A Targetable Fluorescent Probe for Imaging Hydrogen Peroxide in the Mitochondria of Living Cells

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Synthetic Materials and Methods. All reactions were carried out under a dry nitrogen atmosphere. (4-Iodobutyl)triphenylphosphonium (IBTP)[1] and 2-(2,4-dihydroxybenzoyl)benzoic acid (**1**)[2] were synthesized according to literature methods. Silica gel P60 (SiliCycle) was used for column chromatography. Analytical thin layer chromatography was performed using SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. MitoTracker Deep Red and LysoTracker Red were purchased from Invitrogen (Carlsbad, CA). ^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra were collected in $CDCl_3$ or 9:1 $CDCl_3$/CD_3OD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC on a Bruker AV-300, AVQ-400, or DRX-500 spectrometer at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard δ notation of parts per million using the peak of residual proton signals of $CDCl_3$ as an internal reference. Mass spectral analyses were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley. Microwave reactions were performed using a CEM Intelligent Explorer/Discover (Matthews, NC).

Fmoc-piperazine rhodol (3). 2-(2,4-Dihydroxybenzoyl)benzoic acid (**1**, 1.24 g, 4.8 mmol) and 1-(3-hydroxyphenyl)-piperazine (**2**, 853 mg, 4.8 mmol) were added to a pressure flask and dissolved in 20 mL of TFA. The reaction was stirred for 3 hours at 95 °C. After cooling, the reaction mixture was poured into 300 mL of ether. The resulting precipitate was collected, immediately redissolved in methanol, and then evaporated to dryness under reduced pressure to yield a red solid. The crude product was carried on without further purification. The crude fluorophore (1.09 g), Fmoc-Cl (845 mg, 3.27 mmol), and $NaHCO_3$ (686 mg, 8.16 mmol) were added to a dry Schlenk tube. Then, 20 mL of dry acetonitrile was added and the reaction stirred under a nitrogen atmosphere at room temperature for 3 hours. The product was then extracted into ethyl acetate, washed with water, and dried under reduced pressure. Purification by flash chromatography (1:1 hexanes/ethyl acetate) provided **3** as a red solid (654 mg, 39% overall yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.99 (1H, d, *J* = 7.6 Hz), 7.74 (2H, d, *J* = 7.6 Hz), 7.57-7.66 (2H, m), 7.55 (2 H, d, *J* = 7.6 Hz), 7.37 (2H, t, *J* = 7.2 Hz), 7.29 (2H, t, *J* = 7.2 Hz), 7.14 (1H, d, *J* = 7.6 Hz), 6.71 (1H, d, *J* = 2.0 Hz), 6.65 (1H, d *J* = 2.0 Hz), 6.49-6.63 (4H, m), 4.47 (2H, d, *J* = 6.4 Hz), 4.22 (1H, t, *J* = 6.4 Hz), 3.55 (4H, bs), 3.14 (4H, bs). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.03, 159.59, 155.23, 152.82, 152.65, 152.58, 152.41, 143.76, 141.29, 134.92, 129.63, 129.15, 128.81, 127.73, 127.07, 127.00, 125.06, 124.82, 124.18, 129.97, 112.59, 112.18, 110.35, 109.87, 102.82, 102.33, 67.37, 47.99, 47.25, 43.20 (broad multiplet). HR-FABMS: calculated for [M^+] 623.2171, found 623.2182.

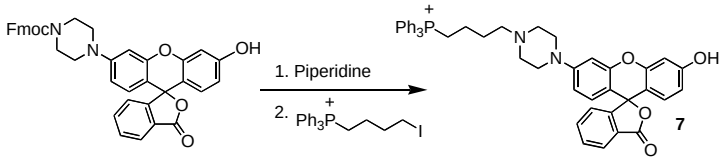
Fmoc-piperazine rhodol triflate (4). Rhodol **3** (400 mg, 0.64 mmol), *N*-phenyl bis(trifluoromethanesulfonamide) (458 mg, 1.28 mmol), and sodium carbonate (340 mg, 3.21 mmol) were added to a dry Schlenk tube. Then, 8 mL of dry DMF was added via syringe and the reaction stirred under a nitrogen atmosphere at room temperature for 12

hours. The reaction mixture was then extracted into ethyl acetate, washed with water, and dried under reduced pressure. Column chromatography (1:1 hexanes/ethyl acetate) afforded **4** as a white solid (222 mg, 46% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.04 (1H, d, *J* = 7.2 Hz), 7.76 (2H, d, *J* = 7.6 Hz), 7.69 (1H, dt, *J* = 1.2, 7.6 Hz), 7.64 (1H, dt, *J* = 7.6, 1.2 Hz), 7.57 (2H, d, *J* = 7.2 Hz), 7.38 (2H, t, *J* = 7.2 Hz), 7.30 (2H, dt, *J* = 1.2, 7.2 Hz), 7.23 (1H, d, *J* = 2.4 Hz), 7.16 (1H, d, *J* = 7.2 Hz), 6.94 (1H, dd, *J* = 2.4, 8.8 Hz), 6.88 (1H, d, *J* = 8.8 Hz), 6.70 (1H, d, *J* = 2.0 Hz), 6.66 (1H, d, *J* = 8.8 Hz), 6.61 (1H, dd, *J* = 2.0, 8.8 Hz), 4.48 (2H, d, *J* = 2.4 Hz), 4.23 (1H, t, *J* = 2.4 Hz), 3.56 (4H, bs), 3.16 (4H, bs). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 169.18, 155.09, 152.77, 152.49, 152.18, 151.85, 149.93, 143.83, 141.31, 135.37, 130.14, 130.06, 128.73, 127.73, 127.07, 126.36, 125.23, 124.86, 123.87, 119.98, 119.86, 116.52, 112.78, 110.42, 108.77, 102.23, 81.96, 67.29, 47.87, 47.30, 43.33 (broad multiplet). HR-FABMS: calculated for [MNa^+] 777.1494, found 777.1501.

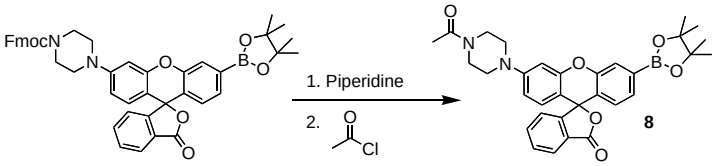
Fmoc-piperazine rhodol boronate (5). Triflate **4** (71 mg, 0.28 mmol), Pd (dppf) Cl_2•CH_2Cl_2 (68 mg, 0.08 mmol), potassium acetate (82 mg, 0.8 mmol), and 10 mL of toluene were added to a dry pressure tube in an inert atmosphere glove box. The pressure tube was then brought out of the box and microwave-heated for 4 hours at 110 °C. After cooling the reaction to room temperature, the contents of the pressure flask were washed into a round bottom flask with dichloromethane and evaporated to dryness. Purification by column chromatography (1:1 hexanes/ethyl acetate) delivered **5** as a white solid (151 mg, 74% yield). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.02 (1H, d, *J* = 6.4 Hz), 7.77 (3H, t, *J* = 7.6 Hz), 7.56-7.68 (4H, m), 7.37-7.45 (3H, m), 7.33 (2H, t, *J* = 8.0 Hz), 7.22 (1H, d, *J* = 6.8 Hz), 6.81 (1H, d, *J* = 8.0 Hz), 6.69 (2H, d, *J* = 7.6 Hz), 6.59 (1H, dd, *J* = 2.4, 8.8 Hz), 4.50 (2H, d, *J* = 6.8 Hz), 4.26 (1H, t, *J* = 6.4 Hz), 3.60 (4H, bs), 3.16 (4H, bs), 1.35 (12H, s). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 169.65, 155.09, 153.44, 152.69, 152.31, 150.84, 143.88, 141.34, 153.09, 129.70, 129.27, 128.73, 128.04, 127.75, 127.25, 127.09, 126.37, 125.07, 124.90, 123.82, 123.44, 121.60, 120.01, 112.27, 109.47, 102.60, 84.20, 82.85, 67.29, 48.13, 47.34, 24.86. (no signal for carbon attached to boronate observed). HR-FABMS: calculated for [MNa^+] 733.3082, found 733.3085.

