



Article

Profiling 26S Proteasome Activity of *Plasmodium falciparum* Monitored by a Live-Cell Assay

Adriana F. Gonçalves ^{1,2,*}, Ana Lima-Pinheiro ^{1,2}, Belém Sampaio-Marques ^{1,2} and Pedro E. Ferreira ^{1,2,*}

¹ Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, 4710-057 Braga, Portugal

² Biomaterials, Biodegradables and Biomimetics Research Group (3B's)-PT Government Associate Laboratory, Life and Health Sciences Research Institute (ICVS), 4710-057 Braga, Portugal

* Correspondence: id10896@alunos.uminho.pt (A.F.G.); pedroferreira@med.uminho.pt (P.E.F.)

Abstract

Malaria remains a major global health challenge, driven in part by widespread antimalarial drug resistance in *Plasmodium* parasites. Artemisinin-based combination therapies (ACTs) are currently the first-line treatment; however, resistance has also emerged. Artemisinin damages parasite proteins, promoting their ubiquitination and subsequent proteasomal degradation. Because inhibitors of the *Plasmodium* 26S proteasome synergize with artemisinin, the proteasome has emerged as a promising drug target, yet tools to monitor its function in live parasites remain limited. Here, we generated a *P. falciparum* line expressing green fluorescent protein fused to a destabilization domain (GFP-DD) to assess proteasome activity and combined it with MitoTracker™ staining. In the absence of the stabilizing ligand Shield-1, the GFP-DD reporter is rapidly degraded by the proteasome. Using fluorescence microscopy and flow cytometry, we show that GFP-DD fluorescence provides a quantitative, inverse readout of proteasomal activity, increasing upon ligand-mediated stabilization or pharmacological inhibition with MG132. Shield-1 titration identified an optimal stabilization range, and MG132 induced a dose-dependent fluorescence increase. This work establishes a practical live-cell platform to probe ubiquitin–proteasome system function, with potential applications in future phenotypic screening and antimalarial resistance studies.

Keywords: malaria; *Plasmodium falciparum*; drug resistance; proteasome; genetic engineering

1. Introduction

Malaria remains a global health challenge, with *Plasmodium falciparum* as the most lethal species [1]. A major obstacle to controlling malaria is the ability of the protozoan *Plasmodium* spp. parasites to gain resistance to all commercially available antimalarials [2]. Nowadays, artemisinin-based combination therapies (ACTs) are the first-line treatment for non-complicated malaria [3]. Resistance to artemisinin and its derivatives (ARTs), the cornerstone of ACTs, has emerged, threatening treatment efficacy [4]. Mutations in the propeller domain of kelch13 have been frequently associated with this resistance. However, the exact mechanism by which these mutations confer resistance remains unclear [4,5], being mainly associated with alterations in hemoglobin uptake and digestion, as well as antioxidant response, DNA repair, and cell stress pathways [6]. ARTs are activated by the cleavage of their endoperoxide bridge upon reaction with FeII-heme due to hemoglobin degradation. In this activated state, artemisinin reacts with susceptible groups of biomolecules, leading to generalized oxidative

Academic Editor: Michael Stear

Received: 15 January 2026

Revised: 13 February 2026

Accepted: 20 February 2026

Published: 24 February 2026

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stress and cellular damage, and, finally, to the parasite's death. ARTs are rapidly metabolized, making it necessary to add a longer-lasting partner, creating the ACTs [7,8].

ART-induced proteotoxic stress involves the ubiquitin-proteasome system (UPS), which is essential for parasite protein homeostasis [9,10]. Targeting the parasite proteasome has emerged as a promising strategy, particularly in combination therapies [2]. The damaged proteins resulting from ARTs reactions are subsequently ubiquitinated and targeted for degradation by the 26S proteasome. In this context, the parasite's proteasome not only mediates the cellular stress response but also serves as a key vulnerability and thus a promising drug target [8,10,11].

While various assays have been developed to study the UPS and proteasome function in *P. falciparum*, few are compatible with live-cell and dynamic monitoring of proteasomal activity. The green fluorescent protein–destabilization domain (GFP-DD) system, which involves a fusion of green fluorescent protein (GFP) with a destabilization domain (DD) derived from the immunophilin protein-folding chaperone FKBP12, enables conditional regulation of protein stability by adding a small-molecule ligand, Shield-1, adapting the system originally described by Banaszynski et al. (2006) [12]. In the absence of Shield-1, the DD-tagged protein is rapidly degraded by the proteasome; however, when Shield-1 is present, degradation is blocked, allowing for the cellular accumulation of GFP to be observed in real time by fluorescence detection [9,12]. The DD system was adapted for use in *P. falciparum* to regulate protein abundance and gene function studies, particularly in gametocyte commitment and sexual differentiation [13], and it has been validated in *P. falciparum* for post-translational control of gene expression [14,15].

