Supporting information appendix for

Real-time monitoring of peroxiredoxin oligomerization dynamics in living cells

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Supplementary methods

Ferrous oxidation of xylenol orange (FOX) assay

Reduced Prx2 or mCER-Prx2 (40 µM) was mixed with 40 µM H_2O_2 in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl at 25°C. After 15, 30, 60 and 300 seconds of reaction, 10 µL of the reaction was mixed with 490 µL of the FOX assay reaction mix (100 µM xylenol orange, 250 µM ammonium ferrous sulfate, 100 mM sorbitol and 25 mM H_2SO_4), and incubated for 30 min at room temperature in darkness. At the end of the reaction, absorbance at 560 nm was measured, and the H_2O_2 concentration was calculated based on a H_2O_2 standard curve.

Thioredoxin/thioredoxin reductase/NADPH-coupled assay of Prx2 and mCER-Prx2 variants

The assay was done as described in (1), with the following modifications: the assay was performed in an Omega plate reader (BMG Labtech) at 30°C and 250 µL final volume, and contained 200 µM NADPH, 2 µM thioredoxin reductase (TrxR) from *Corynebacterium glutamicum* (2), 10 µM thioredoxin from *E. coli* (3) and 200 µM H_2O_2. After 3 minutes of pre-incubation time, 0.5 µM of peroxiredoxin was added to initiate the reaction. The H_2O_2 reduction coupled to NADPH oxidation ($\Delta\epsilon_{340}$ = 6220 M^{-1} cm^{-1}) was measured by following the decrease in absorption at 340 nm. The path length was autocorrected with the Omega plate reader software.

In vitro plate reader polarization measurements

For measuring the concentration dependence of fluorescence polarization as facilitated by purified mCER-Prx2 and mCER-Prx2(C51S), 13 dilutions were prepared (5 µM, 3 µM, 1.98 µM, 1.31 µM, 0.86 µM, 0.57 µM, 0.38 µM, 0.25 µM, 0.16 µM, 0.11 µM, 0.07 µM, 0.05 µM, 0.031 µM). The proteins were diluted in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 10 mM dithiothreitol (DTT) in a final volume of 20 µL. The mixture was incubated for 10 min and then measured in a black/clear 384-well plate (BD Falcon) using the PHERAstar FS microplate reader (BMG Labtech) equipped with a fluorescence polarization filter, excitation at 430 nm and emission at 480 nm. 500mP of polarization with a gain of 50% were used to adjust the initial measurement to the well containing 8 µM of mCER-Prx2. Polarization was calculated using MARS data analysis software (BMG Labtech) according to the following equation:

$$[(I_{\parallel} - G \times I_{\perp}) / (I_{\parallel} + G \times I_{\perp})] \times 1000 = mP$$

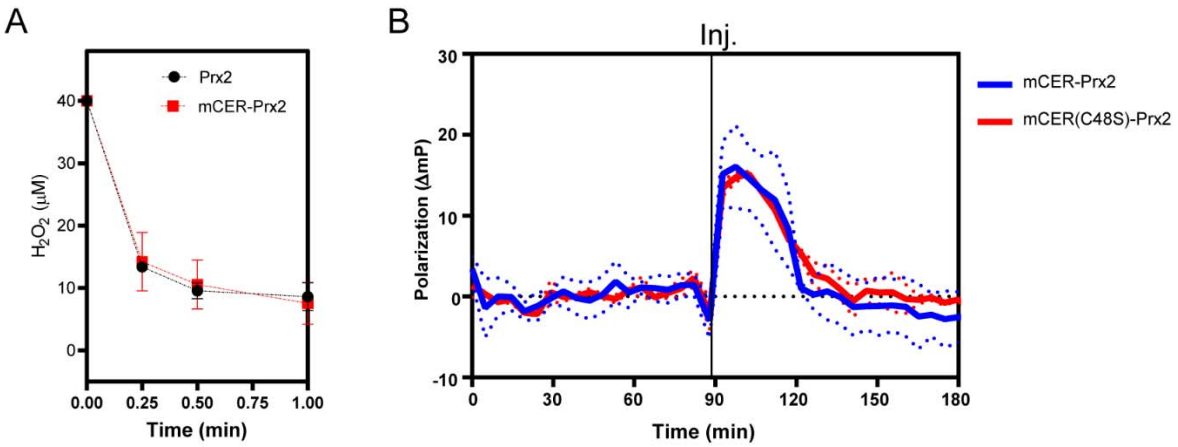
The G factor (compensation factor for the plate reader) was set automatically for each measurement based on gain adjustment settings.

