Supporting Information

Enhancing Photostability of Red Fluorescent Proteins through FRET with Si-Rhodamine for Dynamic Super-Resolution Fluorescence Imaging

Xuelian Zhou,[ab] Lu Miao,*[a] Wei Zhou,[ab] Yonghui Chen,[a] Yiyan Ruan,[a] Xiang Wang,[a] Guangying Wang,[ab] Pengjun Bao,[a] Qinglong Qiao*[a] and Zhaochao Xu *[ab]

[a] Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China

[b] School of Chemistry, Dalian University of Technology, 2 Linggong Road, Dalian 116024, China

*Corresponding authors

E-mail address: miaolu@dicp.ac.cn; qqlqiao@dicp.ac.cn; zcxu@dicp.ac.cn

Contents

Materials and methods

Materials and instruments.

Unless otherwise specifically stated, all reagents were purchased from commercial suppliers (Sigma-Aldrich, Innochem and Aladdin) and used without further purification. All water used was from a Millipore water purification system with a minimum resistivity of 18.0 MΩ · cm. Fluorescence measurements were performed on an Agilent CARY Eclipse fluorescence spectrophotometer. Confocal images were performed on Laser Scanning Confocal Microscope (Andor iQ 3.2) with 100×oil-immersion objective lens (laser combination: 561; 640 nm). Super-resolution images were obtained with Nikon N-SIM 5.0 Super-Resolution Microscope System with a motorized inverted microscope ECLIPSE Ti2-E, a 100×/ NA 1.49 oil immersion TIRF objective lens (CFI HP), and an ORCA-Flash 4.0 SCMOS camera (Hamamatsu Photonics K.K.). Laser combination: 488; 561; 640 nm.

Design and construction of plasmids.

The plasmid pET22b-HaloTag7-His6 was commercially synthesized. Plasmids pCMV-mCherry-tubulin, pCMV-H2B-pSNAPf (#101124), and pCMV-Tomm20-mApple (#54955) were purchased from Addgene. All primers were synthesized by General Bio in China. Plasmid constructions were confirmed by DNA sequencing.

The construction of bacterial expression plasmids. The sequences encoding mApple were separately PCR amplified from pCMV-Tomm20-mApple with various modifications: addition of 5 amino acids to N-terminal of mApple(5aa+mApple), addition of 5 amino acids to C-terminal of mApple (mApple+5aa), wild mApple, deletion of 2 amino acids of mApple C- terminus (mAppleΔ2C), and deletion of 4 amino acids of mApple C- terminus (mAppleΔ4C). The construction of pET22b-HA-L-His6 and pET22b-AH-L-His6 involved using pET22b-HaloTag7-His6 as the vector. After single enzyme digestion of pET22b-HaloTag7-His6 by (Xho Ⅰ or EcoR Ⅰ) , 5aa+mApple and mApple+5aa were inserted to the C or N-terminus of it using the One Step Cloning Kit (YEASEN), respectively. The

construction of pET22b-AH-0-His6, pET22b-AH-1-His6, and pET22b-AH-3-His6 involved reverse PCR amplification from pET22b-HaloTag7-His6 to generate linearized vectors with differentially modified Halo sequences:wild Halo; deletion of 3 amino acids of Halo N-terminus(HaloΔ3N); deletion of 7 amino acids of Halo N-terminus (HaloΔ7N). Subsequently, mApple, mAppleΔ2C, and mAppleΔ4C were connected to these linearized vectors using the One Step Cloning Kit (YEASEN). The construction of the mCherry series fusion proteins (pET22b-CH-L-His6, pET22b-CH-0-His6, pET22b-CH-1-His6, pET22b-CH-2-His6) paralleled to that of mApple.

The plasmid for mammalian expression is based on a vector containing the standard CMV promoter. The various fusion proteins were fused to the C-terminus of the targeting protein of different organelles by single enzyme digestion and then One Step Cloning. Mitochondrial outer membrane, Tom20: pCMV-Tomm20-mApple (#54955); Nucleus, H2B: pCMV-H2B-pSNAPf (#101124).

Protein expression and purification.

