Title

Mechanistic investigation of mEos4b reveals a strategy to reduce track interruptions in sptPALM

Author list

Elke De Zitter¹, Daniel Thédié², Viola Mönkemöller¹, Siewert Hugelier¹, Joël Beaudouin², Virgile Adam², Martin Byrdin², Luc Van Meervelt¹, Peter Dedecker¹, Dominique Bourgeois²

Institutions

1 - Department of Chemistry, KU Leuven, Heverlee, Belgium.

2 - Univ. Grenoble Alpes, CEA, CNRS, IBS, Grenoble, France

Corresponding authors:

D.B. (dominique.bourgeois@ibs.fr), P.D. (peter.dedecker@kuleuven.be)

Abstract

Green-to-red photoconvertible fluorescent proteins repeatedly enter dark states, causing interrupted tracks in single-particle-tracking localization microscopy (sptPALM). We identified a long-lived dark state in photoconverted mEos4b that results from isomerization of the chromophore and efficiently absorbs cyan light. Addition of weak 488-nm light swiftly reverts this dark state to the fluorescent state. This strategy largely eliminates slow blinking and enables the recording of significantly longer tracks in sptPALM with minimum effort.

Main text

Fluorescent proteins (FPs) and in particular green-to-red photoconvertible fluorescent proteins (PCFPs) have become indispensable tools for advanced imaging such as single-molecule localization microscopy (SMLM) or single-particle tracking photoactivated localization microscopy (sptPALM).¹ Both techniques are limited by blinking, a process in which the fluorophores stochastically enter reversible dark states. PCFPs display blinking on multiple timescales, arising from different underlying photochemical processes.² Fluorescence intermittencies shorter than the typical exposure times used in these imaging methodologies (~tens of milliseconds), such as caused by intersystem crossing to the triplet state, reduce the number of photons emitted during a single exposure. Intermittencies longer than the exposure time, in contrast, cause severe complications such as multiple counting of target molecules in quantitative SMLM and track interruptions in sptPALM.³ However, the mechanistic origin of long-lived dark states in PCFPs has remained unclear and there is presently no strategy to eliminate them.

We set out to investigate the nature of long-lived fluorescence intermittencies in photoconverted (red) mEos4b, the latest variant in a series of highly popular green-to-red PCFPs.⁴ Individual mEos4b molecules were immobilized in a polyacrylamide (PAA) matrix, converted to the red state using 405-nm illumination, and the fluorescence emission visualized in time using a widefield microscope with single-molecule (SM) sensitivity and 561-nm excitation. The SM fluorescence traces (Fig. 1A) displayed long-lived intermittencies, and histograms of their duration (Supplementary Fig. 1) revealed the presence of at least two dark states, as previously reported in mEos2 or Dendra2^{5,6} (Supplementary Note 1).

46 While the shorter-lived dark state was insensitive to the intensity of 561-nm illumination
47 (Supplementary Fig. 2), the rate at which the longer-lived dark state returned to the
48 emissive state increased with the illumination intensity, reaching a saturation regime at ~ 0.8
49 s^{-1} above a power density of $\sim 1.5 \text{ kW/cm}^2$ (Fig. 1B), indicating sensitivity to 561-nm light.
50 The shorter-lived dark state has previously been assigned to a putative radical state
51 displaying a distorted chromophore in mEos variants.⁶ With a typical duration $< 100 \text{ ms}$, this
52 state only causes fluorescence interruptions of at most a few frames, which can be handled
53 by current processing software.^{5,7-9} In contrast, the longer-lived dark state may last for
54 several seconds, and is thus much more problematic. Previous work had reported the
55 existence of this state, including a hint of 405-nm light sensitivity^{5,6} but its nature remained
56 elusive.

57 To identify the molecular mechanism underlying long-lived blinking, we first linked the
58 single-molecule observations with ensemble-level measurements carried out at higher
59 concentrations of mEos4b. Illumination of mEos4b samples in PAA with 561-nm light also
60 revealed the formation of a long-lived dark state displaying similar 561-nm laser power
61 dependence as that observed with single-molecule data, suggesting that the same state is
62 observed (Fig. 1B). Importantly, the increased lifetime of the dark state at low illumination
63 intensity facilitated the measurement of its absorption spectrum (Supplementary Fig. 3). In
64 the absence of light, the lifetime of the dark state reached $\sim 2 \text{ hrs}$ (Supplementary Fig. 4).

65 Next, we set out to visualize the structural change associated with slow blinking using
66 kinetic crystallography.¹⁰ To this aim we generated crystals of red-state mEos4b and
67 illuminated them at 561 nm. The resulting changes in spectra (Fig. 1C) were very similar to
68 those observed in PAA, supporting that the long-lived dark state could be accumulated *in*
69 *crystallo* (Supplementary Note 2). Hence, we selected a single crystal and illuminated one
70 part at 561 nm, leaving the other part unexposed. X-ray diffraction on the flash-frozen
71 crystal revealed that both parts contained a mixture of the mEos4b green and red forms
72 (Supplementary Fig. 5), but that the illuminated part additionally contained a fraction of
73 mEos4b in the long-lived dark state (Fig. 1D). Using difference refinement, we selectively
74 extracted the structure of this species, denoted *Red_{off}* (Fig. 1D and Supplementary Fig. 6).
75 The chromophore in *Red_{off}* adopts a strongly bent or “frustrated” *trans* configuration, being
76 forced away from a more canonical *trans* configuration (Fig. 1E) to avoid steric clashes with
77 the nearby Ile157 (Supplementary Fig. 7), and is also found to be highly dynamic
78 (Supplementary Note 3, Supplementary Fig. 8).

