Supporting Information

Probing Subcellular Organic Hydroperoxides Formation via a Genetically Encoded Ratiometric and Reversible Fluorescent Indicator

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General Considerations

Materials

Enzymes were ordered from New England Biolabs (NEB) or Life Technologies. Primers were ordered from Eurofins MWG Operon. Chemicals used were purchased from Sigma Aldrich or Fisher Scientific.

Equipment

PCR and site-directed mutagenesis were performed using C1000 Thermal Cycler (Bio-Rad). Protein purifications were all performed on an AKTA UPC 900 system (GE healthcare). *In vitro* fluorescent property study was performed with Varian Cary Eclipse Fluorescence Spectrophotometer. Confocal imaging and time-lapse monitoring were carried out with Leica TCS SP2 AOBS Laser Scanning Confocal Microscope.

Supplementary Methods

1. Site-directed mutagenesis towards a ratiometric OHSer

Since the ratiometric Hyper probe contains a cpYFP while our OHSer bears a cpVenus, we first examined several non-conserved amino acids between cpVenus and cpYFP as an attempt to obtain a ratiometric OHSer through site-directed mutagenesis (Figure S1). Three mutant forms of OHSer were created that gradually changed cpVenus on OHSer towards cpYFP, which were named OHSer-1 (Y154F), OHSer-2 (Y154F, Q264K), and OHSer-3 (Y154F, Q264K, H343D), respectively. These OHSer variants showed distinctive pH profiles (Figure S2), but none of the variants exhibited significant ratiometric fluorescence property.

2. Construction of ratiometric OHSer (rOHSer)

Polymerase incomplete primer extension method (PIPE)[1] was utilized to construct rOHSer. pET28a plasmid containing Xc-OhrR gene was converted into a linear vector with overhangs at 5' and 3' using PCR amplification with the primers *OhrR-5'-R* and *OhrR-3'-F*. (**Table S1**) The cpYFP gene was directly amplified from Hyper[2] with the primers *cpYFP-5'-F* and *cpYFP-3'-R*, also with overhangs corresponding to the linear vector. The cpYFP insert and linearized OhrR vector were mixed and transformed into *E. coli* DH10B strain according to the previously reported protocol. [1] The resulting plasmids harvested from picked colonies were sequenced, from which the correct pET28a-rOHSer construct was identified and amplified

for further usage. The inactive mutant of rOHSer (rOHSer-mut) was generated by 2 mutations at Cys 22 and 127, both mutated to serine by mutagenesis PCR.

The mammalian plasmid was constructed by the following subcloning procedure: The pCMV-Tag1 plasmid was digested and the fragment of rOHSer gene was amplified by PCR with primers containing *EcoRI/HindIII* cutting sites. After enzyme digestion, the insert gene was cloned into the *EcoRI/HindIII* site on pCMV-Tag1 to produce the final plasmid pCMV-rOHSer for rOHSer expressing in mammalian cells. The cpYFP gene fragment was treated similarly to yield the control plasmid pCMV-cpYFP. For generating subcellular-targeting rOHSer variants, the gene fragment encoding the corresponding signal peptides were incorporated into the forward primer used for rOHSer PCR amplification, which were subsequently cloned into pCMV-Tag1 to form various subcellular targeting plasmids. (**Table S1**)

3. Protein expression and purification.

The pET28a-rOHSer plasmid was transformed into *E. coli* BL21 (DE3) cells and grown in LB to OD_{600}= 0.6 at 37 °C followed by the addition of 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) for induction overnight at 30 °C. Cells were harvested and frozen at -80 °C followed by thaw and cell lysis with sonication. The expressed protein was purified from the supernatant with a Histrap affinity chromatography (GE Healthcare) using FPLC system (GE Healthcare) and finally exchanged into a buffer containing 1 M Tris-HCl (pH 7), 200 mM NaCl with the utilization of a 30 kDa filter (Qiagen).