The GFP-DD system has previously been applied in *P. falciparum* as a qualitative reporter of proteotoxic stress and proteasome perturbation [9,12]. In the present study, we do not introduce GFP-DD as a novel reporter; rather, we refine its application by implementing a quantitative, viability-controlled framework that enables robust interpretation of proteasome activity in live parasites. Importantly, the stabilizing ligand Shield-1 has been shown to negatively affect parasite growth and intraerythrocytic development [14], underscoring the need for independent viability control in GFP-DD-based analyses. To address this, MitoTracker™ was incorporated as an orthogonal marker for parasite identification and viability, particularly under conditions in which Shield-1 exposure or proteasome inhibition induces rapid cellular stress or parasite death. MitoTracker™ has been validated as a reliable indicator of viability in live asexual blood-stage *P. falciparum* parasites in flow cytometry-based assays [16].

Here, we demonstrate that GFP-DD fluorescence intensity correlates with proteasomal activity in live parasites, either through stabilization by Shield-1 or inhibition by proteasome inhibitors such as MG132. Although MG132 has been reported to affect multiple proteolytic pathways in *P. falciparum*, including hemoglobin degradation, it is also a well-established inhibitor of the UPS [17]. In this context, interpretation of MG132 effects relies on the fact that the GFP-DD reporter is a selective substrate of the 26S proteasome, such that changes in GFP-DD fluorescence directly reflect modulation of proteasome-dependent degradation. While this study does not include a drug-screening campaign, the flow cytometry-based platform described here is designed to support future phenotypic screening and comparative analyses of compounds that modulate parasite proteasome function. Collectively, this work represents a conceptual and technical advance in the application of live-cell proteostasis reporters for antimalarial drug research.

2. Results

In the absence of the stabilizing ligand, Shield-1, the GFP fused to the DD is rapidly degraded by the proteasome. The addition of Shield-1 prevents degradation and results in detectable green fluorescence (Figure 1A).

The phenotype of the transgenic parasite line expressing constitutive GFP-DD was examined by fluorescence microscopy using an Olympus BX61 widefield upright microscope equipped with a 100× objective, a FITC channel, and an APC channel. Parasites were imaged under two conditions: without Shield-1 and following treatment with Shield-1 for 24 h. MitoTracker™ (1.6 µM) was added 30 min before imaging in both conditions. In the absence of Shield-1, only minimal GFP fluorescence was observed, whereas Shield-1-treated parasites showed a marked increase in green fluorescence using identical exposure settings (Figure 1B). These observations indicate that the DD system is functioning correctly, with efficient degradation of the fusion protein when Shield-1 is not present.