Supplementary table

Table 1. Primers used for mutagenesis of mCER-Prx2 or roGFP2-Prx2

Mutation	Primer (5'-3')
C172S Forward	GGGGAAGTTTCTCCCGCTGGC
C172S Reverse	ATGCTCGTCTGTGTACTGG
C51S Forward	ACTTTTGTGTCCCCCACCGAG
C51S Reverse	GAAGTCCAGAGGGTAGAAAAAG
A82E,T85E Forward	CGGGGGGTGTTGATCCACTCCAGGTGCTCGAACTGAGAGTCCACCG
A82E,T85E Reverse	CGGTGGACTCTCAGTTCGAGCACCTGGAGTGGATCAACACCCCCCG
S76D Forward	GTGAAGTGCTGGGCGTCGACGTGGACTCTCAGTTCAC
S76D Reverse	GTGAACTGAGAGTCCACGTCGACGCCCAGCACTTCAC
C172A Forward	GCCAGCGGGAGCAACTTCCCCATGCTCGTCT
C172A Reverse	AGACGAGCATGGGGAAGTTGCTCCCGCTGGC

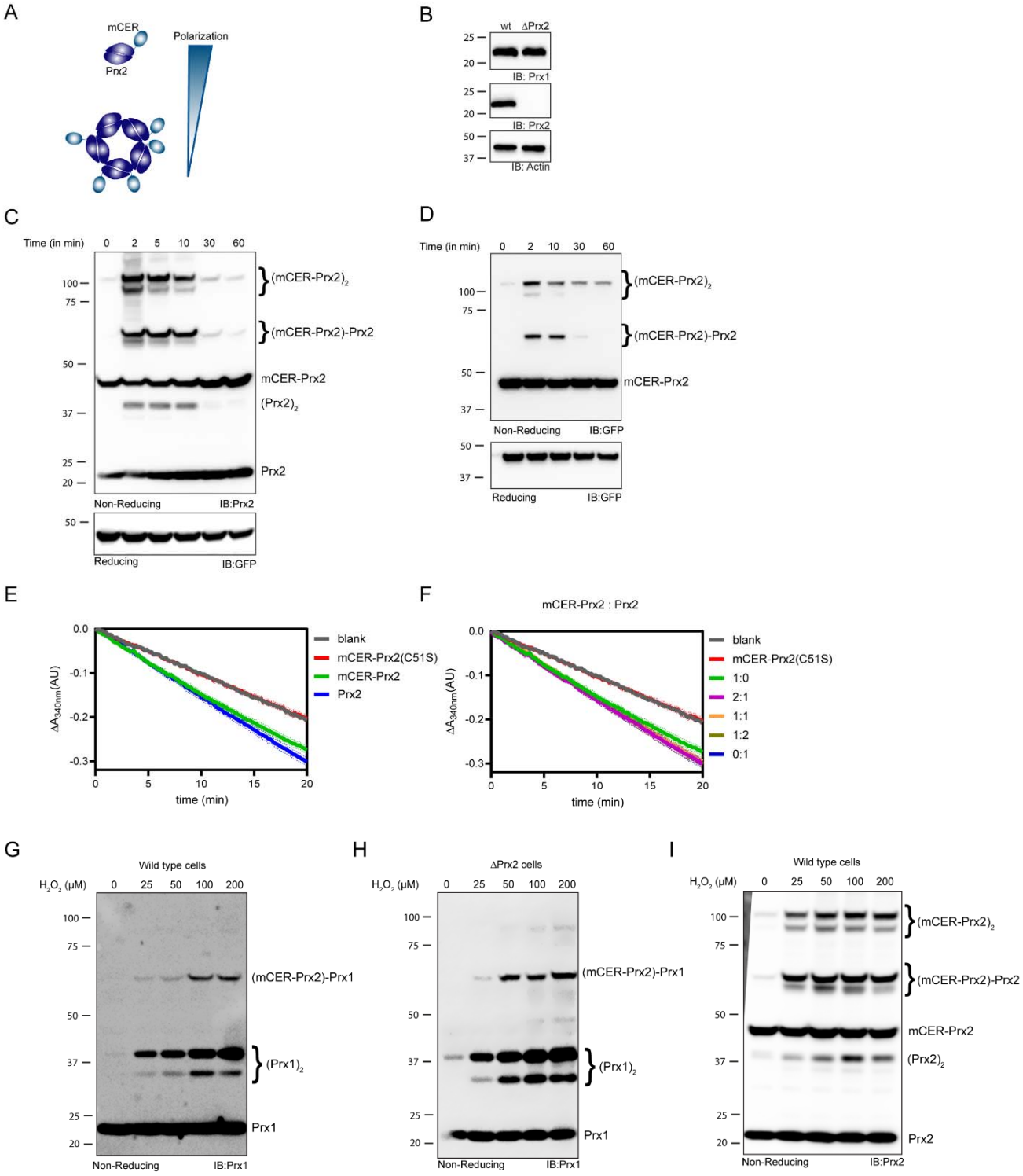
Supplementary Figure 1



(*A*) Recombinant Prx2 and mCER-Prx2 do not differ in H_2O_2 reactivity. Purified Prx2 and mCER-Prx2 (40 µM) were incubated with an equimolar amount of H_2O_2. The remaining concentration of H_2O_2 was determined with the FOX assay at the indicated time points. Based on 3 biological replicates.

(*B*) The surface exposed cysteine Cys-48 of mCER does not affect the probe response. Comparison of the polarization change induced by H_2O_2 (100 µM) in cells expressing either mCER-Prx2 or mCER(C48S)-Prx2. Based on 3 biological replicates with 6 technical replicates each.

Supplementary Figure 2



(*A*) Schematic depiction of fluorophore crowding in dimers vs. decamers. Assuming a mCER-Prx2:Prx2 molar ratio of 1:1, the average dimer will carry one fluorophore and the average decamer five fluorophores. The crowding of fluorophores in the decamer supports energy transfer and hence fluorescence depolarization.

