SUPPLEMENTARY INFORMATION

Monitoring methionine sulfoxide with stereospecific mechanism-based fluorescent sensors

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Supplementary Results

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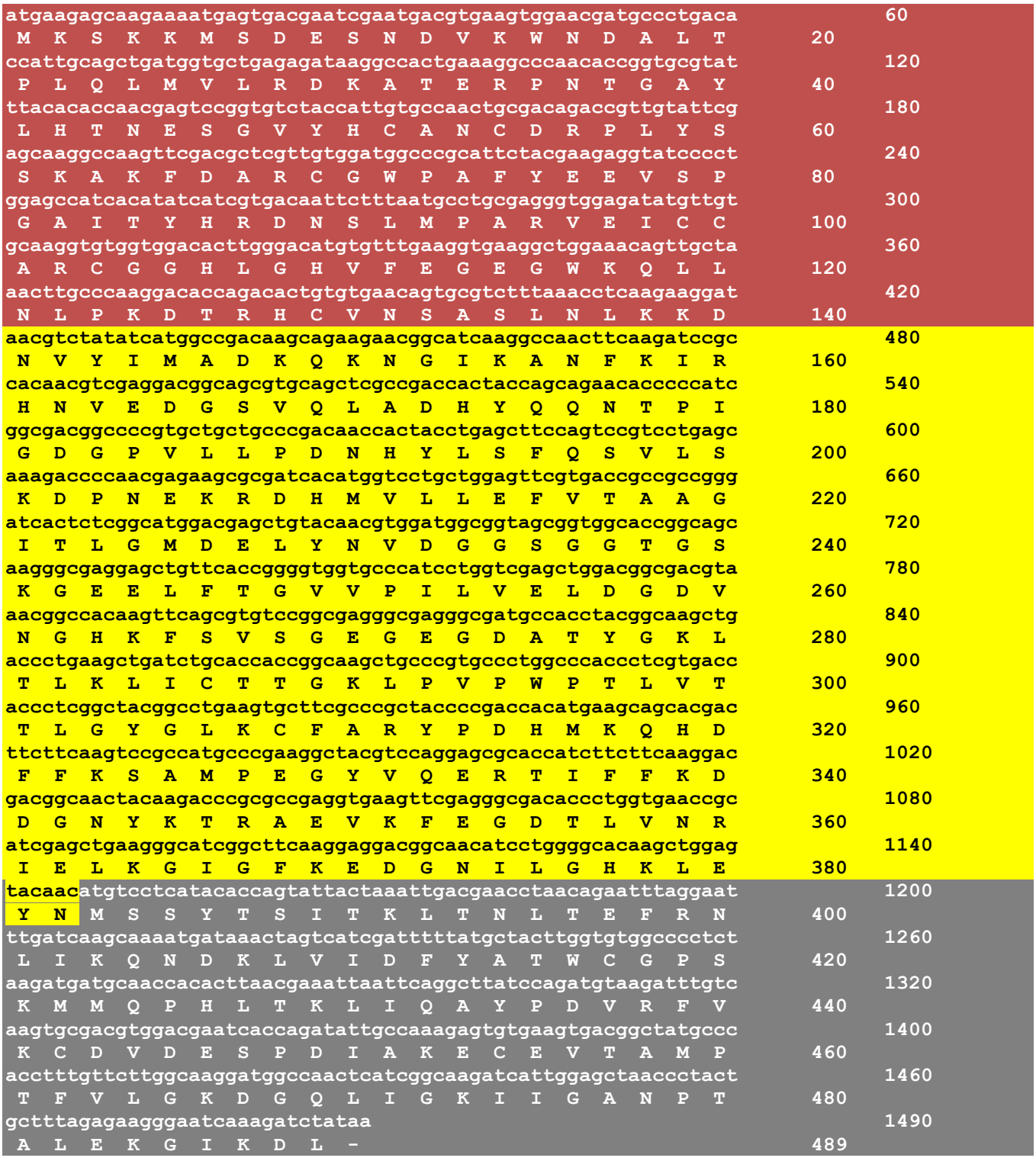
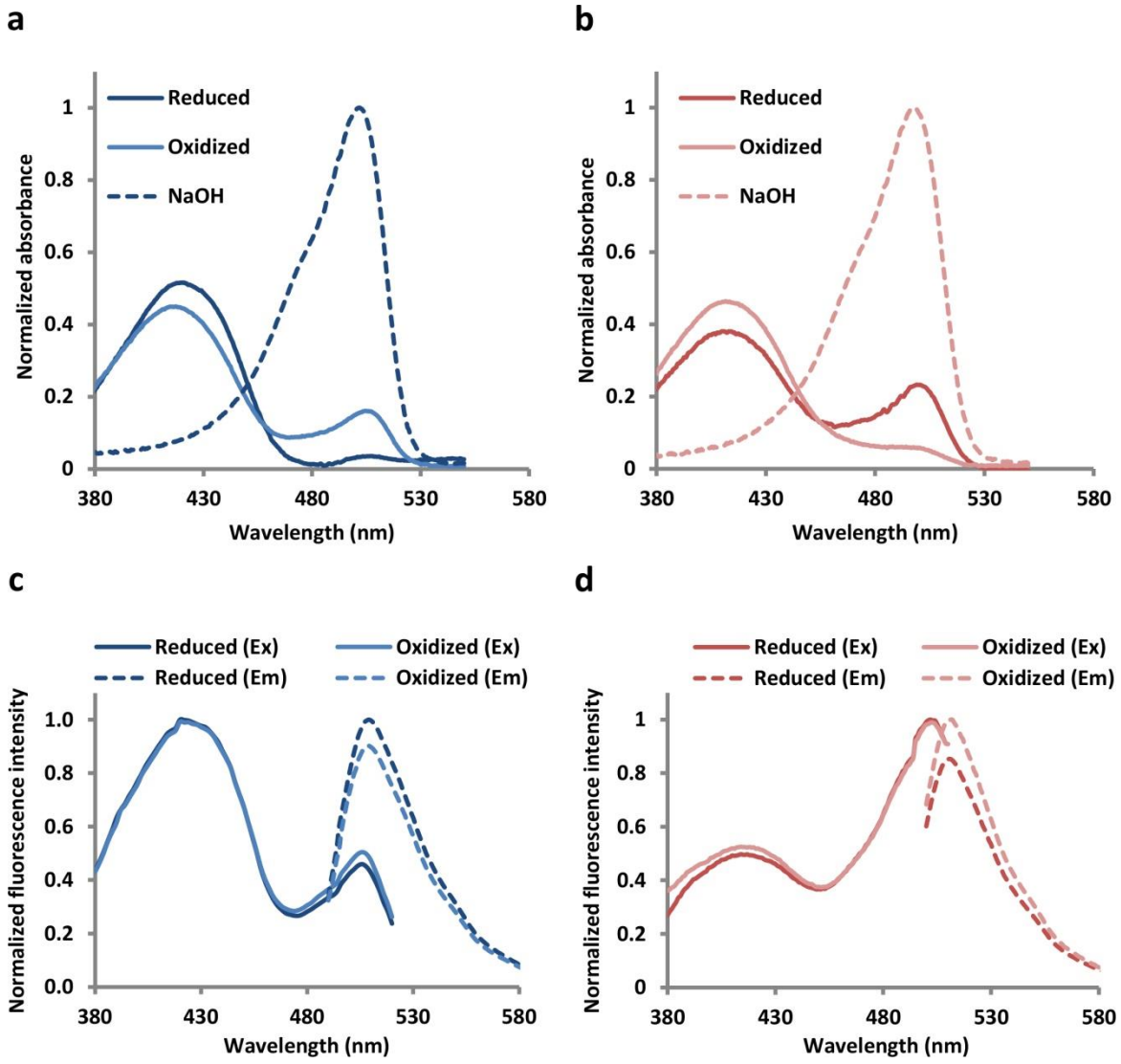
Supplementary Fig. 1. MetSOx DNA and protein sequences.

MSRA, cpYFP and Trx1 moieties are highlighted in *blue*, *yellow* and *grey*, respectively.



