Near-infrared STED nanoscopy with an engineered bacterial phytochrome

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The near infrared (NIR) optical window between the cutoff for hemoglobin absorption at 650 nm and the onset of increased water absorption at 900 nm is an attractive, yet largely unexplored, spectral regime for diffraction-unlimited super-resolution fluorescence microscopy (nanoscopy). We developed the NIR fluorescent protein SNIFP, a bright and photo-stable bacteriophytochrome, and demonstrate its use as a fusion tag in live-cell microscopy and STED nanoscopy. We further demonstrate dual color red-confocal/NIR-STED imaging by co-expressing SNIFP with a conventional red fluorescent protein.

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Within the near-infrared (NIR) optical window at around 650–900 nm, light scattering, autofluorescence and light absorbance is strongly reduced in mammalian cells and tissues. Therefore, this spectral region is preferable for deep-tissue imaging. Phototoxic effects, even at high irradiation intensities, are generally alleviated at long wavelengths, rendering the NIR regime attractive for live-cell microscopy and particularly for live-cell diffusion-unlimited super-resolution (nanoscopy) applications that generally require the application of higher light doses.

Indeed, substantial efforts have been undertaken to generate organic dyes that can be used for live-cell super-resolution microscopy in the NIR regime. Likewise, fluorescent proteins (FPs) are routinely used for live-cell nanoscopy and extensive, albeit unsuccessful, efforts have been undertaken to generate FPs of the green fluorescent protein (GFP) family that are excited within the NIR optical window. To date, no GFP-like FP with an excitation maximum above 611 nm (TagRFP657 and E2-Crimson) or an emission maximum above 686 nm (mNeptune81–Q159C) has been reported. For red fluorescent proteins (emission at around 650–670 nm) including TagRFP657, mNeptune11,12, mGarnet13, and most recently mGarnet214 were used for stimulated emission depletion (STED) nanoscopy. However, the excitation wavelengths used were below 650 nm and thus outside of the NIR optical window.

Recently, NIR fluorescent proteins based on phytochromes, whose excitation and emission maxima are within the NIR optical window, have been developed and applied in fluorescence microscopy. Phytochromes are found in bacteria, cyanobacteria, fungi, algae, and plants, but not in mammals. They rely on linear tetrapyrole molecules, such as phycocyanobilin, phycoerythrin, or biliverdin, as external chromophores. Most phytochromes share a structurally conserved photosensory core module (PCM) of 55–58 kDa that is composed of a PAS (Per-ARNT-Sim), a GAF (cGMP phosphodiesterase-adenylate cyclase) domain which are connected by alpha-helical linkers. Of the phytochromes, the bacterial phytochromes stand out, because they utilize the far-red absorbing biliverdin as a chromophore. As a product of the heme degradation pathway, biliverdin is ubiquitous in many eukaryotic organisms, and in addition, it is readily taken up by mammalian cells when exogenously applied. Several NIR FPs were engineered from different bacteriophytochromes, and exhibit relatively low fluorescence brightness in mammalian cells, limiting their suitability for imaging. To date, none of these proteins have been used for nanoscopy.

In this study, we generated the new bacteriophytochrome-based, bright and photostable NIR FP SNIFP (STED Near Infrared Fluorescent Protein) that proved to be a suitable fusion tag when expressed in human cells. We demonstrate the use of SNIFP for live-cell NIR-STED (NIR-STIMulated Emission Depletion) nanoscopy, with all three wavelengths (excitation, emission, and STED) in the NIR optical window.

**Results**

**Generation of the NIR fluorescent protein SNIFP.** As a starting scaffold, we chose the PCM of the *Deinococcus radiodurans* bacteriophytochrome (DR_A0050). The NIR FPs WiPhy17, WiPhy218, IFP1.419, and IFP2.020 are variants of the truncated PCM of this bacteriophytochrome, containing only the PAS and the GAF domains. We revisited the question on the most suitable truncation or extension of the PCM to generate a NIR FP usable for live-cell imaging. To this end, we first introduced the monomerization mutations F145S, L311E, and L314E, and codon optimized the PCM sequence for expression in human cells. We generated four different variants (W1–W4), with W1 being the longest (530 amino acids (aa)) and W4 the shortest variant (321 aa) (Supplementary Fig. 1; Supplementary Table 1).