(*B*) Immunoblot confirming the lack of Prx2 protein expression in the Prx2 KO (ΔPrx2) HEK293 cell line.

(*C*) Time course following a 100 µM H_2O_2 bolus treatment of HEK293 cells stably expressing mCER-Prx2, analyzed by anti-Prx2 immunoblotting. $(mCER\text{-}Prx2)_2$ and $(Prx2)_2$ indicate the respective covalent (disulfide-linked) homodimers, and (mCER-Prx2)-Prx2 indicates the corresponding covalent (disulfide-linked) heterodimer.

(*D*) Time course following a 100 µM H_2O_2 bolus treatment of HEK293 cells stably expressing mCER-Prx2, analyzed by anti-GFP immunoblotting.

(*E*) Comparison of Prx2 and mCER-Prx2 activity coupled to the thioredoxin/thioredoxin reductase/NADPH system. The decrease in A_{340}, reflecting NADPH oxidation, was monitored after the addition of Prx2 or mCER-Prx2 to the reaction mixture, containing thioredoxin, thioredoxin reductase, NADPH and H_2O_2. The mCER-Prx2(C51S) protein was used as a negative control. Based on three replicates.

(*F*) Mixtures of Prx2 and mCER-Prx2 (yielding heterodimers) exhibit a peroxidatic activity not significantly different from Prx2 peroxidatic activity. The decrease in A_{340}, reflecting NADPH oxidation, was monitored after the addition of Prx2 or mCER-Prx2 to the reaction mixture, containing thioredoxin, thioredoxin reductase, NADPH and H_2O_2. The mCER-Prx2(C51S) protein was used as a negative control. Based on three replicates.