4. Fluorescent property characterization.

All the fluorescence measurements were performed at room temperature on a Varian Cary Eclipse Fluorescence Spectrophotometer with a 10 mm × 2 mm quartz cuvette. The samples were excited at 500 nm and the emission spectrum was scanned from 505 nm to 580 nm with the excitation and emission slit widths set as 2.5 nm and 5 nm, respectively. The protein samples in the buffer containing 1 M Tris-HCl (pH 7) and 200 mM NaCl were pre-reduced overnight, buffer-exchanged to remove excessive reductants, and then used for following experiments. For generation of excitation and emission spectra, rOHSer was treated with reductant or OHP overnight. For generation of response curves, rOHSer was incubated with different concentrations of TBHP for 20 minutes prior to measurement. For selectivity assay, rOHSer was

incubated with various oxidants for 30 min at room temperature before data collection. For reversibility assay, the same samples of rOHSer were treated with 10 μM TBHP and 10 mM NAC alternately, with ultrafiltration-based buffer exchange in between to remove excess oxidants or reductants. For other fluorescent measurements, excitation spectra were recorded with a fixed the emission wavelength at 520 nm and the excitation and emission slit widths set as 2.5 nm and 5 nm, respectively. All experiments were performed in triplicate.

5. Live cell fluorescence imaging.

HeLa cells were plated onto Lab-Tek™ 8-well-chambered cover glasses in DMEM containing 10% fetal bovine serum. 14 hours after plating, the cells were transfected with pCMV-rOHSer, pCMV-cpYFP, or corresponding subcellular targeting rOHSer plasmids using Lipofectamine2000 transfection reagent according to the manufacturer's protocol. After incubation of the transfected cells for 24 hours, the chambers were washed with PBS, followed by the addition of various reagents. For oxidants treatment, the reagents were pre-dissolved in corresponding amounts of PBS and added into chambers. For high glucose treatment, corresponding amounts of D-glucose were added into low-glucose medium and cells were incubated with these medium for certain time specified. (In both conditions osmolarity was kept constant) Then confocal fluorescence imaging was performed under Leica TCS SP2 AOBS Laser Scanning Confocal Microscope with 40×oil-immersion objective lens. The acquisition of image data and synchronization of the illumination were performed with sequential excitation at 405 nm and 488 nm, and a fixed emission range from 500 nm to 560 nm.

The analysis and calculation of imaging data was performed using ImageJ. Background was subtracted and the fluorescence intensity of same cells was used to calculate the change in the emission ratio with dual excitations. The pseudocolor image of the same cell was generated by calculating the pixel-by-pixel ratio of the 488 nm excitation image by the 405 nm excitation image and converting the images into HSV color space. All the experiments were performed in triplicate.

References

1. H. E. Klock, E. J. Koesema, M. W. Knuth and S. A. Lesley, *Proteins: Struct., Funct., Bioinf.*, 2008, **71**, 982-994.
2. V. V. Belousov, A. F. Fradkov, K. A. Lukyanov, D. B. Staroverov, K. S. Shakhbazov, A. V. Terskikh and S. Lukyanov, *Nat. Methods*, 2006, **3**, 281-286.

Supplementary Table

Table S1. Primers used for the construction of rOHSer plasmids.

Description	Sequence (5'-3')
OhrR-5'-R	GCCGGCGGAACCTGCTTTGCTACGCAGTG
OhrR-3'-F	GGCACCGGTGCCGTCCCGGAACAGGTGTTCT
cpYFP-5'-F	GCAGGTTCCGCCGGCTACAACAGCGA
cpYFP-3'-R	GACGGCACCGGTGCCGTTGTACTCCAG
pCMV-rOHSer-F	GTGTACAGAATTCATGGATACCACGACCGCAACGAC
pCMV-rOHSer-R	GTAGGCAAAGCTTACCCGCGCCCAGGGAACTGC
Nu-rOHSer-F	GTGTACAGAATTCATGAAGAGGCCTGCGGCTACCAAAAAAGCAGGCCAGGC AAAGAAGAAGAAACTTGATACCACGACCGCAACGAC
Mem-rOHSer-F	GTGTACAGAATTCATGATGGGCTGCATCAACAGCAAGCGCAAGGACAACCTG AACGACGATACCACGACCGCAACGAC
Mito-rOHSer-F	GTGTACAGGATCCATGGATACCACGACCGCAACGAC
Mito-rOHSer-R	GTAGGCAGATATCACCCGCGCCCAGGGAACTGCGCAGT

Supplementary Figures

Figure S1. Sequence alignment between Venus (from OHSer) and YFP (from Hyper). Highlighted amino acids were selected to generate the three mutated OHSer variants via site-directed mutagenesis.