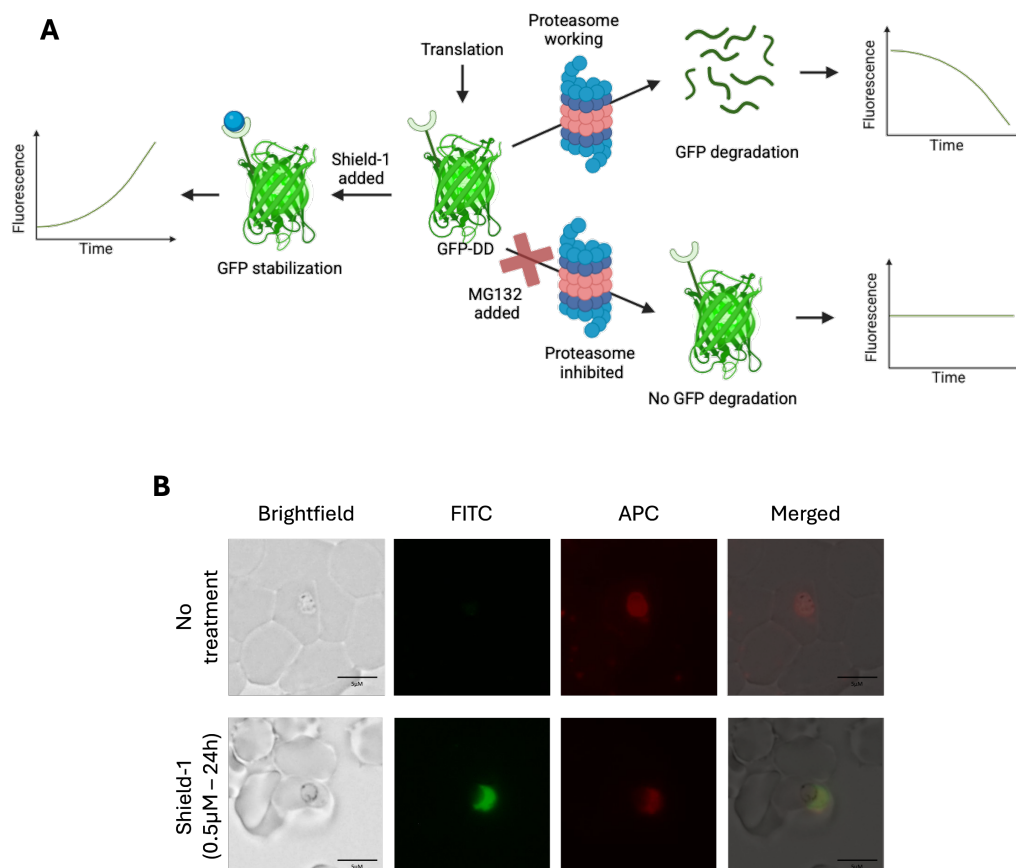


Figure 1. Scheme and Fluorescence Microscopy. **(A)** The GFP coupled with DD is targeted for degradation mediated by the proteasome in the absence of the protective ligand (Shield-1). In the presence of Shield-1, the protein is stabilized. Adapted from Bridgford et al., 2018 [9]. **(B)** Parasites stained with MitoTracker™ (1.6 µM) observed under fluorescence microscopy without treatment and with Shield-1 (0.5 µM), under light microscopy, under fluorescence microscopy FITC and APC, and merged. In both analyses, the same fluorescence exposure time was used.

Flow cytometry analysis began with the establishment of appropriate gating parameters. Cultures were analyzed using an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) with FITC (405/488 nm) and APC (650/780 nm) detection channels. An unstained parasite sample was first used to adjust voltage settings (Figure 2A). Subsequently, single-color controls defined each fluorescence channel: MitoTracker™-only parasites were used to set the APC gate (Figure 2B), and GFP-expressing parasites were used to determine the FITC gate (Figure 2C). A dual-positive sample containing both GFP and MitoTracker™ fluorescence was then analyzed to validate the combined gating scheme (Figure 2D). Viable GFP-negative parasites were quantified in the Q1 quadrant (no GFP). Viable GFP-positive parasites were quantified in the Q2 quadrant (GFP folded). Unviable GFP-positive parasites were quantified in the Q3

quadrant (GFP-positive, MitoTracker™-negative events). Q4 quadrant monitors debris, non-infected erythrocytes, and/or dead parasites. MitoTracker™ staining was used to identify viable parasites and to exclude uninfected erythrocytes, debris, and non-viable events from the analysis. Loss of MitoTracker™ signal reflects dissipation of mitochondrial membrane potential and correlates with parasite death. Because GFP and MitoTracker™ Red exhibit non-overlapping excitation and emission spectra, this dual-parameter approach enabled robust discrimination of viable GFP-positive parasites from non-viable populations, ensuring that GFP-DD fluorescence was quantified exclusively within living parasites.