All proteins in vitro expression of in this study was achieved by transforming the pET22b-His6 vector into Escherichia coli BL21 (DE3) cells. In brief, the plasmid carrying the target protein X, designated as pET22b-X-His6, was transformed into BL21 (DE3) cells. Subsequently, the transformed cells were transferred to 5 mL LB medium containing 100 μg/mL ampicillin (Sangon Biotech) and cultured overnight at 37°C. The following day, a concentrated bacterial culture was inoculated at a 1:100 ratio into 300 mL LB medium supplemented with 100 μg/mL ampicillin, and the cells were further cultured at 37°C until the OD600 reached 0.6-0.8. At this point, 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression, and the culture was continued at 18°C for 20 hours. The harvested cells were resuspended in 10 mL PBS buffer (20 mM, pH 7.4, 0.3 M NaCl, 1 mM PMSF) and subjected to ultrasonication (Noise Isolating Tamber, JY92-IIN, Ninbo, China). Following the manufacturer's instructions, cell lysates were purified using Ni-NTA resin (GE Healthcare). Elution was performed using a gradient of imidazole concentrations (10-500 mM) in PBS buffer (20 mM, pH 7.4, 0.3 M NaCl). Washing buffer consisted of 20 mM PBS, 0.3 M NaCl, 10 mM imidazole, pH 7.4, and elution buffer consisted of 20 mM PBS, 0.3 M NaCl, 250 mM imidazole, pH 7.4. The collected target

proteins were concentrated using a Millipore Corporation centrifugal device with a 10 kD molecular weight cutoff, and excess imidazole was removed by washing the concentrated fraction three times with PBS buffer (20 mM, pH 7.4, 0.3 M NaCl). The purified proteins were quantified using the Bradford reagent (Sangon Biotech).

Preparation of TMSiR-labeled Halo-RFP fusion proteins and HaloTag.

Proteins (10 μM) were incubated with 2 equivalents of Halo-TMSiR dye at 37°C with gentle agitation for 1 hour. The mixture was then subjected to multiple rounds of filtration and washing using a Millipore Corporation filtration device with a 10 kD molecular weight cutoff and PBS buffer at 4°C to remove excess small molecules. Covalent linkage of small molecules was confirmed by SDS-PAGE analysis. The purified concentrate was diluted with PBS buffer for spectral measurements.

Protein characterization (SDS PAGE).

The purified fusion protein and the fusion protein labeled with NAP-488 dye, each at a concentration of 1 μg, were dissolved in 10× SDS sample buffer and boiled for 10 minutes. The samples were then loaded onto a 12% acrylamide gel for protein band separation in SDS-PAGE buffer. Before Coomassie Brilliant Blue (Sangon Biotech) staining, the gel was scanned using UV for fluorescence imaging.

Fluorescence spectral measurements.

The purified protein samples were diluted into a buffer containing PBS (20 mM, pH 7.4) to prepare a final concentration of 2 μM solution. Subsequently, a specific volume was aliquoted into 100 μL quartz microcuvettes for fluorescence spectra acquisition using a fluorescence spectrophotometer (Agilent CARY Eclipse) in vitro. To determine the actual Förster Resonance Energy Transfer (FRET) efficiency of different fusion proteins, emission spectra within the wavelength range of 565-800 nm were recorded upon excitation at 561 nm. The FRET efficiency (E) was calculated using the following standard formula:

$$E = 1 - \frac{I_{D-A}}{I_D},$$

where I_D is the fluorescence intensity of the donor alone and I_{D-A} is the fluorescence intensity of the donor in the presence of the acceptor.

Photobleaching measurement of purified proteins.

The purified protein samples were diluted into a buffer containing PBS (20 mM, pH 7.4) to prepare a final concentration of 2 μM solution. For each experimental run, 100 μL of the sample was dispensed into a sealed quartz microcuvette and covered. A 561 nm external laser light source with export power of 36 mW was employed to irradiate the sample continuously for 100 minutes. Fluorescence spectra changes were recorded at 10-minute intervals to generate a photobleaching curve. Throughout this duration, the distance between the microcuvette and the light source was maintained at 20 cm. Fluorescence spectra were acquired by collecting emission within the wavelength range of 565-800 nm under excitation at 561 nm. The construction of the photobleaching curve was based on the maximum emission intensity of both donor and acceptor. The photobleaching determination of TMSiR labeling to HaloTag followed the same principles; the difference lied in the utilization of an external laser light source at 640 nm (export power 160 mW) for continuous photobleaching.

Reactive oxygen species measurement of purified proteins.