MitoPY1 (6). Boronate **5** (35 mg, 48 μmol) was dissolved in 15% piperidine in acetonitrile and stirred at room temperature for 30 minutes. The reaction was then dried under reduced pressure and brought into an inert atmosphere glovebox, where IBTP (55 mg, 96 μmol), sodium bicarbonate (30 mg, 240 μmol), and 5 mL of acetonitrile were added. The contents were then stirred at room temperature for 24 hours inside the glove box. The reaction was then removed from the glove box, filtered, and dried under reduced pressure. Purification by column chromatography (4.5:4.5:0.5 dichloromethane/ethyl acetate/methanol) furnished MitoPY1 as a light pink solid (35 mg, 76% yield). ^{1}H NMR ($CDCl_3$/10% CD_3OD, 300 MHz): δ 7.96 (1H, d, *J* = 7.2 Hz), 7.76-7.83 (3H, m), 7.55-7.75 (15H, m), 7.35 (1H, dd, *J* = 8.0, 1.2 Hz), 7.09 (1H, d, *J* = 7.2 Hz), 7.71 (1H, d, *J* = 7.6 Hz), 6.67 (1H, d, *J* = 3.2 Hz), 6.60 (1H, d, *J* = 8.8 Hz), 6.56 (1H, dd, *J* = 2.0, 8.8 Hz), 3.36-3.47 (2H, m), 3.23-3.29 (4H, m), 2.74-2.81 (4H, m), 2.62-2.70 (2H, m), 1.88-1.94 (2H, m), 1.65-1.76 (2H, m), 1.29 (12H, s). ^{13}C NMR ($CDCl_3$/10% CD_3OD, 125 MHz): δ 170.08, 153.13, 152.27, 150.08, 135.30, 135.28, 133.55, 133.47, 130.64, 130.54, 129.81, 129.17, 128.64, 127.09, 126.16, 124.97, 123.85, 123.36, 121.31, 118.00, 117.32, 84.23, 83.36, 74.96, 56.25, 52.33, 49.95, 24.61, 20.07. (no signal for

carbon attached to boronate observed). ^{31}P NMR ($CDCl_3$/10% CD_3OD, 162 MHz): δ 23.80. HR-FABMS: calculated for [M^+] 827.3781, found 827.3780.



MitoPY1ox (7). Rhodol **3** (124 mg, 0.20 mmol) was dissolved in 15% piperidine in acetonitrile and stirred at room temperature for 30 minutes. The solvent was removed and the contents were brought into an inert atmosphere glovebox, where IBTP (226 mg, 0.39 mmol), sodium bicarbonate (167 mg, 1.99 mmol), and 6 mL of DMF were added. The reaction was then stirred at room temperature for 96 hours inside the glove box, removed from the glove box and dried under reduced pressure. Purification by column chromatography (gradient from 8.5:1.5:0.01 dichloromethane/methanol/water to 8.5:1.5:0.01:0.005 dichloromethane/methanol/water/HCl, then a second column gradient from 9:1 to 8.5:1.5 dichloromethane/methanol) furnished MitoPY1ox as a red solid (30 mg, 22% yield). ^{1}H NMR (CD_3OD, 500 MHz): δ 8.03 (1H, d, *J* = 7.0 Hz), 7.89 (4H, dt, *J* = 2.0, 6.5 Hz), 7.73-7.83 (12H, m), 7.66-7.73 (2H, m), 7.18 (1H, d, *J* = 6.5 Hz), 6.88 (2H, d, *J* = 5 Hz), 6.84 (1H, d, *J* = 9.5 Hz), 6.63 (1H, d, *J* = 2.5 Hz), 6.56 (1H, dd, *J* = 2.0, 9.0 Hz), 3.42-3.54 (6H, m), 2.89 (4H, bs), 2.71-2.78 (2H, m), 1.90 (2H, quintet, *J* = 7.5 Hz), 1.74 (2H, quintet, *J* = 7.5 Hz). ^{13}C NMR (10% CD_3OD/D_2O, 125 MHz): δ 171.71, 156.18, 154.19, 153.59, 135.06, 135.03, 133.38, 133.30, 130.30, 130.20, 129.68, 129.44, 127.01, 126.53, 118.51, 117.83, 112.45, 111.34, 102.87, 100.17, 56.02, 51.71, 48.51, 29.19, 19.76. ^{31}P NMR (CD_3OD, 162 MHz) δ 23.80. HR-FABMS: calculated for [M^+] 717.2877, found 717.2875.



ContPY1 (8). Boronate **5** (10 mg, 13 μmol) was dissolved in 15% piperidine in acetonitrile and stirred at room temperature for 30 minutes. The reaction was then dried under reduced pressure. Acetyl chloride (2 μL, 27 μmol), cesium carbonate (9 mg, 27 μmol), and 3 mL of acetonitrile were added and the contents were then stirred at room temperature for 30 minutes at room temperature. The reaction was then filtered and dried under reduced pressure. Purification by column chromatography (20:1 dichloromethane/ methanol) furnished ContPY1 as a light pink solid (1.8 mg, 25% yield). ^{1}H NMR ($CDCl_3$/10% CD_3OD, 400 MHz): δ 8.00 (1H, d, *J* = 6.4 Hz), 7.69 (1H, s), 7.63 (1H, dt, *J* = 1.6, 7.2 Hz), 7.59 (1H, dt, *J* = 1.6, 7.6 Hz), 7.39 (1H, d, *J* = 8.0 Hz), 7.10 (1H, d, *J* = 6.8 Hz), 6.76 (1H, d, *J* = 7.6 Hz), 6.66 (1H, d, *J* = 8.8 Hz), 6.60 (1H, dd, *J* = 2.0, 9.2 Hz), 3.71-3.78 (2H, m), 3.61 (2H, t, *J* = 4.8 Hz), 3.25 (2H, t, *J* = 4.8 Hz), 3.22 (2H, t, *J* = 4.8 Hz), 2.12 (3H, s), 1.32 (12H, s) ^{13}C NMR ($CDCl_3$/10% CD_3OD, 100 MHz): δ 169.78, 169.45, 153.25, 152.31, 150.78, 133.07, 129.74, 129.25, 128.76, 127.96, 127.18, 126.28, 125.06, 123.80, 123.38, 121.47, 112.24, 102.63, 84.22, 74.88, 48.45, 48.17, 45.81, 41.01, 24.57, 21.16. (no signal for carbon attached to boronate observed). HR-FABMS: calculated for [M^+] 553.2523, found 553.2521.

Spectroscopic Materials and Methods. Millipore water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES buffer, pH 7. Absorption spectra were recorded on a Varian Cary 50 spectrophotometer (Walnut Creek, CA) and fluorescence spectra were recorded on a Photon Technology International Quanta Master 4 L-format scanning spectrofluorometer (Lawrenceville, NJ) equipped with an LPS-220B 75-W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photon-counting/analog photomultiplier detection unit, and MD5020 motor driver. Samples for absorption and emission measurements were contained in 1-cm × 1-cm quartz cuvettes (1.5-mL volume, Starna, Atascadero, CA). Fluorescence quantum yields were determined by reference to fluorescein in 0.1M NaOH ($\Phi = 0.95$).

Preparation and Staining of Cell Cultures. HEK293 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and glutamine (2 mM). CHO.K1 cells were cultured in DMEM with F-12 supplements, glutamax (Invitrogen), and 5% FBS. Cos-7 cells were cultured in DMEM with high glucose, glutamax, 10% FBS, and non-essential amino acids (Invitrogen). HeLa cells were cultured in DMEM with high glucose, glutamax, and 10% FBS. Two days before imaging, cells were passaged and plated on 18-mm glass coverslips. For all experiments, solutions of dyes (from 5 mM stocks in DMSO) were made in DBPS with calcium chloride and magnesium chloride (Sigma). H_2O_2 was added by bath application to the medium from a 100 mM aqueous stock. For paraquat treatment, HeLa cells were cultured as described above. One day prior to imaging, 1 mM paraquat was added to cells from a 0.5 M stock solution in water. An equal amount of water was added to control cells at the same time. Cells were then incubated at 37 °C, 5% CO_2. After 24 hours, the media was exchanged for DPBS with 5 μM dye and incubated for 1 hour.

Fluorescence Imaging Experiments. Confocal fluorescence imaging studies were performed with a Zeiss LSM510 NLO Axiovert 200 laser scanning microscope and a 63x Achroplan IR water-immersion objective lens. Excitation of PY-loaded cells at 510 nm was carried out with an Ar laser and emission was collected using a META detector between 527-580 nm. MitoTracker Deep Red was excited with a 633-nm line and emission was collected between 666-698 nm. LysoTracker Red was excited with a 543-nm line and emission was collected between 580-644 nnm. Excitation of Hoechst 33342 was carried out using a MaiTai two-photon laser at 780-nm pulses (mode-locked Ti:sapphire laser, Tsunami Spectra Physics) and emission was collected between 452-537 nm. Image analysis was performed in Adobe Photoshop.

Flow Cytometry Experiments. Cells were treated as described in figure legends and analyzed on a Beckman-Coulter EPICS XV-MCL flow cytometer in the Flow Cytometry Facility at the University of California, Berkeley.

References

1. Lin, T.; Hughes, G.; Muratovska, A.; Blaikie, F.; Brookes, P.; Darley-Usmar, V.; Smith, R. A. J.; Murphy, M. P. *J. Biol. Chem.* **2002**, *277*, 17048-17056.
2. Chang, C. J.; Nolan, E. M.; Jaworski, J.; Okamoto, K.; Hayashi, Y.; Sheng, M.; Lippard, S. J. *Inorg. Chem.* **2004**, *43*, 6774-6779.

