 0.05 μM [85]. Taking into account the Avogadro constant and an erythrocyte cytosol volume of 81 fL during the ring stage [85], this translates into 2.8 × 10^3 molecules of GSSG per cell. Thus, regulatory or potentially protective protein glutathionylations are very unlikely to accumulate in the presence of hGrx1 unless they are kinetically uncoupled [21]. In summary, our study shows once again the advantage of non-invasive redox measurements as compared to traditional invasive methods [51, 83], revealing a highly reducing erythrocyte cytosol that prevents an accumulation of oxidized protein species.
Various aspects of the so-called oxidative stress hypothesis in malaria parasites have been iterated in reviews during the past decades [14,15,86,87], although direct experimental data that support specific mechanisms are rather scarce (e.g. Refs. [35,37,88]). Furthermore, blood-stage development was shown to be inhibited not only by oxidizing but also by reducing agents [89]. Thus, the classification of “damaging oxidants” versus “protective antioxidants” is an incorrect simplification of the complex redox biochemistry of the host-parasite unit. Here, we showed that a sudden +30 mV increase of the redox potential in the erythrocyte cytosol is actually a physiological and not a protective process during trophozoite development. We exclude a pH effect because, using the glutathione Nernst equation, an increase of +30 mV would require a drop of the pH below 6.8. Furthermore, our value is in accordance with invasive glutathione measurements for Percoll-purified trophozoite/schizont iRBC that suggested an increase of +25 mV as compared to uninfected erythrocytes [90]. Most important, we found no intrinsic redox imbalance in sickle cell trait iRBC as compared to wild-type iRBC. We therefore consider it unlikely that a biologically relevant difference of the steady-state redox potential between sickle cell trait erythrocytes and wild-type erythrocytes occurs within the measuring range of roGFP2 (∼320 to −240 mV) in otherwise unperturbed infected cells. Nevertheless, we do not exclude that specific redox modifications, which are kinetically uncoupled from the glutathione or roGFP2 pool, might impair the growth and sequestration of *P. falciparum* in infected sickle trait erythrocytes. This might include a higher susceptibility of parasitized sickle trait erythrocytes to lipid peroxidation brought about by elevated levels of free iron, heme and globin-based radicals [35,39,40]. To address these questions, measurements with Fe(II) and Fe(III)-specific probes and indicators of lipid peroxidation would be needed. We also did not consider external oxidative challenges, such as those elicited by the host immune response, to which parasitized sickle trait erythrocytes might be more susceptible. Furthermore, it has been shown that sickle hemoglobin can modulate the host’s immune response [47]. Thus, future experiments in sickle trait iRBC are necessary to mimic oxidative challenges of the host’s immune system.

The oxidative stress hypothesis has been evoked as an underlying cause for the broad range of physiological aberrations displayed by parasitized sickle trait erythrocytes, including impaired cytoadhesion to microvascular endothelial cells [43,45], delayed and reduced export of parasite-encoded proteins to the erythrocyte plasma membrane [91,92], aberrant actin reorganization [35,93], reduced intracellular multiplication particularly under low oxygen tension [41,42], inhibition of parasite translation by host microRNA [94], and modulation of the host’s immune response [46,47]. Our finding of comparable stage-specific redox potentials in parasitized wild-type and sickle cell trait erythrocytes, now casts doubts on general oxidative stress playing a significant role in the malaria-protective function of sickle trait hemoglobin and related hemoglobinopathies.