DCFH-DA (purchased from Aladdin) was subjected to deacetylation to prepare DCFH. The DCFH-DA was dissolved in HPLC-grade DMSO to prepare a stock solution with a concentration of 10 mmol/L. Under dark conditions, the DCFH-DA solution was treated with 0.01 M NaOH for 30 min to initiate a deacetylation at room temperature, leading to the generation of DCFH. DCFH was diluted with PBS to a concentration of 40 μM and aliquoted in light-protected containers, then stored at -80°C with a shelf life of one month.And then the purified protein samples were diluted into a buffer containing PBS (20 mM, pH 7.4) to prepare a final concentration of 2 μM solution. Before each test, 100 μL of the sample was dispensed into a quartz microcuvette, and simultaneously, 2 μM of DCFH was promptly added and sealed. In a dark environment, using an external 561 nm laser light source with an output power of 36 mW, the sample underwent continuous photobleaching for 10 minutes. Fluorescence spectra changes were recorded at 1-minute intervals. Throughout this period, the distance between the microcuvette and

the light source was maintained consistent with the distance for photobleaching, at 20 cm. Fluorescence spectra were acquired by collecting emission spectra within the wavelength range of 475-600 nm under excitation at 470 nm. Simultaneously, spectra for the blank DCFH sample (2 μM, 100 μL) were recorded. The kinetics of reactive oxygen species reactions were determined based on the emission intensity of DCFH at 530 nm.

Cell Culture.

HeLa (HeLa cyton gartleri) cells were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences. HeLa cells were grown in Dulbecco's modified Eagle′s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% antibiotics (Hyclone) which were cultured in a humidified atmosphere of 5% CO_2 at 37℃. Cells were passaged 2 times a week. Before the imaging experiments, HeLa cells were seeded on glass bottom cell culture dish (Nest, polystyrene, Φ 15 mm) for 24 h to reach 60-80% confluency and transfected the following day with 0.5 μg DNA using polyethylenimine (PEI, Polyscience) according to the manufacturer's protocol to express fusion proteins. Cells were imaged 24-48 h after transient transfection.

Confocal fluorescence imaging and photobleaching kinetics measurement.

Prior to imaging, cells were labeled with 0.5 μM Halo-TMSiR for a minimum of 30 minutes. Subsequently, they were washed three times in serum-free DMEM and incubated at 37°C in culture medium for 15 minutes. Live HeLa cells expressing RFP-HaloTag fusion proteins were then imaged using a Laser Scanning Confocal Microscope (Andor iQ 3.2) in cell growth medium. Identification of cells expressing the RFP-HaloTag fusion protein was accomplished by imaging with a 561 nm laser (export power: 33 mW; collection: 580-650 nm), while simultaneously using a 640 nm laser (export power: 33 mW; collection: 660-700 nm) for imaging to determine the localization of the dye ligand in the cell nucleus or mitochondria.

Cells with accurate organelle and dye localization were selected, and continuous illumination with a 561 nm laser at full power (33 mW, 100 ms exposure time) was applied for 20 minutes, with images

recorded every 30 seconds (collection: 580-650 nm). Over time, a series of images were collected to generate photobleaching curves. The final images were shown on the same intensity scale.

Structured Illumination Microscopy (SIM) photobleaching dynamics and long-term dynamic imaging.

As previously described, labeling and washing of HeLa cells with TMSiR dye were performed. SIM imaging of the cells was conducted using the Nikon N-SIM 5.0 Super-Resolution Microscope System, with simultaneous excitation by 561 nm and 640 nm lasers. Cells exhibiting accurate organelle and dye localization were selected for the determination of photobleaching kinetics.

For SIM photobleaching images of mCherry fusion proteins, imaging was conducted with a 561 nm laser at 50% power (0.27 kW/cm^2, collection wavelength: 570-640 nm) and 200 ms exposure time. A total of 121 frames were recorded within 20 minutes with a 10-second interval between each frame. To match the rapid photobleaching of mApple fusion proteins, SIM photobleaching for mApple series was performed using a 561 nm laser at 30% power (0.16 kW/cm^2, collection wavelength: 570-640 nm) and 200 ms exposure time. Similarly, 121 frames were recorded within 20 minutes with a 10 s interval between each frame. The final SIM images were displayed with the same intensity levels.

For dual-color SIM imaging of mitochondria and endoplasmic reticulum, HeLa cells were co-transfected with pCMV-Tom20-CH-1 and EGFP-Sec61β plasmids. After 24-48 hours of protein expression, cells were labeled with TMSiR dye. Imaging utilized a 488 nm laser (10% power, collection wavelength: 500-545 nm) and a 561 nm laser (10% power, collection wavelength: 570-640 nm), with each having a 200 ms exposure time. A total of 121 frames were recorded within 30 minutes with a 15-second interval between each frame.