Fluorescence activated cell sorting (FACS) revealed that the shortest variant W4 comprising only the PAS and the GAF domain, resulted in the highest fluorescence signal in *Escherichia coli* cells (Supplementary Table 1) and we thus continued to focus on this variant. Guided by crystal structures of the PCM (PDB 4O0P and 4O0I)21 and previous mutational analysis22, we chose a number of positions in the chromophore binding pocket for saturation mutagenesis (among others positions 206, 207, 208, 209, 216, 263, and 270) and combined it with polymerase chain reaction (PCR)-mediated random mutagenesis. The plasmid libraries were expressed in *E. coli* cells and screened in several consecutive rounds for fluorescence brightness by FACS analysis and automated microscopy.

We identified three mutations (D207L, Y263F, and G270R) that increased the fluorescence brightness of W4 in *E. coli* cells further. The positions 207 and 263 have been repeatedly identified as important for the fluorescence properties of bacteriophytochromes, whereas the position 270 has not been discussed. Gly270 is located between the alpha-helix 7 and the beta sheet 10 of the GAF-domain. The substitution of the glycine by the larger arginine is likely to influence the positioning of the highly conserved His260 and consequently the network of hydrogen bonds in the chromophore surrounding. To evaluate the influence of the mutation G270R, we compared four W variants, namely W4-Y263FS (W4.20), W4-Y263FS, G270R (W4.33), W4-Y263FS, D207L (W4.34), and W4-Y263FS, D207L, G270R (W4.35). For all four variants, the excitation (697–705 nm) and emission (720–732 nm) maxima are within the near infrared window (Supplementary Table 2; Supplementary Fig. 2), and they behave as monomers on semi-native polyacrylamide gels (Supplementary Fig. 3a). In *E. coli* cells, W4.34 and W4.35 were the brightest variants and showed a higher stability at pH values above 6.5 (Supplementary Table 2; Supplementary Fig. 3b). W4.35, which differs from W4.20 at one position (D207L), exhibited the highest extinction coefficient (~149,200 M−1 cm−1) and a slightly higher cellular brightness in mammalian cells (Supplementary Table 2). Because W4.35 proved to be suitable for STED nanoscopy (see below), we named this protein SNIFP (STED Near Infrared Fluorescent Protein).

**SNIFP as a fusion tag in live cell imaging.** In order to evaluate the usability of SNIFP for live cell imaging, we fused it to the centromere protein C (SNIFP-CENPC) (Fig. 1a), to a core histone (SNIFP-HIST1H2Bbn) (Fig. 1b), to a subunit of the nuclear pore (SNIFP-NUP50) (Fig. 1c), to caveolin 1 (CAV1-SNIFP) (Fig. 1d), to keratin (KRT18-SNIFP) (Fig. 1e), to vimentin (VIM-SNIFP) (Fig. 1f), to a microtubule associated protein (SNIFP-MAP2) (Fig. 1g), and targeted it to the endoplasmic recticulum (ER) (Fig. 1h) and to the peroxisomes (Fig. 1i). These fusion proteins could be readily imaged in living human cells using a confocal microscope with a standard 633 nm excitation line (Fig. 1a–i), demonstrating that SNIFP is a suitable probe for far-red live cell microscopy. However, this excitation wavelength, which is the most red-shifted laser line in most current commercial confocal instruments, is outside the NIR window. Since it is shifted by ~60 nm to the blue compared to the excitation maximum of SNIFP, it is also not optimal for its excitation. To fully benefit from the spectral properties of SNIFP, we employed an excitation laser line of 676 nm in a dedicated NIR confocal microscope. Thereby, we could image more than a thousand consecutive images of living cells expressing VIM-SNIFP (Fig. 1, k).