What could be the reason for the altered redox state during trophozoite development and what can we learn about the glutathione metabolism of the host-parasite unit? The glutathione redox potential depends on both the GSH:GSSG ratio and the total glutathione concentration. Both terms can be separated in the Nernst equation [77], resulting in alternative scenarios to interpret the +30 mV jump in the iRBC based on conflicting invasive glutathione measurements [87]. An initial study was performed without thiol derivatization and suggested an efflux of GSSG from the iRBC [95], whereas subsequent, tightly controlled studies showed that glutathione is effluxed in its reduced form from the parasite and the iRBC through the new permeation pathways [27,96]. Another LC-MS study suggested that GSH and GSSG are both effluxed [90]. It seems unlikely that the observed differences in GSH versus GSSG efflux were related to the detected jump in erythrocyte redox potential because all studies analyzed mature trophozoite iRBC. Our roGFP2 measurements revealed that the steady-state redox potential in the parasite cytosol remained constant during the whole 48 h replicative cycle, suggesting that both terms of the Nernst equation also remained constant. Thus, even if the parasite loses more glutathione during trophozoite development, e.g. through the secretory pathway,
This loss is compensated by the de novo synthesis of GSH [96]. Taking into account our measured redox potentials and an average glutathione concentration of 5.1 mM [27], we can estimate for the parasite cytosol at pH 7.2 [78] a GSH:GSSG ratio between $9.4 \times 10^3$ and $1.8 \times 10^4$ and a GSSG concentration between 0.28 and 0.54 μM (Table S2). Since the volume of the parasite’s cytosol increases roughly from 5 fl (ring stage) to 30 fl (schizont stage), the parasite has to synthesize about $10^5$ molecules GSH just to maintain the glutathione concentration in this compartment (in addition to the glutathione that is transported to other organelles and is lost through the secretory pathway). The redox potential in the parasitophorous vacuole was highly similar in early and late trophozoites, which might indicate a rather constant loss of GSH and GSSG through the secretory pathway (see also below). Furthermore, it seems plausible to assume that human glutathione reductase remains active during the transition from early to late trophozoite iRBC and therefore maintains a very high GSH:GSSG ratio in the erythrocyte. Thus, the jump in erythrocyte redox potential can be predominantly explained by a drastic efflux of GSH from late trophozoite iRBC at a constant (or even slightly increased) GSSG concentration in accordance with the data by Barrand et al. [27] (Fig. 6). In contrast, a significant loss of GSSG, as suggested previously [90,95], would lower the erythrocyte GSSG concentration and result in an even more negative redox potential. Based on an average total glutathione concentration of 1.8 mM [24–26], the detected jumps in redox potential translate at a constant GSSG concentration into a drop of the GSH concentration in the erythrocyte cytosol by 46–63% (Table S2). While this scenario would be considered as a so-called oxidative stress in other systems, it is a normal physiological process throughout the parasite’s intraerythrocytic development. In summary, a stage-dependent comparison of the redox potentials in the parasite cytosol, the parasitophorous vacuole, and the erythrocyte cytosol supports a drastically increased efflux of GSH from the iRBC as the cause for the physiological jump in the erythrocyte redox potential during the transition from early to late trophozoites (Fig. 6). We acknowledge that our study was conducted at an ambient oxygen tension of 21% and not at the oxygen concentration of 5% present in venular capillaries, the part of the circulatory system where parasitized erythrocytes sequester to avoid splenic clearance mechanisms [97]. If a lower oxygen concentration alters the outcome of our findings remains to be seen.

The detected higher but still reducing steady-state redox potential in the parasitophorous vacuole has not only parasite-specific but also general implications as this compartment is at the interface between the erythrocyte and the secretory pathway. So far there are only few studies on the redox state of the secretory pathway. Hence, it remains controversial whether GSH and GSSG act as a major electron donor and acceptor for oxidative protein folding in the endoplasmic reticulum [83, 98, 99]. On the one hand, an accumulation of glutathione and an extremely oxidizing GSH:GSSG ratio of 5:1 was reported for the endoplasmic reticulum [100–102], whereas a more recent study contradicted an accumulation of glutathione and supported a diffusion-controlled import of GSH into the secretory pathway [103]. Nothing is known about the redox state of the Golgi apparatus [83]. Assuming a distance between the plasma membrane and the parasitophorous vacuolar membrane around 50 nm, early and late trophozoite volumes of 20 and 28 fl. [85], the calculated redox potentials at pH 7.2, and a putative glutathione concentration of 5.1 mM [27], we can estimate for the parasitophorous vacuole a GSH:GSSG ratio around $3 \times 10^3$, a GSSG concentration of 2 μM, and a GSSG content around $2 \times 10^3$ molecules (Table S2). However, as also pointed out for previous redox measurements in the secretory pathway of opisthokonts [83], these values have to be interpreted with great care since it remains to be shown that the measured steady-state redox potential in the parasitophorous vacuole really reflects the glutathione and not another redox couple. If the steady-state redox potential reflects the glutathione couple, the redox state could be maintained by (i) an oxidoreductase, such as imported human or exported parasite glutathione reductase, and/or (ii) the export of GSSG into the erythrocyte or parasites. The insertion of channel proteins into the parasitophorous vacuolar membrane during trophozoite development might allow an equilibration with the erythrocyte glutathione pool and might explain the similar redox potentials in both compartments in late trophozoite iRBC (Fig. 6). Thioredoxin 2 of Plasmodium, which is attached to the luminal site of the protein exporting translocon in the parasitophorous vacuolar membrane, has been postulated to reduce disulfide bonds of exported proteins to allow their transport in an unfolded state [7]. However, to the best of our knowledge, it remains to be shown whether structural disulfide bonds in exported proteins such as the erythrocyte membrane protein 1 [10] are reversibly formed and broken. The intermediate redox potential in the parasitophorous vacuole might facilitate these processes.

