Going live: a comparative analysis of the suitability of the RFP derivatives RedStar, mCherry and tdTomato for intravital and in vitro live imaging of Plasmodium parasites

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Technical Report ((5477 words))

**Going live: a comparative analysis of the suitability of the RFP derivatives
RedStar, mCherry and tdTomato for intravital and *in vitro* live imaging of
Plasmodium parasites**

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Keywords: RedStar, mCherry, tdTomato, *in vitro* live imaging, intravital
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FP fluorescent protein

GFP green fluorescent protein

MFI mean fluorescence intensity

P Plasmodium

RFP red fluorescent protein

Abstract

Fluorescent proteins have proven to be important tools for *in vitro* live imaging of parasites and for imaging of parasites within the living host by intravital microscopy. We observed that a red-fluorescent transgenic malaria parasite of rodents, *Plasmodium berghei*-RedStar, is suitable for *in vitro* live imaging experiments but bleaches rapidly upon illumination in intravital imaging experiments using mice. Therefore, we have generated two additional transgenic parasite lines expressing the novel red-fluorescent proteins tdTomato and mCherry, which have been reported to be much more photostable than first- and second-generation red fluorescent proteins including RedStar. We have compared all three red fluorescent parasite lines for their use in *in vitro* live and intravital imaging of *P. berghei* blood and liver parasite stages, using both confocal and wide-field microscopy. While tdTomato bleached almost as rapidly as RedStar, mCherry showed improved photostability and was bright in all experiments performed.

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1 Introduction

The first fluorescent protein to be employed for live imaging experiments was the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* in 1994 [1]. Shortly after, the red fluorescent protein (RFP) DsRed was discovered in the coral *Discosoma sp.* and subsequently made available for transgenic expression in various other cell types including mammalian cells and parasites [2]. Numerous derivatives of RFP were created, such as the smaller, monomeric variant mRFP1 [3] or the fast-fluorescing RedStar [4]. Since there are many GFP-expressing cell lines and mouse strains, these RFP derivatives are extremely useful for the analysis of interactions between parasites and their hosts [5-8]. We study the development of the *Plasmodium* parasite, the causative agent of malaria, in the mammalian host. An infection of the mammalian host begins after a bite of an infected *Anopheles* mosquito. During the blood meal, sporozoites, that are present in the salivary gland of the mosquito, are injected into the dermis of the mammalian host. They subsequently enter blood vessels [9, 10] and travel through them to the liver, where they invade hepatocytes and develop into thousands of daughter cells, the merozoites. Merosomes, small vesicles filled with merozoites, bud from the infected hepatocyte and are released into the blood stream [8, 11, 12]. Eventually the merosomes rupture and release merozoites, which then infect erythrocytes [13]. Within the erythrocytes, the parasite either replicates asexually or develops into male or female

gametocytes that can be taken up by the mosquito during a blood meal. After fertilization in the mosquito midgut, the newly formed ookinetes transmigrate through the midgut epithelium and form cysts under the basal lamina. Within these oocysts, sporozoites develop and are liberated into the haemocoel of the insect. Sporozoites eventually penetrate the salivary glands of the mosquito, ready to be injected into the next vertebrate host.

The development of tools for genetic modification of malaria parasites [14] and the availability of fluorescent reporter proteins such as GFP and RFP have greatly improved the study of the complex interactions of the parasite with cells of their hosts [5, 9]. A RedStar-expressing variant of the rodent parasite strain *P. berghei* has been generated that is very well suited for *in vitro* live imaging of the parasite development in hepatoma cell culture lines [8].

In 2004, Tsien et al. used directed mutation as well as iterative somatic hypermutation to enhance mRFP fluorescence [15, 16]. Some of the resulting red fluorescent proteins were reported to be much brighter and more photostable than the conventional RFPs dsRed or RedStar [16]. However, these data were generated using isolated recombinant fluorescent proteins in solution. To test the suitability of these new RFP variants in parasites, we generated transgenic, clonal *P. berghei* lines expressing the two most promising candidates, tdTomato and mCherry.