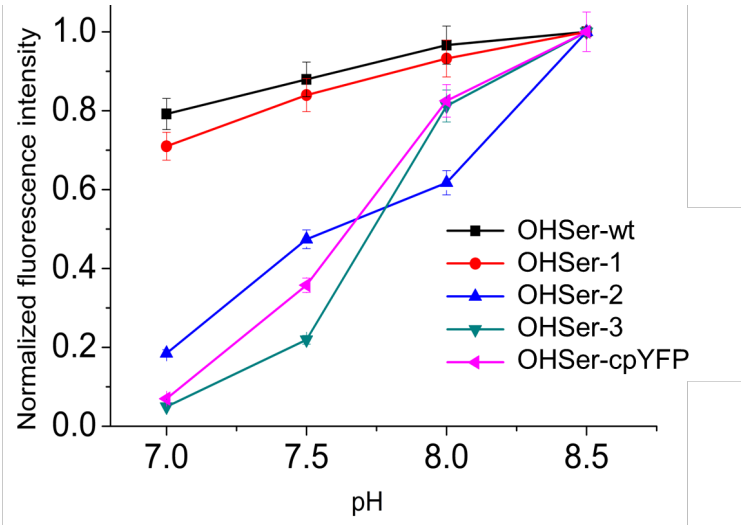

Figure S2. The pH dependent fluorescence of wild-type and mutant OHSer variants between pH 7 and pH 8.3. The fluorescence was normalized by setting the mean fluorescence at pH 8.3 as 1. Error bars represents ±s.d..

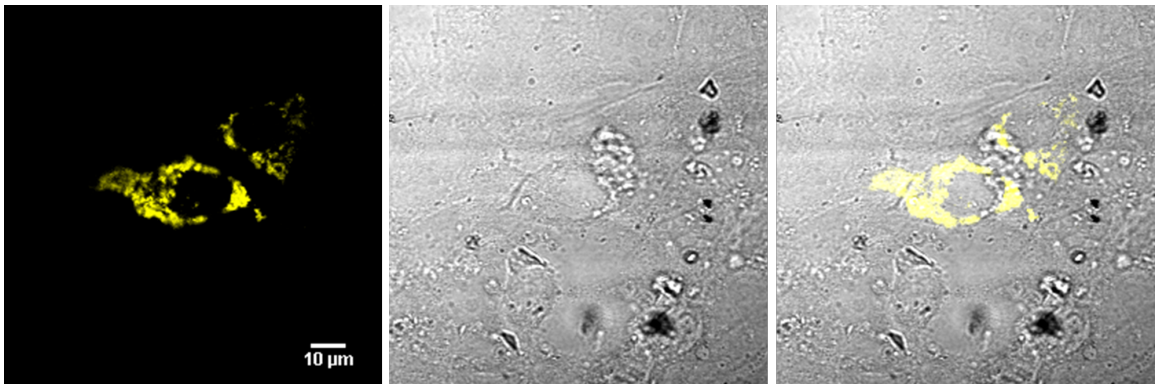

Figure S3. Confocal images of mitochondria-targeting rOHSer. The plasmid carrying mitochondria-targeting rOHSer (pcDNA-mito-rOHSer) was transfected into HeLa cells to express the mito-rOHSer protein. Confocal fluorescence imaging showed that mito-rOHSer was specifically targeted to mitochondria of HeLa cell. From left to right: mito-rOHSer fluorescence, brightfield, merged image of mito-rOHSer signal and brightfield. Scale bar represents 10 μm.

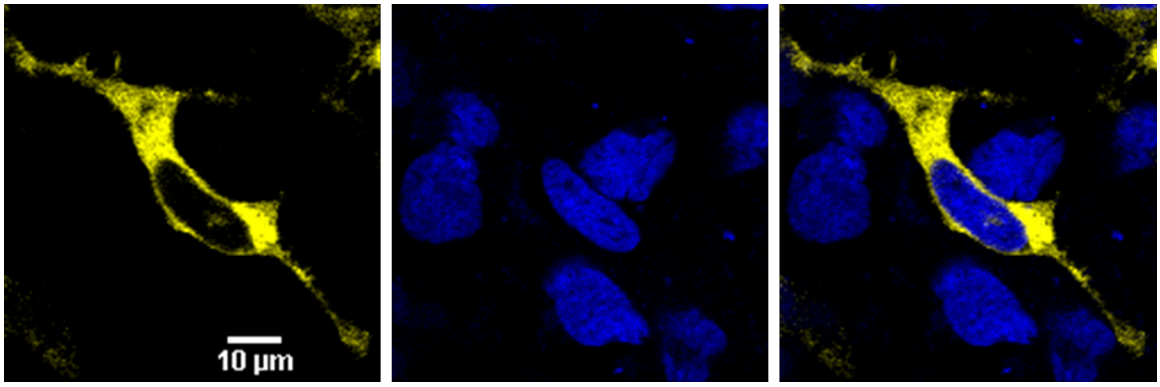

Figure S4. Confocal images of membrane-targeting rOHSer. The plasmid carrying the membrane-targeting rOHSer (pCMV-mem-rOHSer) was transfected into HeLa cells to express the mem-rOHSer protein. Confocal fluorescence imaging showed that mem-rOHSer was specifically targeted into outer membrane region of HeLa cell. From left to right: mem-rOHSer fluorescence, nucleus dyed by DAPI, merged image of mem-rOHSer signal and DAPI. Scale bar represents 10 μm.