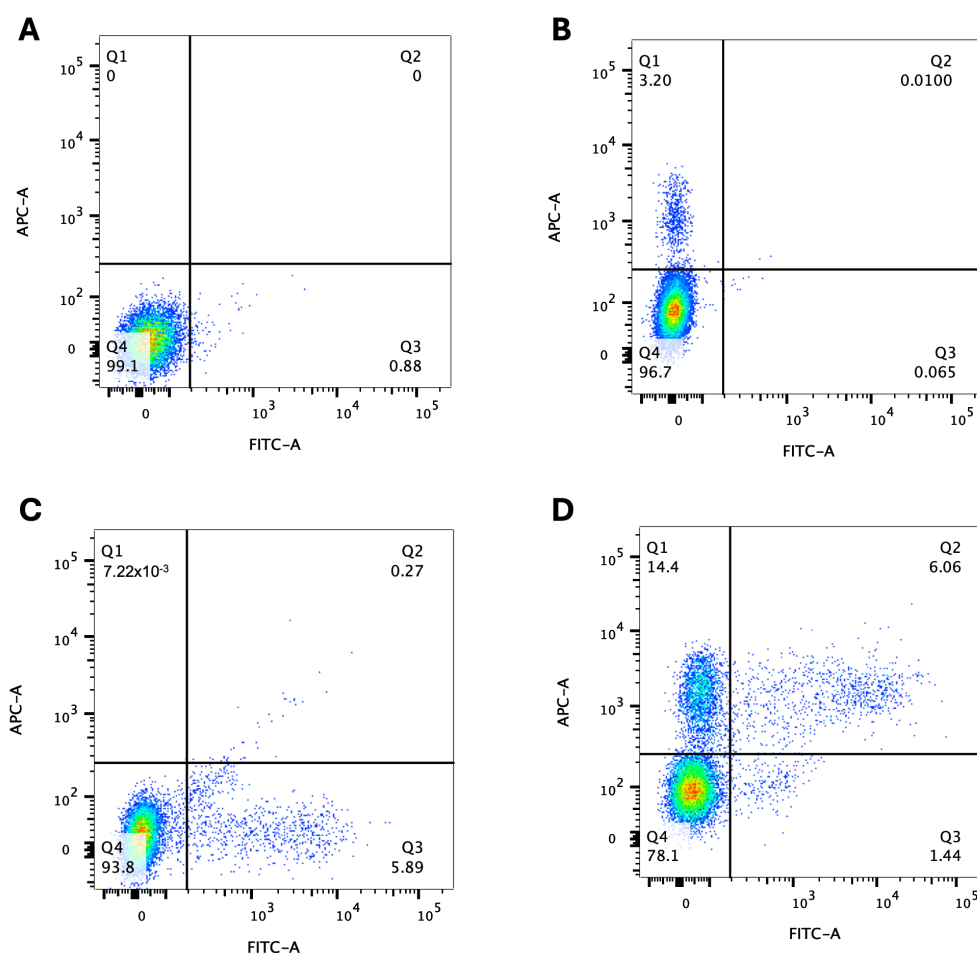


Figure 2. Flow cytometer correct gating. (A) The baseline population was established using unstained parasites (parental line *Dd2* unstained). (B) MitoTracker™-labeled parasites identified the APC-positive region (parental line *Dd2* stained). (C) Parasites displaying only green fluorescence defined the FITC-positive gate (*Dd2* GFP-DD). (D) A double-positive sample confirmed the accuracy of the combined gating parameters (*Dd2* GFP-DD stained).

After establishing appropriate gating parameters, we optimized the stabilizing ligand concentration. To identify the amount of Shield-1 required for maximal stabilization of the GFP-DD reporter, cultures were exposed to increasing ligand concentrations and analyzed by flow cytometry after 24 h. GFP fluorescence increased with Shield-1 concentration, reaching a maximum of 4-fold the baseline between 1 and 2.5 μ M, after which it decreased and returned to baseline levels (Figure 3A). In parallel, APC fluorescence gradually declined as Shield-1 concentrations rose, with a 50% reduction in parasite viability ($50\% \pm 1.7\%$ SEM) observed at 20 μ M of Shield-1 (Figure 3B). Treatment with MG132 resulted in a concentration-dependent increase in GFP-DD fluorescence. While MG132 is not a proteasome-exclusive inhibitor, the

accumulation of GFP-DD specifically reports impaired degradation of a 26S proteasome substrate, indicating functional inhibition of proteasome-mediated turnover.

After identifying the optimum Shield-1 concentration, we examined the response of the GFP-DD reporter to monitor proteasome inhibition. Parasites were first maintained in 1 μM Shield-1 for 24 h. After ligand washout, trophozoites were incubated for 3 h under six conditions: MCM alone, 1 μM Shield-1, and the proteasome inhibitor MG132 at 0.1, 0.5, 2, and 5 μM . Samples were collected at 0, 0.5, 1, and 3 h; for the baseline measurement (0 h), only the MCM condition was analyzed. All samples were stained with MitoTracker™ for 30 min at 37 °C before acquisition.

As expected, fluorescence without Shield-1 was lower compared to cultures with the ligand, showing about a 20% reduction from baseline ($20\% \pm 0.8\%$ SEM), consistent with proteasomal degradation of the unstable GFP fusion. MG132 treatment induced a concentration-dependent increase in GFP-DD fluorescence relative to baseline, with maximal increases observed at higher concentrations (Figure 3C,E), indicating decreased degradation of the reporter under proteasome-inhibitory conditions. Additionally, Shield-1 caused an initial mitochondrial hyperpolarization, and APC fluorescence showed a transient rise at 30 min followed by a decline at 3 h (Figure 3D,F), suggesting that the reporter may not be fully stabilized early on and that prolonged exposure could harm parasite viability. The distribution of parasites across quadrants was monitored throughout all experiments and conditions (Figure S1A–D). We observed that the reduction in viable parasites resulted from experimental handling and was consistent across all quadrants.