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2 **Material and methods**

Generation of transgenic *P. berghei* lines expressing red fluorescent proteins

(RedStar, tdTomato, mCherry): The RedStar-expressing *P. berghei* line has been generated previously and was named *P. berghei*-RFP in a previous publication [8]. Since all parasite lines discussed in the present paper express RFP derivatives we have re-named the parasite line expressing the RFP variant RedStar as *P. berghei*-RedStar. Two additional transgenic *P. berghei* lines expressing other red fluorescent proteins were generated by subcloning the tdTomato and mCherry coding sequences from the supplied pRSET-B plasmids [16]; these sequences were subsequently inserted into the *Plasmodium* expression plasmid pL0017 (www.mr4.org), replacing the GFP coding sequence (Supplementary Figure 1A). All genes encoding fluorescent proteins are under the control of the promoter region of the constitutively expressed *P. berghei* eukaryotic elongation factor 1 alpha (*eef1aa*). The pL0017 plasmid contains target sequences for integration into the c- or d-ssu-rRNA locus of the genome and the *tgdhfr/ts* selectable marker cassette, which allows selection of transfected parasites using pyrimethamine. Plasmids were transfected into blood stage *P. berghei* wildtype schizonts of the ANKA strain using standard methods for genetic modification of *P. berghei* [17]. After pyrimethamine selection of the transgenic parasites, the parent lines were cloned by injection of single infected detached hepatocytes or merozoites into mice. Briefly, HepG2 cells were

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6 infected with transgenic sporozoites and the resulting detached infected
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8 hepatocytes were collected 65 hours later. Individual detached cells were
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10 isolated and injected intravenously into naïve mice. The cloned parasites from
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12 the resulting blood stage infections were collected and used for further analysis.
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14 Correct integration of the genes encoding the fluorescent proteins into the
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16 genome was analyzed by PCR (Supplementary Figure 1B). The two new
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18 transgenic parasite lines that were generated are referred to as *P. berghei*-
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20 tdTomato and *P. berghei*-mCherry.
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25 **Culture of HepG2 cells:** HepG2 cells were obtained from the European cell
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27 culture collection and cultured in Minimum Essential medium with Earle's salts
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29 supplemented with 10% FCS, 1% L-glutamine and 1% Penicillin/Streptomycin
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31 (all purchased from PAA Laboratories, Austria). The cells were kept at 37°C and
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33 5% CO₂ and were split by trypsinization.
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37 **Preparation of *P. berghei* parasites for *in vitro* live imaging:** To analyze liver
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39 stage parasites, 1×10^5 HepG2 cells were seeded into glass bottom dishes (WillCo
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41 Wells BV, The Netherlands). *P. berghei* sporozoites were isolated from salivary
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43 glands of infected *Anopheles stephensi* mosquitoes 18-25 days after an
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45 infectious blood meal. Approximately 2×10^4 sporozoites were added to the
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47 HepG2 cells and incubated for a period of 2 h. After this period, the sporozoite-
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49 containing medium was removed and the cells were incubated in fresh culture
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51 medium at 37°C and 5% CO₂. For photostability experiments, parasites were
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53 imaged at 48 h post infection as described below. For imaging of the different
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liver stages, GFP-expressing HepG2 cells were infected, and nuclei were stained with 1 μ g/ml Hoechst 33342 at different time points after infection as described previously [8].

For imaging of blood stage parasites, a droplet of blood was taken from the tail vein of infected female NMRI mice, diluted with PBS and put on a microscope slide coated with 0.5 mg/ml concanavalin A. A cover slip was placed on top and sealed to the slide to prevent dehydration of the cells. Parasites were then imaged as described below.

Preparation of *P. berghei* parasites for intravital imaging: For imaging of liver stages, $4-8 \times 10^5$ *P. berghei* sporozoites were collected from salivary glands of infected *A. stephensi* mosquitoes and injected intravenously into female NMRI mice. At 44 h post infection, the mice were anesthetized with rompun/xyzazine and prepared for imaging of the parasites in the liver as described previously [18].

For imaging of blood stages, female NMRI mice were infected by intraperitoneal injection of blood stage parasites. At a parasitemia of 10-15%, the mice were anesthetized and prepared for imaging of the liver as described [18], allowing infected erythrocytes adhering near the liver capsule to be imaged.

Preparation of *P. berghei* parasites for imaging of fixed samples: 1×10^5

HepG2 cells were seeded on cover slips in 24 well plates. The cells were infected with *P. berghei* sporozoites as described for *in vitro* live imaging. At 48

h post infection, the culture medium was removed and the cells were fixed in 4% paraformaldehyde for 20 minutes. They were washed three times with PBS and then either directly mounted on microscope slides with Dako Fluorescent Mounting Medium (Dako, Cambridgeshire, UK) or additionally incubated with methanol for 10 minutes. The methanol-treated cells were again washed three times with PBS and then mounted on slides.