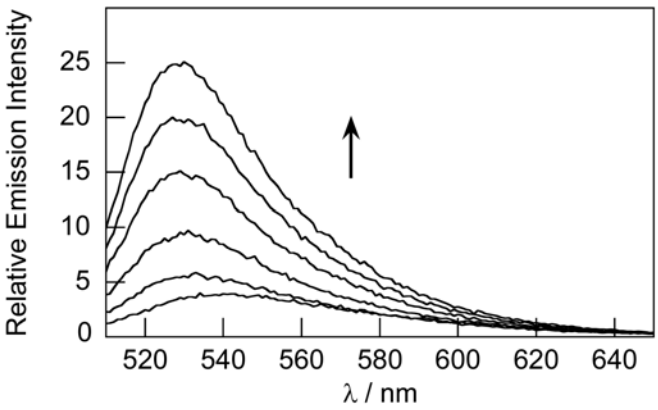


Figure S1: Fluorescence turn-on response of 5 μM MitoPY1 to H_2O_2. Data were acquired at 25 ºC in 20 mM HEPES, pH 7, with excitation at λ = 503 nm. Emission was collected between 510 and 750 nm. Time points represent 0, 5, 15, 30, 45, and 60 minutes after the addition of 100 μM H_2O_2. Reactions are not complete at these time points.

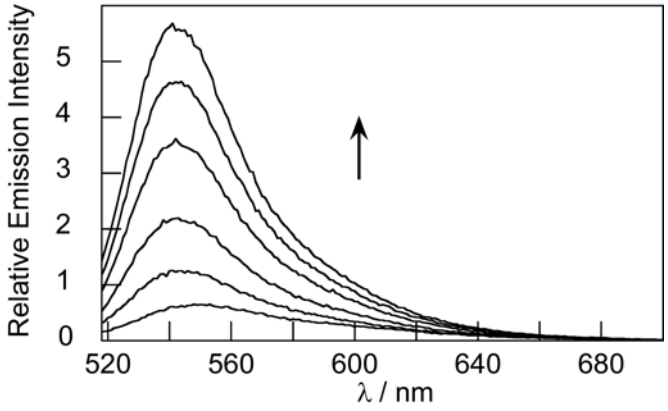


Figure S2: Fluorescence turn-on response of 5 μM ContPY1 to H_2O_2. Data were acquired at 25 ºC in 20 mM HEPES, pH 7, with excitation at λ = 510 nm. Emission was collected between 518 and 750 nm. Time points represent 0, 5, 15, 30, 45, and 60 minutes after the addition of 100 μM H_2O_2. The boronate dye features three major visible region absorption bands (λ_{abs} = 404 nm, ε = 4,100 $M^{-1}cm^{-1}$; 490 nm, ε = 15,600 $M^{-1}cm^{-1}$; 510 nm, ε = 16,130 $M^{-1}cm^{-1}$) and a weak emission centered at λ_{em} = 549 nm (Φ = 0.018). Reaction of ContPY1 with H_2O_2 triggers conversion to the corresponding rhodol, which possesses one major absorption band at 515 nm (ε = 38,200 $M^{-1}cm^{-1}$) and enhanced emission (λ_{em} = 543 nm, Φ =0.388). Reactions are not complete at these time points.

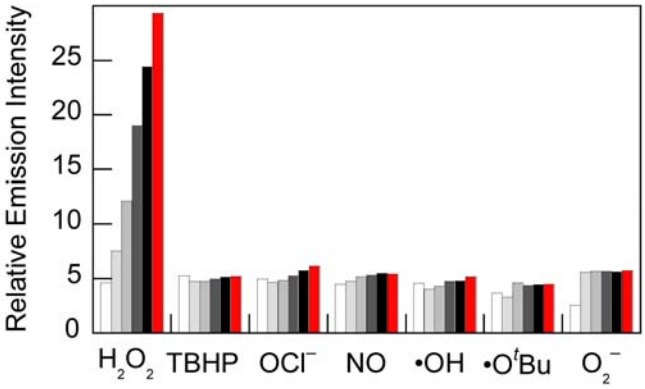


Figure S3. Fluorescence responses of 5 μM ContPY1 to various reactive oxygen species (ROS). Bars represent relative responses at 0, 5, 15, 30, 45, and 60 min after addition of each ROS. Data shown are for 10 mM O_2^- (with 10 μM catalase), 200 μM NO, and 100 μM for all other ROS. Data were acquired at 25 ºC in 20 mM HEPES, pH 7, with excitation at λ = 510 nm and emission collected between 518 and 750 nm.

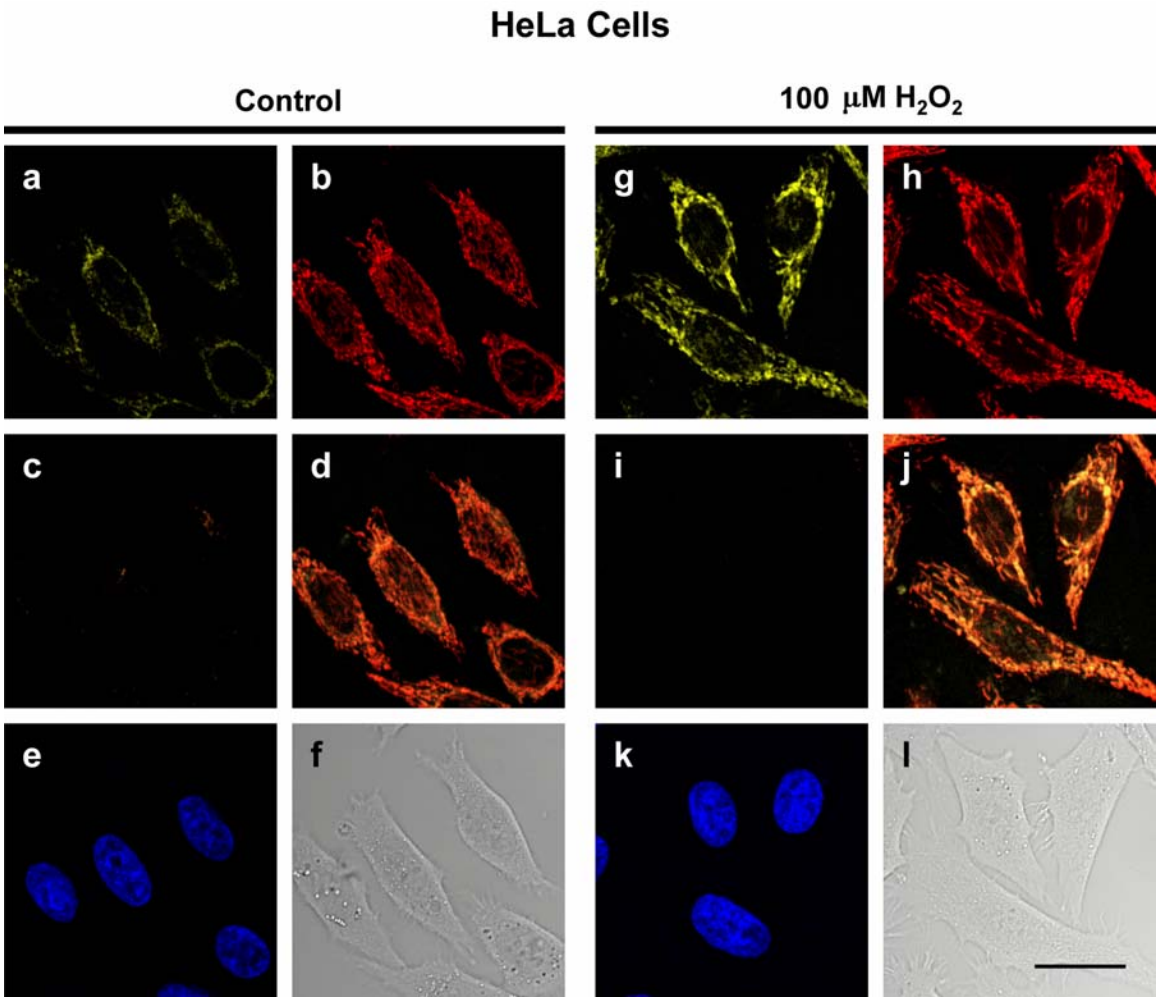


Figure S4: HeLa cells (on two coverslips each in separate petri dishes) were incubated with 5 μM MitoPY1 in DPBS for 20 minutes. 100 μM H_2O_2 was then added to one of the petri dishes. At 20 minutes, 50 nM MitoTracker Deep Red, 500 nM Lysotracker Red, and 1 μM Hoechst 33342 were added to both dishes. After an additional 20 minutes (40 minute stimulation total), the two coverslips were put in a single, new petri dish filled with fresh DPBS and confocal images were taken (3.2 μsec pixel time, 100% laser power for 514 line, 4.1% laser power for the 633 line, 26% laser power for the 543 line, scanned using 12-bit multi-track scan mode using a constant receiver gain). Shown above are signals from MitoPY1 (a), MitoTracker Deep Red (b), LysoTracker Red (c), an overlay of MitoTracker Deep Red and MitoPY1 (d), Hoechst (e), and a brightfield image (f) of cells not treated with H_2O_2, as well as signals from MitoPY1 (g), MitoTracker Deep Red (h), LysoTracker Red (i), an overlay of MitoTracker Deep Red and MitoPY1 (j), Hoechst (k), and a brightfield image (l) of cells treated with H_2O_2. 20 μm scale bar shown for all images.

