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Fluorogenic Detection of Mitochondrial Superoxide in Live Cells

Michael S. Janes[1,2], Dani M. Hill[1], Caleb M. Cardon[1], Kristine M. Robinson[3], Julia R. Walls[1], Wai-Yee Leung[1], Joseph S. Beckman[3,4], Michael J. Ignatius[1]
[1]Molecular Probes, Inc., Eugene, Oregon,
[2]Molecular and Cellular Biology Program, Oregon State University, Corvallis, Oregon
[3]Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon
[4]Linus Pauling Institute, Oregon State University, Corvallis, Oregon

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Abstract

In an otherwise tightly coupled electron transport chain, approximately 1-3% of mitochondrial oxygen consumed is incompletely reduced and leads to the production of reactive oxygen species (ROS). While not “harvested” for oxidative phosphorylation, these “leaky” electrons can quickly interact with molecular oxygen to form superoxide anion, the predominant ROS in mitochondria. MtDNA lacks protective histones and has minimal repair mechanisms rendering mitochondria particularly vulnerable to oxidative damage. Superoxide has been implicated in various diseases including hypertension, atherosclerosis, diabetes, Parkinson’s, and amyotrophic lateral sclerosis.

We have developed a novel fluorogenic dye, MitoSOX™ Red reagent, for the highly selective detection of mitochondrial superoxide in live cells. The assumption that mitochondria serve as the major intracellular source of ROS has been based largely on experiments with isolated mitochondria rather than with live cells. To establish selectivity for superoxide, we employed various cell-free systems to generate ROS and radical nitrogen species. The probe was readily oxidized by superoxide but not by other oxidative species. The oxidation product becomes highly fluorescent upon binding nucleic acids and oxidation of the probe was prevented by superoxide dismutase. The probe is permeable to live cells and is rapidly and selectively targeted to mitochondria. Once in the mitochondria, the compound is oxidized by superoxide and red fluorescence is easily visible as distinct reticula.

MitoSOX Red reagent may enable researchers to delineate artifacts of isolated mitochondrial preparations from direct measurements of superoxide generated in the mitochondria of live cells. It may also provide a valuable tool in the discovery of agents that modulate oxidative stress in various pathologies.

MitoSOX Red reagent competes efficiently with superoxide dismutase for superoxide

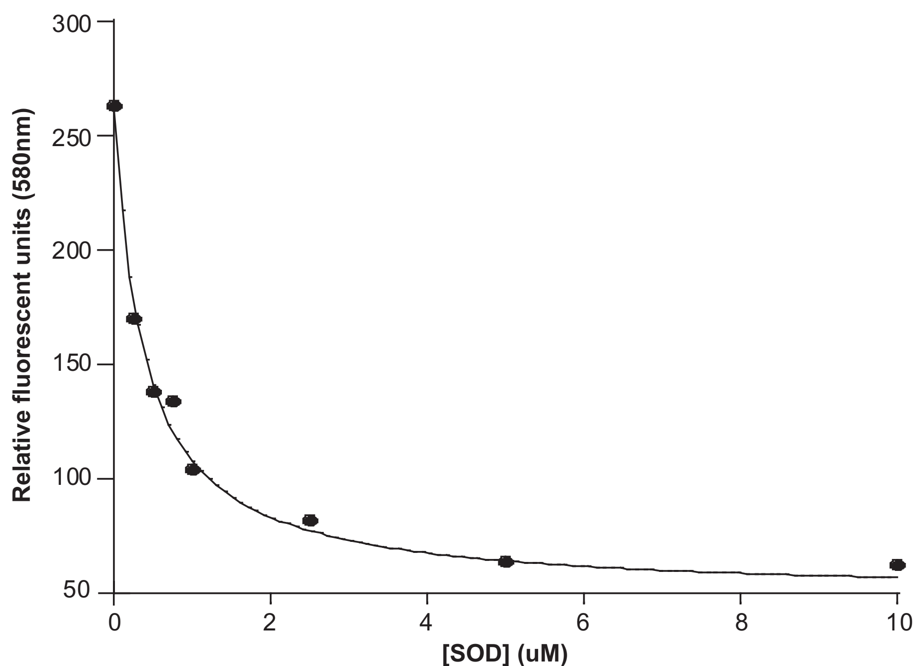
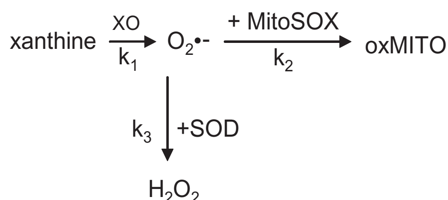