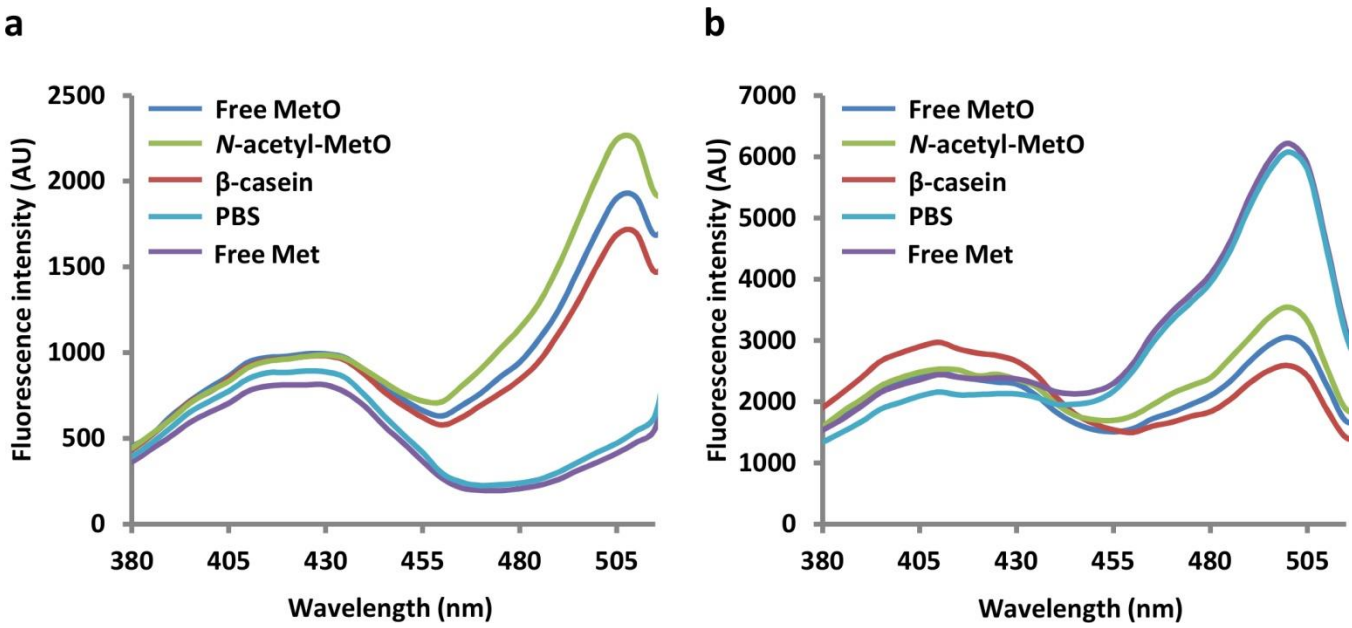
Supplementary Fig. 2. MetROx DNA and protein sequences.

MSRB, cpYFP and Trx3 moieties are highlighted in *red*, *yellow* and *dark grey*, respectively.



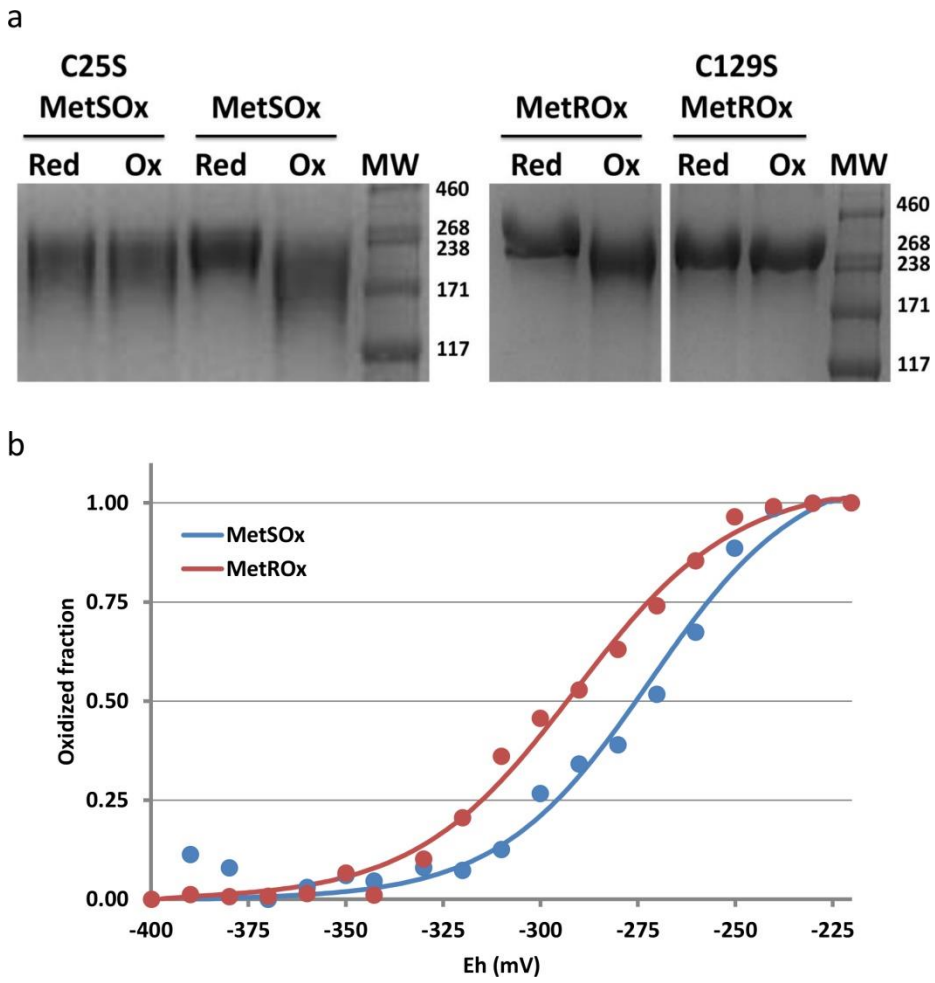
Supplementary Fig. 3. Absorbance spectra of MetSOx and MetROx and fluorescence spectra of C25S MetSOx and C129S MetROx.

(**a**) Absorbance spectra of reduced, oxidized and denatured MetSOx. Extinction coefficients for absorbance at 425 and 505 nm (or 500 nm for denatured) were estimated by measuring absorbance spectra at 3 protein concentrations. (**b**) Absorbance spectra of reduced, oxidized and denatured MetROx. Extinction coefficients for absorbance at 410 and 500 nm were estimated by measuring absorbance spectra at 3 protein concentrations. (**c**) Excitation (Ex) (*full line*) and emission (Em) (*dashed line*) spectra of reduced (*dark blue*) and oxidized inactive C25S MetSOx (*light blue*). Spectra were normalized to the oxidized form. (**d**) Excitation (Ex) (*full line*) and emission (Em) (*dashed line*) spectra of reduced (*dark red*) and oxidized inactive C129S MetROx (*pink*). Spectra were normalized to the reduced form. Sensors were reduced with 10 mM DTT and oxidized with 10 μM MRP4.