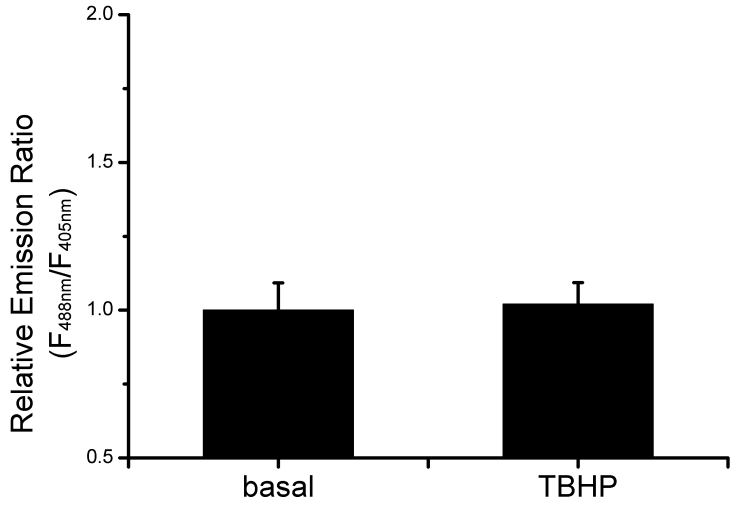

Figure S5. The fluorescence intensity change of rOHSer-mut with OHP treatment. HeLa cells expressing rOHSer-mut were treated with 100 μM TBHP or without the treatment (basal condition) for 30 minutes before the fluorescence being measured by confocal imaging. The relative emission ratio was the ratio of fluorescent intensity with 488 nm excitation by that with 405 nm excitation. All error bars represent ±s.d.

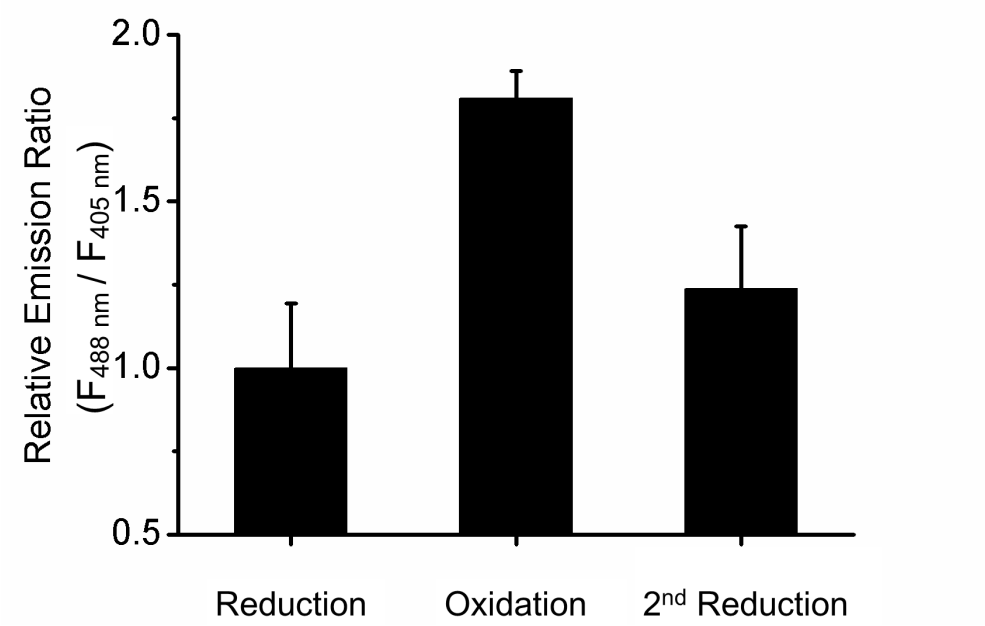

Figure S6. Reversibility of rOHSer probe inside living cells. HeLa cells expressing rOHSer were consecutively treated with reduction (10 mM NAC, 4h), oxidation (100 μM TBHP, 30 min), and a second reduction (10 mM NAC, 4h) with the fluorescence measured by confocal imaging after each treatment. The relative emission ratio was the ratio of fluorescent intensity with 488 nm excitation by that with 405 nm excitation. All error bars represent ±s.d..