Wide-field imaging: Wide-field microscopy was performed with a Zeiss Observer.Z1 inverted microscope. A Zeiss 63x Plan-Apochromat 1.4 Oil objective was used and images were acquired with the Zeiss Axiovision software version 4.6. Fluorescence was excited by a Kuebler Codix HXP120 lamp (Leistungselektronik Jena, Germany) in combination with the Zeiss filter set 43 (1114-101), which passed the emitted light through a 605/70 nm bandpass filter before detection via a QuantEM:512SC EMCCD camera (Spectra Services, USA). During image collection, cells were kept in a CO₂ incubator at 37°C and mice were placed on a heating platform in order to maintain their body temperature under anesthesia.

Confocal imaging: For brightness and photostability experiments, confocal microscopy was performed with a Zeiss Observer.Z1 inverted microscope in an LSM 5 Live imaging setup. A Zeiss 63x Plan-Apochromat 1.4 Oil objective was used and images were acquired with the Zeiss Efficient Navigation 2008 software. Red fluorescence was excited by a CompassTM 561nm 40mW diode pumped laser (Coherent, USA) and emission light passed through a 575 nm

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longpass filter before detection via a CCD line detector. The master gain was set to 30 and the pinhole to 1 Airy unit. During imaging, cells were kept in a CO₂ incubator at 37°C and mice were placed on a heating platform in order to maintain their body temperature under anesthesia.

Images of liver stage parasites were acquired with an Olympus IX81 microscope in a confocal laser scanning setup. An Olympus 60x Plan-Apochromat 1.35 Oil objective was used and images were acquired with the Olympus Fluoview software version 1.7b. Fluorescence was excited by a 405 nm diode laser (Olympus, Germany), a 488 nm argon laser (Olympus, Germany) and a 559 nm diode laser (NTT Electronics, USA) and emission light passed through a 405/Mar/5437635 filter set before detection. Cells were kept in a CO₂ incubator at 37°C.

Determination of mean fluorescence intensity and photostability: Infected cells were exposed to 90 seconds of constant wide-field or confocal illumination. Every two seconds the mean red fluorescence intensity of the parasite was measured in Grey for wide-field imaging and on a 256-grey scale for confocal imaging. The initial mean fluorescence intensity was set to 100% and the drop in intensity was calculated in relation to this value. For *in vitro* live imaging, 15 to 22 parasites of each *P. berghei* strain were examined; for intravital imaging, 3 to 6 parasites were analyzed.

Imaging of *P. berghei*-RedStar and -mCherry parasites in mosquito stages: Parasites in midguts (oocyst stage) and salivary glands (sporozoite stage) of

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6 infected mosquitoes were either imaged in the intact mosquito or in PBS after
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8 dissection of the midgut or the salivary glands. Images were acquired in the
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10 wide-field microscopy setup described above.
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12 **Data analysis:** Statistical analysis was performed with the Prism software
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14 version 4.0a using the two-tailed unpaired t test. Curve fits were also carried out
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16 with the Prism software.
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19 20 21 22 **3 Results** 23

24 Since our research is mainly focused on the interactions of the malaria parasite
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26 with its host during its development in the liver, most of the imaging analyses
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28 presented concentrate on liver stage parasites. However, corresponding
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30 experiments have been performed for blood stage parasites (Supplementary
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32 Tables 1 and 2).
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35 We used *P. berghei* parasites constitutively expressing RedStar to analyze
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37 parasite development in liver cells by confocal laser scanning microscopy
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39 (Figure 1A). Clearly, *P. berghei*-RedStar parasites show bright fluorescence in
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41 *in vitro* live imaging experiments. In contrast, imaging of these parasites within
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43 the livers of living animals resulted in rapid bleaching (Figure 1B). After only 90
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45 seconds of exposure to 3% laser intensity, the RedStar parasites were hardly
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47 detectable. To analyze the dynamics of host-parasite interactions *in vivo*, for
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49 example the interaction between infected hepatocytes and host immune cells,
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51 long exposure times are a prerequisite and thus *P. berghei*-RedStar parasites are
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not suitable for such experiments. Therefore, we tested two alternative red fluorescent proteins for the expression in murine malaria parasites. These proteins, tdTomato and mCherry, have been reported to show bright fluorescence and enhanced photostability [16].