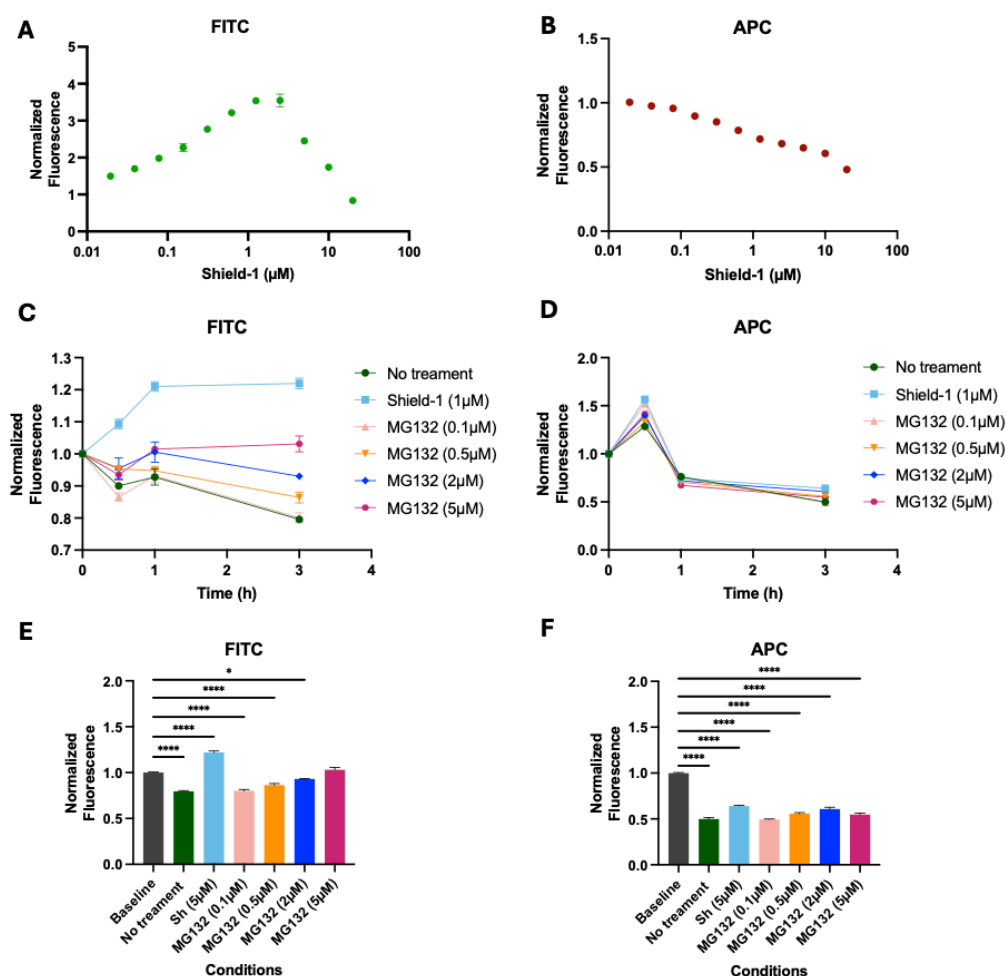


Figure 3. Flow cytometry characterization of the GFP-DD reporter. (A) FITC and (B) APC analysis of the dose–response of GFP-DD fluorescence after 24 h of treatment with varying Shield-1 concentrations. (C) FITC and (D) APC analysis of the GFP-DD fluorescence profiles in parasites exposed

to increasing concentrations of the proteasome inhibitor MG132 following Shield-1 washout. (C) FITC and (D) APC normalized fluorescence $\pm$ SEM were analyzed comparing baseline (0 h) and the following conditions at 3 h: no treatment, Shield-1 (5 μ M), MG132 (0.1, 0.5, 2, and 5 μ M). (E) FITC and (F) APC statistical analysis of the previously mentioned conditions compared to the baseline using one-way ANOVA. * $p < 0.05$, **** $p < 0.0001$.

3. Discussion

In this study, we evaluated the GFP-DD reporter as a functional tool for monitoring proteasome activity in *P. falciparum*. The destabilization-domain strategy, originally developed by Banaszynski et al. (2006) [12], enables conditional regulation of protein stability through the Shield-1 ligand. In the absence of Shield-1, DD-tagged proteins undergo rapid proteasomal degradation, whereas upon Shield-1 binding, the fusion protein is protected from turnover, stabilizing the protein. Fluorescence microscopy and flow cytometry measurements showed a low GFP signal after Shield-1 removal, indicating efficient proteasomal degradation of the GFP-DD fusion. The addition of Shield-1 restored GFP fluorescence, confirming ligand-dependent stabilization of the reporter. Titration experiments revealed that GFP accumulation peaks at Shield-1 concentrations between 1.25 and 2.5 μ M, with a subsequent decline in fluorescence at higher concentrations. A critical consideration when applying FKBP-based destabilization domain systems in *P. falciparum* is the biological impact of the stabilizing ligand itself. Shield-1 exposure has been reported to reduce parasite growth and delay intraerythrocytic development even at low micromolar concentrations [14]. Our data are consistent with these observations and demonstrate that GFP-DD fluorescence cannot be interpreted independently of parasite viability, underscoring the importance of viability-controlled analysis.