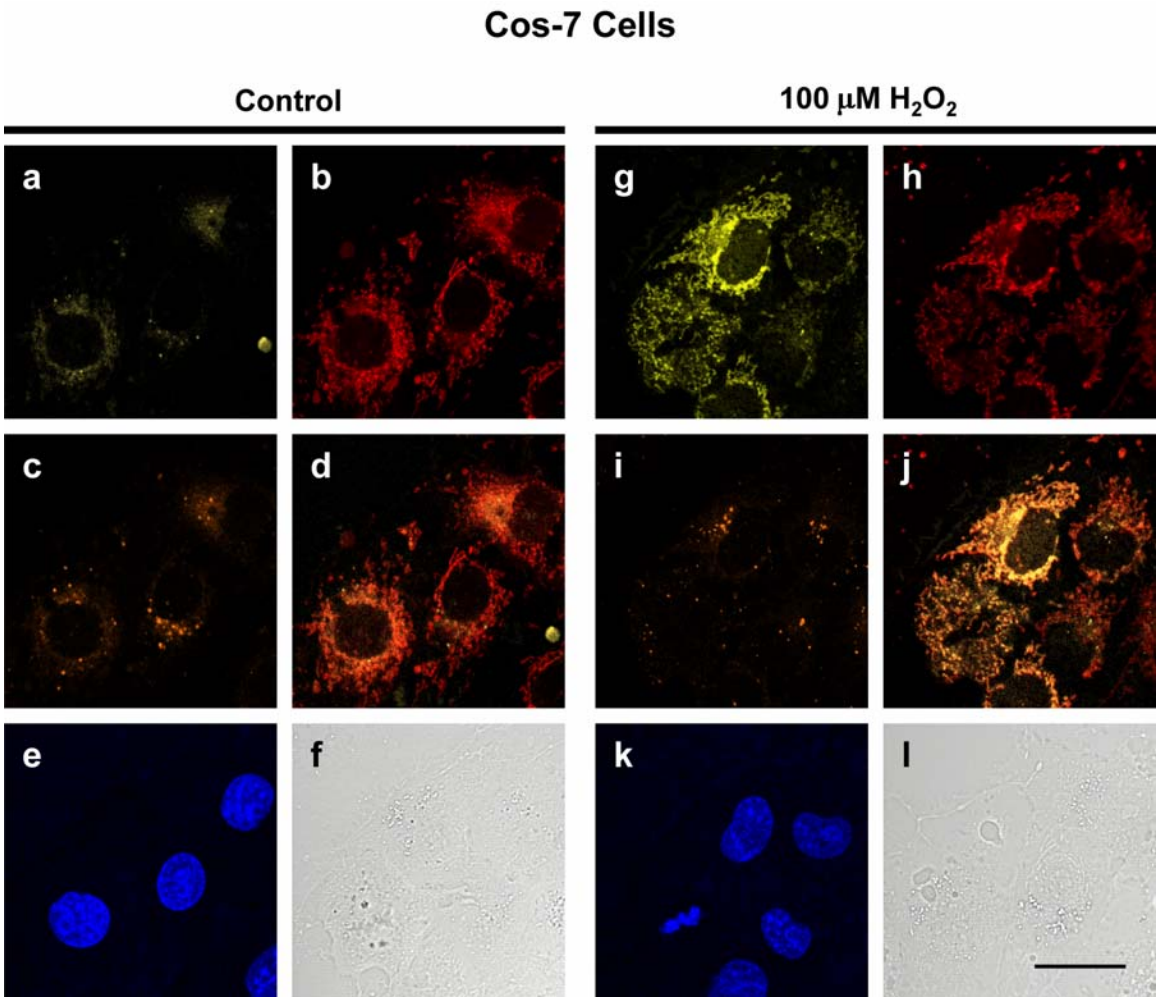


Figure S5: Cos-7 cells (on two coverslips each in separate petri dishes) were incubated with 5 μM MitoPY1 in DPBS for 20 minutes. 100 μM H_2O_2 was then added to one of the petri dishes. At 20 minutes, 50 nM MitoTracker Deep Red, 500 nM Lysotracker Red, and 1 μM Hoechst 33342 were added to both dishes. After an additional 20 minutes (40 minute stimulation total), the two coverslips were put in a single, new petri dish filled with fresh DPBS and confocal images were taken (3.2 μsec pixel time, 100% laser power for 514 line, 4.1% laser power for the 633 line, 26% laser power for the 543 line, scanned using 12-bit multi-track scan mode using a constant receiver gain). Shown above are signals from MitoPY1 (a), MitoTracker Deep Red (b), LysoTracker Red (c), an overlay of MitoTracker Deep Red and MitoPY1 (d), Hoechst (e), and a brightfield image (f) of cells not treated with H_2O_2, as well as signals from MitoPY1 (g), MitoTracker Deep Red (h), LysoTracker Red (i), an overlay of MitoTracker Deep Red and MitoPY1 (j), Hoechst (k), and a brightfield image (l) of cells treated with H_2O_2. A 20 μm scale bar is shown for all images.

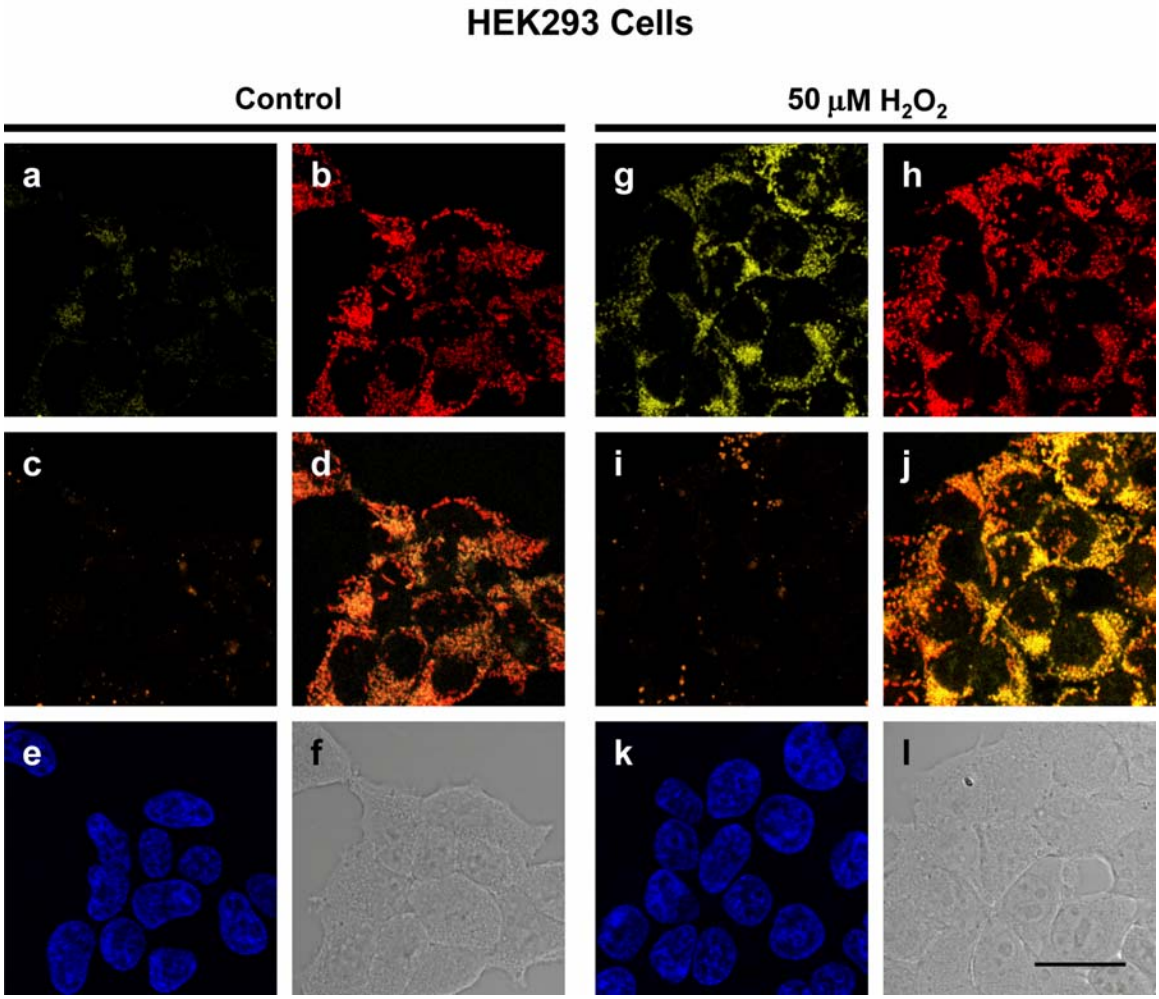


Figure S6: HEK293 cells (on two coverslips each in separate petri dishes) were incubated with 5 μM MitoPY1 in DPBS for 20 minutes. 50 μM H_2O_2 was then added to one of the petri dishes. At 20 minutes, 50 nM MitoTracker Deep Red, 500 nM Lysotracker Red, and 1 μM Hoechst 33342 were added to both dishes. After an additional 20 minutes (40 minute stimulation total), the two coverslips were put in a single, new petri dish filled with fresh DPBS and confocal images were taken (3.2 μsec pixel time, 100% laser power for 514 line, 4.1% laser power for the 633 line, 26% laser power for the 543 line, scanned using 12-bit multi-track scan mode using a constant receiver gain). Shown above are signals from MitoPY1 (a), MitoTracker Deep Red (b), LysoTracker Red (c), an overlay of MitoTracker Deep Red and MitoPY1 (d), Hoechst (e), and a brightfield image (f) of cells not treated with H_2O_2, as well as signals from MitoPY1 (g), MitoTracker Deep Red (h), LysoTracker Red (i), an overlay of MitoTracker Deep Red and MitoPY1 (j), Hoechst (k), and a brightfield image (l) of cells treated with H_2O_2. A 20 μm scale bar is shown for all images.

