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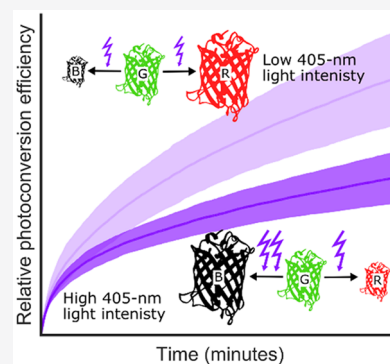


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Supporting Information

ABSTRACT: Green-to-red photoconvertible fluorescent proteins (PCFPs) are widely employed as markers in photoactivated localization microscopy (PALM). However, their highly complex photophysical behavior complicates their usage. The fact that only a limited fraction of a PCFP ensemble can form the photoconverted state upon near-UV light illumination, termed photoconversion efficiency (PCE), lowers the achievable spatial resolution in PALM and creates undercounting errors in quantitative counting applications. Here, we show that the PCE of mEos4b is not a fixed property of this PCFP but strongly depends on illumination conditions. Attempts to reduce long-lived blinking in red mEos4b by application of 488 nm light lead to a reduction of the PCE. Furthermore, the PCE of mEos4b strongly depends on the applied 405 nm power density. A refined photophysical model of mEos4b accounts for the observed effects, involving nonlinear green-state photobleaching upon violet light illumination favored by photon absorption by a putative radical dark state.



For now more than a decade, super-resolution microscopy has provided unprecedented insights into biological processes.¹ Photoactivation localization microscopy (PALM) is a single-molecule-based localization technique that typically takes advantage of so-called phototransformable fluorescent proteins (PTFPs).² Major advantages of using PTFPs are the possibility to work on living cells, the ideal specificity of labeling, and the option to label endogenous targets of interest with genome editing techniques.² Green-to-red photoconvertible fluorescent proteins (PCFPs) of the mEos family are often used as PTFP markers in PALM because of their relatively high brightness as well as high monomeric character and also because successfully labeled samples can be easily detected by monitoring green-state fluorescence prior to PALM data collection.³ However, a disadvantage of mEos-based PCFPs is their highly complex photophysical behavior (Figure S1).^{4–7}

One property essential to the success of PALM experiments using PCFPs is the so-called photoconversion efficiency (PCE),^{8,9} sometimes also termed signaling efficiency.¹⁰ Typically, upon prolonged violet light illumination, green-to-red photoconversion remains incomplete, meaning that from a pool of PCFPs, only a limited fraction can ever be imaged in the red channel of the microscope.^{4,9,11,12} In addition to unsuccessful protein folding or chromophore maturation, incomplete PCE's can be attributed to possible subpopulations within the PCFP pool unable to photoconvert, rapid photobleaching in the red state before detection can be made, or premature photobleaching in the green state before photoconversion occurs.^{4,9,11,12} In general, green-to-red photoconversion, the mechanism of which remains incompletely

understood,³ is in competition with other photophysical pathways that upon absorption of a violet photon may lead to reversible dark-state formation or photobleaching.⁹

A limited PCE is highly detrimental to PALM experiments. First, it limits the achievable spatial resolution of nanoscopy images due to a reduced apparent labeling efficiency, compromising the high labeling density necessary to satisfy the Nyquist criterium.⁸ Second, it complicates counting by quantitative PALM (qPALM).¹² In this technique the stoichiometry of protein complexes, typically nonresolvable spatially, can in principle be determined by counting fluorescence bursts from each complex.^{12–16} However, a limited PCE results in severe undercounting errors. Such errors can in theory be corrected for based on the expected binomial distribution of measured stoichiometries, but this comes at the cost of increased uncertainty of the extracted values, notably when a distribution of stoichiometries is inherently present in the sample.^{12,16}

PCEs of various PCFPs have been reported in several papers,^{9,10,12,13,17,18} usually relying on the use of biological templates.¹⁴ The measured values, however, tend to differ widely for a single PCFP, e.g., from ~1%¹⁰ to ~90%¹⁷ for mEos2. One hypothesis to account for such variability could be