Figure 1. Xanthine oxidase was used to produce superoxide. Increasing amounts of Cu,Zn superoxide dismutase (SOD) were added to inhibit oxidation of MitoSOX Red reagent (oxMITO).



The competition between SOD and MitoSOX Red reagent for superoxide can be modeled by the following rate law:

$$K = \frac{k_1 * k_2[\text{oxMITO}]}{k_2[\text{oxMITO}] + k_3[\text{SOD}]} + C$$

The data was fit to the above equation and solved to determine $k_2 = 7x10^7 M^{-1}s^{-1}$, the rate constant for the reaction between superoxide and MitoSOX Red reagent. SOD reacts with superoxide $\sim 1.8x10^9 M^{-1}s^{-1}$. The approximate rate constant of $7x10^7 M^{-1}s^{-1}$ demonstrates the rapidity of the reaction between MitoSOX Red reagent and superoxide and exacerbates the ability of MitoSOX Red reagent to detect superoxide by efficiently competing with SOD.

MitoSOX Red reagent is selectively oxidized by superoxide

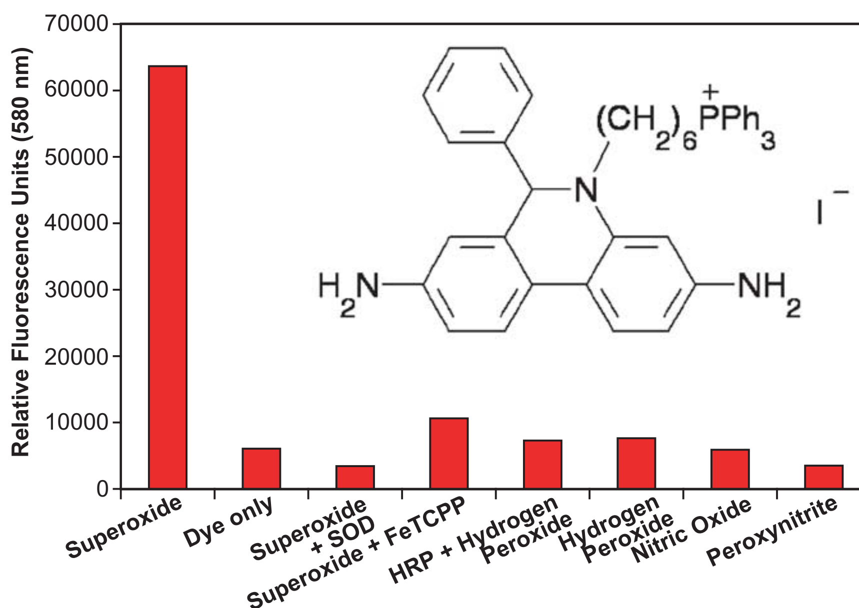


Figure 2. The superoxide probe at 10 µM was exposed to various ROS/RNS generating systems. Samples were incubated at 37°C for 10 min, excess DNA was added (additional 15 min), and fluorescence was measured at 580 nm. Approximately 200 µM superoxide was generated with 50 mU/mL xanthine oxidase (~0.48 nM steady state). Superoxide was scavenged with 37.5 µg/mL superoxide dismutase (SOD) followed by addition of substrate and enzyme as described above. Both 1 mM hydrogen peroxide alone and 50 µM hydrogen peroxide in a peroxidase reaction with 0.2 U/mL horseradish peroxidase (HRP) is shown. Nitric oxide was formed by using the nitric oxide donor DEANO (500 µM), which forms two nitric oxide molecules per molecule of DEANO. Concentrated peroxynitrite from a sodium hydroxide stock was added to a vortexing reaction mixture for a final concentration of 50 µM. The chemical structure of MitoSOX Red reagent prior to oxidation is shown as a graph inset.