We explored the activity of 26S proteasome through the monitoring of GFP-DD using MG132, a widely used proteasome inhibitor in *P. falciparum* research [17]. MG132 is known to exert pleiotropic effects in *P. falciparum*, including inhibition of proteases involved in hemoglobin degradation. However, the specificity of our readout is defined by the reporter rather than by the inhibitor. Because GFP-DD degradation occurs through the 26S proteasome, increased GFP-DD fluorescence under MG132 treatment reflects inhibition of proteasome-dependent degradation, irrespective of additional off-target effects. This reporter-based specificity allows MG132 to be used as a functional probe of proteasome activity without requiring inhibitor exclusivity. As expected, MG132 treatment resulted in a concentration-dependent increase in GFP fluorescence, which exceeded the baseline fluorescence levels observed in Shield-1-free parasites. This suggests that this reporter is highly sensitive to disruptions in the ubiquitin-proteasome system and can effectively monitor proteasomal activity. Although proteasome inhibition is known to induce downstream endoplasmic reticulum stress responses, the aim of this work was not to characterize secondary stress pathways but to establish a reliable, quantitative live-cell readout of proteasome activity. ER stress markers report downstream consequences of proteasome dysfunction and do not substitute for independent viability control. By integrating MitoTracker™ Red as a validated viability marker, we address the primary confounding factor affecting GFP-DD interpretation in *P. falciparum*, namely ligand- and inhibitor-induced parasite death.

Collectively, these results validate GFP-DD as a functional biosensor for proteasome activity in *P. falciparum*. The system provides a dynamic, quantifiable readout that responds both to ligand-dependent stabilization and to pharmacological inhibition of proteasomal degradation. This tool has significant potential not only for studying parasite proteostasis capacity across different parasite strains, including variation in protein turnover and proteasome responsiveness, but also for profiling antimalarial compounds targeting protein degradation pathways. Future studies combining GFP-DD alongside

complementary redox sensors or stress markers could further illuminate how proteostasis integrates with broader aspects of parasite physiology, offering insights into the molecular mechanisms that govern parasite survival and drug resistance.

4. Materials and Methods

4.1. *P. falciparum* Cell Culture

4.1.1. Culture Maintenance

P. falciparum cultures were maintained on a t25 flask at 4% hematocrit in 5 mL of MCM (malaria culture medium) [RPMI, 1640 (Gibco, Grand Island, NY, USA) with 2 mM L-glutamine, 200 μ M hypoxanthine, 0.25 μ g/mL gentamycin, 25 mM HEPES, 0.2% NaHCO₃, and 0.25% Albumax II (Life Technologies, Carlsbad, CA, USA)] under a controlled atmosphere of 5% O₂/5% CO₂/90% N₂ at 37 °C in a CO₂ incubator (Thermo Fisher Scientific, Waltham, MA, USA). Medium was changed every other day, and the cultures' healthiness and parasitemia were monitored regularly by microscopy, using a blood smear, fixed with methanol (100%), and colored with 10% Giemsa's azur eosin methylene blue solution (Merck, Darmstadt, Germany) for 20 min. Parasitemia was calculated by dividing the number of parasitized erythrocytes by the total counted erythrocytes.

4.1.2. Plasmid Construction

Cloning strategies were designed in silico with the software ApE. To construct a plasmid bearing a GFP-DD complex, two plasmids were used: pDC2-cyto-roGFP2-Grx1 [18] and GDV1-GFP-DD [13] (kindly provided by Till Voss). The first was digested with AvrII and XhoI restriction enzymes (New England Biolabs—NEB, Ipswich, MA, USA) to obtain the pDC2 vector. A PCR was performed with the second one, using the Supreme NZY-Proof 2x Colourless Master Mix enzyme (NZYTech, Lda, Lisbon, Portugal) and primers listed in Table 1. Electrophoresis was used to verify the digestion and PCR correctness, and the desired bands were extracted with the NZYGelpure kit (NZYTech, Lda, Lisbon, Portugal). Then, the fragment was ligated to the plasmid using T4 DNA Ligase (Thermo Scientific, Waltham, MA, USA). The ligation product was transformed into competent *E. coli* cells (NZY5 α —NZYTech, Lda, Lisbon, Portugal). Subsequently, the plasmid was extracted using the NZYMiniprep kit (NZYTech, Lda, Lisbon, Portugal) and the NZYMaxiprep kit (NZYTech, Lda, Lisbon, Portugal) for transfection.