For dual-color SIM imaging of mitochondria and lysosomes, HeLa cells were transfected with pCMV-Tom20-CH-1 plasmid and labeled with 0.5 μM TMSiR dye and 0.5 μM Lyso-tracker green dye for 30 minutes to 1 hour. After three washes with DMEM, imaging was performed in serum-containing DMEM using a 488 nm laser (10% power, collection wavelength: 500-545 nm) and a 561 nm laser

(10% power, collection wavelength: 570-640 nm), each with a 200 ms exposure time. A total of 121 frames were recorded within 30 minutes with a 15 s interval between each frame.

Quantification and statistical analysis.

The generation of all spectral curves and photobleaching kinetics curves in this study was processed using Origin 2018 software. FRET efficiency and half-life calculations were conducted using Microsoft Excel. Fluorescence intensity data for live cell imaging were obtained from the built-in analysis software of the imaging system. The fitting of photobleaching kinetics curves for live cells was analyzed using the single-exponential function in Origin 2018 software. Statistical analyses related to mitochondrial events were performed using GraphPad Prism 8. p values were calculated using GraphPad unpaired two-tailed t-test (ns, no significant difference, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$).

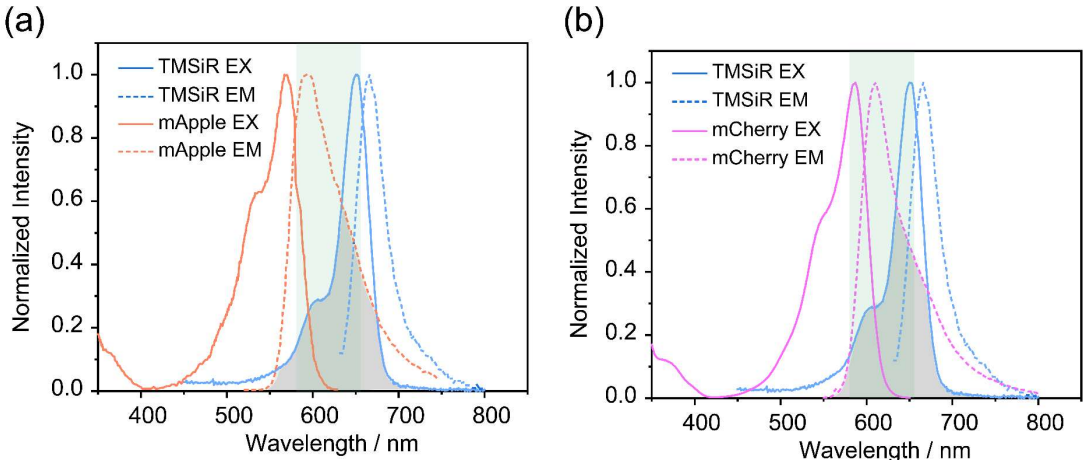

Figure S1. Normalized excitation (EX) and emission (EM) spectra of donor red fluorescent proteins (a) mApple and (b) mCherry, along with acceptor dye TMSiR. The gray region indicates the overlap between the emission spectrum of the donor RFP and the excitation spectrum of the acceptor TMSiR. The green region indicates the range of confocal imaging channels.

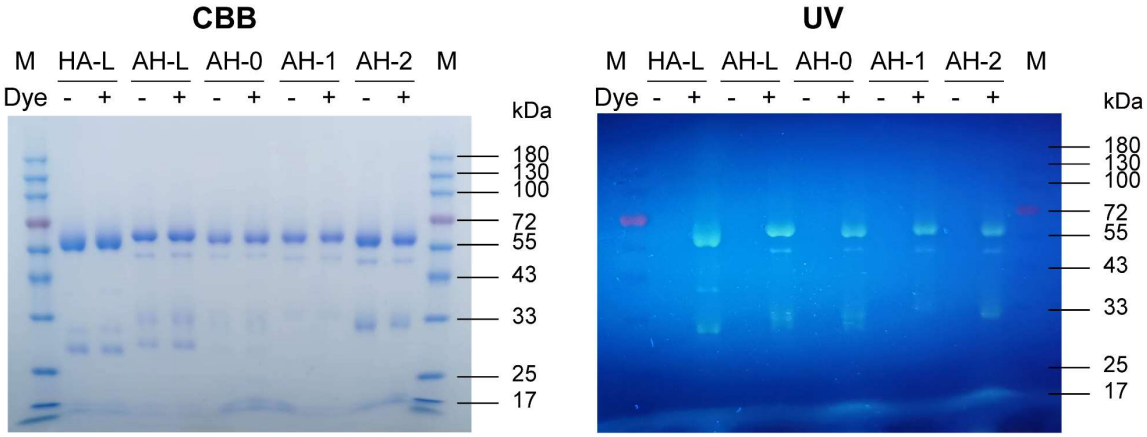

Figure S2. The full gel electrophoresis of purified mApple series fusion proteins labeled with or without NAP-488 dye. CBB: gel stained with Coomassie Brilliant Blue; UV: Fluorescence image excited with UV.