We generated two clonal transgenic *P. berghei* lines (*P. berghei*-tdTomato and *P. berghei*-mCherry; Supplementary Figure 1), which carry the coding sequences of these reporter proteins integrated into the genome. Human hepatoma cells were infected with *P. berghei*-RedStar, -tdTomato and -mCherry sporozoites, and parasites were imaged by confocal laser scanning microscopy at 48 h post infection using a laser intensity of 0.5% (Figure 2A, upper panel). tdTomato and mCherry parasites are clearly much brighter than RedStar parasites. When imaged at a laser intensity of 3%, which is optimal for *P. berghei*-RedStar, tdTomato and mCherry parasites are drastically overexposed (Figure 2A, lower panel). Because of the profound difference in fluorescence intensity of the parasites from the different transgenic lines, it was not possible to perform photostability measurements always under the same settings (laser intensity, exposure time) for all three lines. The details of the settings are provided in Figure 2B and in Supplementary Table 1. Comparison of absolute fluorescence measured in mean fluorescence intensity (MFI) revealed that tdTomato and mCherry are significantly brighter even when imaged at a lower laser intensity than RedStar expressing parasites (Figure 2B).

For determination of the photostability during intravital imaging of the parasite liver stage, NMRI mice were injected intravenously with *P. berghei* sporozoites of the respective parasite lines. At 44 h post infection, the livers of these mice were surgically exposed for analysis by intravital microscopy. Comparable to the results obtained during *in vitro* live imaging, *P. berghei*-RedStar parasites were less bright than parasites of the *P. berghei*-tdTomato and -mCherry lines and thus a higher laser intensity was needed to achieve similar fluorescence intensities (Figure 3, Supplementary Table 2). Upon illumination, a subset of bright *P. berghei*-RedStar parasites initially bleach rapidly but a low level of fluorescence remains that is then lost at a much slower rate (Figure 3, first panel). A similar bleaching behavior has been described previously for tagRFP, a red fluorescent protein derived from *Entacmaea quadricolor* [19]. Most of the observed *P. berghei*-RedStar parasites, however, did not clearly show this rapid biphasic bleaching. Their absolute fluorescence intensity (on a 256-grey scale) was already weak at the beginning of the experiment, either due to variation of expression levels or their location in different focal planes (Supplementary Figure 2). For these parasites, the drop in fluorescence was comparatively low. Whatever the explanation for the different fluorescence intensity and bleaching behavior of this RFP derivate is, it is clear that *P. berghei*-RedStar parasites are not suitable for intravital imaging experiments lasting for several hours.

Although tdTomato has been reported to have enhanced photostability, *P. berghei*-tdTomato parasites bleached equally as fast as RedStar parasites in

intravital imaging experiments. After 90 seconds of illumination, nearly 50% of the initial fluorescence intensity was lost (Figure 3, second panel). Moreover, the parasites observed in the liver of the infected mouse had a low fluorescence intensity even at the beginning of illumination. In contrast to *P. berghei*-RedStar and -tdTomato, *P. berghei*-mCherry parasites were bright and hardly bleached over the course of the experiment. After 90 seconds of illumination, more than 90% ($91\pm2\%$) of the initial fluorescence intensity still remained (Figure 3, third panel).

All photostability data obtained for liver and blood stage parasites are summarized in Figure 4. For confocal imaging (Figure 4A), it can be concluded that mCherry is by far the best option for the analysis of liver and blood stage parasites by *in vitro* live imaging and for the analysis of liver stage parasites by intravital imaging. In contrast to the clear difference in bleaching between liver stages of *P. berghei*-RedStar and *P. berghei*-mCherry, intravital imaging of the blood stages of these parasites showed comparable bleaching features of for both RFP derivatives. However, since mCherry is much brighter than RedStar (Figure 2B and Supplementary Table 2), it is also the protein of choice for the intravital analysis of parasites in red blood cells. The jags in the individual bleaching curves of the blood stages (Figure 4, on the right) result from the small size of the parasites. Even a slight movement of the anesthetized animal causes a shift of the infected red blood cell out of the focal plane, which in turn results in a drop in recorded fluorescence. Still, the curves allow for an estimation of the

bleaching behavior of the fluorescent proteins expressed by the blood stage parasites analyzed in the living host.