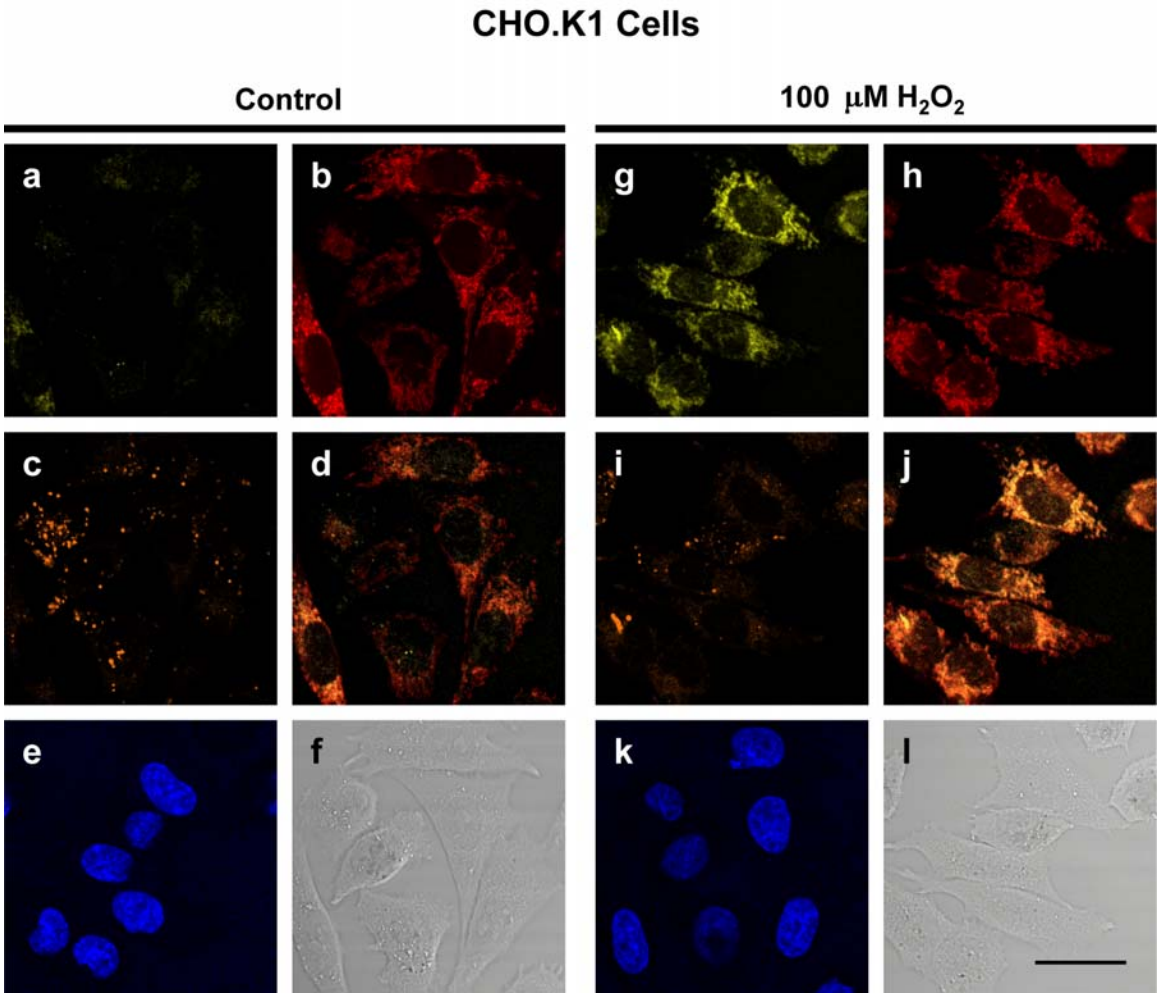


Figure S7: CHO.K1 cells (on two coverslips each in separate petri dishes) were incubated with 5 μM MitoPY1 in DPBS for 20 minutes. 100 μM H_2O_2 was then added to one of the petri dishes. At 20 minutes, 50 nM MitoTracker Deep Red, 500 nM Lysotracker Red, and 1 μM Hoechst 33342 were added to both dishes. After an additional 20 minutes (40 minute stimulation total), the two coverslips were put in a single, new petri dish filled with fresh DPBS and confocal images were taken (3.2 μsec pixel time, 100% laser power for 514 line, 4.1% laser power for the 633 line, 26% laser power for the 543 line, scanned using 12-bit multi-track scan mode using a constant receiver gain). Shown above are signals from MitoPY1 (a), MitoTracker Deep Red (b), LysoTracker Red (c), an overlay of MitoTracker Deep Red and MitoPY1 (d), Hoechst (e), and a brightfield image (f) of cells not treated with H_2O_2, as well as signals from MitoPY1 (g), MitoTracker Deep Red (h), LysoTracker Red (i), an overlay of MitoTracker Deep Red and MitoPY1 (j), Hoechst (k), and a brightfield image (l) of cells treated with H_2O_2. A 20 μm scale bar is shown for all images.

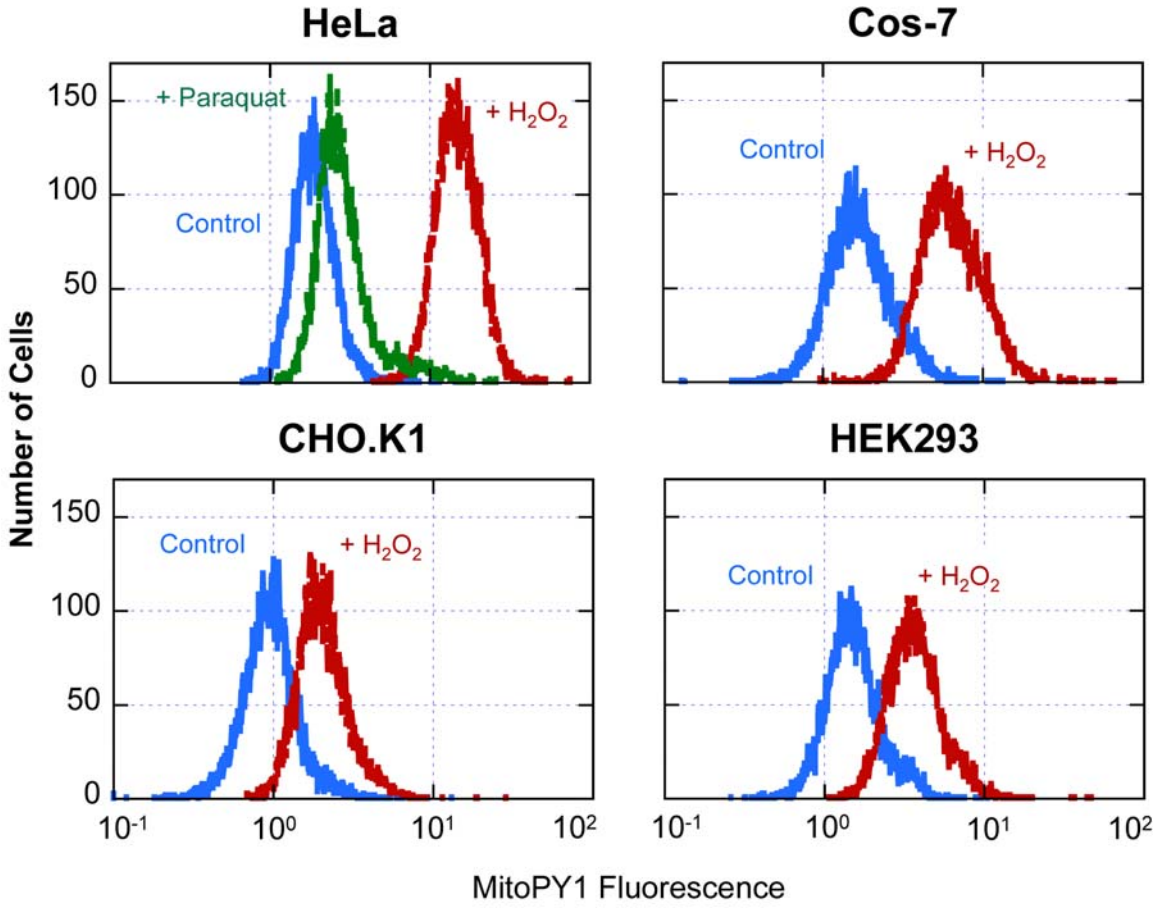


Figure S8: HeLa, Cos-7, CHO.K1, and HEK293 cells were grown in 35 mm tissue culture plates. Cells were washed with DBPS, detached with trypsin, and pelleted by centrifugation. The supernatant was removed and the pellets were each redissolved in 1 mL of 5 μM MitoPY1 in DPBS. The cell suspensions were then each split in half and incubated at 37 ºC for 20 minutes. H_2O_2 (50 μM for the HEK293, 100 μM for all other cells) was then added to one of the aliquots of each cell type and the cells incubated for a subsequent 40 minutes at 37 ºC. The cells were then subjected to analysis by flow cytometry using excitation by a 488 nm laser and collection by a 525 mm band pass filter (687 volts, 1.0 receiver gain). The paraquat-treated HeLa cells were analyzed identically as control cells except they were pretreated with 1 mM Paraquat the previous day. The data represents at least 10,000 cells for each analysis.