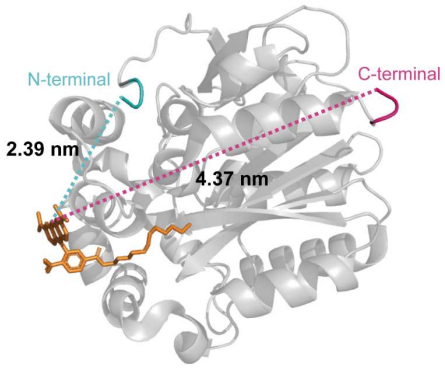

Figure S3. X-ray structure of the HaloTag7 labeled with TMSiR (PDB: 6y7a). The green and red dashed lines in the figure represent the distances from the fluorophore to the N-terminus and C-terminus, respectively.

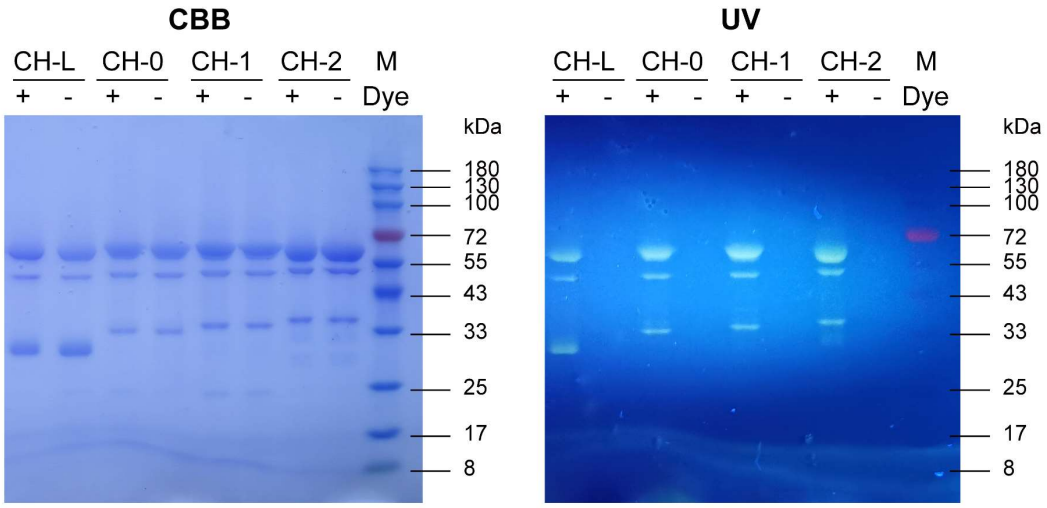

Figure S4. The full gel electrophoresis of purified mCherry series fusion proteins labeled with or without NAP-488 dye. CBB: gel stained with Coomassie Brilliant Blue; UV: Fluorescence image excited with UV.

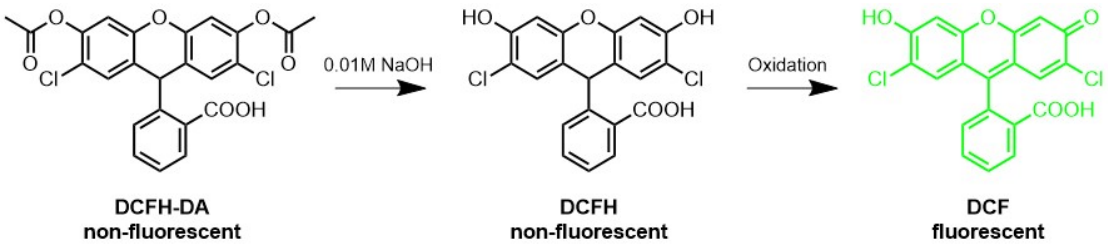

Figure S5. Protocol for DCFH detection of reactive oxygen species (ROS).[1]