79 The structural signature of the *Red_{off}* dark state is reminiscent of the photochromism
80 observed in reversibly switchable fluorescent proteins (RSFPs).^{11,12} In RSFPs, illumination in
81 the main absorption band populates a nonfluorescent, spectroscopically blue-shifted state,
82 corresponding to a protonated *trans* chromophore. The fluorescent state can then be
83 efficiently recovered by illumination in the blue-shifted band. In mEos4b, the dark state
84 absorbs maximally at $\sim 475 \text{ nm}$ and we observed prompt recovery of red fluorescence upon
85 405-nm, 473-nm or 488-nm illumination scaling with absorbance at these wavelengths (Fig.
86 1F, Supplementary Fig. 9). This finding suggested that long-lived intermittencies in SMLM
87 and sptPALM experiments could be readily suppressed by weakly illuminating the sample
88 with 488-nm light. Laser illumination at this wavelength is available on most SMLM
89 instruments, interferes minimally with green-to-red photoconversion (peaking at $\sim 400 \text{ nm}$),
90 and can be applied continuously or in a pulsed manner (Supplementary Fig. 10,
91 Supplementary Note 4). Weak illumination at 488-nm of single mEos4b molecules
92 embedded in PAA indeed revealed a clear suppression of the long intermittencies by about

one order of magnitude (Fig. 2A), leading to a pronounced increase in the number of molecules that displayed long on-times (Fig. 2B). Similar results were obtained in fixed HeLa cells expressing mEos4b in the cytosol (Supplementary Fig. 11). All mEos variants responded to 488-nm light in a similar manner, but PAmCherry, mMaple3 or Dendra2 remained insensitive, pointing to different blinking mechanisms in these FPs (Fig. 2C, Supplementary Note 5). Addition of 488-nm light at the used power densities did not reduce the number of photons collected from red mEos4b molecules in each frame, and therefore the localization precision was preserved (Supplementary Fig. 12 and Supplementary Fig. 13). The total number of photons detected before irreversible photodestruction was also maintained (Supplementary Fig. 14), nor did the weak illumination affect the photobleaching rate of green mEos4b molecules (Supplementary Note 6, Supplementary Fig. 15). We did observe a slightly higher rate of green-to-red photoconversion (Supplementary Fig. 16), which we found beneficial as it reduced the need for additional phototoxic 405-nm light.¹³

Recent efforts have focused on increasing single-particle track lengths using organic dyes,^{14,15,16} multiple copies of FPs in combination with sparse labelling¹⁷ or FRET-enhanced photostability.¹⁸ However none of these methods offer the generality of using single FPs in live-cell compatible environments. We reasoned that our strategy could provide longer and more informative single-molecule tracks in standard sptPALM experiments based on labelling with EosFP variants. To this aim, we explored the diffusion behaviour of Microtubule-Associated Protein 4 (MAP4) fused to mEos4b and expressed in COS-7 cells.¹⁹ Visual inspection of raw sptPALM data suggested that much longer mEos4-MAP4 tracks could be obtained when a small amount of 488-nm light (0.75 - 4.80 W/cm²) was added in continuous mode (Fig. 2D, Supplementary Fig. 17, Supplementary Video 1 and Supplementary Video 2), taking care that the average number of fluorescence spots per recorded frame was approximately kept the same in both conditions. Quantitative evaluation of the data indicated a 44 % decrease in the absolute number of tracks upon addition of 488-nm light, and a concomitant increase of the average track length by 65 %, with 11 to 27 fold more tracks lasting longer than 2 s (Fig. 2E, Supplementary Table 1). These results confirm that fewer tracks are prematurely terminated due to blinking when 488-nm light is applied.

Although the long-lived dark state can be excited by 405-nm light (Supplementary Fig. 18), illumination at this wavelength is constrained by the desirable rate of green-to-red photoconversion and generates phototoxicity. Illumination at 473 nm, however, is expected to work about as efficiently as 488-nm light to suppress long-lived blinking, despite a possible stronger residual crosstalk with photoconversion.

For comparison, we also collected sptPALM data under the same experimental conditions using the photoactivatable organic dye PA-JF549¹⁴ and a MAP4-HaloTag fusion (Supplementary Fig. 19, Supplementary Video 3 and Supplementary Note 7). Although PA-JF549 was mostly found at microtubules, considerably more background staining was observed as compared to mEos4b, JF503, or JF549 (Supplementary Fig. 20 and Supplementary Fig. 21). A detailed analysis of the track lengths (Supplementary Fig. 22) revealed that 7.7% of the PA-JF549 tracks lasted longer than 2 seconds, compared with 0.13% for MAP4-mEos4b without 488-nm light and 3.4% with 488-nm light. We concluded that addition of weak 488-nm light provides a means to approach with a PCFP the very long tracks measured with a highly performant dye, while retaining the advantages of full genetically-encoded labelling.