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Encouraged by the superior photostability of SNIFP upon confocal imaging, we next explored the suitability of SNIFP for STED nanoscopy. To identify the optimal STED wavelength, we first determined the fluorescence signal induced by anti-Stokes excitation at different STED wavelengths (820–870 nm, provided by a tunable pulsed (80 MHz) Ti:Sapphire laser) on VIM-SNIFP in living cells. We found the wavelength of 860 nm to be optimal for STED nanoscopy of SNIFP, because this wavelength combines efficient stimulated emission with a low level of anti-Stokes excitation (Supplementary Fig. 4).

We recorded STED images of living human HeLa cells expressing VIM-SNIFP or SNIFP-NUP50, demonstrating a clear resolution improvement compared to conventional confocal microscopy (Fig. 2a–c). All images display raw data. To quantify the resolution improvement, we determined the FWHM (Full Width at Half Maximum) of three neighboring averaged intensity profiles across the vimentin filaments. In the confocal case, the

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FWHM was measured as ~280 nm (Supplementary Figs. 5 and 6), which is close to the theoretical confocal resolution of 260 nm. In the STED recordings, the FWHM values were consistently around 80 nm (Fig. 2b; Supplementary Figs. 5 and 6), which corresponds to a ~3.5-fold improvement in resolution. We could record more than ten consecutive STED images, which is comparable to the number of recordings achievable with the far-red fluorescent protein mGarnet2 at a similar resolution using shorter wavelengths (Fig. 2e; Supplementary Fig. 7). SNIFP targeted peroxisomes exhibited vivid inner-cellular movements.
when imaged in the STED mode (Fig. 2f; Supplementary Fig. 8). As we added 25 µM biliverdin to the growth medium, we next ask the question if imaging is also possible without additional biliverdin. We found that both confocal as well as STED imaging is possible, albeit at a reduced signal-to-noise ratio (Supplementary Fig. 9). We conclude that addition of biliverdin is beneficial.

Dual color red-confocal/NIR-STED. Since SNIFP covers the NIR spectral regime, but leaves the spectrum below 676 nm uncharted, SNIFP can be readily teamed with conventional red fluorescent proteins to enable red/NIR dual color imaging. To evaluate this option, we targeted the red fluorescent protein mCherry26 having an excitation maximum at 587 nm, to the ER and co-expressed VIM-SNIFP. This co-imaging allowed the mCherry signal in the confocal, and the SNIFP signal in the super-resolved STED mode (Fig. 2d), demonstrating live cell imaging with two fluorophores emitting above 600 nm. Numerous additional suitable NIR fluorescent proteins are available that can be discriminated by, for example, the fluorescence lifetime or the excitation wavelength, also dual color NIR STED nanoscopy in living cells should be readily possible.

Discussion

In conclusion, we engineered the bright and photostable bacteriophage SNIFP and demonstrate its utility for STED nanoscopy in the NIR spectral regime. As the NIR regime is particularly attractive for imaging living cells and for focusing deep into tissue, this is expected to pave the way towards multi-color live-cell deep tissue NIR nanoscopy.

Methods

Constructs for bacterial expression and mutagenesis. For expression in E. coli cells, the respective coding sequences were cloned into a pbad/HisB expression plasmid (Addgene plasmid #14382). The sequences were PCR amplified, digested (EcoRI and SalI) and ligated into the digested (EcoRI and XhoI) pbad/HisB vector. PCR-based random error-prone mutagenesis, site-directed mutagenesis and multiple site mutagenesis were performed according to standard protocols27,28.