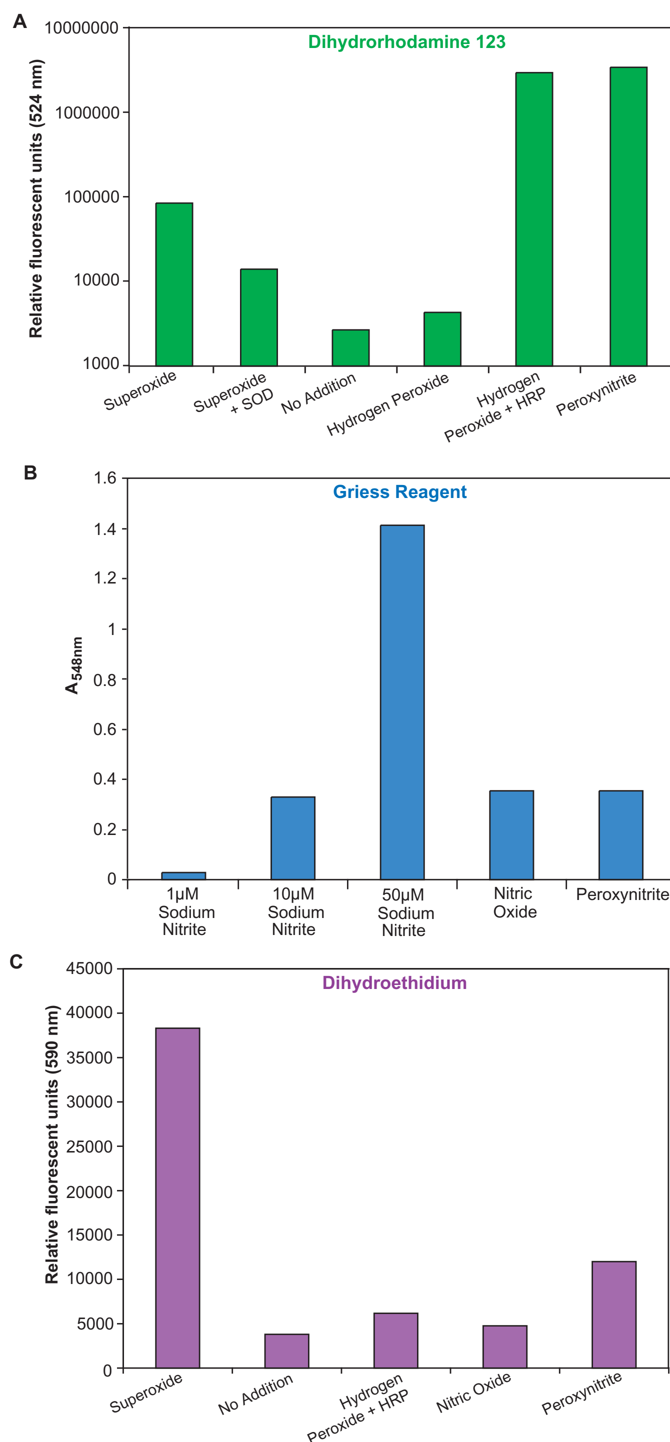


Figure 3. Oxidants were generated as described in figure 2 unless specified below and measured with (A) 10µM dihydrorhodamine 123 (DHR 123), (B) the Griess nitrite determination kit, or (C) 10µM dihydroethidium (DHE). The Griess reagent was used per manufacturer's recommendations and excess DNA addition was necessary with DHE only. Nitric oxide was formed by using the nitric oxide donor DEANO (10µM) and peroxynitrite was added at 50 µM for DHR 123 or 5µM for the Griess determination. These probes also served as positive controls for oxidant generation for comparison with MitoSOX Red reagent selectivity data.