We observed multiple diffusion regimes displaying the same distributions of diffusion coefficients with or without 488-nm light, supporting that weak 488-nm light did not influence diffusion of the MAP4-mEos4b fusion (Supplementary Fig. 23A-D and Supplementary Table 2). The heterogeneity in MAP4 dynamics could be visualized by inspection of long tracks (Fig. 2F, Supplementary Video 4). Importantly, the additional long tracks obtained in the presence of 488-nm light provided much better sampling of the diffusion state changes (Fig. 2G, Supplementary Fig. 24). In the presence of nocodazole, the microtubular structure disappeared, resulting in faster movement of the MAP4-mEos4b fluorophores and shorter tracks (Supplementary Fig. 25, Supplementary Fig. 26, Supplementary Fig. 23 and Supplementary Table 2).

Although addition of 488-nm light eliminated a large fraction of the long intermittencies in mEos4b, the suppression was not complete. This observation can be explained by the tentative photophysical scenario depicted on Supplementary Fig. 27 and Supplementary Note 8, involving an additional non-emissive state, which also accounts for the saturation behavior observed in Fig 1B. We also observed that addition of 488-nm light resulted in an increased number of short intermittencies (Supplementary Fig. 28) by a factor 2 to 3. Monte Carlo simulations (Supplementary Note 9 and Supplementary Fig. 29) show however that despite this increase, the connectivity of the reconstructed long tracks is significantly better than in the absence of 488-nm light. The simulations therefore suggest that 488-nm illumination provides not only longer but also more reliable tracks.

In conclusion, our work has revealed that long-lived intermittencies observed in mEosFP-like PCFPs predominantly result from photochromism as seen in reversibly switchable or biphotochromic FPs²⁰ and can be drastically reduced by “rational microscope engineering” based on mechanistic studies. Since light sources at or near 488-nm are present on most instruments capable of sptPALM, illumination at these wavelengths is likely to be readily applicable, providing a strong improvement in data quality with minimum effort. Our blinking suppression strategy should be similarly useful in increasing the accuracy of molecular counting in quantitative SMLM imaging.

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Authors contributions

D.B. and P.D. designed the project with E.D.Z.. E.D.Z., V.A., M.B. and D.B. carried out mechanistic investigations. D.T. performed in vitro single-molecule and ensemble

experiments with contributions from E.D.Z. and D.B.. V.M. initiated application to sptPALM. V.M., S.H. and P.D. performed and analyzed sptPALM experiments. S.H., V.M., and D.T. performed JF-dyes experiments and analysis. D.T. and J.B. performed fixed cells experiments. D.B. performed Monte Carlo simulations with assistance of S.H.. L.V.M. assisted in crystallographic analysis. E.D.Z., D.T., P.D. and D.B. wrote the manuscript with input from all authors.

Competing financial interests statement

The authors declare no competing financial interests.

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Figure legends

Figure 1. mEos4b mechanistic studies. (A) Single molecule trace of red mEos4b showing long intermittencies (seconds) under 561-nm illumination (0.5 kW/cm^2). (B) Comparison of recovery rates from the long-lived dark state obtained from single-molecule and ensemble data. Values represent mean $\pm$ sd ($n=3$ separate preparations of the purified FP). (C) Evolution (red to black, arrows) of absorption (solid lines) and fluorescence (dashed lines) emission spectra from crystalline mEos4b during 561-nm illumination. (D) Comparison between the crystallographic structures of bright red mEos4b (magenta, Red_{on}) and the 561-nm light-induced long-lived dark state (grey, Red_{off}) in the $F_{obs, illuminated} - F_{obs, non-illuminated}$ electron difference density map contoured at 3 r.m.s.d. (negative density in orange, positive density in blue). The chromophore and important residues of the chromophore pocket are shown. (E) Comparison between the crystallographic structures of the Red_{off} state in mEos4b (grey) and the off-switched structure of green IrisFP (PDB ID: 2VVI).²⁰ Arrows indicate the shift in imidazolinone ring and consecutive distortion of the chromophore in Red_{off} compared to off-switched IrisFP. (F) Ensemble photoswitching of red mEos4b under 561- and 488-nm light (80 and 5 W/cm^2 , respectively). Upon illumination at 488 nm, fluorescence recovery occurs in a few tens of milliseconds. Values represent mean $\pm$ sd ($n=3$ separate preparations of the purified FP). The red curve shows a fit with a monoexponential model, yielding a rate of 45 s^{-1} . Data in (A), (C) and (D) are representative of >10 , >5 and 2 experiments, respectively.