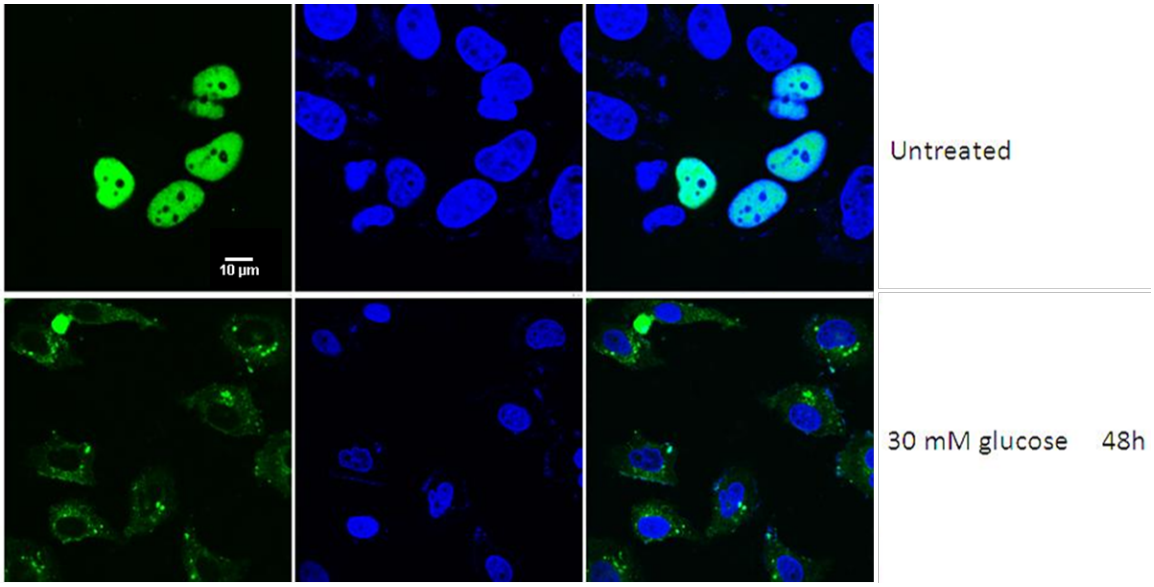

Figure S7. Intracellular localization of p53 under normal and stress conditions. HeLa cells expressing a p53-GFP reporter were treated with 30 mM D-glucose for 48 h before being visualized under confocal fluorescence microscopy. Untreated p53-GFP-bearing HeLa cells were used as a control which showed that p53 were exclusively located with cell nucleus. By contrast, the presence of glucose significantly exported p53 into cytosol. From left to right: p53-GFP fluorescence, nucleus stained by DAPI and merged images of p53-GFP and DAPI. Scale bar represents 10 μm.