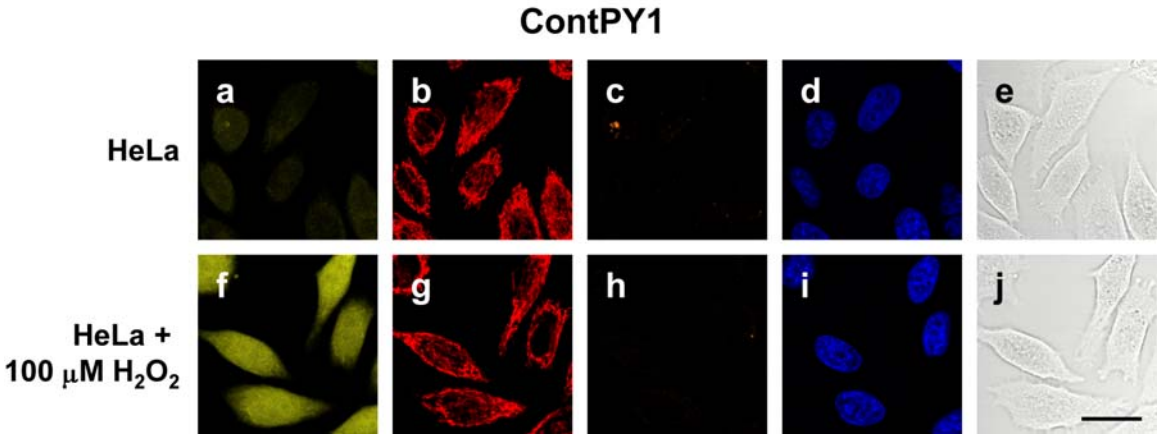


Figure S9: HeLa cells (on two coverslips each in separate petri dishes) were incubated with 5 μM ContPY1 in DPBS for 20 minutes. 100 μM H_2O_2 was then added to one of the petri dishes. At 20 minutes, 50 nM MitoTracker Deep Red, 500 nM Lysotracker Red, and 1 μM Hoechst 33342 were added to both dishes. After an additional 20 minutes (40 minute stimulation total), the two coverslips were put in a single, new petri dish filled with fresh DPBS and confocal images were taken (3.2 μsec pixel time, 100% laser power for 514 line, 4.1% laser power for the 633 line, 26% laser power for the 543 line, scanned using 12-bit multi-track scan mode using a constant receiver gain). Shown above are signals from ContPY1 (a), MitoTracker Deep Red (b), LysoTracker Red (c), Hoechst (d), and a brightfield image (e) of cells not treated with H_2O_2, as well as signals from ContPY1 (f), MitoTracker Deep Red (g), LysoTracker Red (h), Hoechst (i), and a brightfield image (j) of cells treated with H_2O_2. A 20 μm scale bar is shown for all images.

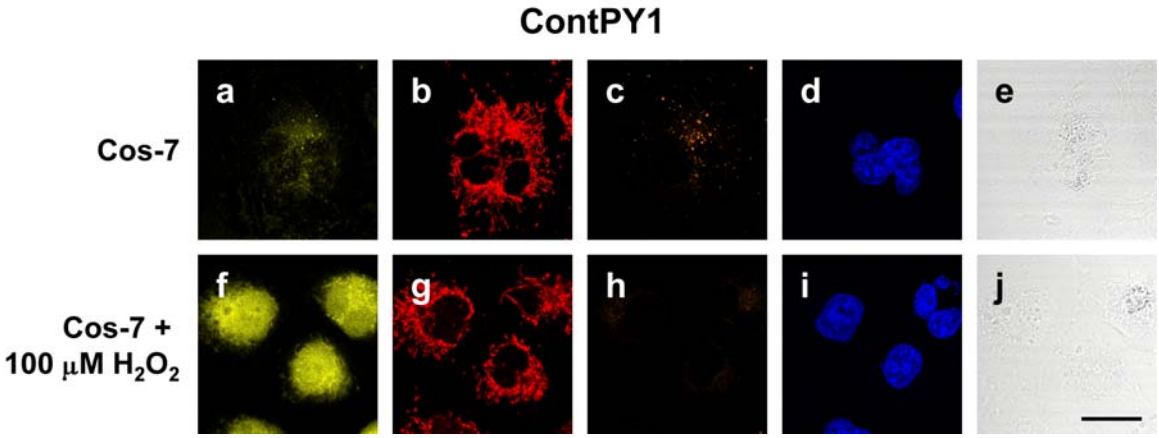


Figure S10: Cos-7 cells (on two coverslips each in separate petri dishes) were incubated with 5 μM ContPY1 in DPBS for 20 minutes. 100 μM H_2O_2 was then added to one of the petri dishes. At 20 minutes, 50 nM MitoTracker Deep Red, 500 nM Lysotracker Red, and 1 μM Hoechst 33342 were added to both dishes. After an additional 20 minutes (40 minute stimulation total), the two coverslips were put in a single, new petri dish filled with fresh DPBS and confocal images were taken (3.2 μsec pixel time, 100% laser power for 514 line, 4.1% laser power for the 633 line, 26% laser power for the 543 line, scanned using 12-bit multi-track scan mode using a constant receiver gain). Shown above are signals from ContPY1 (a), MitoTracker Deep Red (b), LysoTracker Red (c), Hoechst (d), and a brightfield image (e) of cells not treated with H_2O_2, as well as signals from ContPY1 (f), MitoTracker Deep Red (g), LysoTracker Red (h), Hoechst (i), and a brightfield image (j) of cells treated with H_2O_2. A 20 μm scale bar is shown for all images.

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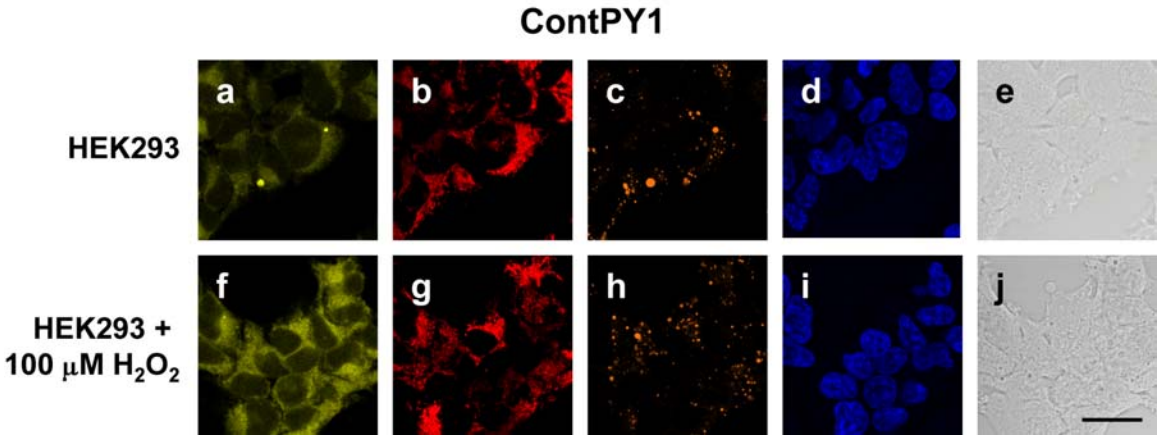

Figure S11: HEK293 (on two coverslips each in separate petri dishes) were incubated with 5 μM ContPY1 in DPBS for 20 minutes. 100 μM H_2O_2 was then added to one of the petri dishes. At 20 minutes, 50 nM MitoTracker Deep Red, 500 nM Lysotracker Red, and 1 μM Hoechst 33342 were added to both dishes. After an additional 20 minutes (40 minute stimulation total), the two coverslips were put in a single, new petri dish filled with fresh DPBS and confocal images were taken (3.2 μsec pixel time, 100% laser power for 514 line, 4.1% laser power for the 633 line, 26% laser power for the 543 line, scanned using 12-bit multi-track scan mode using a constant receiver gain). Shown above are signals from ContPY1 (a), MitoTracker Deep Red (b), LysoTracker Red (c), Hoechst (d), and a brightfield image (e) of cells not treated with H_2O_2, as well as signals from ContPY1 (f), MitoTracker Deep Red (g), LysoTracker Red (h), Hoechst (i), and a brightfield image (j) of cells treated with H_2O_2. A 20 μm scale bar is shown for all images.