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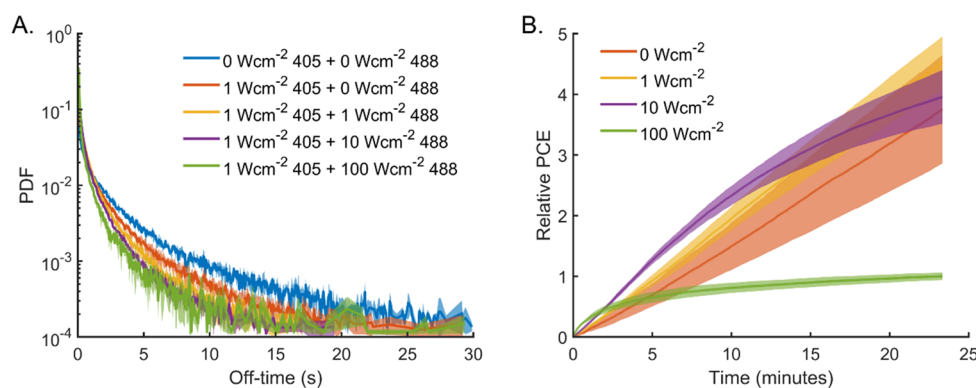


Figure 1. Off-time reduction by 488 nm light is offset by a reduced photoconversion efficiency. Off-time histograms (A) and the relative PCE (B) of mEos4b molecules embedded in PAA under alternating illumination with 500 W/cm² 561 nm light (70 ms) and 1 W/cm² 405 nm light plus 0–100 W/cm² 488 nm light (8.2 ms). Data are normalized to the final number of photoconverted molecules under 100 W/cm² 488 illumination. Data represent mean \pm s.d. of ≥ 3 measurements.

that the employed templates might induce biases due to factors such as template heterogeneity, reduced tumbling of the labels in fixed samples or uncontrolled energy transfer mechanisms between neighboring PCFPs.^{11–13} However, the possibility that PCEs may depend on the employed illumination conditions, notably the applied power densities, has not been put forward. Here, we investigate the effects of 405 and 488 nm light illumination on the PCE of mEos4b, a now popular mEos variant initially designed to withstand chemical fixation.¹⁹

The study was initially stimulated by the prospect of extending to qPALM applications a blinking reduction strategy that we recently introduced in the context of single-particle-tracking PALM (sptPALM).⁵ In this technique, 488 nm light is used in addition to readout (561 nm) and very weak activating (405 nm, of the order of mW/cm²) light to increase the length of single-molecule tracks through a reduction of fluorescence intermittencies in red mEos4b molecules.⁵ The mechanistic interpretation of the effect is that long off-times in mEos-based PCFPs originate at least in part from 561 nm induced *cis*–*trans* isomerization of the chromophore and that back-isomerization to the fluorescent *cis* state is efficiently promoted by 488 nm light.⁵ Thus, we wondered whether the same strategy could be used to improve counting accuracy with qPALM by reducing long-lived blinking expected to be at the origin of overcounting errors.

We first performed simulations to check whether long-lived off-time reduction effectively improves counting, deliberately ignoring the rich photophysics of mEos4b in its green state.^{4,6} The qPALM data were simulated by using the photophysical model depicted in Figure S2A and Table S1, in the absence or the presence of additional 488 nm light. Then, keeping in mind that widely different strategies have been proposed to process qPALM data,²⁰ we analyzed the data using either the off-times thresholding method of Lee et al.¹⁵ or the blinking statistics method of Heilemann et al.^{13,16,17,21} The results shown in Figures S3 and S4 revealed that whereas the accuracy of stoichiometry retrieval by off times thresholding benefited from long-lived dark-state reduction, this was not observed in the case of blinking statistics analysis (Supporting Information, Note 1). However, importantly, we noticed that slight measurement errors in the blinking propensity (*p*-value) of mEos4b measured in the monomeric state propagated to very large errors when evaluating complexes of high stoichiometries (Figure S4F). Overall, the performed simulations encouraged

us to experimentally test the potential benefit of additional 488 nm light for counting purposes.