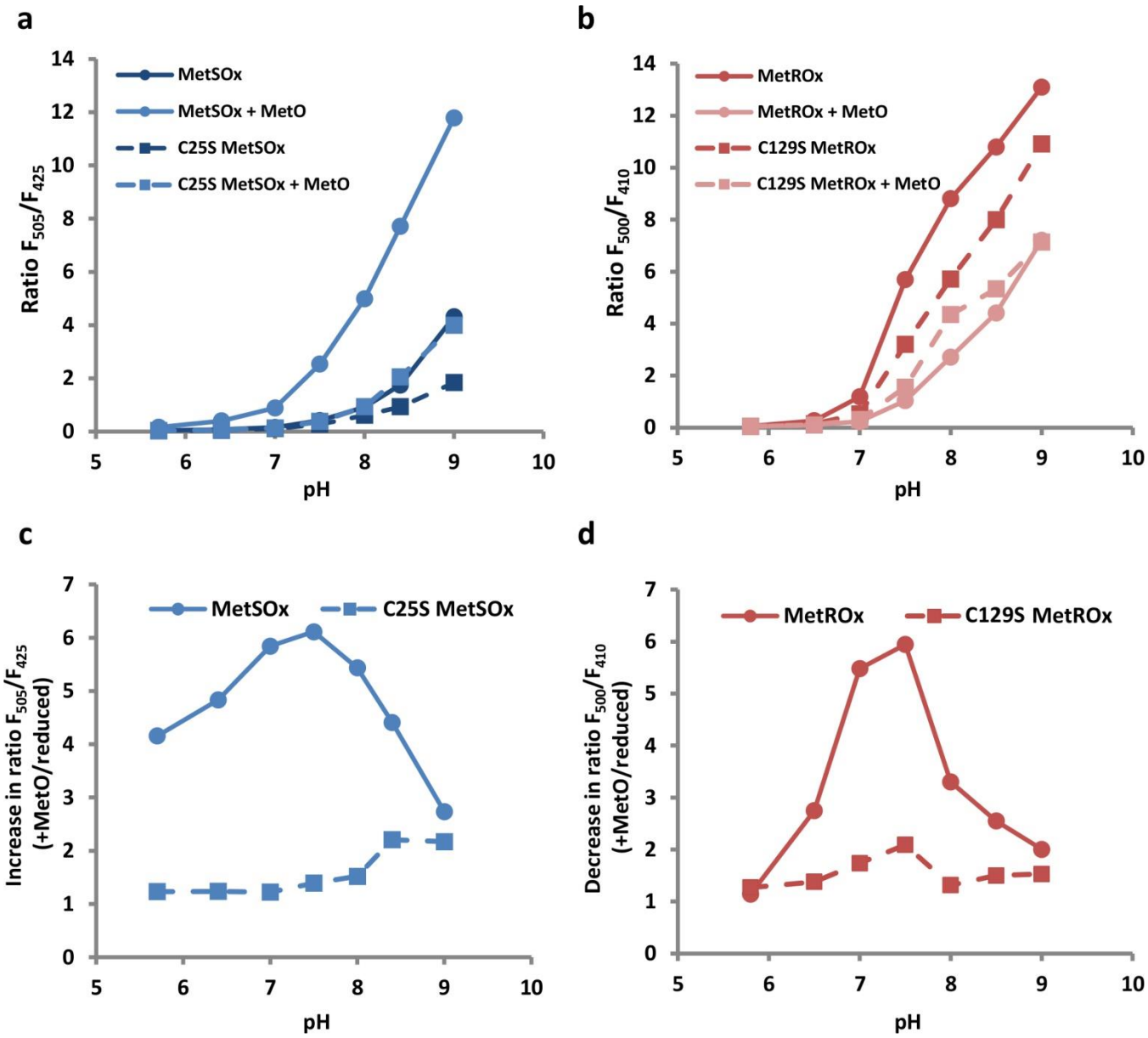
Supplementary Fig. 4. Spectra of MetSOx and MetROx incubated with MetO-containing substrates.

Excitation spectra of MetSOx (**a**) and MetROx (**b**) with various MetO-containing substrates. Reduced sensors (1 µM) were incubated with free L-Met-*R,S*-O (100 µM), oxidized β-casein (10 µM), *N*-acetyl-L-Met-*R,S*-O (10 µM), PBS or free L-Met (100 µM) for 1h.



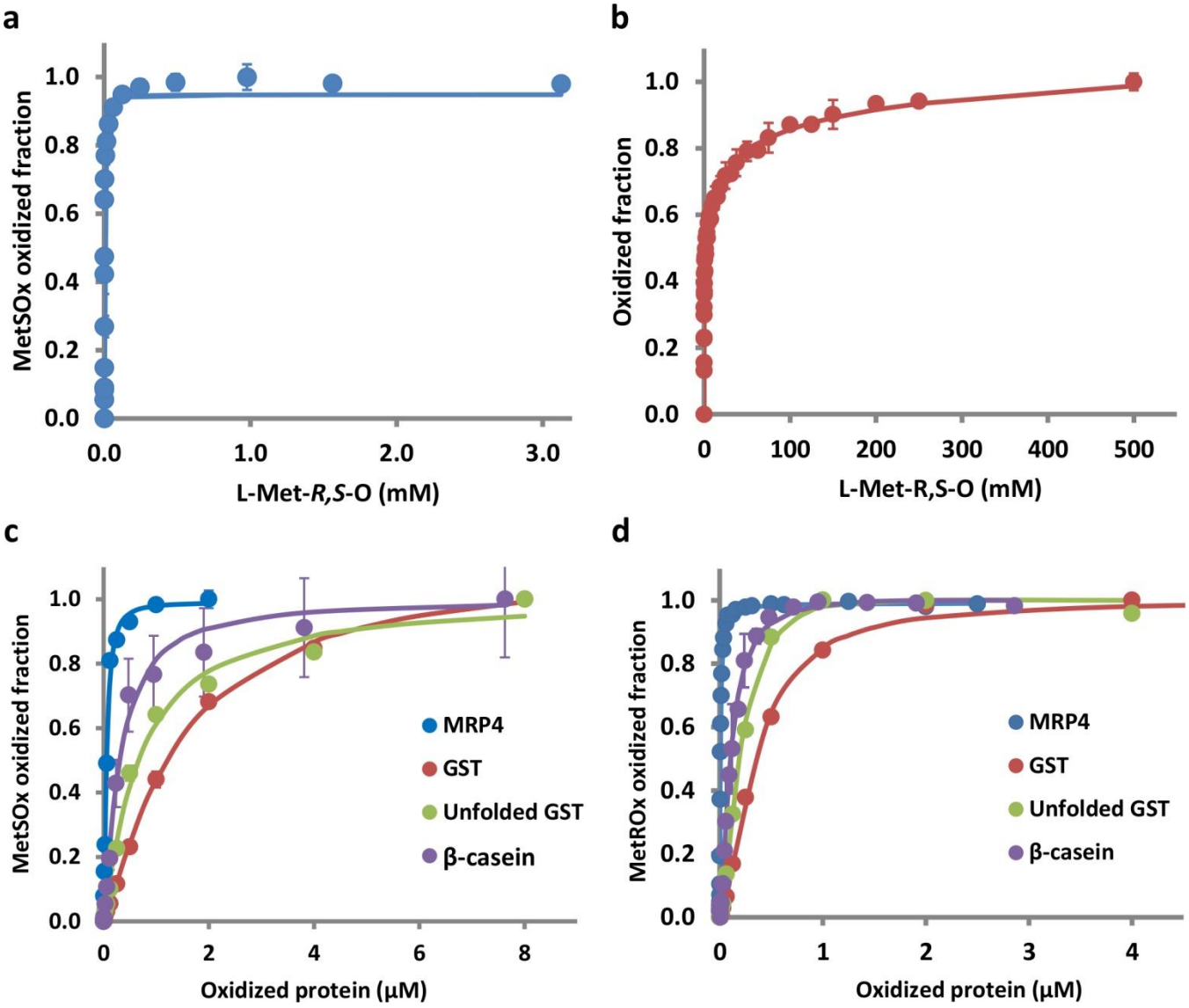
Supplementary Fig. 5. Redox characterization of MetSOx and MetROx.

(**a**) Cys alkylation assay. Reduced or oxidized MetSOx (left panel) and MetROx (right panel), and their inactive versions were alkylated with Mal-PEG and loaded on 7% Tris-Acetate gels. Mal-PEG added 5 kDa per alkylated Cys. (**b**) Redox midpoints of MetSOx and MetROx at pH 7.0 were determined by fluorescence measurements after incubation of oxidized sensors with mixture of oxidized and reduced DTT (10 mM) at defined E_h. The percentages of oxidized fraction as a function of E_h were fitted to the Nernst equation (n = 2) using nonlinear regression. E_m values were -276 ± 6 mV and -293 ± 4 mV for MetSOx and MetROx, respectively. Data presented are representative of 3 replicates.