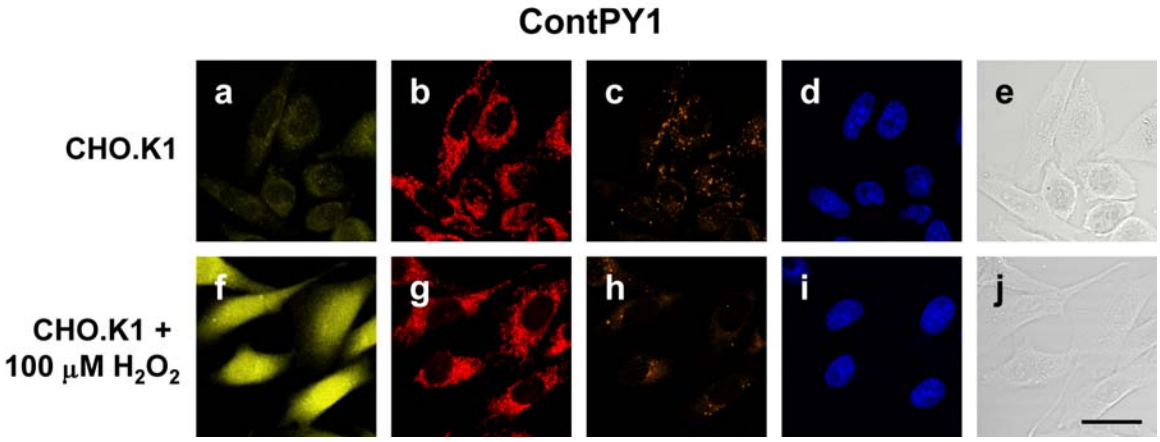

Figure S12: CHO.K1 cells (on two coverslips each in separate petri dishes) were incubated with 5 μM ContPY1 in DPBS for 20 minutes. 100 μM H_2O_2 was then added to one of the petri dishes. At 20 minutes, 50 nM MitoTracker Deep Red, 500 nM Lysotracker Red, and 1 μM Hoechst 33342 were added to both dishes. After an additional 20 minutes (40 minute stimulation total), the two coverslips were put in a single, new petri dish filled with fresh DPBS and confocal images were taken (3.2 μsec pixel time, 100% laser power for 514 line, 4.1% laser power for the 633 line, 26% laser power for the 543 line, scanned using 12-bit multi-track scan mode using a constant receiver gain). Shown above are signals from ContPY1 (a), MitoTracker Deep Red (b), LysoTracker Red (c), Hoechst (d), and a brightfield image (e) of cells not treated with H_2O_2, as well as signals from ContPY1 (f), MitoTracker Deep Red (g), LysoTracker Red (h), Hoechst (i), and a brightfield image (j) of cells treated with H_2O_2. A 20 μm scale bar is shown for all images.

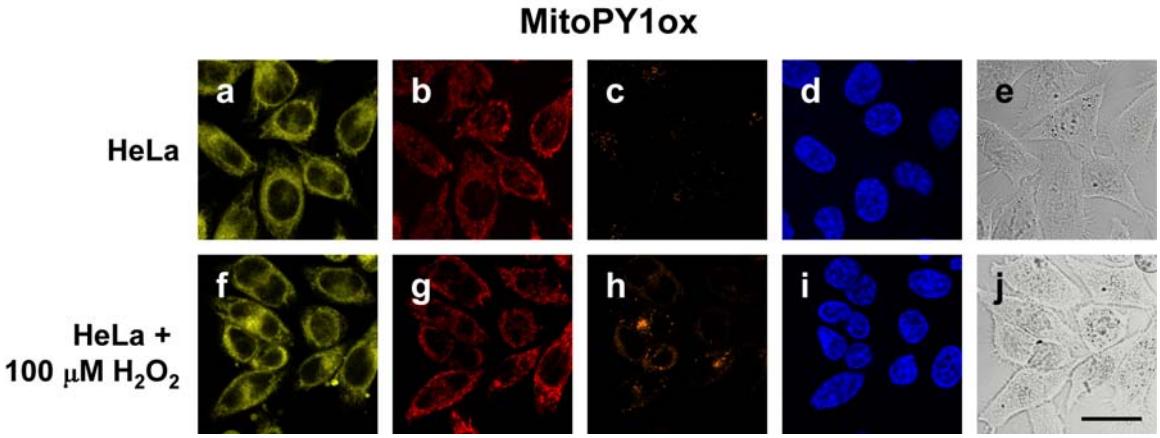

Figure S13: HeLa cells (on two coverslips each in separate petri dishes) were incubated with 5 μM MitoPY1ox in DPBS for 20 minutes. 100 μM H_2O_2 was then added to one of the petri dishes. At 20 minutes, 50 nM MitoTracker Deep Red, 500 nM Lysotracker Red, and 1 μM Hoechst 33342 were added to both dishes. After an additional 20 minutes (40 minute stimulation total), the two coverslips were put in a single, new petri dish filled with fresh DPBS and confocal images were taken (3.2 μsec pixel time, 100% laser power for 514 line, 4.1% laser power for the 633 line, 26% laser power for the 543 line, scanned using 12-bit multi-track scan mode using a constant receiver gain). Shown above are signals from MitoPY1ox (a), MitoTracker Deep Red (b), LysoTracker Red (c), Hoechst (d), and a brightfield image (e) of cells not treated with H_2O_2, as well as signals from MitoPY1ox (f), MitoTracker Deep Red (g), LysoTracker Red (h), Hoechst (i), and a brightfield image (j) of cells treated with H_2O_2. A 20 μm scale bar is shown for all images.

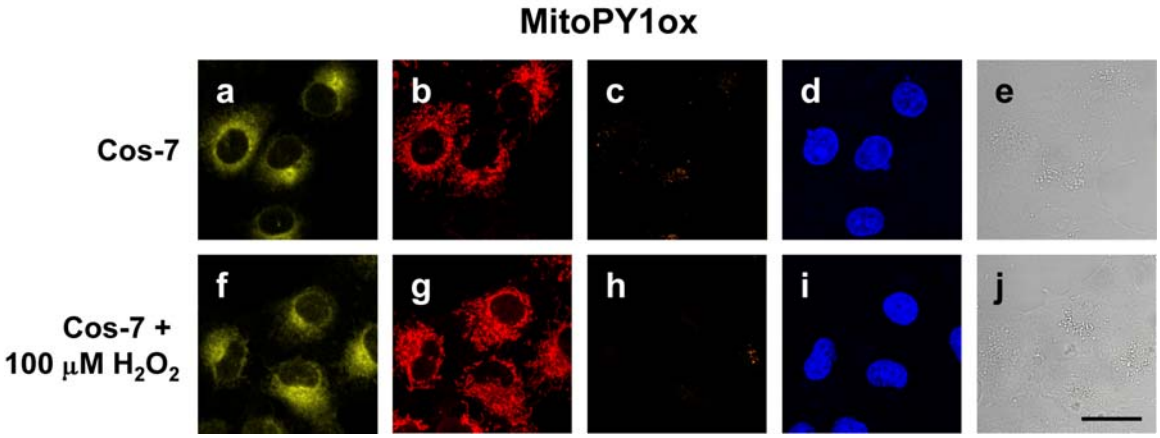

Figure S14: Cos-7 cells (on two coverslips each in separate petri dishes) were incubated with 5 μM MitoPY1ox in DPBS for 20 minutes. 100 μM H_2O_2 was then added to one of the petri dishes. At 20 minutes, 50 nM MitoTracker Deep Red, 500 nM Lysotracker Red, and 1 μM Hoechst 33342 were added to both dishes. After an additional 20 minutes (40 minute stimulation total), the two coverslips were put in a single, new petri dish filled with fresh DPBS and confocal images were taken (3.2 μsec pixel time, 100% laser power for 514 line, 4.1% laser power for the 633 line, 26% laser power for the 543 line, scanned using 12-bit multi-track scan mode using a constant receiver gain). Shown above are signals from MitoPY1ox (a), MitoTracker Deep Red (b), LysoTracker Red (c), Hoechst (d), and a brightfield image (e) of cells not treated with H_2O_2, as well as signals from MitoPY1ox (f), MitoTracker Deep Red (g), LysoTracker Red (h), Hoechst (i), and a brightfield image (j) of cells treated with H_2O_2. A 20 μm scale bar is shown for all images.