mEos4b proteins were immobilized in polyacrylamide (PAA) gel, and fluorescence traces of photoconverted molecules were recorded by using 500 W/cm² 561 nm light under increasing 488 nm light intensities with wide-field illumination. First, we validated that 488 nm illumination reduces the off-time duration of red mEos4b in the presence of weak 405 nm laser light (1 W/cm², which can be taken as representative for qPALM experiments)²² (Figure 1A). We noticed, however, that the reduction in off-times was not as pronounced as with 405 nm light alone when using similar intensities (Figure S5). The mechanism behind this experimental observation remains to be deciphered, but it could result from the presence of one or several long-lived dark states, in addition to the switched-off protonated *trans* chromophore, which would be reactive to 405 nm but insensitive to 488 nm light.

Next, we assessed the effect of 488 nm illumination on the PC rate and efficiency. Although absolute PCEs could not be reliably extracted using our experimental setup (Supporting Information, Note 4), we could compare relative values over time at different illumination intensities. We thus measured the numbers of photoconverted molecules at the end of experiments by using 405, 488, and 561 nm light illumination and compared them with those using only 405 and 561 nm light. Figure 1B shows that although low 488 nm light intensity (1 W/cm²) had a minimal effect on the PC kinetics within the time window of the experiment, high 488 nm light intensity (100 W/cm²) drastically decreased the PCE. Because the bleaching time and photon budget of photoconverted red molecules were not reduced by 488 nm illumination (Figure S6), green-state photophysics is likely at the origin of the decrease in PCE. Using simulations with the current model of mEos4b photophysics,⁴ we found that the decrease can be explained by green-state bleaching by the 488 nm light (Figure S7). Importantly, the dependence of the PCE on 488 nm light is only observed in the presence of significant 405 nm light (~ 1 W/cm²) (Figure S7). This is explained by the fact that in such case photoconversion (mostly driven by 405 nm light) and green-state photobleaching (driven by all lasers) become strongly decoupled. Thus, under very weak 405 nm light ($\ll 1$ W/cm²) often used in sptPALM to achieve strong single-molecule sparsity,⁵ the PCE is low⁴ but there is little additional penalty in using 488 nm light to reduce red-state

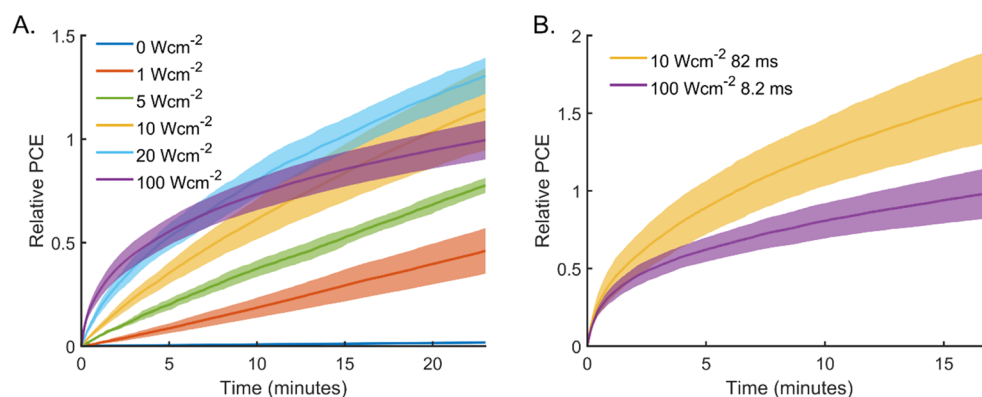


Figure 2. High 405 nm illumination intensities reduce the photoconversion efficiency of mEos4b under PALM imaging conditions. (A) Relative PCE of mEos4b molecules embedded in PAA under alternating illumination with 500 W/cm² 561 nm light (70 ms) and 1–100 W/cm² 405 nm light (8.2 ms). (B) Relative PCE of mEos4b molecules embedded in PAA under alternating illumination with 500 W/cm² 561 nm light (70 ms) and 10 or 100 W/cm² 405 nm light for 82 and 8.2 ms, respectively. Data are normalized to the final number of photoconverted molecules under 100 W/cm² 405 nm illumination. Data represent mean \pm s.d. of ≥ 3 measurements.