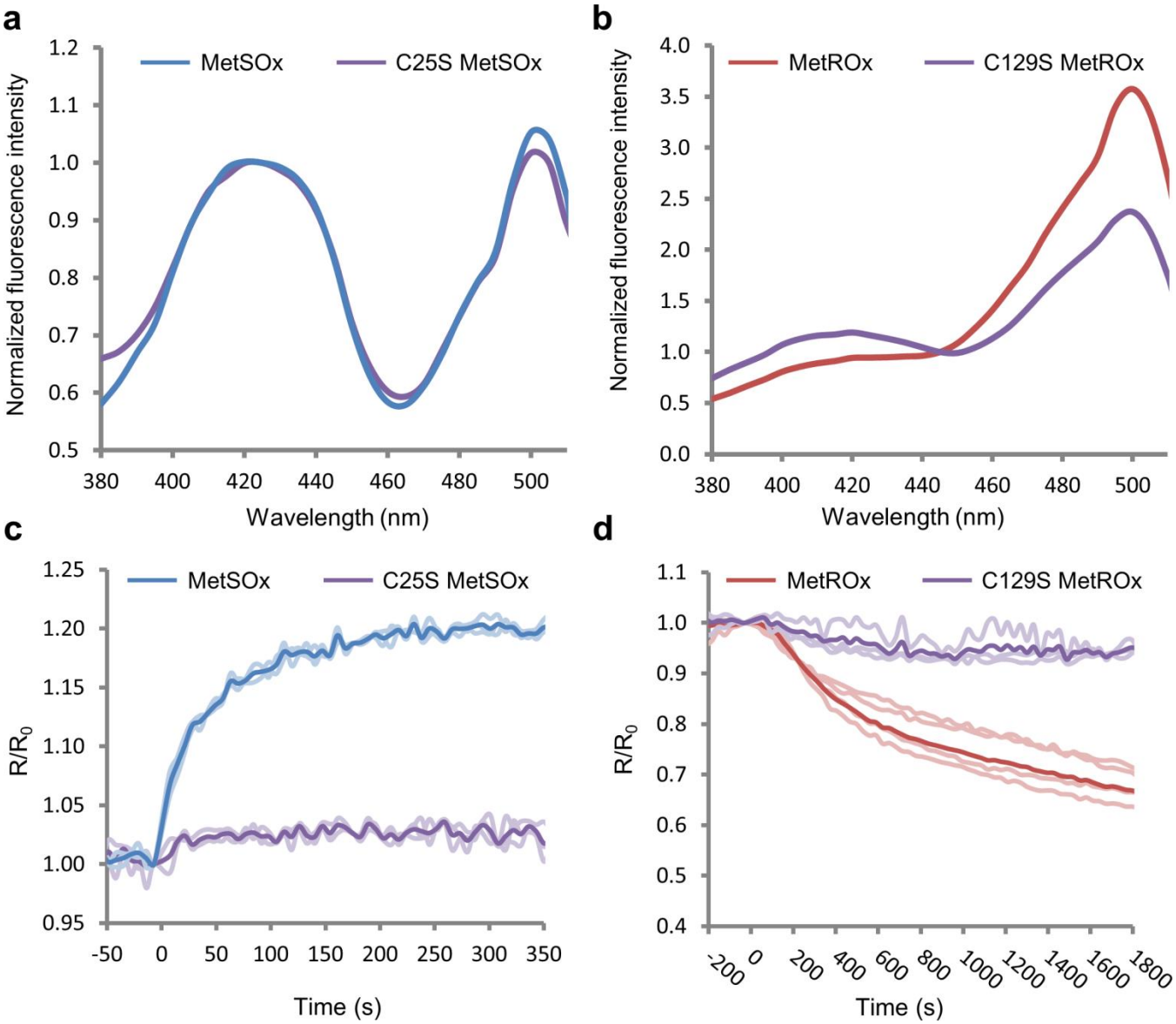
Supplementary Fig. 6. pH-dependence of MetSOx and MetROx.

The ratio and dynamic range of MetSOx and its inactive C25S MetSOx form as a function of pH (**a**, **c**). Dynamic range was calculated by dividing the measured ratio of the oxidized sensors (+MetO) by the measured ratio of the reduced sensors. The ratio and dynamic range of MetROx and its inactive C129S MetROx form as a function of pH (**b, d**). Dynamic range was calculated by dividing the measured ratio of the reduced sensors by the measured ratio of the reduced sensors (+MetO).



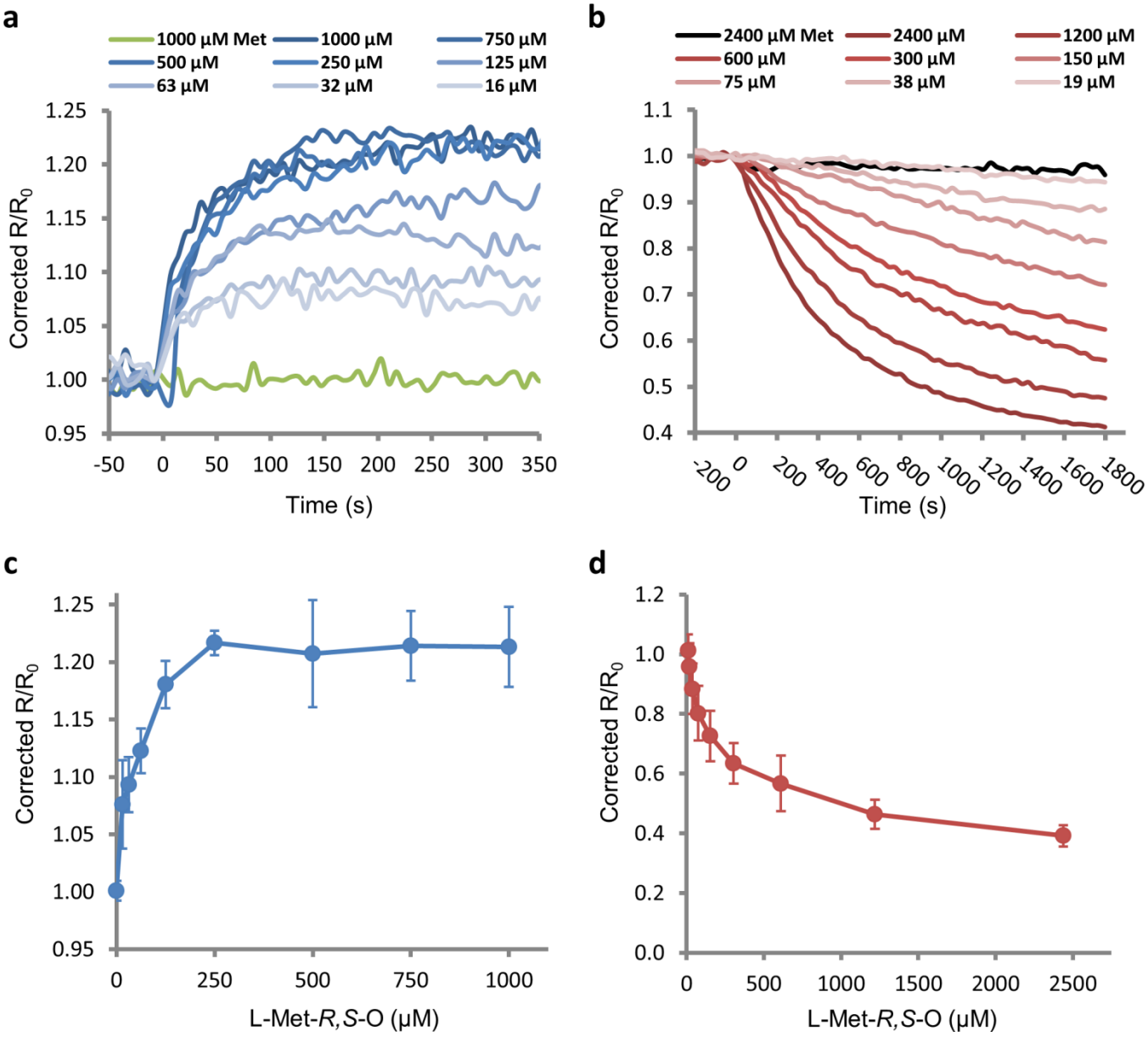
Supplementary Fig. 7. Saturation curves of MetSOx and MetROx to MetO-containing substrates.