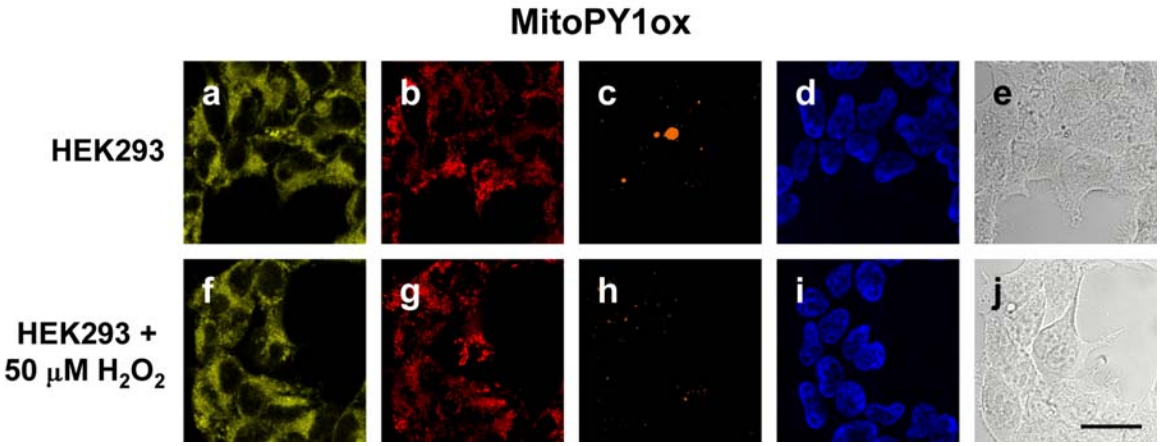

Figure S15: HEK293 cells (on two coverslips each in separate petri dishes) were incubated with 5 μM MitoPY1ox in DPBS for 20 minutes. 50 μM H_2O_2 was then added to one of the petri dishes. At 20 minutes, 50 nM MitoTracker Deep Red, 500 nM Lysotracker Red, and 1 μM Hoechst 33342 were added to both dishes. After an additional 20 minutes (40 minute stimulation total), the two coverslips were put in a single, new petri dish filled with fresh DPBS and confocal images were taken (3.2 μsec pixel time, 100% laser power for 514 line, 4.1% laser power for the 633 line, 26% laser power for the 543 line, scanned using 12-bit multi-track scan mode using a constant receiver gain). Shown above are signals from MitoPY1ox (a), MitoTracker Deep Red (b), LysoTracker Red (c), Hoechst (d), and a brightfield image (e) of cells not treated with H_2O_2, as well as signals from MitoPY1ox (f), MitoTracker Deep Red (g), LysoTracker Red (h), Hoechst (i), and a brightfield image (j) of cells treated with H_2O_2. A 20 μm scale bar is shown for all images.

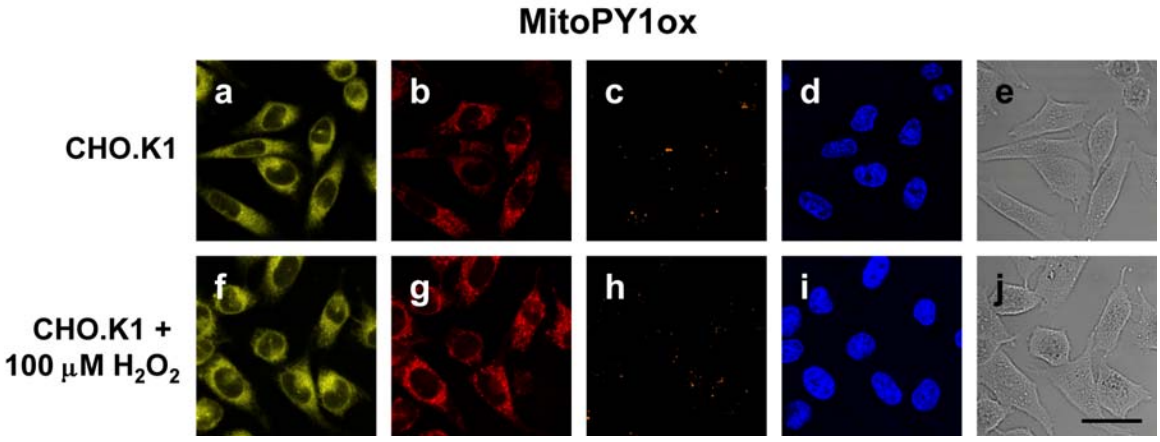

Figure S16: CHO.K1 cells (on two coverslips each in separate petri dishes) were incubated with 5 μM MitoPY1ox in DPBS for 20 minutes. 100 μM H_2O_2 was then added to one of the petri dishes. At 20 minutes, 50 nM MitoTracker Deep Red, 500 nM Lysotracker Red, and 1 μM Hoechst 33342 were added to both dishes. After an additional 20 minutes (40 minute stimulation total), the two coverslips were put in a single, new petri dish filled with fresh DPBS and confocal images were taken (3.2 μsec pixel time, 100% laser power for 514 line, 4.1% laser power for the 633 line, 26% laser power for the 543 line, scanned using 12-bit multi-track scan mode using a constant receiver gain). Shown above are signals from MitoPY1ox (a), MitoTracker Deep Red (b), LysoTracker Red (c), Hoechst (d), and a brightfield image (e) of cells not treated with H_2O_2, as well as signals from MitoPY1ox (f), MitoTracker Deep Red (g), LysoTracker Red (h), Hoechst (i), and a brightfield image (j) of cells treated with H_2O_2. A 20 μm scale bar is shown for all images.

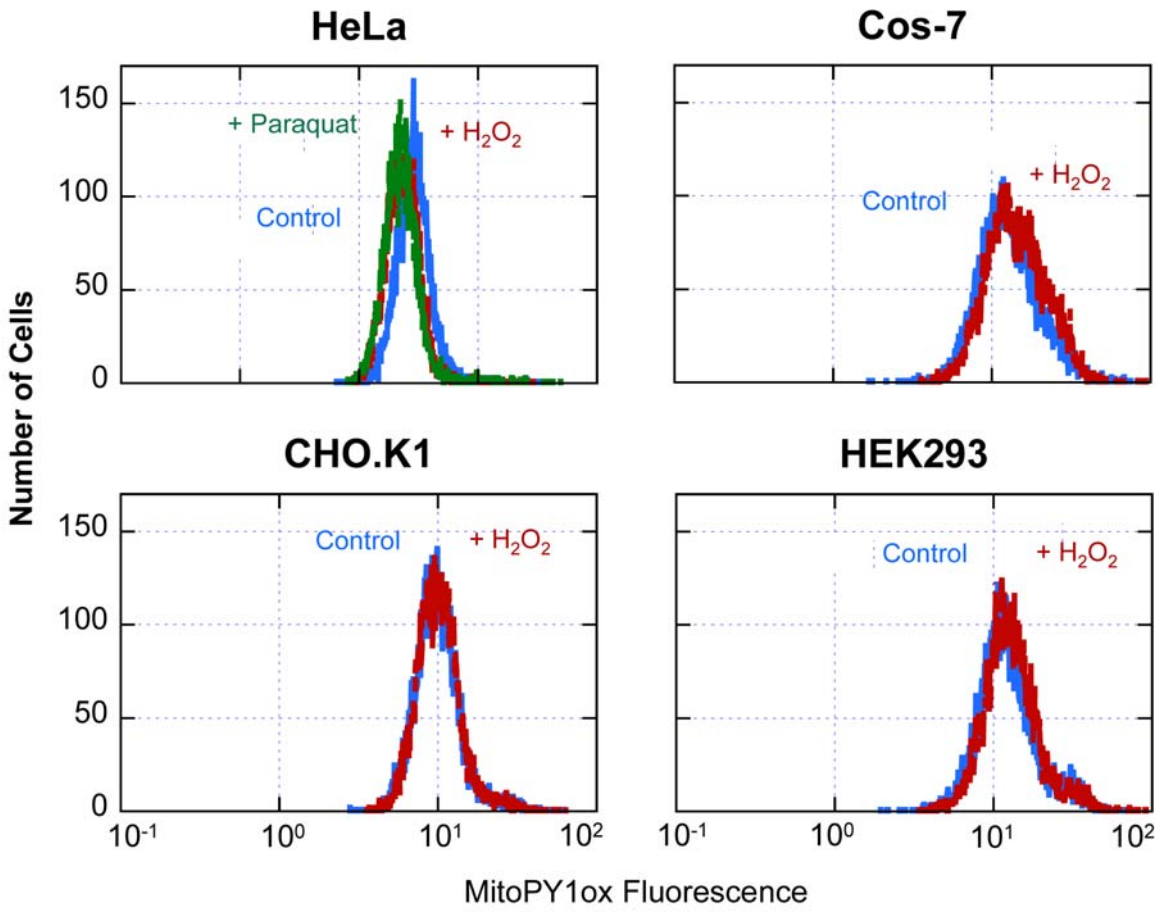

Figure S17: HeLa, Cos-7, CHO.K1, and HEK293 cells were grown in 35 mm tissue culture plates. Cells were washed with DBPS, detached with trypsin, and pelleted by centrifugation. The supernatant was removed and the pellets were each redissolved in 1 mL of 5 μM MitoPY1ox in DPBS. The cell suspensions were then each split in half and incubated at 37 ºC for 20 minutes. H_2O_2 was then added to one of the aliquots (50 μM for the HEK293, 100 μM for all other cells) and the cells incubated for a subsequent 40 minutes at 37 ºC. The cells were then subjected to analysis by flow cytometry using excitation by a 488 nm laser and collection by a 525 mm band pass filter (687 volts, 1.0 receiver gain). The paraquat-treated HeLa cells were analyzed identically as control cells except they were treated with 1 mM paraquat the previous day. The data represents at least 10,000 cells for each analysis.