MitoSOX Red reagent oxidation can be prevented by addition of superoxide scavengers in live cell mitochondria

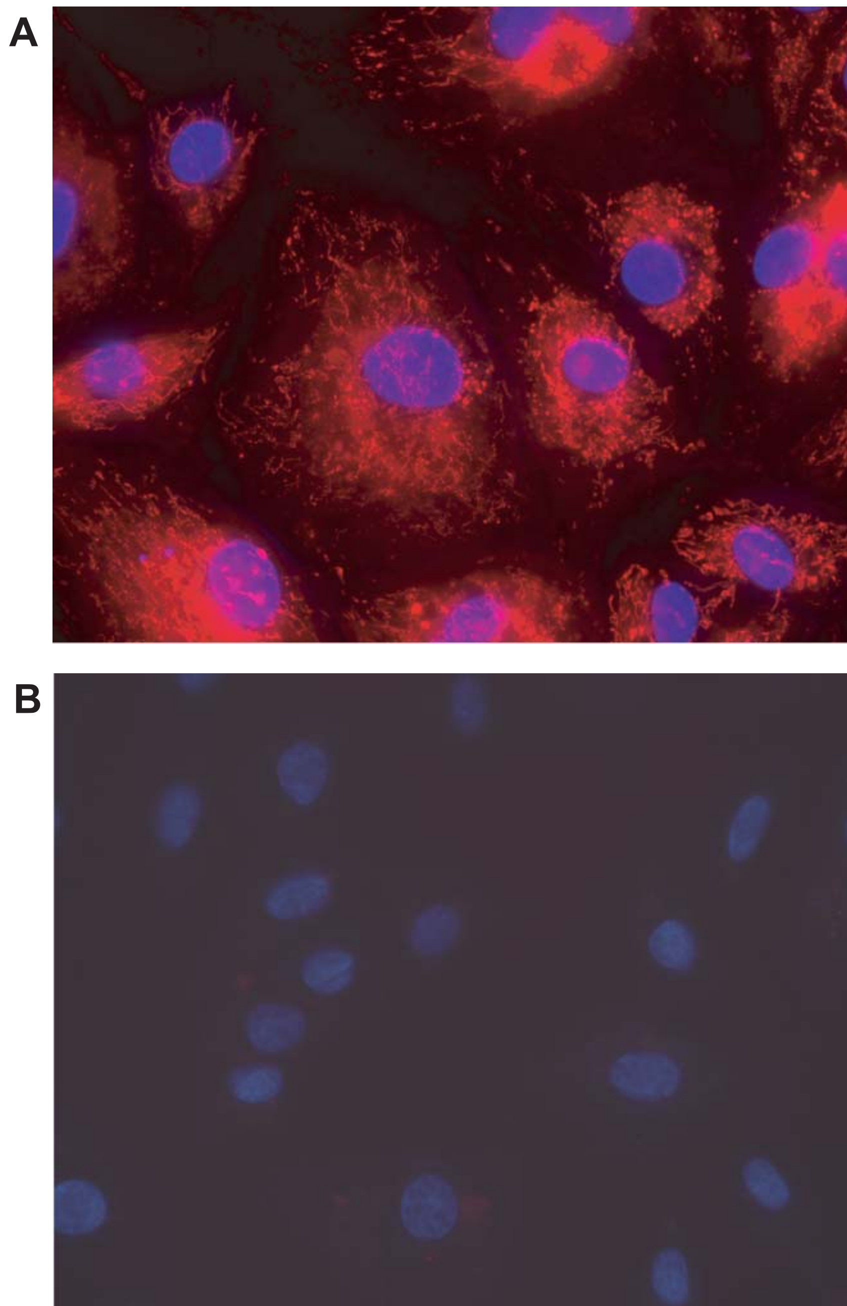


Figure 4. Bovine pulmonary artery endothelial (BPAE) cells pre-incubated for 10 min with (*A*) no superoxide scavenger, (*B*) 10 µM FeTCPP. Cells were labeled with 5 µM MitoSOX Red reagent for 10 min, washed, and counterstained with blue-fluorescent Hoechst 33342 nucleic acid dye.