In conclusion, non-invasive roGFP2 measurements revealed a much more reducing steady-state redox potential of the erythrocyte and parasite cytosol than deduced from invasive glutathione measurements. Parasite-dependent modifications of its host cell result in a physiological +30 mV jump in erythrocyte redox potential around 28 hpi that can be explained by a drastic efflux of GSH. Most important, there was no difference between the stage-dependent steady-state redox potentials of infected wild-type or sickle trait erythrocytes in contrast to the oxidative stress hypothesis. The steady-state redox potential of the parasitophorous vacuole was similar to the more oxidizing redox potential of late trophozoite- or schizont-stage iRBC and had an estimated GSH:GSSG ratio around $3 \times 10^3$ with implications for oxidative protein folding and redox regulation. Follow-up studies are necessary to address

![Fig. 6. Schematic model describing the increase in redox potential in P. falciparum infected erythrocytes during parasite maturation. Left panel: The parasite exports glutathione into the erythrocyte compartment, which together with host cell glutathione, maintains a reducing environment until early trophozoite development. Right panel: At the trophozoite stage, the parasite begins to establish new permeation pathways in the erythrocyte plasma membrane, which allow for the efflux of reduced glutathione, resulting in an increase in the erythrocyte redox potential in late trophozoites and schizonts. The average redox potentials of sub-compartments are indicated. RBC, red blood cell; RBCM, red blood cell plasma membrane; PVM, parasitophorous vacuolar membrane; PV, parasitophorous vacuole; PPM, parasite plasma membrane, GSH, reduced glutathione; N, nucleus; HbAA, a wild type erythrocyte; HbAS, a sickle cell trait erythrocyte.](image-url)
other hemoglobinopathies, to improve the temporal resolution of the
roGFP2 measurements, and to unravel the underlying mechanisms of
the jump in erythrocyte redox potential and the loss of GSH.

Author contributions

M.H., J.K., and C.P.S. conducted experiments. M.H., J.K., M.D. and
M.L. analyzed the data and wrote the manuscript. M.H., J.K., C.P.S., M.
D. and M.L. designed the experiments.

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Availability of data and materials

All data needed to evaluate the conclusions in the paper are present
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analyzed during the current study are available from the corresponding
author on request.

Declaration of competing interest

The authors declare that they have no known competing financial
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Data availability

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Appendix A. Supplementary data