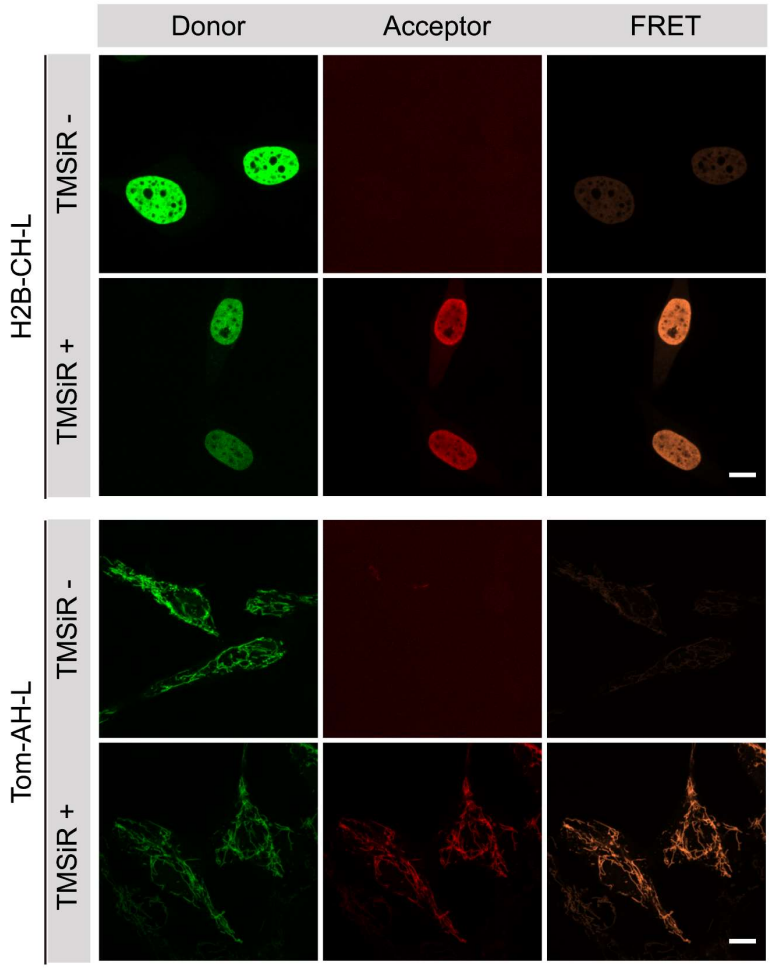

Figure S6. HeLa cells expressing the H2B-CH-L, Tom-AH-L and H2B-Halo (labeled with TMSiR) fusion proteins were imaged with confocal microscopy through the donor channel (excitation: 561 nm; collection: 580-650 nm), acceptor channel (excitation: 640 nm; collection: 660-700 nm), and FRET channel (excitation: 561 nm; collection: 660-700 nm), respectively. Scar bar: 10 μm

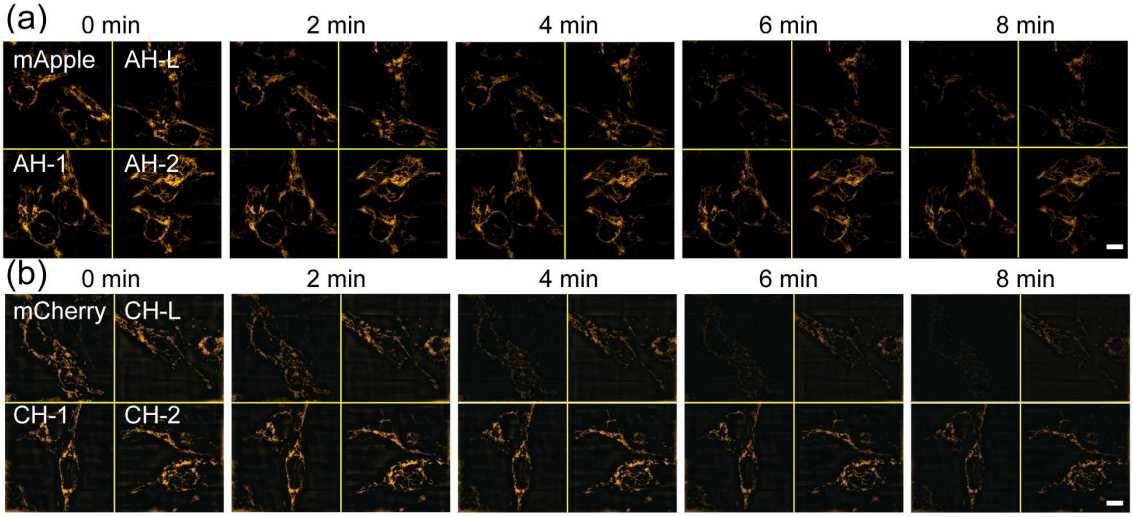

Figure S7. (a), (b) Time series SIM images of mApple series FRET pairs (a) and Cherry series FRET pairs (b) fused to Tom20 in hela cell under continuous illumination with 561 nm laser over time. Laser export power: mApple (0.16 kW/cm^2)/mCherry (0.27 kW/cm^2). The quantitative evolution of fluorescence signals over time is represented by the photostability curves in Fig 4e, g. Scar bar: 10 μm