Table 1. Primer sequences.

Name	Sequence
P1	CTAATAGAAATATATCACCTAGGATGAGTAAAGGAGAAGAAGCT
P2	AATATATTATATAACTCGAGTCATTCCAGTTTGTAGAAGCT

4.1.3. *P. falciparum* Transfection

The first step to perform the transfection of the plasmid GFP coupled with DD into the parasites is synchronization. For this, sorbitol (5%) was used to synchronize the parasites in the ring stage. During the maturation of the intraerythrocytic stages of the parasite, there is a remodeling of the erythrocyte with parasite molecules being exported and incorporated into its plasma membrane. In the late-stage parasites, these molecules make the red blood cells (RBCs) permeable to sorbitol, leading to hypotonic lysis of infected erythrocytes and consequently to their elimination [19].

The next day, transfection was performed by electroporation in uninfected RBCs (uRBCs) [20] and added to infected RBCs (iRBCs) with trophozoite-stage parasites. First, uRBCs are washed with cytomix [10 mM/L K₂HPO₄/KH₂PO₄, 120 mM/L KCl, 0.15 mM/L CaCl₂, 5 mM/L MgCl₂, 25 mM/L HEPES, 2 mM/L EGTA, adjusted with 10 M/L KOH to

pH 7.6]. Then, they were added to the solution containing 50 µg of plasmid in cytomix and transferred to a Gene Pulser/MicroPulser™ Electroporation Cuvette, 0.2 cm gap (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and electrically shocked on the Gene Pulser Xcell™ (Bio-Rad Laboratories, Inc., Hercules, CA, USA) electroporator with a voltage of 0.31 kV and a capacitance of 950 µF. The time constant should be 10–14 ms. Afterwards, the mixture was washed with MCM to remove lysed RBCs and then inoculated with the *P. falciparum* Dd2 strain, a multi-drug-resistant strain derived from an Indochina isolate [21]. The next day, blasticidin (BSD, 2.5 µg/mL—Gibco, Grand Island, NY, USA) was added to the culture to begin the selection of the genetically modified parasites. Based on the emergence of drug-resistant parasites relative to the initial parasite population, the estimated transfection efficiency was within the expected range for episomal plasmid transfection in *P. falciparum* (approximately 10^{-6} – 10^{-5}). Drug-resistant parasites became detectable after approximately three weeks of continuous selection, without the need for limiting dilution cloning. The reporter construct is maintained episomally and therefore requires continuous drug selection for stable expression. Under sustained selection, fluorescence levels and parasite growth remained stable throughout the experimental period. In the absence of drug pressure, episomal plasmids in *P. falciparum* are progressively lost during replication, leading to a gradual reduction in reporter-positive parasites. Thus, the reporter line is stable under continuous selection but is not genetically fixed.

4.2. Fluorescence Microscopy

Fluorescence microscopy was used after the selection to assess whether the plasmid was functioning in the parasite. For this, the parasites were synchronized at the ring stage as described in Section 4.1.2. Then, the culture was divided, and Shield-1 (0.5 µM) was added to one half, and the other was maintained in MCM. After an incubation of 24 h, MitoTracker™ Deep Red FM (1.6 µM—Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA) was added and incubated for 30 min at 37 °C in the dark. Afterward, blood drops from both cultures were prepared on slides and covered with coverslips. Parasites were then observed under a 100× objective with and without fluorescence excitation on a GFP-emitting channel in the fluorescence microscope.

4.3. Flow Cytometry

Different concentrations of Shield-1 were tested. First, parasites were synchronized at the ring stage as previously mentioned (Section 4.1.2). Then, parasites at 5% parasitemia and 2% hematocrit were added to the plate previously prepared with serial dilutions of Shield-1, starting at 20 µM, 100 µL of the mixture (iRBCs, RBCs, and MCM) to each well. The plate was incubated for 24 h in the CO₂ incubator. Subsequently, parasites were incubated with MitoTracker™ (1.6 µM) at 37 °C for 30 min in the dark. Then, 20 µL of parasites marked with MitoTracker™ were added to 300 µL of PBS 1x and passed through the flow cytometer at 405/488 nm and 650/780 nm.

Shield-1 (1 µM) was added to the ring-stage culture and incubated for 24 h. The next day, parasites at the trophozoite stage were concentrated using the autoMACS® Pro Separator (Miltenyi Biotec, Bergisch Gladbach, Germany). Herein, different-stage parasites are separated using magnetic beads. *P. falciparum* parasites degrade and feed on hemoglobin when they infect the RBC, which generates a toxic iron-containing heme moiety. This toxicity is avoided by transforming it into hemozoin, an inert crystal polymer, stored in their food vacuole. The metal in it has an oxidative state different from the one in heme, conferring a paramagnetic property absent in uRBCs. With the maturation of parasites, the amount of hemozoin increases. Therefore, the latest stages can be separated by passing the culture through a column containing magnetic beads. When placed on a magnetic holder, the magnetic beads trap late-stage parasites inside the column [22].