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References

[1] H. Sies, V.V. Belousov, N.S. Chandel, M.J. Davies, D.P. Jones, E.G. Mann, M.
P. Murphy, M. Yamamoto, C. Winterbourn, Defining roles of specific reactive
oxygen species (ROS) in cell biology and physiology, Nat. Rev. Mol. Cell Biol. 23
(7) (2022) 499–515, https://doi.org/10.1038/s41580-022-00456-z.
[2] J.A. Inlay, The molecular mechanisms and physiological consequences of
oxidative stress: lessons from a model bacterium, Nat. Rev. Microbiol. 11 (7)
(2013) 443–454, https://doi.org/10.1038/nrmicro3032.
[3] A.J. Meyer, A. Dreyer, J.M. Ugalle, E. Feitoza-Ararajo, K.J. Dietz, M.
Schwarzlander, Shifting paradigms and novel players in Cys-based redox
regulation and ROS signaling in plants - and where to go next, Biol. Chem. 402
(3) (2021) 399–423, https://doi.org/10.1515/hz-2020-0291.
[4] M.B. Toledano, A. Delaunay-Moisan, C.E. Outten, A. Igbaria, Functions and
regulation and ROS signaling in plants - and where to go next, Biol. Chem. 402
(3) (2021) 399–423, https://doi.org/10.1515/hz-2020-0291.
[5] M.B. Toledano, A. Delaunay-Moisan, C.E. Outten, A. Igbaria, Functions and
regulation and ROS signaling in plants - and where to go next, Biol. Chem. 402
(3) (2021) 399–423, https://doi.org/10.1518/hz-2020-0291.
[6] M.B. Toledano, A. Delaunay-Moisan, C.E. Outten, A. Igbaria, Functions and
regulation and ROS signaling in plants - and where to go next, Biol. Chem. 402
(3) (2021) 399–423, https://doi.org/10.1518/hz-2020-0291.
[7] M.B. Toledano, A. Delaunay-Moisan, C.E. Outten, A. Igbaria, Functions and
regulation and ROS signaling in plants - and where to go next, Biol. Chem. 402
(3) (2021) 399–423, https://doi.org/10.1518/hz-2020-0291.
Redox Biology 58 (2022) 102536

M. Haag et al.

[31] M. Rahbari, S. Rahlfis, E. Jortzik, I. Bogeski, K. Becker, H\textsubscript{2}O\textsubscript{2} dynamics in the malaria parasite Plasmodium falciparum, PLoS One 12 (4) (2017), e0174837, https://doi.org/10.1371/journal.pone.0174837.

[32] M. Rahbari, S. Rahlfis, J.M. Przyborski, A.S. Khuch, N.H. Hunt, D.A. Fidoek, G. E. Grau, K. Becker, Hydrogen peroxide dynamics in subcellular compartments of malaria parasites using genetically encoded redox probes, Sci. Rep. 7 (1) (2017), 100, https://doi.org/10.1038/s41598-017-00649-4.

[33] S. S. Siddiqui, C. Giannangelo, A. De Paoli, A.K. Schuh, K.C. Heimsch, D. Anderson, S. M. Taylor, C. Cerami, R.M. Fairhurst, Hemoglobinopathies: slicing the Gordian knot of Plasmodium falciparum malaria pathogenesis, PLoS Pathog. 5 (9) (2009), e1003327, https://doi.org/10.1371/journal.ppat.1003327.

[34] S. M. Taylor, C. Cerami, R.M. Fairhurst, The molecular pathobiology of cell membrane stress in sickle cell disease: an overview of erythrocyte redox metabolism and iron: the sickle red cell as a model, Free Radic. Biol. Med. 24 (6) (1998) 658–673, https://doi.org/10.1016/S0891-5849(98)00039-1.

[35] S. M. Taylor, C. Cerami, R.M. Fairhurst, Plasmodium falciparum infected red blood cells, ACS Infect. Dis. 8 (1) (2022) 210–226, https://doi.org/10.1021/acsinfecdis.0c00050.

[36] S. M. Taylor, C. Cerami, R.M. Fairhurst, Oxidative stress and characterization of a superfolder green fluorescent protein, Nat. Biotechnol. 14 (3) (1996) 315, https://doi.org/10.1038/nbt0396-315.

[37] J. Schindelin, I. Arganda-Carreras, E. Frise, T. Arie, M. Preibisch, S. Saalfeld, B. Schmid, J.Y. Tinevez, D.J. White, C.T. Dooley, T.M. Dore, G.T. Hanson, W.C. Jackson, S.J. Remington, R.Y. Tsien, F. Knop, R. H. Klompe, C. A. MacRaild, J. Wu, X. Wang, Y. Dong, J.L. Vennerstrom, J. Rogerson, J.G. Beeson, B.S. Crabb, P.R. Gilson, T.F. de Koning-Ward, PTEX is an essential nexus for protein export in malaria parasites, Nature 511 (7511) 1987, https://doi.org/10.1038/nature13555.