Figure 2. Increase in mEos4b tracklength upon 488-nm light illumination. (A) Intermittency histograms for red mEos4b under sole 561-nm light (0.5 kW/cm^2 , violet curve), or combined 561- and 488-nm light (15, 60 or 120 W/cm^2 at 0.125 duty cycle, pulsed mode, blue, green and red curves, respectively. PDF: Probability Density Function. The fraction of intermittencies longer than 4 frames decreases as 488-nm illumination is increased (Inset). (B) Cumulative distribution of single-molecule on-times longer than a given time (corrected for intermittencies shorter than 4 frames) under sole 561-nm light or combined 561- and 488-nm light. The inset shows the minimum on-times displayed by a given fraction of the data (1%, circle; 5%, square; 10%, triangle), as a function of the applied 488-nm laser power density. (C) Effect of 488-nm light (120 W/cm^2 at 0.125 duty cycle) on various FPs commonly used in sptPALM experiments. Values in (A) and (B) and their insets, and in (C) represent mean $\pm$ sd ($n=3$ separate preparations of the purified FP). * $P < 0.05$ (independent one-sample t-tests). (D) Image of COS-7 live cell labeled with MAP4-mEos4b. Left: conventional image and PALM reconstruction, middle and right: single particle trajectories longer than 1 s in the absence and presence of 488-nm light (4.80 W/cm^2 , CW mode). Each image shows the same field of view (scale bar on top of left image). Data are representative of ≥ 3 experiments (E) Cumulative

distribution of track lengths from MAP4-mEos4b tracking experiments, in the absence (violet) or presence (green) of additional 488-nm illumination (4.80 W/cm², CW mode). Values represent mean $\pm$ sd (n = 5 cells). (F) Panel of diffusion patterns identified amongst the large set of long tracks that become visible upon addition of 488-nm light, color coded according to step length (red to blue for respectively small to large steps per frame), overlaid on raw data from randomly chosen single frames. Data are representative of ≥ 5 experiments (G) Swarm and box plots of the amount of state changes extracted by the HMM-Bayes software in the presence (left) and absence (right) of 488-nm light (4.80 W/cm², CW mode, ***: χ^2 test with $P < 10^{-7}$, box plot blue line, box and whiskers show respectively mean, 25-75 quartiles and adjacent points. The number of tracks are $n_0 = 6013$ and $n_{4.8} = 3272$, respectively, collected from 5 independent cells in each condition).

294 Online Methods

295 Cloning, protein expression and purification

296 mEos4b was expressed from a pRSETb plasmid (Invitrogen) in JM109(DE3) cells (Promega,
297 Leiden, The Netherlands) in 1 l LB containing ampicillin. After four days of growth at 21°C,
298 the cells were harvested by centrifugation (20 minutes at 5000 rpm), resuspended in TN
299 buffer (100 mM Tris, 300mM NaCl, pH 7.4) and lysed using a French pressure cell press
300 (1150 psi). After spinning down the cell debris (20 minutes at 8500 rpm), a Ni-NTA affinity
301 chromatography was carried out using a HisTrap FF crude column (GE Healthcare, Diegem,
302 Belgium) coupled to an Akta Prime system (GE Healthcare, Diegem, Belgium) and TN
303 buffer supplemented with 500 mM imidazole. The sample was then loaded onto a HiLoad
304 Superdex 200pg 16/600 column (GE Healthcare, Diegem, Belgium) coupled to an Akta
305 Purifier 10 system (GE Healthcare, Diegem, Belgium) for size-exclusion chromatography.
306 Elution was performed using 0.1xTN buffer. Finally, the protein was concentrated using a
307 Vivaspin 10K MWCO column.

308
309 CD86-mEos4b was cloned by replacing mEos2 by the gene coding for mEos4b in the CD86-
310 mEos2 construct (Addgene # 98284),²¹ using the Gibson Assembly® Master Mix (NEB,
311 Ipswich, Massachusetts, United States). The MAP4-mEos4b construct was generated by
312 replacing mCherry for the mEos4b gene in the mCherry-MAP4-N-10 construct, purchased at
313 Addgene (plasmid # 55076), using the AgeI and NotI restriction sites.

314 The MAP4-HaloTag construct was generated by replacing mEos4b for the HaloTag
315 gene (Addgene # 29644) in the MAP4-mEos4b construct using the BamHI and NotI
316 restriction sites

317 Crystallization

318
319 Green mEos4b crystals were grown in sitting drops in which 1 µl of protein was mixed with
320 1 µl precipitant (100 mM HEPES pH 7.5 and 30 % PEG 1000) equilibrated against 100 µl of
321 precipitant. Rod-shaped crystals ($0.1 \times 0.1 \times 0.5 \mu\text{m}^3$) grew in a few weeks. Subsequently,
322 crystals were exposed to sunlight to photoconvert mEos4b to the red state, as described by
323 Ando et al.²²

324 Cell culture and transfection

325 mEos4b fused to CD86 in HeLa cells

326
327 HeLa cells were grown in DMEM growth media containing 10% FBS and maintained at
328 37°C in 5% CO₂. Cells were seeded in a tissue-culture treated 6 well plate and transfected
329 using X-tremeGENE 9 (Sigma-Aldrich, St. Louis, Missouri, United States). Three days later,
330 cells were trypsinated and replated in a sticky-Slide VI 0.4 (Ibidi GmbH, Martinsried,
331 Germany) mounted with a 24X50mm #1 Menzel coverslip (Fisher scientific, Illkirch, France)
332 coated with fibronectin (Sigma-Aldrich, St. Louis, Missouri, United States). 5 hours later,
333 cells were rinsed with PBS and fixed with a mix of 4% formaldehyde (stock 16%,
334 Thermofisher, Waltham, Massachusetts, United States) and 0.1% glutaraldehyde (stock 25%,
335 Sigma-Aldrich, St. Louis, Missouri, United States) in PBS. Cells were then washed and kept
336 in PBS for imaging.