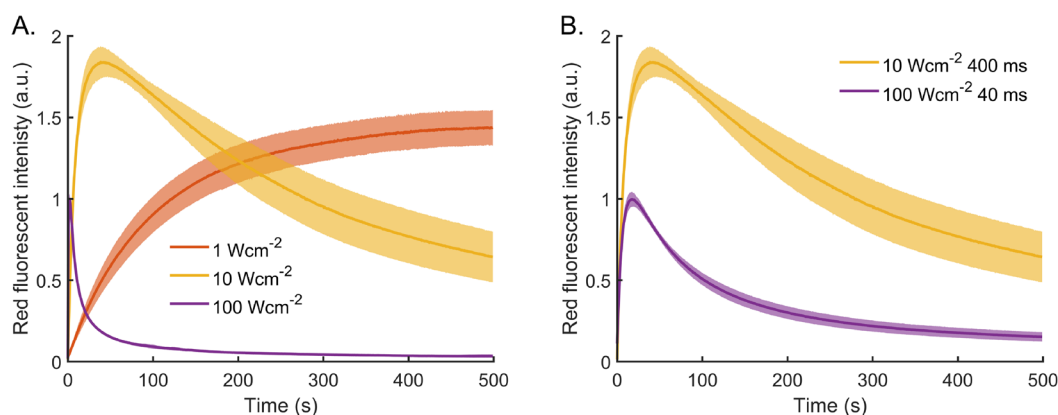


Figure 3. High 405 nm light illumination intensities reduce the photoconversion efficiency of mEos4b in the absence of 561 nm light illumination. Ensemble fluorescence time traces of mEos4b molecules embedded in PAA. The red fluorescent intensity was measured every 500 ms by illumination with 8 W/cm² 561 nm light for 20 ms. (A) Samples were exposed to 400 ms pulses of 405 nm light (1–100 W/cm²). (B) Samples were exposed to 40 and 400 ms pulses of 100 and 10 W/cm² 405 nm, respectively. Data represent mean \pm s.d. of ≥ 3 measurements.

intermittencies. Overall, these results show that 488 nm light illumination is not beneficial in qPALM measurements with mEos4b, as the gain in red-state off-time reduction is relatively limited and the PCE becomes compromised in the presence of $> \sim 1$ W/cm² 405 nm light.

Intrigued by the finding that 488 nm light drastically reduces the mEos4b PCE in the presence of 405 nm light, we set out to investigate how the intensity of the 405 nm light itself affects the PCE in the absence of 488 nm light. We first hypothesized that the PCE could increase with increasing 405 nm light because 405 nm induced photoconversion and photobleaching rates would evolve in proportion if based on single-photon mechanisms while bleaching of green molecules by the 561 nm laser⁴ would be reduced due to shorter acquisition times. To test this, we acquired single-molecule data at varying 405 nm power densities, similar to the 488 nm illumination experiments described above. Whereas low levels of 405 nm light (1 W/cm²) increased the PCE as compared to readout photoconversion only, in line with our previous findings,⁴ we found that high 405 nm intensities (100 W/cm²) substantially decreased the PCE despite a higher initial photoconversion rate (Figure 2A). Ensemble measurements where we monitored the cumulative fluorescence of the mEos4b red state under identical light illumination conditions as in the

single-molecule case revealed a similar relation between the 405 nm light intensity and the PCE (Figure S8). To evaluate whether the decrease in PCE at high 405 nm light intensity could be due to increased bleaching of red molecules, we examined the single-molecule bleaching time and photon budget histograms of the red state (Figure S9). These histograms reveal that red-state bleaching was not significantly increased when raising the 405 nm light power density, suggesting that, again, green-state photophysics are at the origin of the decreased PCE under increased 405 nm light intensities.