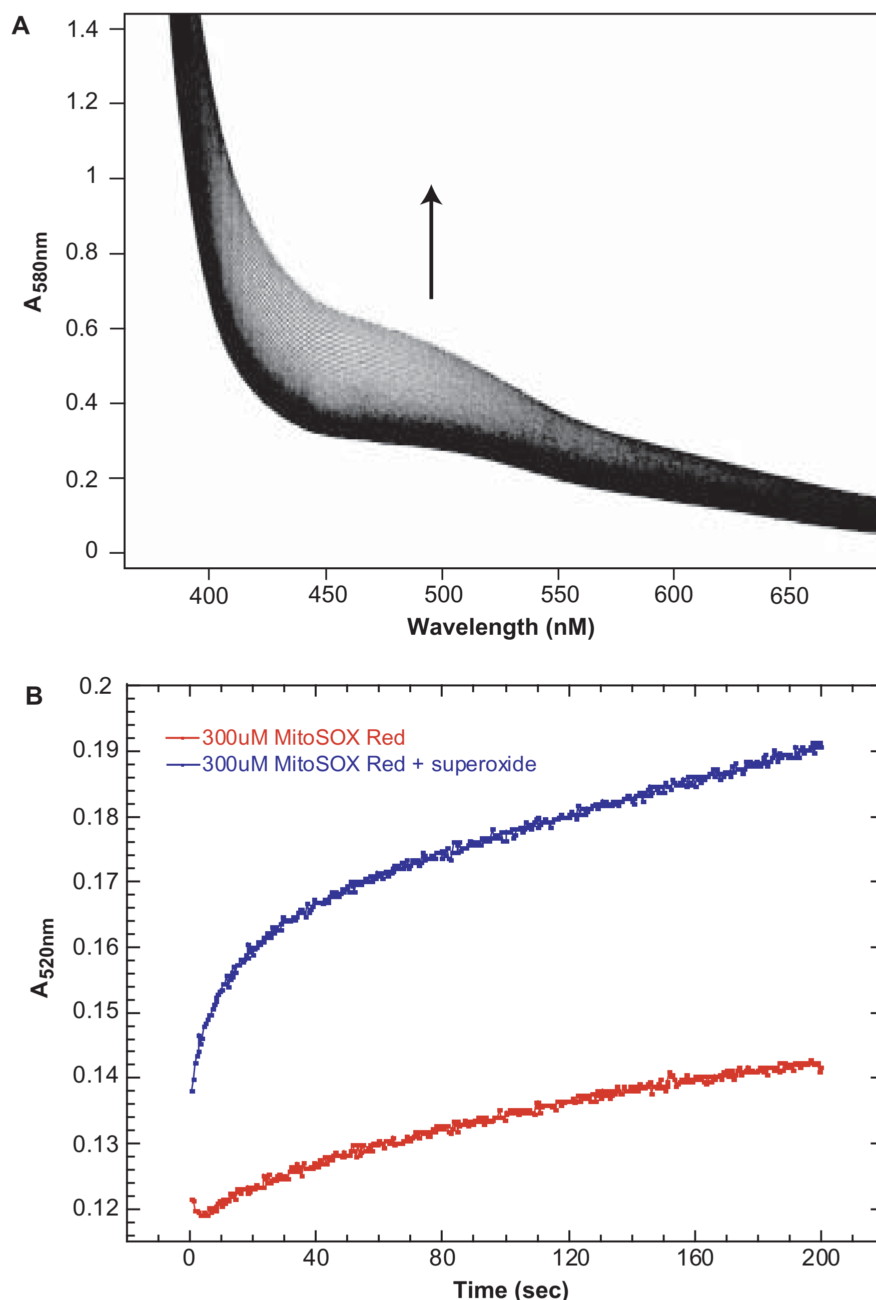


Figure 5. (A) Stopped flow spectroscopy of MitoSOX Red reagent oxidation as indicated by an increase in absorbance at 520 nm for ~4 min after exposure to (A and B-blue trace) superoxide and 510 nm light or (B-red trace) 510 nm light only. Approximately 80% of the probe oxidation was the result of oxidation by superoxide.

Pre-oxidized MitoSOX Red reagent does not accumulate in the mitochondria of live cells

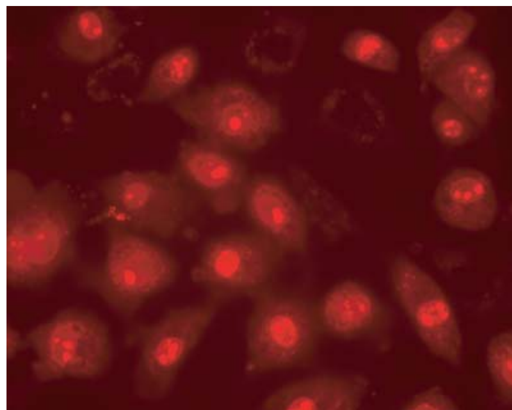