337 mEos4b fused to MAP4 in COS-7 cells

338
339 COS-7 cells were grown in DMEM growth media containing 10% FBS and maintained at
340 37°C in 5% CO₂. Cells were seeded in sterile 35 mm glass-bottom imaging dishes and
341 transfected using FuGENE (Promega) following the manufacturer's protocol. After
342 incubation for 24 h, the cells were washed twice with Hank's Balanced Salt Solution (HBSS,

14065, Life Technologies) buffer for live cell imaging and subsequently imaged in HBSS buffer at room temperature.

Nocodazole experiments

COS-7 cells expressing mEos4b–MAP4 were treated with nocodazole (10 µg/ml) in DMEM for 90 min at 37°C. Afterwards they were washed twice with HBSS buffer and subsequently imaged in HBSS at room temperature.

Halo-tag fused to MAP4 in COS-7 cells and staining with Janelia Fluor Halo-tag ligands

COS-7 cells were grown in DMEM growth media containing 10% FBS and maintained at 37°C in 5% CO₂. Cells were seeded in sterile 35 mm glass-bottom imaging dishes and transfected using FuGENE (Promega) following the manufacturer's protocol. After incubation for 24 h, the cells were washed twice with PBS and stained with 100nM (for imaging) or 5nM (for tracking) Janelia Fluor Halo-tag ligands (PA-JF 549 and JF 503) in DMEM for 1h and then washed three times with HBSS including two steps of about 10 min at 37°C to remove unbound dyes.

Mutagenesis and screening

Site saturation mutagenesis was performed using a modified QuikChange protocol²³. The mEos4b point mutants were expressed in JM109 cells. Screening for altered blinking/photoswitching behavior was performed on a home-built colony imaging system containing a 300 W Xenon lamp (MAX-302, Asahi Spectra)²⁴. Green colonies were exposed for 60 min to light passing through a 480/40 bandpass filter and then for 10 min to light passing a 400/30 bandpass filter, and this illumination cycle was repeated twice. Every minute an image was acquired through a 530/40 bandpass filter on an EMCCD camera (Cascade 512B, Photometrics).

Colonies showing altered blinking/photoswitching behavior were selected and further investigated using an inverted microscope (Olympus IX71) containing a Sola Light Engine (Lumencor).²⁴ 200 µl cell cultures expressing the mutant proteins were subjected twice to 5 s cyan light (50 and 75 %) and 5 s violet light (5%). Then the proteins were photoconverted using 30 s violet light (100%) and subsequently exposed twice to yellow-green (5 s, 50 and 75 %) and cyan light (5 s, 5%). Every second an image was captured on an EMCCD camera (iXon, Andor).

Microscopy experiments

Ensemble and single-molecule *in vitro* and CD86 data were acquired on a home-built PALM setup based on an Olympus IX81 inverted microscope equipped with a 100× 1.49 NA oil-immersion apochromatic objective lens (Olympus, Japan). Widefield illumination was achieved by focusing the diode-pumped solid state 405-nm (CrystaLaser, USA), 488-nm (Spectra Physics, USA) and 561-nm (Cobolt Jena, Sweden) laser beams to the back focal plane of the objective. Intensities of laser illuminations at the sample were tuned by an acousto-optical tunable filter (AOTF, AA Opto Electronic, France). Fluorescence images were acquired with an Evolve 512 back-illuminated EMCCD camera (Photometrics, USA) controlled by the Metamorph software (Molecular Devices, USA).

mEos4b immobilization for in-vitro experiments

Purified mEos4b was diluted to either micromolar (ensemble) or nanomolar (single-molecule) concentrations in a 15% polyacrylamide (PAA) gel. The mEos4b-containing PAA was then spread to form a uniform thin layer on a coverslip thoroughly cleaned using a UV Ozone Cleaning System (HELIOS-500, UVOTECH Systems). Polymerization was initiated using 1% APS (Ammonium Persulfate) and 1% TEMED (Tetramethylethylenediamine), and the gel was let to harden at room temperature for 5 minutes.

In-vitro single-molecule experiments

PALM data were collected using an exposure time of 70 ms, under continuous 561-nm illumination only at different laser power-densities (0.2, 0.5, 1, 2, 3, 4 and 6 kW/cm²) or under 561-nm illumination at 0.5 kW/cm² interleaved with pulsed 488-nm illumination for 10 ms per frame at different power-densities (0, 15, 60 and 120 W/cm²). These power-densities provide the same photon doses as obtained with 1.9, 7.5 and 15 W/cm² applied continuously (CW mode). Illumination at 488-nm was applied between camera exposures to avoid any contribution of the green mEos4b molecules to the recorded signal. Experiments were performed in triplicate.