In contrast to the case of 488 nm light, the dependence of the mEos4b PCE on 405 nm light cannot be explained by the current photophysical model of this PCFP (Figure S7).⁴ To explain the decrease of the PCE at high 405 nm light intensity, 405 nm photons must bleach the green state in a nonlinear manner; that is, bleaching must require the subsequent absorption of two (or more) photons. To verify this hypothesis, we compared the PCEs under 10 and 100 W/cm² 405 nm light while keeping the integrated illumination dose constant by adjusting the exposure time (82 ms for 10 W/cm² and 8.2 ms for 100 W/cm²). As expected, application of short exposures of 100 W/cm² 405 nm light decreased the mEos4b PCE as compared to longer exposures to 10 W/cm²,

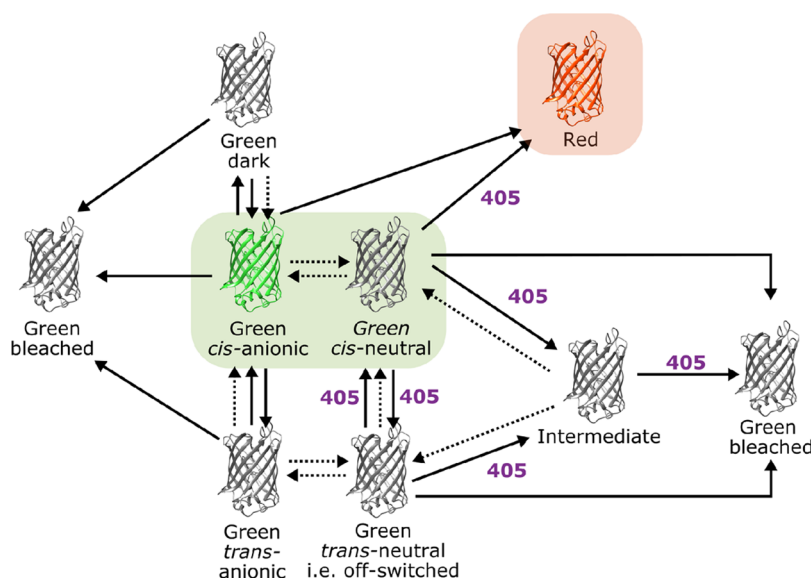


Figure 4. Tentative updated model of mEos4b green-state photophysics. An intermediate state, downstream of the neutral green chromophore, thermally recovers or absorbs a second 405 nm photon leading to nonlinear bleaching at high 405 nm power densities. Single-photon bleaching may occur from every light-absorbing state. Solid arrows indicate photoinduced transitions; dotted arrows indicate thermal transitions or pH-dependent equilibria.

while this did not affect the red-state photophysics (Figure 2B and Figure S10).

Next, we aimed to identify the photophysical state(s) of green mEos4b involved in nonlinear bleaching by 405 nm light. Violet light is predominantly absorbed by the protonated states of the green chromophore,⁶ which makes the *cis*-protonated and *trans*-protonated (i.e., off-switched) states likely starting points for the mechanism at play. The *trans*-protonated state can be populated by intense 561 nm illumination,⁴ as used in PALM measurements to reach single-molecule sensitivity, which made us wonder whether 405 nm light also induced nonlinear bleaching in the absence of 561 nm light. To test this, we monitored the progression of ensemble mEos4b red fluorescence under 1, 10, and 100 W/cm² 405 nm illumination and weak exposure to 561 nm light (8 W/cm²) (Figure 3A). Sun et al.⁷ reported that the ensemble red fluorescence intensity of mEos3.2 under constant 405 and 561 nm illumination follows a three-state model: green → red → bleached. Such a model implies that the level of the maximum recorded red fluorescence increases with the applied 405 nm light intensity as bleaching by the 561 nm laser lowers the peak height at low 405 nm intensities. However, the experimentally measured maximum intensity under 100 W/cm² 405 nm light reached a significantly lower value as compared to illumination with 1 and 10 W/cm² 405 nm light (Figure 3A and Supporting Information, Note 3), suggesting that the model of Sun et al. is insufficient to account for our data. To further validate this finding, we monitored the ensemble red-state fluorescence under illumination with either 10 or 100 W/cm² 405 nm light while keeping the integrated dose of 405 nm light constant. The data again revealed that high-intensity 405 nm light decreased the red fluorescence maximum intensity (Figure 3B). Altogether, these ensemble measurements are consistent with the notion that 405 nm light induces nonlinear photobleaching of the mEos4b green state independently of the presence of additional 561 nm light. We speculate that, upon absorption of a 405 nm photon in either the *cis*-neutral or *trans*-neutral chromophore state, an