Free L-Met-*R,S*-O dose response of MetSOx (**a**) and MetROx (**b**). MetO concentration was from 12 nM to 3 mM for MetSOx and from 24 µM to 100 mM for MetROx. Dose responses of MetSOx (**c**) and MetROx (**d**) to oxidized proteins. Reduced MetO sensors were incubated with oxidized MRP4 (8 nM to 2 µM), oxidized GST (8 nM to 8 µM), unfolded oxidized GST (8 nM to 8 µM) and oxidized β-casein (6 nM to 7.6 µM) for 1h. The oxidized fraction was calculated from the ratio of fully reduced and maximal oxidation state observed. Data presented are the means (n = 3) ± SD and are representative of 3 replicates.



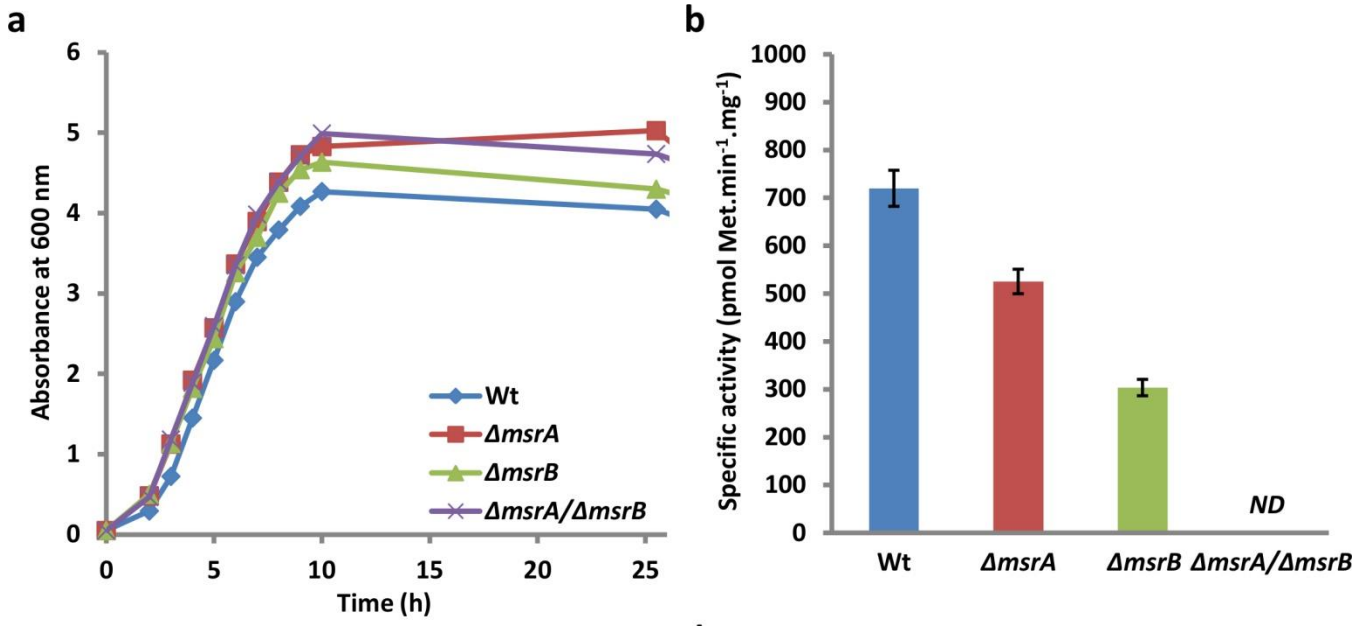
Supplementary Fig. 8. Use of inactive version of MetSOx and MetROx in *E. coli*.

(**a**) Excitation spectra of *E. coli* cells expressing MetSOx and its inactive C25S MetSOx form. Fluorescence intensity was normalized by the intensity at 425 nm. (**b**) Excitation spectra of *E. coli* cells expressing MetROx and its inactive C129S MetSOx form. Fluorescence intensity was normalized by the intensity at 445 nm. (**c**) Kinetics of the response of MetSOx and C25S MetSOx in *E. coli* suspensions to free MetO. Cells in M9 media were incubated with 125 µM free MetO. The ratio of fluorescence $F_{505\ nm}/F_{425\ nm}$ was normalized by the value at t = 0 s. (**d**) Kinetics of the response of MetROx and C129S MetSOx in *E. coli* suspensions to free MetO. Cells in M9 media were incubated with 150 µM free MetO. The ratio of fluorescence $F_{500\ nm}/F_{410\ nm}$ was normalized by the value at t = 0 s. Light and dark colors correspond to replicate measurements and average, respectively. Data presented are representative of 3 replicates.



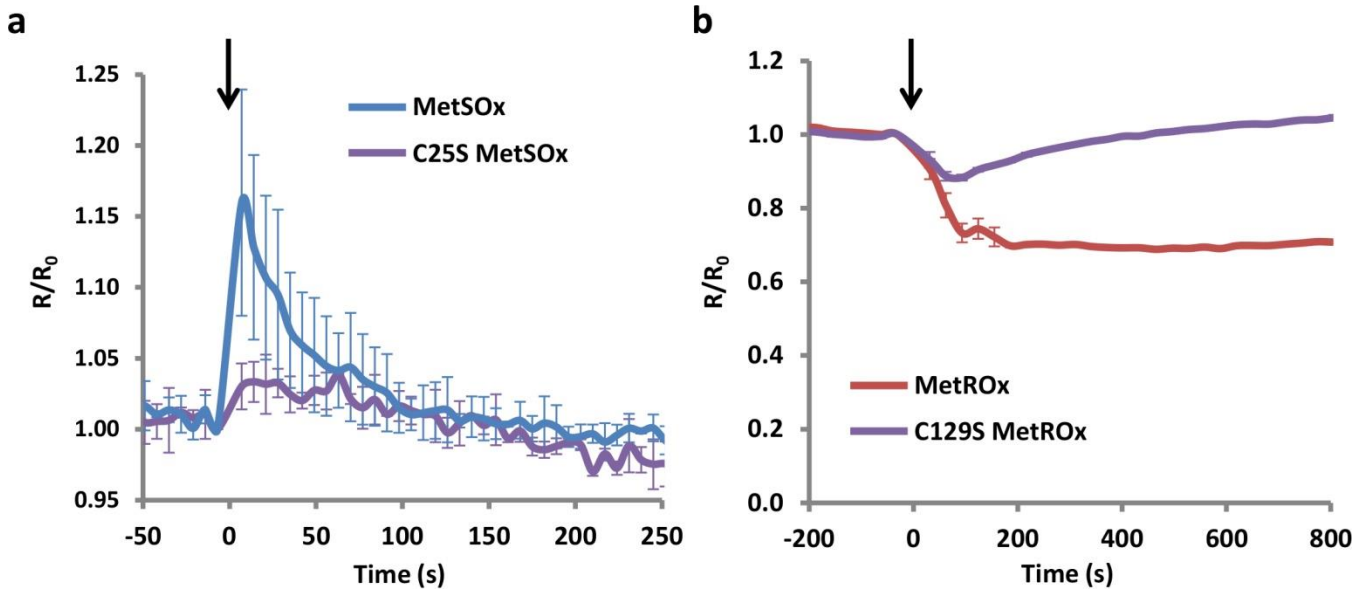
Supplementary Fig. 9. MetO calibration in *E. coli*.

Kinetics of the response of MetSOx (**a**) and MetROx (**b**) expressed in *E. coli* to increasing concentrations of free MetO. Cells in M9 media were incubated with free MetO or free Met. The ratio of fluorescence was normalized by the value at t = 0 s and corrected by the normalized ratio of the inactive sensor. This experiment was done twice (**a**) and thrice (**b**). The ratio was calculated after complete kinetics corrected by the ratio obtained with inactive sensors (panels **a** and **b**). Free L-Met-*R,S*-O dose response of MetSOx (**c**) and MetROx (**d**) in *E. coli*. MetO concentration was from 16 µM to 1,000 µM for MetSOx and from 19 µM to 2,400 µM for MetROx. Data presented are the means (n = 3) ± SD.