In-vitro single-molecule experiments on JF dyes

A construct encoding Halo-tag fused to the Nrf2 protein in pET28a was transformed in *E. Coli* BL21, and protein expression induced with 250 μ M IPTG overnight at 20°C. Cells were harvested in lysis buffer (NaH₂PO₄ 50 mM, imidazole 10 mM, Triton X-100 0.05%, pH 7.5), lysed by sonication, and the protein purified on a Ni-NTA affinity column. The protein was finally dialyzed overnight in Tris 50 mM, Glycerol 5%, pH 7.5. The Halo-tag Nrf2 fusion was then mixed in equimolar amounts with the PA-JF549 dye and incubated at 4°C for 20 min. The protein was subsequently adsorbed on glass between two sealed coverslips, and PALM data were acquired using similar conditions to the mEos4b experiments described in the previous paragraph (70 ms exposure time, 561-nm illumination at 0.5 kW/cm², no 488-nm illumination). Experiments were performed in triplicates, and analyzed in the same way as the mEos4b data.

Determination of on-switching rates from single-molecule experiments

Data were processed as described in ref ⁷. Briefly, after localization of the single molecules using the Thunderstorm plugin for ImageJ,²⁵ their fluorescence traces were computed and off-times were extracted and plotted as a histogram. Although off-times histograms could be precisely fit with a three-exponential model (Supplementary Fig. 1B, Supplementary Note 1), the extracted rates could not be directly related to the rates defining the kinetic scheme of Supplementary Fig. 27. Therefore, we used the single-molecule fluorescence traces to reconstitute fluorescence decay curves, by counting the number of fluorescent and dark molecules at each time-point after activation. The curves obtained were similar to those obtained from ensemble-level experiments, and were fitted using the same kinetic model (see below). The first point of the decay curves was however not taken into account, as we found that this point was affected by residual false positive localizations detected on single frames.

In-vitro ensemble fluorescence

For ensemble measurements, samples were first illuminated with 405-nm light (1 W/cm²) for 1 min to prompt photoconversion. To assess the photoswitching behavior of red mEos4b (Supplementary Fig. 18), alternating 561-nm light (25 W/cm²) and 405-nm light (1 W/cm², 60 seconds off, 30 seconds on) were used. To test the fluorescence recovery induced by illumination at 488-nm (Fig. 1F), red mEos4b was alternately illuminated with 561-nm light (80 W/cm², 8.5 seconds) and 488-nm light (5 W/cm², 1.5 seconds). To measure the

dependence of the on-switching rate on the 561-nm laser power (Fig. 1B), single on-off switching cycles were acquired under the same conditions as described above for single-molecule experiments, except that measurements at much lower power-densities of the 561 nm laser (2, 5, 10 and 20 W/cm²) could be performed, as single-molecule sensitivity was not required. Experiments were performed in triplicate.

Determination of on-switching rates from in-vitro ensemble experiments

Analysis of the on-off switching cycles was carried out on a small, homogenous region of the sample. For each applied 561-nm laser power, the corresponding fluorescence decay profile was fitted with a kinetic model including photobleaching and two reversible dark-states. The (thermally driven) recovery rate of the short-lived dark state was fixed to 16 s⁻¹ as fitted on the single-molecule data, to prevent fitting of several non-independent constants. This value was also found consistent with ref ^{5,6}. The fitted recovery rate then corresponds to an effective, slow on-switching rate whose value is affected by the power-dependent build-up of a potential extra dark state *Dark*₂ (Supplementary Fig. 27)

In-crystallo ensemble fluorescence

To assess the photoswitching behavior of crystalline mEos4b, crystals were mounted between two coverslips in a drop of mother liquor and placed on the PALM microscope. The same illumination scheme was used as described above for the *in-vitro* ensemble fluorescence experiments, using 70 W/cm² of 561-nm laser light for off-switching and 0.3 W/cm² of 405-nm light for on-switching. The higher power density of 561-nm light and lower power density of 405-nm light as compared to that used for ensemble experiments in PAA was chosen to compensate for the high optical density of crystals at 561 nm (Supplementary note 2). Data analysis was carried out on a small region of the crystal. Experiments were performed in triplicate.

sptPALM data acquisition and analysis

sptPALM acquisition

Imaging of the transfected cells was done on a commercial Cell TIRF DX83 microscope (Olympus) using a 100x (NA 1.49) oil-immersion objective and EMCCD camera (Hamamatsu, C9100-23B EM X, pixel size 160 nm). The power of the 405-nm laser was set at 0.4 – 0.6 % (0.3 – 0.6 mW/cm²) in the absence of 488-nm light and 0 - 0.5% (0 - 0.34 mW/cm²) in its presence, that of the 488-nm laser at 5 – 10 – 25 – 35% (0.75 W/cm², 1.40 W/cm², 2.50 W/cm² and 4.80 W/cm², CW mode) and that of the 561-nm laser at 40% (55 W/cm²) in a HILO (Highly Inclined and Laminated Optical) illumination configuration. The used 488-nm power densities provided photon doses that fall on the low range of the ones used during in-vitro experiments (see online Methods section on *In vitro single molecule experiments*). Because the 488-nm light also resulted in residual green-to-red conversion (Supplementary Fig. 16), the intensity of the 405 nm light was reduced to maintain the approximate same number of active molecules per frame (i.e. emitter density) between the measurement with and without addition of 488-nm light. The exposure time was 40 ms with an EM gain of 500. On each cell we recorded two datasets, one with and one without 488-nm light. On half of the cells, the dataset with 488-nm light was recorded first, while on the other half the dataset without 488-nm light was recorded first. The appearance of longer tracks was independent of the order in which these datasets were acquired.