[38] J. Schindelin, I. Arganda-Carreras, E. Frise, T. Arie, M. Preibisch, S. Saalfeld, B. Schmid, J.Y. Tinevez, D.J. White, C.T. Dooley, T.M. Dore, G.T. Hanson, W.C. Jackson, S.J. Remington, R.Y. Tsien, F. Knop, R. H. Klompe, C. A. MacRaild, J. Wu, X. Wang, Y. Dong, J.L. Vennerstrom, J. Rogerson, J.G. Beeson, B.S. Crabb, P.R. Gilson, T.F. de Koning-Ward, PTEX is an essential nexus for protein export in malaria parasites, Nature 511 (7511) 1987, https://doi.org/10.1038/nature13555.

[39] D. G. Silva, E. Belini Junior, E.A. de Almeida, C.R. Bonini-Domingos, Oxidative stress in sickle cell disease: an overview of erythrocyte redox metabolism and iron: the sickle red cell as a model, Free Radic. Biol. Med. 24 (6) (1998) 658–673, https://doi.org/10.1016/S0891-5849(98)00039-1.
M. Hayashi, H. Yamada, T. Mitamura, T. Horii, A. Yamamoto, Y. Moriyama, H. Ostergaard, A. Henriksen, F.G. Hansen, J.R. Winther, Shedding light on transsulphuration pathway results in accumulation of redox metabolites and provides a host-based drug target, Cell Rep. 39 (11) (2022), 110923, https://doi.org/10.1016/j.celrep.2022.110923.

M. Deponte, Glutathione catalysis and the reaction mechanisms of glutathione-dependent enzymes, Biochim. Biophys. Acta 1830 (5) (2013) 3217–3266, https://doi.org/10.1016/j.bbagrm.2012.09.018.

Y. Kuhn, P. Rohrbach, M. Lanzer, Quantitative pH measurements in Plasmodium falciparum-infected erythrocytes using pHluorin, Cell Microbiol. 9 (4) (2007) 1004–1013, https://doi.org/10.1111/j.1462-5822.2006.00647.x.

M. Deponte, Glutathione and glutathione-dependent enzymes, Redox Chem. Biol. 3 (1) (2019) 27–34, https://doi.org/10.1016/j.redox.2019.01.001.

P. Swietach, T. Tiffert, J.M. Mauritz, R. Seear, A. Esposito, C.F. Kaminski, V. Beri, B. Balan, S. Chaubey, S. Subramaniam, B. Surendra, U. Tatu, A disrupted transport (1), Biochem. Cell. Biol. 97 (3) (2019) 270–289, https://doi.org/10.1139/bcb-2018-0189.

J. Zimmermann, J. Oestreicher, F. Geisel, M. Deponte, B. Morgan, An intracellular assay for activity screening and characterization of glutathione-dependent oxidoreductases, Free Radic. Biol. Med. 172 (2021) 340–349, https://doi.org/10.1016/j.freeradbiomed.2021.06.016.

M. Waldecker, A.K. Dasmana, C. Lansche, M. Linke, S. Srismith, M. Cyrklaff, C.P. Sanchez, U.S. Schwarz, M. Lanzer, Differential time-dependent volumetric and dependent oxidoreductases, Free Radic. Biol. Med. 172 (2021) 340–349, https://doi.org/10.1016/j.freeradbiomed.2021.06.016.

J. Oestreicher, B. Morgan, Glutathione: subcellular distribution and membrane transport (1), Biochem. Cell. Biol. 97 (3) (2019) 270–289, https://doi.org/10.1139/bcb-2018-0189.

R.A. Kavishe, J.B. Koenderink, M. Alifrangis, Oxidative stress in malaria and provides a host-based drug target, Cell Rep. 39 (11) (2022), 110923, https://doi.org/10.1016/j.celrep.2022.110923.

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