Protein expression and purification. For spectroscopic measurements in cells, proteins were expressed at 37 °C in E. coli strain BL21-AI (Invitrogen, Carlsbad, CA, USA) transformed with the plasmid pW23h, enabling rhodamine-induced biliverdin synthesis. For cultivation on agar plates and in liquid culture, the LB medium was supplemented with antibiotics (ampicillin, 50 µg/ml and kanamycin, 30 µg/ml) for selection. Arabinose (0.02%) and rhodamine (0.2% in liquid culture, 0.02% in agar dishes) were used for induction of expression. 50 µM FeCl3 and 0.02% in agar dishes) were used for induction of expression. 50 µM FeCl3 and 0.02% in agar dishes) were used for induction of expression. 50 µM FeCl3 and 0.02% in agar dishes) were used for induction of expression.

For protein purification, proteins were expressed in BL21-AI cells (Invitrogen) without the pW23h plasmid. After expression for 20 h at 37 °C followed by 1 h at room temperature, purification was performed by Ni-NTA affinity chromatography (His SpinTrap Kit, GE Healthcare, Little Chalfont, BKM, GB) according to the manufacturer’s instructions with a 30 min binding step. After purification, the protein concentration was determined using the BioRad (Hercules, CA, USA) protein assay and subsequently the solution was saturated 3-fold with biliverdin. With repeated washing steps using Vivaspin 500 columns (Sartorius, Göttingen, DE) unfold biliverdin was washed out and the proteins were taken up in 100 mM Tris-HCl, 150 mM NaCl, pH 7.5.

Constructs for expression in mammalian cells. To target SNIFP to MAP2, Hist1H2BN or the peroxisomes, the coding sequence of SNIFP was amplified using the primers 5′ATCGCGTACGAGCCATCGTGCGCTCTCT3′ and 5′CAGTCGACATCTGAGTCCGGATTCTTTGACTTGACACCTGT3′. The PCR-product was digested with BglII and Nhel.

In case of MAP2 the coding sequence of MAP2 was amplified as described27 and the PCR fragment was digested with Xhol and BamHI and ligated into the digested pEGFP-Tub plasmid, resulting in pEGFP-HIST1H2BN. Subsequently, the SNIFP coding sequence was swapped with the EGFP coding sequence using BglII and Nhel, resulting in pSNIFP-MAP2.

To target Histone-2B, the coding sequence of Histone-2B was amplified as described27. The PCR fragment was digested with Xhol and BamHI and ligated into the digested pEGFP-Tub plasmid, resulting in pEGFP-HIST2BN. Subsequently, the SNIFP coding sequence was swapped with the EGFP coding sequence using BglII and Nhel, resulting in pSNIFP-HIST2BN.

To target peroxisomes, we generated a plasmid expressing SNIFP with the peroxisomal targeting sequence (PTS) at its C-terminus. To this end, we fused the coding sequence of SNIFP by PCR using the primers 5′CGCTGAGATCTGAGTCCGGATTCTTTGACTTGACACCTGT3′ and 5′AACAGGTATCTACGCTTGAGACCTGCTGACACCTGT3′. Subsequently, this PCR-product was swapped with the coding sequence of pEGFP-Tub in pEGFP-Tub (BD Biosciences Clontech) using Nhel and BamHI, resulting in pEGFP-PTS. Subsequently, the coding sequence of SNIFP was swapped with the EGFP coding sequence using BglII and Nhel, resulting in pSNIFP-PTS.

To target SNIFP to Vimentin, the coding sequence of SNIFP was amplified with the primers 5′CCCCCGGGGCGCACTGTCCGCGTACCTCCTC3′ and 5′GCCGGCGCTCATTCTCTGGACACTGG3′. The PCR product was swapped with the mKate2 coding sequence in pmKate2-Vimentin (Evrogen, Moscow, RU) using AgeI/Xmnl and NotI, resulting in pVIM-SNIFP.

For generation of a mouse cytokeratin-18 fusion construct, the SNIFP coding sequence was amplified with the primers 5′ACGGTACCGGCGGCGGCGGGA TCCACCGGTGCGCACTGTCCGCGTACCTCCTC3′ and 5′ATCGCGTACGAGCCATCGTGCGCTCTCT3′. The PCR-product was swapped with the TagRFP coding sequence in pTagRFP-Keratin18 (Evrogen) using KpnI and NotI, resulting in pKRT8-SNIFP.