intermediate state forms with a minimum lifetime in the tens of milliseconds range (Figure 4 and Supporting Information, Note 5). Such a lifetime appears too long to attribute the intermediate to the triplet state.²³ However, a radical state, similar to the one that we pinpointed in an earlier X-ray-based study of IrisFP (a PCFP derived from EosFP), could be invoked.²⁴ This intermediate would thermally relax to the fluorescence state or lead to photobleaching upon absorption of violet light. The precise mechanism of nonlinear bleaching of PCFPs by 405 nm light will now need further dedicated studies. A possible scenario is decarboxylation of Glu212,²⁵ a fully conserved residue in fluorescent proteins of the GFP family, that was found critical for successful photoconversion in PCFPs,²⁶ in line with our previous structural studies of photobleaching in green IrisFP.²⁷ An updated model of mEos4b photophysics is presented in Figure 4, of use for the future design of optimized illumination schemes.

We also observed nonlinear photobleaching by 405 nm light in mEos3.2, pcStar, and Dendra2 (Figure S11), and we anticipate that the described mechanism applies to most commonly used PCFPs of anthozoan origin. Moreover, Renz and Wunder²⁸ reported that the photoactivation efficiency of the photoactivatable FPs PA-GFP and PA-mCherry is reduced by high-intensity 405 nm illumination, which suggests that 405 nm light-induced nonlinear bleaching could be a feature shared by many phototransformable FPs. In the future, it will be interesting to investigate the effects of pH, oxygen removal, and the addition of triplet-state quenchers, reducers, or oxidizers on the nonlinear photobleaching phenomenon.^{29,30} Of particular interest would be the investigation of the effects of reducing thiols, which have been shown to significantly increase red-state blinking in mEos2³¹ and mEos3.2,¹³ possibly resulting from a photoprotective effect that might be beneficial for increasing PCE at high violet light intensities. More generally, how our findings made *in vitro* using PAA gel immobilization can be altered in the many different cellular nanoenvironments encountered in biological samples will need

to be investigated in detail, for example, by using specific nanotemplates.^{14,32}

In conclusion, our results demonstrate that the photoconversion efficiency of mEos4b and other PCFPs strongly depends on the employed illumination conditions. In the presence of 405 nm light the PCE of mEos4b decreases with increasing 488 nm illumination because 488 nm light significantly increases the overall green-state bleaching rate while the overall photoconversion rate remains governed by the applied 405 nm light. Most importantly, high 405 nm light intensities decrease the PCE of mEos4b due to nonlinear bleaching of the green state, possibly through subsequent photon absorptions by a neutral state of mEos4b and a short-lived radical state.

A high PCE is essential to ensure sufficient labeling density in PALM and accurate molecular counting in qPALM. Whereas the use of very low 405 nm light ($\ll 1$ W/cm²) should be avoided whenever possible (depending on single-molecule sparsity) due to accumulating green-state photo-bleaching by readout 561 nm light, high 405 nm light is expected to also yield suboptimal results in the following circumstances: (i) in high-speed PALM,³³ (ii) whenever the photoconversion light is progressively ramped-up in PALM or qPALM, or (iii) whenever short and intense photoconversion light pulses (e.g., in between recorded frames) are used instead of longer and weaker pulses. Finally, this work highlights the complexity and, yet, key importance of green-state photo-physics in photoconvertible fluorescent proteins.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jpcllett.2c00933>.

Materials and methods, detailed notes on the experimental setup and data analysis, and supporting figures (PDF)

Transparent Peer Review report available (PDF)

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Author Contributions

J.W. and D.B. conceived of the project; J.W. and D.T. performed experiments and data analysis; J.W. and D.B. performed simulations; O.G. and D.T. developed data

acquisition schemes; J.W. and D.B. wrote the paper with contributions from all authors.

Notes

The authors declare no competing financial interest.

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