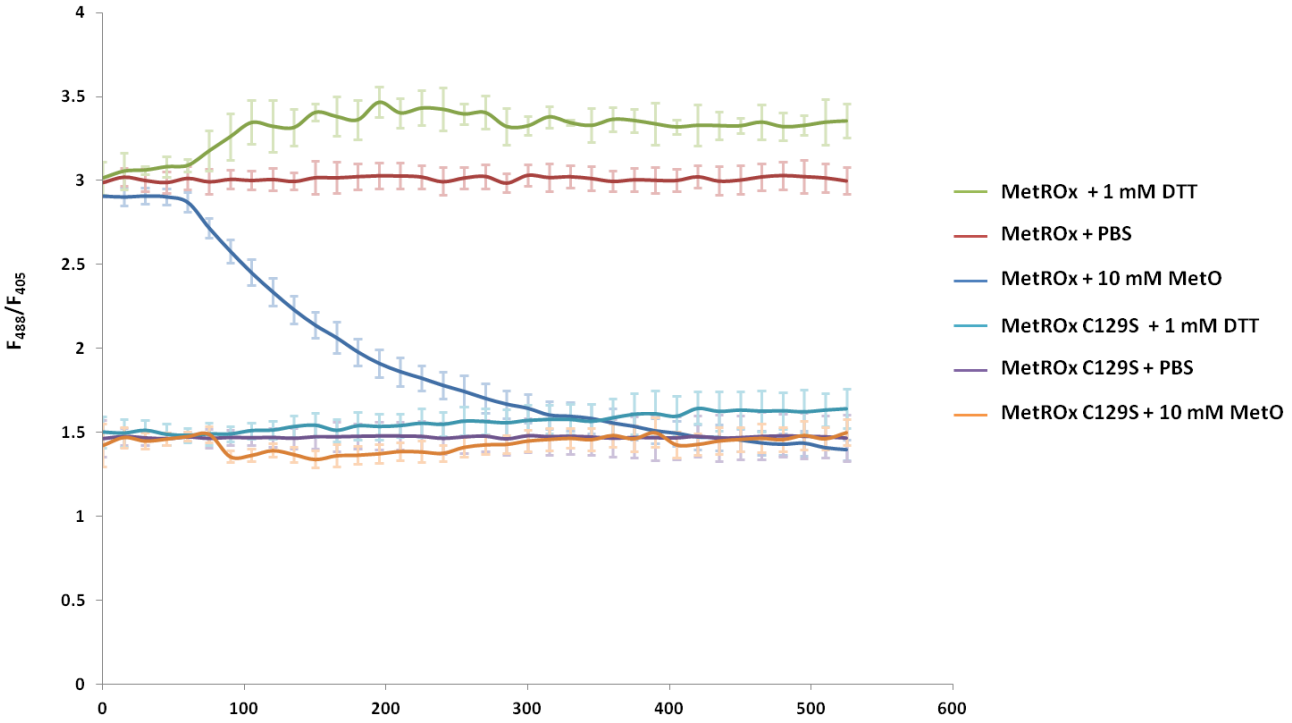
Supplementary Fig. 10. Growth curves and MSR activity of *E. coli* Wt and *msr* mutants.

(**a**) Growth curves of Wt, single Δ*msrA* and Δ*msrB* mutants and the double Δ*msrA*/Δ*msrB* mutant. Strains were grown in LB medium and absorbance values at 600 nm were recorded. (**b**) Total specific MSR activities of Wt and *msr* mutants. After overnight growth, total specific MSR activity was determined using dabsyl-MetO as substrate and an HPLC-based method. *ND*, not detected. Data presented are the means (n = 3) ± SD and are representative of 3 replicates.



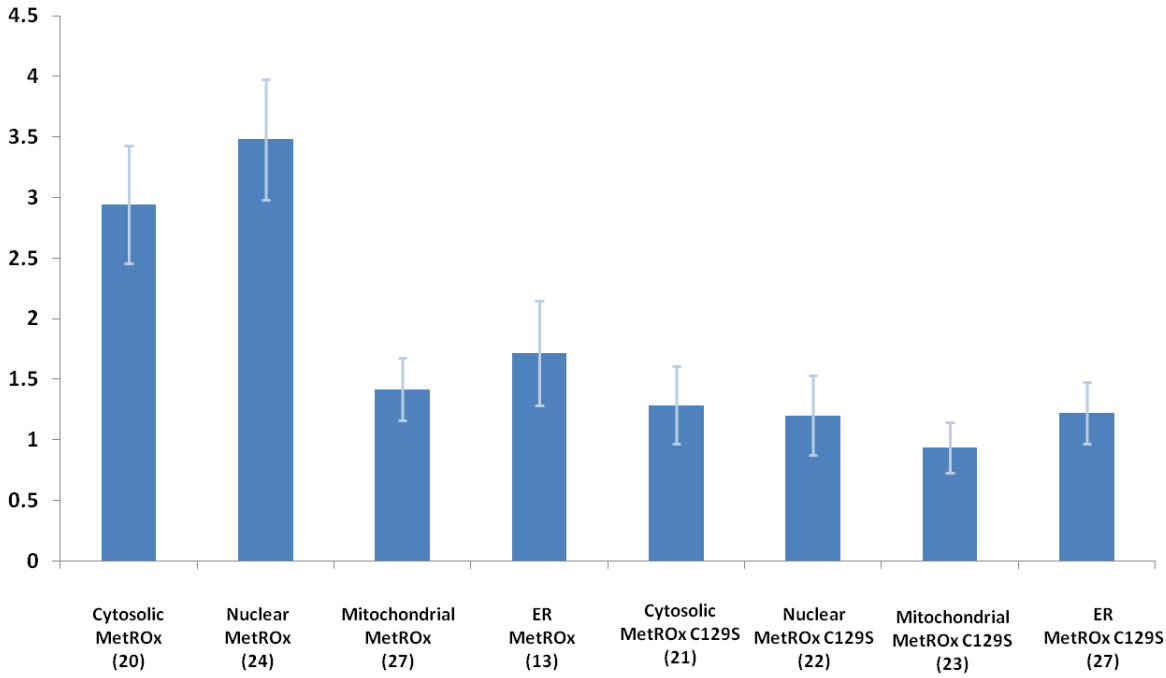
Supplementary Fig. 11. Kinetics of reactivity of *E. coli* Wt and *msr* mutants expressing MetSOx, MetROx or their inactive versions to NaOCl.

(**a**) Dynamics of the intracellular response of *E. coli* Wt mutants expressing MetSOx or C25S MetSOx to 100 µM NaOCl. Strains were grown in LB for 20 h, rinsed and equilibrated in M9 medium, and the ratio $F_{505\ nm}/F_{425\ nm}$ was recorded for cells expressing MetSOx and the inactive C25S MetROx form. (**b**) Dynamics of the intracellular response of *E. coli* Wt MetROx to 40 µM NaOCl. Strains were grown in LB for 20 h, rinsed and equilibrated in M9 medium and the ratio $F_{500\ nm}/F_{410\ nm}$ was recorded for cells expressing MetROx and the inactive C129S MetROx form. Data presented are the means (n = 3) ± SD and are representative of 3 replicates.