To target SNIFP (or mCherry) to the endoplasmic reticulum (ER), the coding sequence of SNIFP was amplified with the primers 5′CTCGAGGT CGACATGCGGCGGACGCCCTC3′ and 5′TTCGCGGCGGACGCCCTC3′. The PCR-products were digested using Sfil and NotI and ligated into the digested pEF/Myc-ER construct (Invitrogen). Resulting in pER-SNIFP (pER-mCherry).

To target Caveolin 1 (Cav1), the coding sequence of SNIFP was amplified using the primers 5′GCCGGCGGCGGCGGCACTGTCCGCGTACCTCCTC3′ and 5′GGCGGGCGGGACGCCCTC3′. The PCR-product was swapped with the coding sequence of TagRFP (TagRFP-N, Evrogen) using AgeI/Xmnl and NotI, resulting in pSNIFP-N. The Sequence of Cav1 (obtained from pDNR223-CAV129) was amplified using the primers 5′TCGGAGCTCATGCAGCAGGCGGCGGACGCCCTC3′ and 5′TCGGAGCTCATGCAGCAGGCGGCGGACGCCCTC3′. The PCR-product was swapped with the Coding sequence of mNeonGreen using Xhol and BamHI and ligated into the digested pSNIFP-N plasmid, resulting in pC1-SNIFP.

To target the centromere protein C (CENP-C), the coding sequence of CENP-C (obtained from pDNR223_CENP-CaC92) was amplified using the primers 5′CATGACATCGGACGTTGCGGCATCTCTGAGCCTC3′ and 5′CAGCATGACATCGGACGTTGCGGCATCTCTGAGCCTC3′. The PCR-product was swapped with the coding sequence of mNeomGreen using Xhol and BamHI and ligated into the digested pEGFP-CENP-C plasmid, resulting in pCENP-C-SNIFP.

To target SNIFP to the endoplasmic reticulum of HeLa cells, the coding sequence of SNIFP was amplified using the primers 5′ATCGCGTACGAGCCATCGTGCGCTCTCCT3′ and 5′ATCGCGTACGAGCCATCGTGCGCTCTCCT3′. The PCR-product was swapped with the mKate2 coding sequence in pmKate2-HeLa (Evrogen, Moscow, RU) using AgeI/Xmnl and NotI, resulting in pVIM-SNIFP.

For generation of a mouse cytokeratin-18 fusion construct, the SNIFP coding sequence was amplified with the primers 5′ACGGTACCGGCGGCGGCGGGA TCCACCGGTGCGCACTGTCCGCGTACCTCCTC3′ and 5′ATCGCGTACGAGCCATCGTGCGCTCTCT3′. The PCR-product was swapped with the TagRFP coding sequence in pTagRFP-Keratin18 (Evrogen) using KpnI and NotI, resulting in pKRT8-SNIFP.
CACC3. This PCR-product was swaped with the coding sequence of EGFP (pEGFP-CENP C) using Xhol/Sall and Nhel, resulting in pSNIFP-CENP.

Viral expression system and particle preparation. To ensure invariant expres- 9sion levels, infection with the expression system simliki forest virus (SFV) was used for comparison of mammalian cellular brightnesses (see Supplementary Table 2). The sequences of proteins of interest (SNIFP, W4.20, W4.33, W4.34) were amplified using the primers 5′-GTAATCTGCGTCGAGCTCAAGC-3′, 5′-TGCGAATTCCTGCGTCGAGCTCAAGC-3′ and 5′-AGCTGGTCGCGGCGGCTTTGACCAAGCTGGT-3′. The PCR-product was swapped with the coding sequence of LA-EYFP (pSCA3-CMV-la-eYFP-con- struct)30, using BarnH and NotI, which results in pSCA3-CMV-SNIFP, pSCA3- CMV-W4.20, pSCA3-CMV-W4.33 and pSCA3-CMV-W4.34.