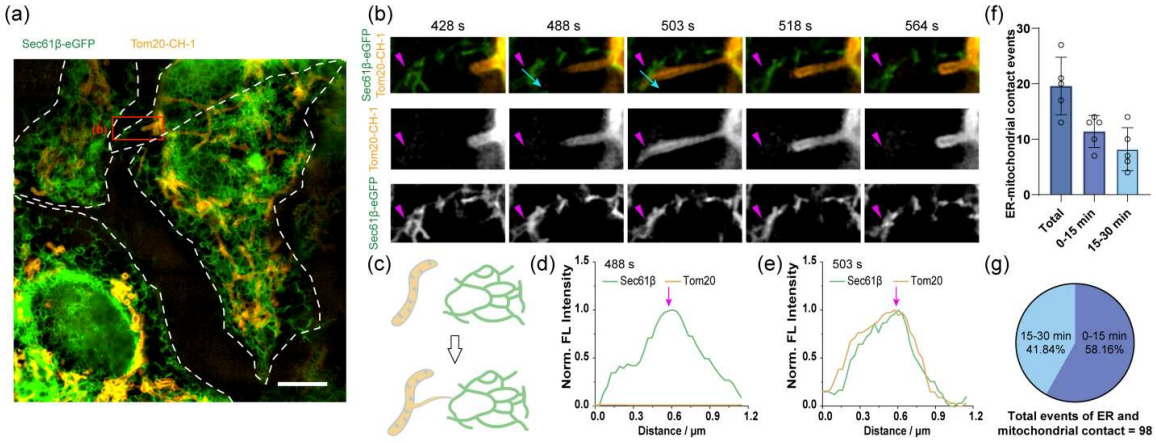

Figure S8. Dual-color SIM images of endoplasmic reticulum and mitochondria contact in Live Hela Cells. (a) Dual-color SIM image of the entire live Hela cell co-transfected with GFP-Sec61β and CH-1-Tom20 (labled with TMSiR). Delayed dual-color SIM images show the merged channels of endoplasmic reticulum (green) and mitochondria (orange). (b) Local magnified images of the solid-boxed region in (a). Contact points between mitochondria and endoplasmic reticulum are highlighted with pink arrows. (c) Model of endoplasmic reticulum and mitochondrial contact. (d), (e) Line scan analysis of relative fluorescence intensity along the blue lines in representative images from endoplasmic reticulum and mitochondrial contact time points (488 s, 503 s) in (b). (f) Quantitative statistics of endoplasmic reticulum and mitochondria contact events occurring in HeLa cells during the total observation period of 30 minutes. The number of events was counted separately for the intervals of 0-15 minutes and 15-30 minutes. (h) Quantification of the percentage of endoplasmic reticulum-mitochondria contact events occurring in 0-15 min and 15-30 min stages.