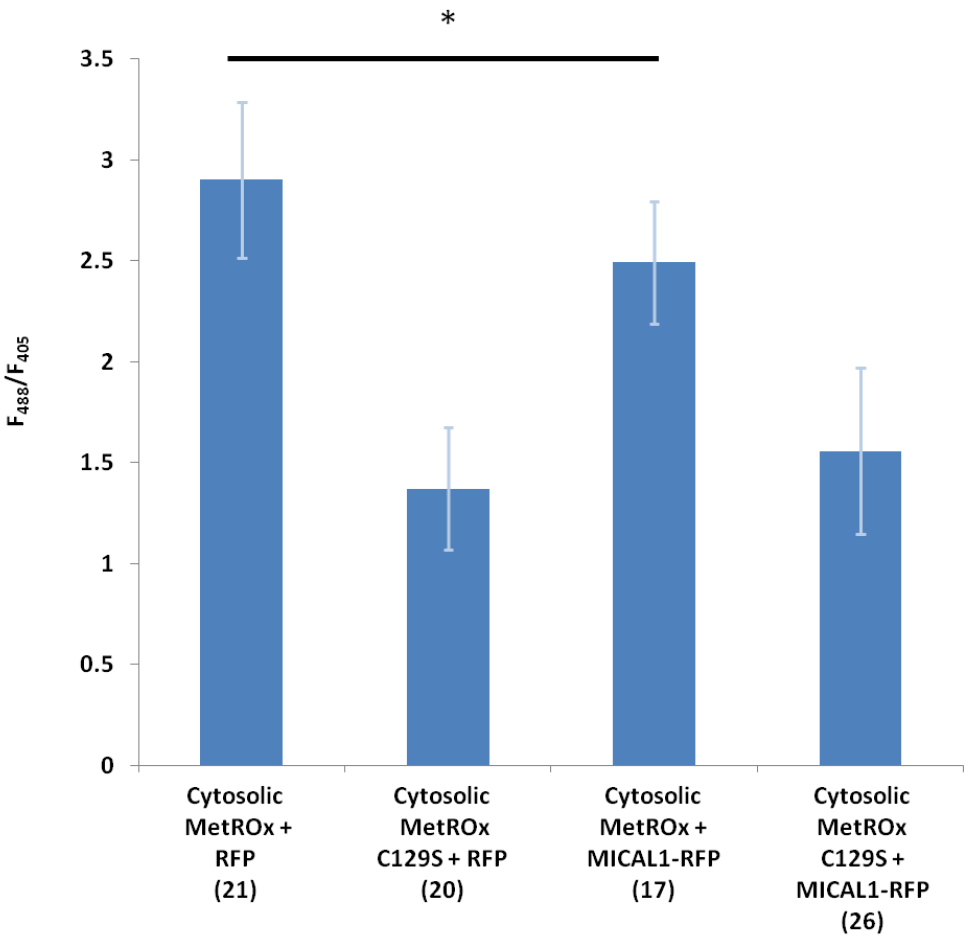
Supplementary Fig. 12. MetROx ratio changes of HEK293 cells in response to MetO and DTT.

HEK293 cells expressing non-targeted MetROx and MetROx C129S were subjected to 1 mM DTT or 10 mM MetO. The corrected ratio values after the equilibrium were ~1.5 for MetO and ~3.5 for DTT. These values represent the fully oxidized and reduced forms of the sensor in the cytosol of mammalian cells. Consistent with **Fig 6b** and by comparison with the data obtained with the recombinant sensors, and similarly to *E. coli* assays, the data allowed proposing an empiric scale wherein MetROx was half and fully oxidized with ~100 µM and ~1,000 µM Met-*R*-O, respectively. Data presented are the means (n = 5-8) ± SD and are representative of 3 replicates.

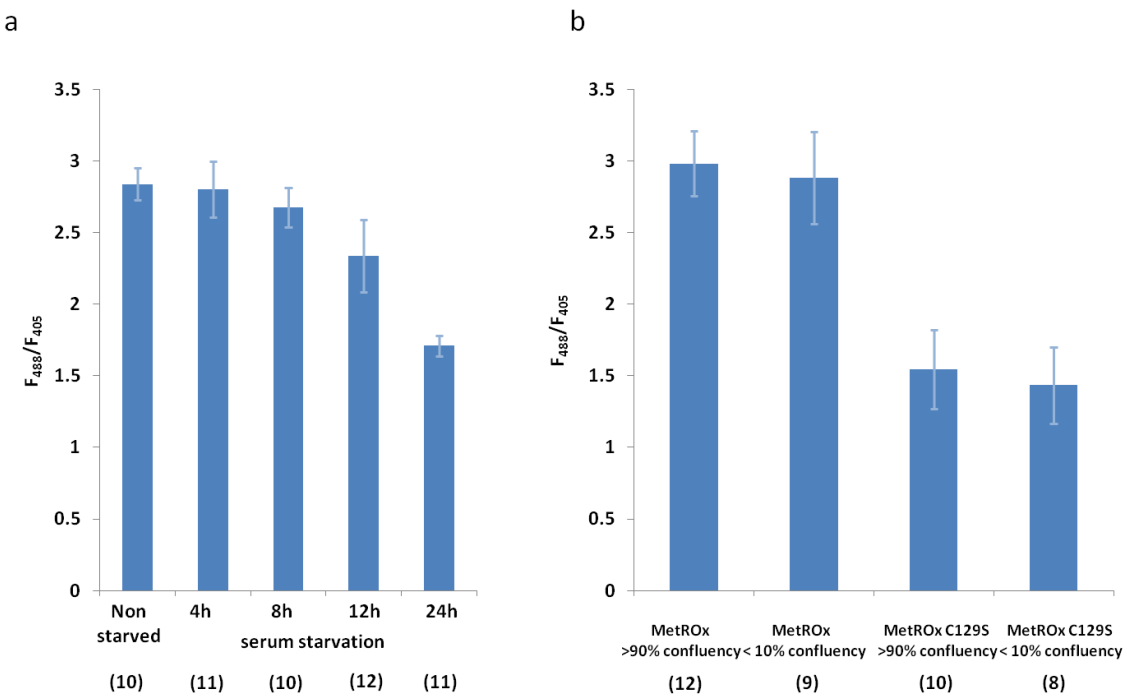


Supplementary Fig. 13. Variation in the MetROx and C129S MetROx fluorescence ratio in different cellular compartments.

HEK293 cells expressing MetROx or C129S MetROx constructs targeted to different cellular compartments were subjected to live cell confocal microscopy and the captured images were analyzed to calculate the MetROx ratio in different compartments. The number of cells used for the analysis is indicated in parentheses. Data presented are the means ± SD and are representative of 3 replicates.



Supplementary Fig 14. MetROx ratio changes upon expression of the constitutively active MICAL1. Fluorescent confocal microscopy images of live HEK293 cells expressing MetROx, or C129S MetROx along with either RFP or the constitutively active MICAL1-RFP were analyzed to calculate the MetROx ratio. Expressing RFP alone altered neither the MetROx nor the inactive mutant C129S MetROx fluorescent ratio (compare values with **Supplementary Fig. 13**). The expression of the constitutively active MICAL1 caused a significant decrease in MetROx fluorescence, but left the inactive sensor unchanged. The number of cells used for the analysis is indicated in parentheses. Data presented are the means± SD, * $p < 0.05$ and are representative of 3 replicates.



Supplementary Fig. 15. MetROx response to serum starvation and confluency.

(a) HEK293 cells expressing MetROx constructs were subjected to serum starvation for indicated time periods followed by live cell confocal microscopy, and the images were analyzed to calculate the MetROx ratio. The number of cells used for the analysis is indicated in parentheses. Data is presented as mean ± SD. **(b)** HEK293 cells expressing MetROx and C129S MetROx were cultured at different confluency for 24 h and then subjected to live cell confocal microscopy. We did not detect any differences in the MetROx ratio below 10% and over 90% confluency. The number of cells used for the analysis is indicated in parentheses. Data presented are the means ± SD and are representative of 3 replicates.

Supplementary Table 1. Spectral properties of MetSOx and MetROx.

			Reduced			Oxidized		
	λ Abs (nm)	λ Em (nm)	ε ($M^{-1}.cm^{-1}$)	Quantum yield	Brightness ($M^{-1}.cm^{-1}$)	ε ($M^{-1}.cm^{-1}$)	Quantum yield	Brightness ($M^{-1}.cm^{-1}$)
MetSOx								
	425	510	20,700	0.08	1,600	17,700	0.08	1,400
	505	516	1,300	0.81	1,000	5,300	0.67	3,500
MetROx								
	410	510	12,500	0.15	1,900	15,700	0.12	1,800
	500	516	5,800	0.71	4,100	2,200	0.67	1,500

Supplementary Table 2. Dose-response of MetSOx and MetROx to MetO-containing substrates.