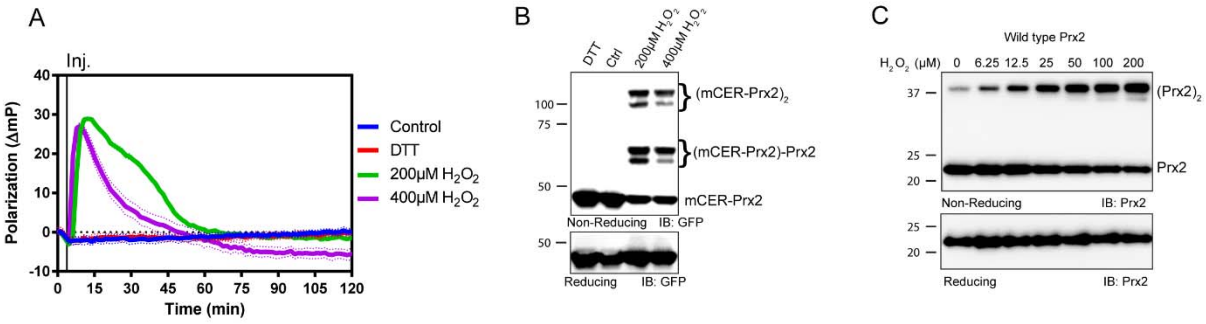
(*G*) Detection of (mCER-Prx2)-Prx1 disulfide-linked heterodimers in Prx2-proficient cells, 2 min after H_2O_2 treatment, analyzed by anti-Prx1 immunoblotting.

(*H*) Detection of (mCER-Prx2)-Prx1 disulfide-linked heterodimers in Prx2-deficient (Prx2-KO) cells, 2 min after H_2O_2 treatment, analyzed by anti-Prx1 immunoblotting.

(*I*) The same experiment as in (G), analyzed by anti-Prx2 immunoblotting.

All immunoblots in this figure are representative of 3 replicates.