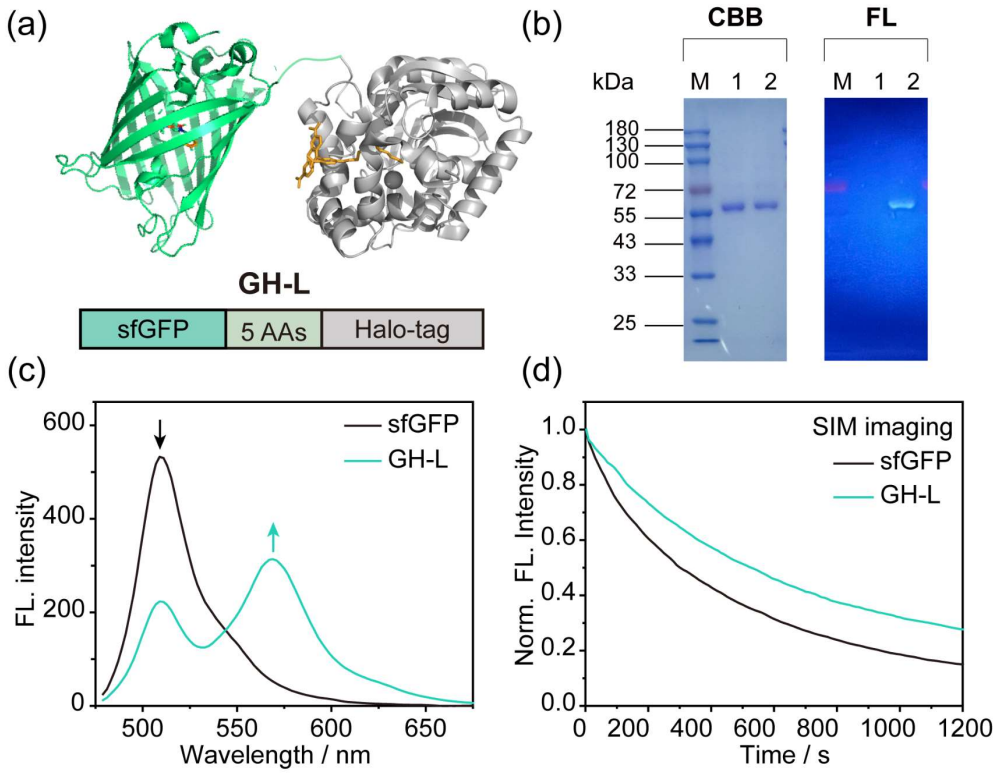

Figure S9. (a) Schematic representation of the structure of Halo-Tag (PDB: 6y7a) fused at the N-terminal of sfGFP (PDB: 2b3p). (b) The 12% SDS-PAGE of purified **GH-L** fusion proteins labeled with or without NAP-488 dye. CBB: gel stained with Coomassie Brilliant Blue; FL: Fluorescence image of the gel excited with UV. (c) Fluorescence emission spectra of **GH-L** with and without the addition of O-rhodamine (λ_{ex} = 470 nm). [protein]: 2 μM. (d) Photobleaching dynamics comparison of sfGFP and GH-L fused to H2B in SIM imaging under continuous illumination with 488 nm laser over time. Each curve represents the average of three independent experiments. Laser power 30%, collection wavelength: 500-545 nm.

Table S1. Comparison of the properties of donor red fluorescent proteins and acceptor dye TMSiR.

	λ_{EX} (nm)	λ_{EM} (nm)	ε ($M^{-1}\cdot cm^{-1}$)	ϕ	photostability[2]
TMSiR	643	662	141000	0.41	-
mCherry	587	610	72000	0.22	87.97 s
mApple	568	592	75000	0.49	28.33 s
mRuby	558	605	112000	0.35	14.38 s
mRFP1	584	607	50000	0.25	9.44 s

λ_{EX}: excitation wavelength (nm)；λ_{EM}: emission wavelength (nm)；ε: extinction coefficient；φ: quantum yield; photostability: the half-time of bleaching under laser scanning illumination is also given for both 200 μW of incident power (Data from Nat. Met. 2016[2]).

Table S2. Amino acid sequences and molecular weights of the linker regions in fusion proteins of mApple and mCherry with different linkers.

	mApple-Linker-Halo Protein squence	MW(Da)
AH-2	……RHSTGGMDFPFDPHYVE……	61490
AH-1	……RHSTGGMDELIGTGFPFDPHYVE……	62060
AH-0	……RHSTGGMDELYKMAEIGTGFPFDPHYVE……	62690
AH-L	……STGGMDELYKATEFHMAEIGTGFPFDPHYVE……	62990
HA-L	……IARWLSTLEISGPPVATMVSKGEENNMAIIKE……	62580
	mCherry-Linker-Halo Protein squence	**MW(Da)**
CH-2	……STGGMDFPFDPHYVE……	61260
CH-1	……STGGMDELIGTGFPFDPHYVE……	61830
CH-0	……STGGMDELYKMAEIGTGFPFDPHYVE……	62450
CH-L	……STGGMDELYKSGLRMAEIGTGFPFDPHYVE……	62860

References

(1) Miljevic, B.; Hedayat, F.; Stevanovic, S.; Fairfull-Smith, K. E.; Bottle, S. E.; Ristovski, Z. D. To Sonicate or Not to Sonicate PM Filters: Reactive Oxygen Species Generation Upon Ultrasonic Irradiation. *Aerosol Science and Technology* **2014**, *48* (12), 1276–1284. https://doi.org/10.1080/02786826.2014.981330.

(2) Cranfill, P. J.; Sell, B. R.; Baird, M. A.; Allen, J. R.; Lavagnino, Z.; de Gruiter, H. M.; Kremers, G.-J.; Davidson, M. W.; Ustione, A.; Piston, D. W. Quantitative Assessment of Fluorescent Proteins. *Nat Methods* **2016**, *13* (7), 557–562. https://doi.org/10.1038/nmeth.3891.