	Apparent stoichiometry[a] (mol of MetO reduced. mol sub^{-1})	$K_{0.5}$ (μM)	Corrected $K_{0.5}$[b] (μM of MetO)	Concentration range		
				Minimum concentration (for oxidized fraction = 0.1) (μM)	Maximum concentration (for oxidized fraction = 0.9) (μM)	Max / Min
MetSOx						
Free MetO	0.5	0.985 ± 0.085	0.492 ± 0.043	0.115	18.000	157
MRP4	4.16 ± 0.16	0.025 ± 0.004	0.105 ± 0.019	0.003	0.250	83
GST	1.21 ± 0.02	1.914 ± 0.156	2.13 ± 0.176	0.169	4.840	29
Unfolded GST	3.25 ± 0.05	1.392 ± 0.187	4.524 ± 0.608	0.082	4.445	55
β-casein	2.49 ± 0.15	0.343 ± 0.045	0.854 ± 0.112	0.037	2.440	66
MetROx						
Free MetO	0.5	352.900 ± 63.690	176.450 ± 31.845	2.100	$165.000x10^{3}$	78,571
MRP4	7.18 ± 0.66	0.006 ± 0.001	0.043 ± 0.007	$6.700x10^{-4}$	0.054	80
GST	0.78 ± 0.02	0.521 ± 0.082	0.406 ± 0.064	0.043	1.900	44
Unfolded GST	4.66 ± 0.24	0.779 ± 0.090	3.631 ± 0.418	0.029	1.085	38
β-casein	3.19 ± 0.34	0.136 ± 0.016	0.434 ± 0.050	0.013	0.555	42

[a] Apparent stoichiometries with the MSR moiety were previously calculated[26]. [b] $K_{0.5}$ were corrected by multiplication with apparent stoichiometry to remove the variation due to the different numbers of MetO reduced in each substrate. Data presented are the means (n = 3) ± SD.

Supplementary Table 3. Oligonucleotides used in this study.

#	Name	Sequence	Purpose
1	MSRA_cpYFP_For	5'-cattatctgagagaaatgaacgtctatatcatggc-3'	Fuse MSRA to cpYFP
2	MSRA_cpYFP_Rev	5'-gccatgatatagacgttcatttctctcagataatg-3'	
3	cpYFP_Trx1_For	5'- caagctggagtacaacatggttactcaattcaaaactgc-3'	Fuse cpYFP to Trx1
4	cpYFP_Trx1_Rev	5'-gcagttttgaattgagtaaccatgttgtactccagcttg-3'	
5	MSRB_cpYFP_For	5'-gtctttaaacctcaagaaggataacgtctatatcatggcc-3'	Fuse MSRB to cpYFP
6	MSRB_cpYFP_Rev	5'-ggccatgatatagacgttatccttcttgaggtttaaagac-3'	
7	cpYFP_Trx3_For	5'-gcacaagctggagtacaacatgtcctcatacaccag-3'	Fuse cpYFP to Trx3
8	cpYFP_Trx3_Rev	5'-ctggtgtatgaggacatgttgtactccagcttgtgc-3'	
9	MetSOx_*Bam*HI_For	5'-ctctatctggatccatgtcgtcgcttatttc-3'	Clone MetSOx in pGEX-4T1
10	MetSOx_*Not*I_Rev	5'-atatttatgcggccgcttaagcattagcagc-3'	
11	MetROx_*Bam*HI_For	5'-cacacacaggatccaagagcaagaaaatg-3'	Clone MetROx in pGEX-4T1
12	MetROx_*Not*I_Rev	5'-atatttatgcggccgcttaagcattagcagc-3'	
13	MetSOx_C25S_For	5'-cacattagcatgtggatctttttggggtac-3'	Cys 25 to Ser site directed mutagenesis (MSRA moiety)
14	MetSOx_C25S_Rev	5'-gtaccccaaaaagatccacatgctaatgtg-3'	
15	MetSOx _C459S_For	5'-ccacttggtgcggtccatctaaaatgattg-3'	Cys 459 to Ser site directed mutagenesis (Trx1 moiety)
16	MetSOx _C459S_Rev	5'-caatcattttagatggaccgcaccaagtgg-3'	
17	MetROx_C129S_For	5'-caccagacactctgtgaacagtgcgtc-3'	Cys 129 to Ser site directed mutagenesis (MSRB moiety)
18	MetROx_C129S_Rev	5'-gacgcactgttcacagagtgtctggtg-3'	
19	MetROx_C420S_For	5'-cttggtgtggcccctctaagatgatgc-3'	Cys 420 to Ser site directed mutagenesis (Trx3 moiety)
20	MetROx_C420S_Rev	5'-gcatcatcttagaggggccacaccaag-3'	
21	EcMSRA_FRT_For	5'-ctatgcttccggtggcagacagacgccaattccgcca attccacagtaacaattaaccctcactaaagggcg-3'	Create FRT cassette for MSRA deletion in *E. coli*
22	EcMSRA_FRT_Rev	5'-atgagtttatttgataaaaagcatctggtttcccccgc cgatgccctgcctaatacgactcactatagggctc-3'	
23	EcMSRB_FRT_For	5'-tcaaccgttgatttcttcgccgttttcgccatcggtaaag cgtaaagaggaattaaccctcactaaagggcg-3'	Create FRT cassette for MSRB deletion in *E. coli*
24	EcMSRB_FRT_Rev	5'-atggctaataaaccttcggcagaagaactgaaaaaaa atttgtccgagattaatacgactcactatagggctc-3'	
25	EcMSRA_For	5'-ctatgcttccggtggcagacagac-3'	Screen MSRA deletion
26	EcMSRA_Rev	5'-atgagtttatttgataaaaagcatctggtttcccccg-3'	
27	EcMSRB_For	5'-tcaaccgttgatttcttcgccgttttc-3'	Screen MSRB deletion
28	EcMSRB_Rev	5'-atggctaataaaccttcggcagaagaactg-3'	
29	MetROx_*Nhe*I_For	5'-gtcagatacgctagcatgaagagcaagaaaatg-3'	Clone MetROx in pEGFP-C3 for cytosolic localization
30	MetROx_*Bam*HI_Rev	5'-ctagacccggcggatccttatagatctttg-3'	
31	Mito_MetROx_For	5'-ccaagatccattcgttgaagagcaagaaaatgag-3'	Fuse Cytochrome C oxidase subunit VIII mitochondrial targeting sequence to MetSOx (with Mito_*Nhe*I_For)
32	Mito_MetROx_Rev	5'-ctcattttcttgctcttcaacgaatggatcttgg-3'	

Supplementary Table 3 (*continued*). Oligonucleotides used in this study.

33	ER_MetROx_For	5'-ccacctccgccaccaagagcaagaaaatgag-3'	Fuse SelM endoplasmic reticulum targeting sequence to MetROx (with ER_*Nhe*I_For)
34	ER_MetROx_Rev	5'-ctcattttcttgctcttggtggcggaggtgg-3'	
35	MetROx_ER_*Bam*HI_Rev	5'-attattattggatccttacagctcgtcctttagatctttgattc-3'	Add KDEL peptide and clone MetROx in pEGFP-C3 for endoplasmic reticulum localization
36	MetROx_Nucleus_For	5'-gggaatcaaagatctagatccaaaaaagaag-3'	Add nuclear localization signal (NLS) from SV40 large T antigen to MetSOx (with Nucleus_*BamHI*_Rev)
37	MetROx_Nucleus_Rev	5'-cttctttttggatctagatctttgattccc-3'	

Supplementary Table 4. Plasmids used in this study.

Name	Description
pGEX-MetSOx	Expression of MetSOx in *Escherichia coli*
pGEX-C25S MetSOx	Expression of inactive C25S MetSOx in *Escherichia coli*
pGEX-MetROx	Expression of MetROx in *Escherichia coli*
pGEX-C129S MetROx	Expression of inactive C129S MetROx in *Escherichia coli*
pCyto-MetROx	Expression of MetROx in cytosol of mammalian cell
pCyto-C129S MetROx	Expression of inactive C129S MetROx in cytosol of mammalian cell
pMito-MetROx	Expression of MetROx in mitochondria of mammalian cell
pMito-C129S MetROx	Expression of inactive C129S MetROx in mitochondria of mammalian cell
pNucleus-MetROx	Expression of MetROx in nucleus of mammalian cell
pNucleus-C129S-MetROx	Expression of inactive C129S-MetROx in nucleus of mammalian cell
pER-MetROx	Expression of MetROx in ER of mammalian cell
pER-C129S-MetROx	Expression of inactive C129S-MetROx in ER of mammalian cell