The results for wide-field *in vitro* live imaging experiments (Figure 4B, upper panels) confirm our earlier observations that under *in vitro* conditions, RedStar is rather photostable [8]. In contrast, intravital imaging of infected liver cells showed a rapid bleaching of RedStar and tdTomato even with the less phototoxic wide-field illumination. In comparison with these proteins, mCherry demonstrated enhanced photostability in intravital liver stage imaging (Figure 4B lower panel, left). Interestingly, bleaching of tdTomato and RedStar in blood stage parasites was much less evident in wide-field intravital microscopy (Figure 4B lower panel, right). However, the absolute fluorescence levels again argue for mCherry as the red fluorescent protein of choice (Supplementary Table 1).

Even after fixation of HepG2 cells infected with fluorescent *P. berghei* parasites, mCherry turned out to be more photostable than RedStar when exposed to laser illumination (Supplementary figure 3). Fixation of RedStar parasites caused a marked decrease in absolute fluorescence (Supplementary figure 3A) and enhanced susceptibility to bleaching (Supplementary figure 3B). In comparison to this, mCherry did not show any loss of fluorescence after PFA fixation (Supplementary figure 3A) and bleached only moderately (Supplementary figure 3B).

4 Discussion

Our observations of the photostability of all three RFP proteins show that in general, they bleach far more rapidly in intravital than in *in vitro* live imaging (Figure 4). The process of bleaching is a poorly understood phenomenon, and we can therefore only speculate about the reasons for the difference in photostability between the fluorescent proteins expressed by liver and blood stage parasites. It is assumed that bleaching is related to the formation of an excited triplet state of the chromophore upon illumination. This excited triplet state can either return to the ground state for another round of excitation or react with other molecules. Such reactions can lead to the covalent modification of the chromophore and therefore the permanent loss of its ability to fluoresce (<http://www.olympusmicro.com/primer/java/fluorescence/photobleaching/index.html>). A prime candidate for such highly reactive molecules would be oxygen since it is often present in a natural triplet state. It has been shown that fluorescent dyes containing aromatic carbon rings bleach in the presence of oxygen [20], and since the chromophores of the red fluorescent proteins have such rings, loss of fluorescence in a similar manner is feasible. Oxygen is far more readily available as a reaction partner within tissues than in cell culture. In most laboratory cultures of mammalian cells such as the HepG2 hepatoma line, oxygen reaches the cells by passive diffusion through the culture medium. This diffusion process becomes less efficient with increasing fluid levels and a level of about 1 mm allows for an optimal oxygen supply for HepG2 cells [21]. In our *in vitro* experimental setup, the level of culture medium in the culture dish was

approximately 8 mm. The oxygen supply within the cells was therefore likely low. In contrast, within the liver tissue, oxygen should be relatively freely available due to distribution via hemoglobin. It is therefore possible that the differing photostability observed could be a consequence of the disparity in oxygen levels in cell culture and tissue. This might also explain the observation that bleaching is usually more pronounced in confocal than in wide-field imaging. In the former, the light source is a laser, and it is possible that this focused, high-energy excitation allows formation of the triplet state more often than does wide-field excitation. An additional aspect that could influence bleaching is that in confocal imaging, only light from the focal plane is detected and bleaching is therefore immediately obvious. In wide-field imaging, in comparison, some degree of bleaching might initially be masked by fluorescence from other focal planes.

Another mechanism that could lead to a loss of fluorescence is closely related: upon interaction of oxygen with the chromophore triplet state, highly reactive singlet oxygen is formed. It will rapidly oxidize molecules containing carbon-carbon double bonds [22] and has been shown to damage cells [23, 24]. However, the detrimental effects of this process are fairly long term and it is thus unlikely to play a role during the observation time of 90 seconds used in the present study.