Supplementary Figure 3

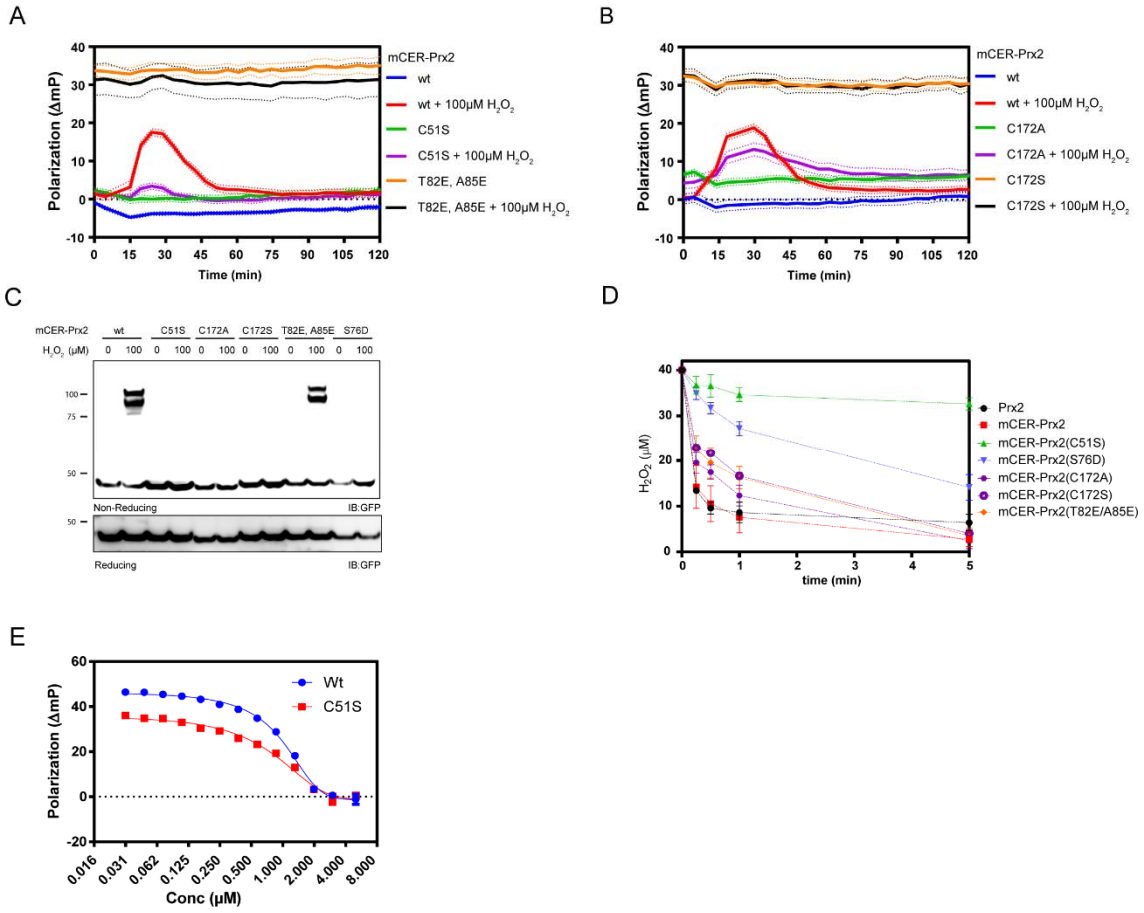


(*A*) Maximal polarization is reached at 200 μM H_2O_2, as shown by comparison with a 400 μM H_2O_2 treatment. The response at 400 μM is affected by hyperoxidation, as explained in the main text. Based on 3 biological replicates with 6 technical replicates each.

(*B*) Maximal disulfide-linked dimer formation is reached at 200 μM H_2O_2, as shown by anti-GFP immunoblotting using the same cells as in (*A*), 2 min after H_2O_2 exposure. Representative of 3 replicates.

(*C*) Covalent dimerization of endogenous Prx2 in HEK293 cells in the absence of mCER-Prx2, 2 min after H_2O_2 exposure. Representative of 3 replicates.

Supplementary Figure 4



(*A*) Fluorescence polarization response facilitated by mCER-Prx2, mCER-Prx2(C51S) and mCER-Prx2(T82E/A85E). Based on 3 biological replicates with 6 technical replicates each.

(*B*) Fluorescence polarization response facilitated by mCER-Prx2, mCER-Prx2(C172A) and mCER-Prx2(C172S). Based on 3 biological replicates with 6 technical replicates each.

(*C*) mCER-Prx2 and mCER-Prx2(T82E/A85E), but not the other mutants, form covalent homodimers upon H_2O_2 exposure. Cells transiently expressing the indicated mCER-Prx2 mutants were analyzed by anti-GFP immunoblotting. Representative of 3 replicates.

(*D*) H_2O_2 reduction kinetics for Prx2, mCER-Prx2 and corresponding mutants. Purified proteins (40 μM) were incubated with an equimolar amount of H_2O_2, and the remaining concentration of H_2O_2 was measured at the indicated time points with the FOX assay. Based on 3 biological replicates.

(*E*) Fluorescence polarization facilitated by recombinant mCER-Prx2 and mCER-Prx2(C51S) proteins at concentrations between 0.031 and 5 μM. Based on 3 biological replicates.

Supplementary references

1. Nelson KJ & Parsonage D (2011) Measurement of peroxiredoxin activity. *Curr Protoc Toxicol* Chapter 7:Unit7 10.
2. Ordonez E*, et al.* (2009) Arsenate reductase, mycothiol, and mycoredoxin concert thiol/disulfide exchange. *J Biol Chem* 284(22):15107-15116.
3. El Hajjaji H*, et al.* (2009) The zinc center influences the redox and thermodynamic properties of Escherichia coli thioredoxin 2. *J Mol Biol* 386(1):60-71.