For virus particle generation, HEK293 cells were co-transfected (using TransIT- 293 transfection reagent, Mirus Bio LLC, Madison, WI, USA) using the pSCA3-CMV- construct encoding the protein of interest and the pSca3 helper plasmid encoding the viral structural proteins31. After transfection, according to manufacturer’s instructions, cells were grown for four days at 37 °C under 90% humidity and 5% CO2. Afterwards, cells were lysed by two freeze-thaw cycles. Cell debris was removed by centrifugation at 17000 rpm for 10 min. The supernatant was centrifuged for 2 h at 47000 g to pellet the SFV particles. The pellet was dissolved in 150 µl TBS-5 (130 mM NaCl, 10 mM KCl, 5 mM MgCl2, 50 mM Tris-HCl, pH 7.8). Before infection, particles were activated with chymotrypsin (PBS, 10 mg/ml chymotrypsin, 10 mM MgCl2, 10 mM CaCl2). Chymotrypsin was inactivated with apotinin (10 µM HEPS, 10 mg/ ml apotinin). One day after seeding in six-well plates, the cells had a density of ~70% and were infected using 5 µl of activated particles per well.

Mammalian cell culture. HeLa (ATCC CCl-2) and U2OS (ATCC HTB-96) cells were transfected with the respective plasmid using TurboFect Kit (Thermo Fisher Scientific). Cells were cultivated in Dulbecco’s Modified Eagle Medium (DMEM-Medium: 4.5 g/l glucose, Glutamax, phenol red, 10% vol/vol FCS, 1 mM sodium pyruvate, 100 µg/ml streptomycin, 100 µg/ml penicillin) on coverslips (for imaging) or without coverslips (for FACS measurements) in six- well plates at 37 °C, 90% humidity and 5% CO2. Approximately, 2 h before imaging or measuring, 25 µM biliverdin was added to the medium.

Semi-native polyacrylamide gel electrophoresis. 1 µg purified protein dissolved in 10% sucrose, 100 mM Tris-HCl, 150 mM NaCl, pH 7.5 was loaded onto 15% polyacrylamide gels containing 0.1% sodiumdodecyl sulfate. As size standards, purified monomeric mIFP32, rsEGFP223, and dimeric dTomato26 were used. Fluorescence was detected with a homemade gel-recording device. To detect the NIR fluorescence, the gel was irradiated with light of 655/40 nm and fluorescence was recorded at 740/40 nm. To detect green fluorescence (rsEGFP2), the gel was irradiated with 470/10 nm and fluorescence was recorded at 505/40 nm. For direct determination of quantum yields, a Quantaurus-QY instrument (PicoQuant GmbH, Berlin, DE) was used. All measurements were performed in replicates.

Microscopic imaging. Cells were imaged in HEPES buffered DMEM (HDMEM) without phenol red, approximately 24 h after transfection and 2 h after adding 25 µM biliverdin to the cultivation medium.

Confocal microscopy (Fig. 1) was performed with a laser raster scanning microscope (Leica TSC SP8, Leica Microsystems, Wetzlar, DE), at room temperature. The microscope was equipped with a 63× NA 1.4–0.7 oil immersion lens. For excitation, light of 633 nm was used. The detection window was between 650 and 800 nm.

StED nanoscopy of cells expressing mGarnet214 fusion proteins was performed using an Abberior STED 775 QUAD scanning microscope (Abberior Instruments GmbH, Göttingen, DE) equipped with an UPlanSApo 100x/1.40 oil objective (Olympus, Tokyo, J). The sample was excited with a 488 nm, 561 nm and 755 nm lasers. The detection window was between 600 and 800 nm.