The sptPALM experiments carried out with JF dyes were performed under the same acquisition setting as the mEos4b sptPALM experiments (7000 frames, 40 ms framerate, 55 W/cm² 561 nm, 0.8 mW/cm² 405 nm). Before SPT acquisition a transfected COS-7 cell was selected by using diffraction-limited JF503 imaging similar to the experiments with

mEos4b (in the green state), to guaranty that the experiments were done on similar transfected cells with an intact flat tubulin structure.

sptPALM analysis and statistical relevance

For track visualization we employed the Localizer software package,²⁶ implemented in Igor Pro (WaveMetrics). For analysis of the localizations, the default settings were used with a generalized likelihood ratio test (GLRT) insensitivity of 25 for segmentation and a standard deviation of the PSF of 1.6 pixels using the Gaussian PSF model (160 nm pixel size). For analysis of the track lengths the parameters jump distance was set to 4 pixels, blinking frames to 4, and the minimum track length to 3.

For quantitative analysis of the diffusion coefficients we applied the HMM Bayes software package,²⁷ which builds on output from the UTrack software package.²⁸ UTrack was applied with the same settings as for the Localizer-based analysis. HMM Bayes was employed with default settings to estimate diffusion coefficients corresponding for each observed track. Only tracks longer than 25 frames were used in the HMM-Bayes evaluation to speed up the analysis. All calculations, including all statistical tests (χ^2 tests), were performed in Matlab R2018a (MathWorks).

Spectroscopic characterization and Crystal preparation

Ensemble and in crystallo absorbance and fluorescence spectra were acquired on a custom-build microspectrophotometer (CAL(AI)²DOSCOPE)²⁹ with the sample placed at the focus of two opposite 15× mirror objectives (Edmund optics, France). A 561-nm laser (Crystal laser, USA), 405-nm laser (New Industries Optoelectronics Tech, PRC) and UV-VIS light source were coupled into the optical path using optical fibers. Two AvaSpec ULS2048 spectrophotometers (Avantes, The Netherlands) collected the transmitted or emitted light while the sample surface could be visualized on a color detector (uEye GigE UI-52405E, IDS, Germany). Every 2 s an absorbance and fluorescence spectrum was acquired using respectively the white light source and the actinic light as excitation light source. The illumination and detection scheme were controlled by a 9518PG pulse generator (Quantum composers, USA) and the Avasoft software (Avantes, The Netherlands). Data analysis was performed in Igor Pro (WaveMetrics).

In vitro ensemble experiments

In vitro ensemble measurements were performed at room temperature on cover slips bearing mEos4b embedded in 1 % PVA in PBS. Photoconverted samples were illuminated for 22.5 s with 561-nm light (28 W/cm²) and 7.5 s with 405-nm light (0.2 W/cm²).

In crystallo experiments

Needle-shaped crystals were mounted between two coverslips in a hanging drop of mother liquor. All experiments were performed at room temperature. One part of a photoconverted red crystal was illuminated with 561-nm light (34 W/cm²) during 43.5 s, leaving the other part unexposed. For the control experiment, a photoconverted red crystal was illuminated in a similar manner and subsequently during 18 s with 405-nm light (1.0 W/cm²). Incomplete photoswitching was deliberately chosen to avoid the built-up of irreversibly photobleached molecules within the crystal.

Of note, we used 405-nm rather than 488-nm light for back switching of *Red_{off}* using our microspectrophotometer. Both wavelengths in fact induce back switching. Whereas the use of significant 405-nm light to promote back switching would be problematic in single molecule

experiments due to coupling with photoconversion, this is not the case in ensemble experiments where samples are photoconverted beforehand.

As the thermal recovery rate from the *Red_{off}* dark state is slow, sufficient time was available to safely trap the proteins in this dark state by soaking them immediately after illumination for a few seconds through a cryoprotectant solution (10% glycerol in 100 mM HEPES pH 7.5 and 30 % PEG 1000), before flash freezing and storing in liquid nitrogen. Of note, the time required for this procedure (< 1 min) likely allowed molecules trapped in the *Dark₂* state to thermally relax to *Red_{on}*.