The question remains why we observe a significantly more rapid bleaching for RedStar and tdTomato than for mCherry. Various mutations were introduced

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6 into both tdTomato and mCherry that lead to pronounced changes in
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8 chromophore architecture. Little is known about the resulting three-dimensional
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10 structure of tdTomato, but in mCherry the chromophore is distinctly distorted in
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12 comparison to the planar architecture of mRFP [25]. It is likely that this can lead
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14 to a different electron density distribution and it is conceivable that this, along
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16 with steric changes, might hinder the ability of the chromophore to react with
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18 triplet oxygen or other molecules.
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22 Comparisons of the brightness and the bleaching features of red fluorescent
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24 protein variants are rarely reported in the literature. The few reports discussing
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26 this topic deal with different sets of fluorescent proteins than used for the present
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28 study and thus cannot be compared directly. However, the published data
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30 suggest that the behavior of fluorescent proteins depends on the experimental
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32 setup and that it is sometimes difficult to predict which RFP variant is suitable
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34 for expression and imaging in different organisms. It is therefore worthwhile to
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36 briefly discuss the previous findings concerning the use of different RFP
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38 variants. In these studies, the difference in brightness between tdTomato,
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40 mCherry and dsRed [16] has been analysed but not that of RedStar. RedStar is
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42 reported to be twenty times brighter than dsRed [4], but this is based on
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44 measurements in bacterial and yeast expression systems, which might differ
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46 from expression in parasites or mammalian cells. Since mCherry showed a
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48 reduced brightness compared to dsRed [16], it is surprising that mCherry
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50 expressed in *P. berghei* is even brighter than RedStar. One possible explanation
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could be that the data of Shaner et al. were obtained using a Xenon arc lamp for excitation of the proteins whereas we used either a HXP120 lamp or laser excitation. For confocal imaging of RFP expressing parasites, we used a laser excitation wavelength of 561 nm, and thus not all RFP derivatives will be excited equally well. However, RedStar and tdTomato with excitation maxima at 558 and 554 nm, respectively are closer to the excitation wavelength of the laser than mCherry with an excitation maximum of 587 nm. Therefore the fixed excitation wavelength of the laser cannot be the reason for the superior brightness of mCherry. Another, more likely explanation is that while mCherry is already fluorescent as a monomer, RedStar and dsRed have to reach a tetrameric state before they can emit fluorescence. It is therefore possible that the tetrameric conformation is not achieved equally well upon expression in different organisms. A similar effect would also explain why in our experiments tdTomato is not, as published, five times brighter than mCherry [16]. tdTomato, unlike the monomeric mCherry, is a linked dimer, and therefore the correct folding of the protein could be more problematic.

5 Concluding remarks

In comparison to RedStar and tdTomato, mCherry is the protein of choice for the analysis of *Plasmodium* liver and blood stages by confocal and wide-field *in vivo* imaging. It generally shows the brightest fluorescence and, more importantly, proves to be far more photostable. This is most impressively

demonstrated during the analysis of the liver stages of the parasite by intravital microscopy. mCherry has recently also been recommended for tomographic imaging where red fluorescent proteins are preferentially used because they emit at wavelengths where tissue absorption is low [26]. Moreover, mCherry is convenient also in the mosquito stages of *P. berghei* because it makes analysis and selection of infected mosquitoes under a fluorescence binocular easier (Supplementary Figure 3).

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The authors have declared no conflict of interest.

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Figure legends

Figure 1: *P. berghei*-RedStar is well suited for *in vitro* live imaging but not intravital imaging of parasite liver stages.

A HepG2-GFP cells (green) were infected with *P. berghei*-RedStar sporozoites. At the indicated time points after infection, nuclei were stained with Hoechst 33342 (blue) and the cells were imaged with a confocal laser-scanning microscope. The parasite (red) progresses from a sporozoite (6 hours post

infection, hpi) to a large schizont (48 hpi) before it divides into merozoites (54 hpi); eventually the infected hepatocyte detaches (65 hpi).

B An NMRI mouse was infected with 5×10^5 *P. berghei*-RedStar sporozoites by intravenous injection. At 44 hours post infection, a liver lobe was surgically exposed and parasites (red) were imaged with a confocal laser scanning microscope. Bars = 10 μ m

Figure 2: In confocal imaging of fluorescent *Plasmodium* liver stage parasites, tdTomato and mCherry are much brighter than RedStar.

HepG2 cells were infected with *P. berghei*-RedStar, -tdTomato and -mCherry sporozoites, respectively. At 48 hours post infection, parasites were imaged by confocal laser scanning microscopy at different laser intensities.

A At the optimal laser intensity for the imaging of tdTomato and mCherry (0.5%), RedStar fluorescence is too weak to be clearly detected, while the optimal setting for RedStar (3%) results in an overexposure for tdTomato and mCherry.

Bar = 10 μ m

B Absolute fluorescence levels are compared at optimal laser intensities (LI, indicated by the boxed numbers). At low LI (0.5%), mCherry is significantly brighter than that of tdTomato (LI 0.5%) and RedStar at higher LI (3%). $n=15$, ** = $p < 0.005$, *** = $p < 0.0001$.