After separation, the eluate was centrifuged at 1500× g for 5 min and washed 3 times with MCM, forming a dark pellet with the parasites. Then, parasites were divided into a 96-well plate previously prepared with the following conditions: MCM, Shield-1 (1 µM), and proteasome inhibitor MG132 (0.1 µM; 0.5 µM; 2 µM; 5 µM) and incubated at 37 °C. For the first reading (0 h), only the MCM condition was used. For this, parasites were incubated with MitoTracker™ (1.6 µM) at 37 °C for 30 min in the dark. Then, 20 µL of parasites marked with MitoTracker™ were added to 300 µL of PBS 1× and passed through the flow cytometer at 405/488 nm and 650/780 nm. The following readings were at 30 min, 1 h, and 3 h, and MitoTracker™ (1.6 µM) was added to all conditions. The data was analyzed using the FlowJo V10 Software (Version 10.8.1—BD Biosciences, San Jose, CA, USA).

4.4. Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 10 software (Dot-matics, Boston, MA, USA). Results were presented as means ± standard error of the mean (SEM). Comparisons between different conditions were made using one-way ANOVA (analysis of variance). Differences were considered significant in the statistical analysis when $p < 0.05$. At least three biological replicates were performed for each assay.

5. Conclusions

Malaria remains a substantial global health threat, despite extensive clinical research and treatment procedures spanning decades. Since the use and effectiveness of experimental vaccines have been minimal, there is heavy dependence on antimalarial drugs for both the prevention and treatment of infected individuals. Nowadays, this scenario is especially worrying in the context of *P. falciparum*, the deadliest of all malaria parasites in humans, resistant to ACTs [3,23]. *Plasmodium* 26S proteasome has emerged as an attractive target for new drugs, since its inhibitors synergize with ARTs, improving its efficacy [24].

Herein, we developed a protocol to monitor proteasome activity in *P. falciparum* through the quantification of the GFP fluorescence in live cells. Using fluorescence microscopy, it was possible to confirm that the DD is working, since there was residual fluorescence when the stabilizer was not present and visible fluorescence when the Shield-1 was added. With cytometry analysis, we managed to robustly monitor 26S proteasome activity through the monitoring of GFP. Dual staining with MitoTracker™ enables total discrimination between uRBCs and death parasites.

The development of a transgene parasite culture expressing the GFP-DD complex can allow us to increase the knowledge of the proteasome function in malaria parasites by testing different drugs or evaluating different mutations in the proteasome.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27052104/s1>.

Author Contributions: Conceptualization, A.F.G. and P.E.F.; methodology, A.F.G., A.L.-P. and B.S.-M.; formal analysis, A.F.G.; investigation, A.F.G. and B.S.-M.; writing—original draft preparation, A.F.G.; writing—review and editing, A.F.G., A.L.-P., B.S.-M. and P.E.F.; supervision, P.E.F. All authors have read and agreed to the published version of the manuscript.

Funding: Financial support was provided by grants from Portuguese National funds, through the Foundation for Science and Technology (FCT)—project UIDB/50026/2020 (DOI 10.54499/UIDB/50026/2020), UID/06304/2025 (<https://doi.org/10.54499/UID/06304/2025>), LA/P/0050/2020 (<https://doi.org/10.54499/LA/P/0050/2020>), PTDC/SAU-PAR/2766/2021 and 2020.02226.CEECIND to P.E.F., 2022.12892.BD (<https://doi.org/10.54499/2022.12892.BD>) to A.F.G.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author(s).

Acknowledgments: We acknowledge the Life and Health Sciences Research Institute (ICVS), University of Minho, for enabling the work presented here. We would also like to thank all the blood donors at Laboratório de Análise Clínicas Vale do Sousa Lda. for their contributions during the preparation of this manuscript authors used ChatGPT-5.2 editing assistance. The authors have reviewed and edited the output and take full responsibility for the content of this publication.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ACTs	Artemisinin-based combination therapies
ANOVA	Analysis of variance
ARTs	Artemisinin and its derivatives
BSD	Blasticidin
DD	Destabilization Domain
GFP	Green Fluorescent Protein
iRBCs	Infected RBCs
MCM	Malaria Culture Medium
RBCs	Red Blood Cells
uRBCs	Uninfected RBCs

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