X-ray diffraction data collection and analysis and structure refinement

X-ray diffraction data of green mEos4b and the illuminated and non-illuminated parts of the red mEos4b crystals were collected at the ID-23-1 beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France), under a nitrogen stream of 100 K with an X-ray wavelength of 1 Å. The data were indexed, integrated and scaled using XDS and XSCALE v. October 15, 2015.³⁰

The structure of mEos4b in its green state was solved via molecular replacement using Phaser³¹ using chain A of mEosFP (PDB ID 1ZUX³²) as a model. Structure refinement was carried out using phenix.refine v.1.11³³ and COOT v. 0.8³⁴ and consisted of bulk-solvent and anisotropic scaling, individual coordinate and isotropic B-factor refinement and occupancy refinement for residues in alternative conformations. After a first round of refinement, the chromophore and the mutations compared to the phasing model were clear in the $F_{obs}-F_{calc}$ difference map. Water molecules were added to the model if they appeared in the mF_o-DF_c difference map contoured at 3.0 r.m.s.d., had acceptable B-factors and were within reasonable distance from chemically relevant groups. The crystallographic dictionary file for the chromophore was generated using eLBOW³⁵ and manually adapted.

The structure of mEos4b in its red state (being the non-illuminated part of the red crystal) was determined starting from the green state model. After one cycle of rigid body refinement, the protein backbone break characteristic of the green-to-red photoconversion was clear in the $F_{obs}-F_{calc}$ electron density map, albeit incomplete, indicating a mixture of the green and red form of mEos4b. Restrained structure refinement was performed as mentioned for the green state above. The red and green state chromophore occupancies converged towards a 60:40 red:green ratio, which is in accordance to the crystal's absorption spectrum. Again, crystallographic library files for the chromophore and non-standard amino acids were generated using eLBOW and manually changed. With the final model, an ensemble refinement³⁶ was carried out using the default parameters except for the fraction of atoms included in the TLS fit being 0.9.

In the 561-nm illuminated part of the red crystal, a significant fraction of mEos4b molecules were still found in the bright red or non-photoconverted green state as was anticipated due to the gentle illumination during photoconversion and photoswitching to avoid unwanted side effects such as photobleaching. To be able to highlight features specific to the *Red_{off}* dark state, we calculated a Bayesian q-weighted³⁷ $F_{obs,illuminated}-F_{obs,non-illuminated}$ electron density difference map,³⁸ using the red state model to calculate phases. The ability to calculate this electron density difference map, which is essentially free of model bias, was possible because both x-ray diffraction data sets were collected from a single crystal, thereby avoiding small differences between crystals that would otherwise severely degrade the quality of the map (such as small differences in unit cell size or green-to-red photoconversion completeness). Based on an occupancy of *Red_{off}* established at 25%, extrapolated structure factor amplitudes

corresponding to the pure Red_{off} dark state were then calculated as described in Coquelle et al.,³⁹ and these were used in further structure refinement of Red_{off} , for which we applied the same strategy as described above for the Red_{on} red state, albeit starting with a rigid body refinement of the red state model. To understand the importance of flexibility and figure out whether the dark state contains multiple flexible states around a single conformation or multiple stable conformations, an ensemble refinement was carried out as described for the red bright state.

To ensure that the dark-state structure obtained mostly originates from Red_{off} and not from irreversibly photobleached states, we performed an extra experiment in which we repeated the same crystal illumination procedure except that before flash-cooling we applied additional 405-nm light to induce recovery of the bright state. Diffraction data of both the illuminated and non-illuminated sides of the crystal were acquired and a q-weighted $F_{obs, illuminated\ 561nm+405nm} - F_{obs, non-illuminated}$ electron density difference map was calculated. This map showed no significant difference between both sides (Supplementary Fig. 30) and hence indicate that the captured structure is that of a reversible dark state.

All data collection and refinement statistics can be found in Supplementary Table 3 and Supplementary Table 4.

Simulations of PALM experiments

PALM experiments were simulated using an in-house made Matlab software (described in Avilov *et al.*⁷ and running on Matlab version 2015b). In brief, the photophysics of single-molecules was simulated according to a two-dark-states model (i.e. taking into account the Red_{on} , Red_{off} , $Dark_1$ and Bleached states of Supplementary Fig. 27). The simulation also included a large panel of experimental parameters, such as fluorophore density, dipole orientation, laser power density and polarization at 405, 488 and 561 nm, Poissonian background noise, objective properties, and EMCCD parameters. For sptPALM simulations, datasets of 12000 frames were generated, using 5000 molecules randomly positioned on a segmented 2D microtubule pattern, and set to diffuse in a confined manner on the pattern with diffusion coefficients of 0.05 $\mu m^2/s$ for half of the molecules and 1 $\mu m^2/s$ for the other half. The frame time, pixel size and labeling density were chosen identical to the experimentally used values. To reproduce the increase in short-lived intermittencies observed in the experimental data, the quantum yield of the transition from the on state to the $Dark_1$ state was raised appropriately.

Statistical analysis

All ANOVA tests performed were one-sided, and used the following assumptions:

- Normal distribution of the response variable
- Equal variances of the tested populations
- Responses for a given group are independent and identically distributed normal random variables

Data availability statement

Crystal structures of mEos4b in its green, red and long-lived dark state are available in the Protein DataBank with accession codes 6GOY, 6GP0, 6GP1, respectively. The main figures of the paper have associated raw data. Raw data for Supplementary Figures are available upon request. All unique biological materials used in this study and the data that support the findings are available from the corresponding authors upon reasonable request.

Reporting summary

Report of data collection, analysis and applied statistical analysis can be found in the Life Sciences Reporting Summary associated to this paper.

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