Spectral characteristics. Absorption and emission spectra were measured on a Varian Cary 4000 UV/vis spectrometer and a Varian Cary Eclipse fluorescence spectrometer. Extinction coefficients were determined in comparison to mEFP (55,000 M−1 cm−1; value based on protein concentration12). Absorption spectra were baseline corrected and normalized to the 280 nm peak. The spectra were corrected according to the Tyr and Trp content. All measurements were performed in replicates, n = 3 for the different pH-conditions, for direct quantum yields. For direct quantum yields, a Quantaurus-QY instrument (PicoQuant, Berlin, DE) was used. All measurements were performed in replicates, n = 3 for W4.20 and W4.35, n = 2 for W4.34 and W4.35. Measurements for pH-stability were performed using a Cytation 3 plate reader (BioTek, Winooski, VT, USA) with fluorescence excitation at 650/19 nm and detection at 670/35 nm. The values of each measurement were normalized to the corresponding maximal signal. For the different pH-conditions, the following buffers were prepared:

pH 3–5:75: 100 mM citric acid, 150 mM NaCl
pH 6–7: 100 mM KH2PO4, 150 mM NaCl
pH 7.5–8.5: 100 mM Tris, 150 mM NaCl
pH 9.9–9.5: 100 mM glycine, 150 mM NaCl

All measurements were performed in replicates, n = 13 for W4.34 and W4.35, n = 8 for W4.20 and W4.33.

FACS. For determination of cellular brightness in mammalian cells, proteins were expressed for ~20 h using the viral expression system as described above. Before the measurements, cells were incubated for ~2 h in 25 µM biliverdin, trypsinized and resuspended in PBS. All measurements were performed in replicates, n = 12 for W4.34 and W4.35, n = 11 for W4.34 and W4.35, and n = 10 for W4.20. For measuring cellular brightness in bacterial cells, proteins of interest and heme oxygenase were expressed in BL21-AI cells in liquid culture with FeCl3 and ALA as described above. Protein expression was followed by protein approximate densitometry. Before the measurements, all samples were washed with PBS. All FACS measurements were performed in multiple independent replicates. n = 2 for W1–W4; n = 6 for W4.20, W4.33 and W4.34, n = 7 for W4.34.

The detection window was between 690 and 766 nm.

Lifetime measurements. The fluorescence lifetimes were measured with a homebuilt microscope that was also used for the STED measurements. The fluorescence was collected with a prototype detector (red enhanced, <50 ps jitter, Micro Photon Device, Bolzano, BZ, IT) and data acquisition was performed by a homebuilt light-time-correlated photon counting (TCSPC) system (PicoQuant GmbH, Berlin, DE). The fluorescence signal was collected from transfected (VIM-SNIFP) mammalian cells, which where incubated in 25 µM biliverdin for ~2 h. The FWHM of the IRF was below 200 ps (count rate 100k), ensuring that lifetimes of >0.6 ns could be reliably measured. All measurements were performed in replicates, n = 7 for W4.20 and W4.35, n = 6 for W4.34 and n = 3 for W4.33.
splitter (BP14581, Thorlabs GmbH). In this case, the laser light reflected from gold nanospheres was collected for overlapping the excitation and depletion beams with a photomultiplier tube (H10723-01, Hamamatsu Photonics Deutschland GmbH).

All laser power values refer to that of the back aperture of the objective lens. To determine bleaching during STED and confocal imaging, the fluorescence signals recorded in a time series were summed up for each individual image. For data analysis, the decay of the summed up fluorescence signal was fitted by a single exponential decay function. From the fit, the background level was estimated. The decay curves shown are normalized to the background and the maximum signal. The reported decay to 1/e was determined on the fit. Each data point represents the average of nine measurements (STED, mGarnet2, Supplementary Fig. S7a, c), eight measurements (STED, SNIFP, Supplementary Fig. S7c, d), five measurements (SNIFP confocal 3 µW, Fig. 1j, k), six measurements (SNIFP confocal, 6 µW, Fig. 1j, k). All imaging parameters are summarized in Supplementary Table 3.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request. The SNIFP sequence is deposited at GenBank. Accession number: MH982583. URL: https://www.ncbi.nlm.nih.gov/nuccore/MH982583.

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