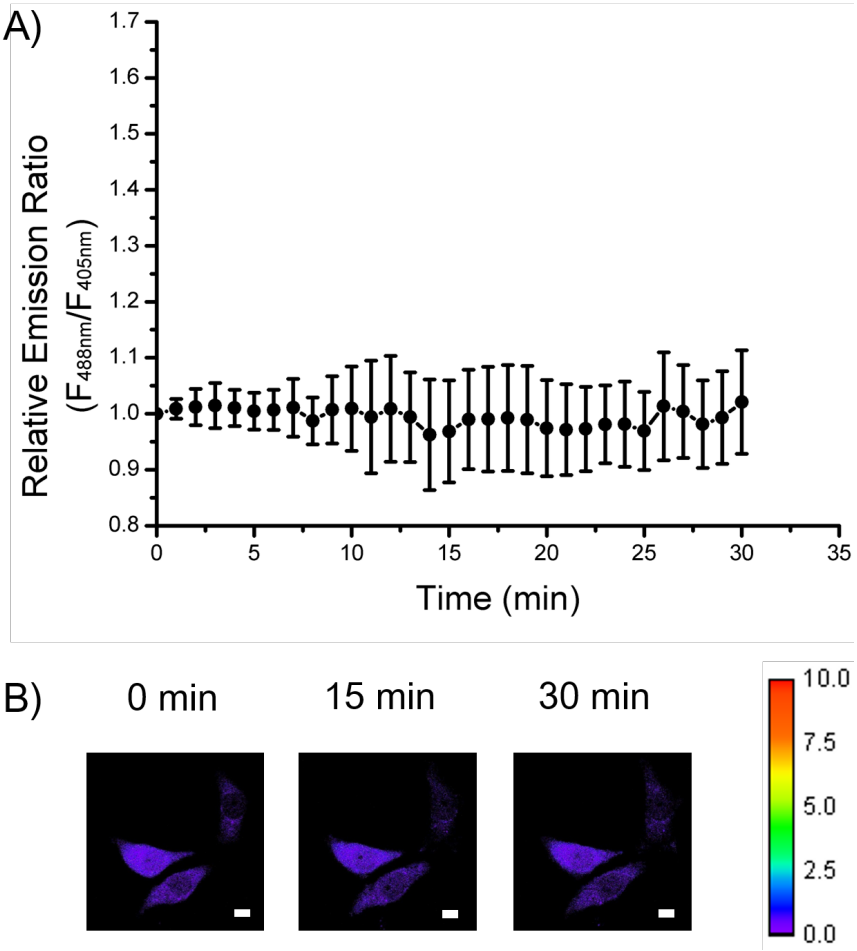

Figure S8. The fluorescence intensity change of rOHSer-mut with excessive glucose treatment. (A) HeLa cells expressing rOHSer-mut were treated with 100 mM D-glucose and the images with excitation at 405 nm or 488 nm were recorded with a 1-min interval over a 30-minute period. The fluorescence ratios of the same cell excited with two different wavelengths at different time were calculated and plotted. The relative emission ratio was the ratio of fluorescent intensity with 488 nm excitation by that with 405 nm excitation. Error bars represent ±s.d.. (B) The pseudocolor ratiometric fluorescence images of representative rOHSer-mut-expressing cells treated with D-glucose for different time. Scale bars represent 10 μm.

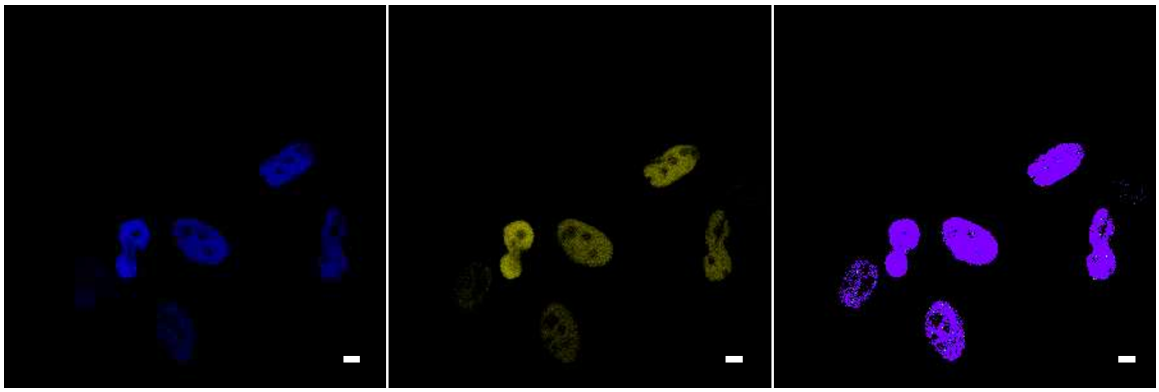

Figure S9. The demonstration of advantages of the ratiometric sensor over intensiometric ones. One major drawback of protein-based fluorescent indicators is the inconsistency of basal fluorescence caused by the difference in protein expression levels. Ratiometric sensors, however, are capable of overcoming such drawback by exhibiting a change in the emission fluorescence ratio between two excitation wavelengths upon recognition of the target molecules. Under the same redox condition, the ratio between two emission wavelengths stays the same, regardless of the absolute fluorescent intensity of the cells, thus normalizing the difference in expression levels of the indicator. For instance, in this figure several cells with different basal fluorescence levels were shown. After generating the pseudocolor ratiometric fluorescence images based on the ratio of fluorescent intensity with 488 nm excitation by that with 405 nm excitation, the intrinsic difference was diminished and cells showed similar fluorescent patterns, confirming the normalizing effect of ratiometric property of rOHSer. From left to right, fluorescent image with 405 nm excitation, fluorescent image with 488 nm excitation, pseudocolor ratiometric fluorescence image. Scale bars represent 10 μm.