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Figure 3: In intravital imaging of *Plasmodium* liver stage parasites, mCherry is far more photostable than RedStar and tdTomato.

NMRI mice were injected intravenously with *P. berghei* sporozoites of the respective parasite lines. At 44 hours post infection, the liver was surgically exposed and parasites were imaged by confocal laser scanning microscopy. The mean fluorescence intensity was measured every two seconds during a 90 second period of constant illumination optimal for image acquisition for the respective red fluorescent protein. The images presented were acquired at the beginning (0 sec) and at the end (90 sec) of the photostability assay. The drops in mean fluorescence intensity were calculated in relation to the initial intensity and are indicated in the graphs (mean and the standard deviation of several cells imaged per experiment are presented). A curve fit was performed (shown as a thin black line) and the bleaching of RedStar and tdTomato was found to fit best a biphasic exponential decay curve ($R^2=0.9906$ and $R^2=0.9978$, respectively). Bars = 10µm. Total numbers of recorded parasites are shown in Supplementary table 1.

Figure 4: Of the red fluorescent parasite strains tested, *P. berghei*-mCherry is best suited for live imaging.

P. berghei-RedStar, -tdTomato and -mCherry blood and liver stage parasites were prepared for *in vitro* live imaging and intravital imaging (for details see material and methods section). Individual fluorescent parasites were exposed to

90 seconds of constant confocal laser scanning (A) or wide-field (B) illumination and the mean red fluorescence was measured every two seconds. The drop in fluorescence intensity is shown as the percentage of the initial mean fluorescence for individual parasites. Total numbers of recorded parasites are shown in supplementary table 1.

Figure 1: Graewe et al

P. berghei-RedStar is well suited for in vitro live imaging but not intravital imaging of parasite liver stages.

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B An NMRI mouse was infected with 5x10⁵ *P. berghei*-RedStar sporozoites by intravenous injection. At 44 hours post infection, a liver lobe was surgically exposed and parasites (red) were imaged with a confocal laser scanning microscope. Bars = 10µm

93x165mm (300 x 300 DPI)

Figure 2: Graewe et al

In confocal imaging of fluorescent *Plasmodium* liver stage parasites, tdTomato and mCherry are much brighter than RedStar.

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B Absolute fluorescence levels are compared at optimal laser intensities (LI, indicated by the boxed numbers). At low LI (0.5%), mCherry is significantly brighter than that of tdTomato (LI 0.5%) and RedStar at higher LI (3%). $n=15$, ** = $p<0.005$, *** = $p<0.0001$.

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Figure 3: Graewe et al

In intravital imaging of Plasmodium liver stage parasites, mCherry is far more photostable than RedStar and tdTomato. NMRI mice were injected intravenously with *P. berghei* sporozoites of the respective parasite lines. At 44 hours post infection, the liver was surgically exposed and parasites were imaged by confocal laser scanning microscopy. The mean fluorescence intensity was measured every two seconds during a 90 second period of constant illumination optimal for image acquisition for the respective red fluorescent protein. The images presented were acquired at the beginning (0 sec) and at the end (90 sec) of the photostability assay. The drops in mean fluorescence intensity were calculated in relation to the initial intensity and are indicated in the graphs (mean and the standard deviation of several cells imaged per experiment are presented). A curve fit was performed (shown as a thin black line) and the bleaching of RedStar and tdTomato was found to fit best a biphasic exponential decay curve ($R^2=0.9906$ and $R^2=0.9978$, respectively). Bars = $10\mu\text{m}$. Total numbers of recorded parasites are shown in Supplementary table 1.

150x160mm (300 x 300 DPI)

Figure 4: Graewe et al

Of the red fluorescent parasite strains tested, *P. berghei*-mCherry is best suited for live imaging. *P. berghei*-RedStar, -tdTomato and -mCherry blood and liver stage parasites were prepared for in vitro live imaging and intravital imaging (for details see material and methods section). Individual fluorescent parasites were exposed to 90 seconds of constant confocal laser scanning (A) or wide-field (B) illumination and the mean red fluorescence was measured every two seconds. The drop in fluorescence intensity is shown as the percentage of the initial mean fluorescence for individual parasites. Total numbers of recorded parasites are shown in supplementary table 1.

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