Figure 6. In the absence of cells, MitoSOX Red reagent (5 µM) was oxidized using superoxide generated from xanthine oxidase at 37°C for 100 min. Live BPAE cells were then incubated in this oxidized probe solution for 5 min at 37°C. Taken together with selectivity and localization data, these results demonstrate the origin of cellular oxidation to be the mitochondria.

Mitochondrial localization of MitoSOX Red reagent compared to dihydroethidium

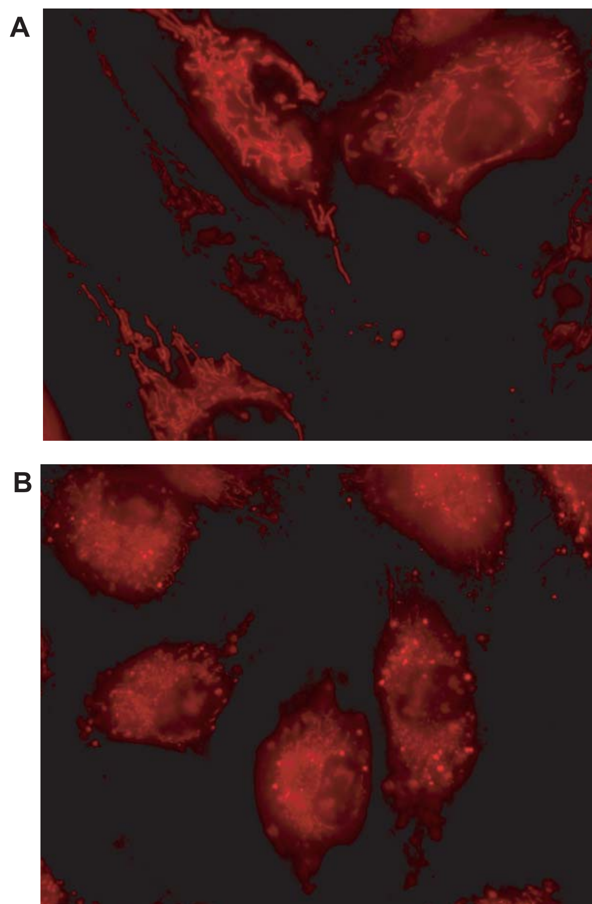


Figure 7. Live BPAE cells were loaded with 5 µM (A) MitoSOX Red reagent or (B) dihydroethidium (DHE) for 5 min at 37°C in D-PBS to compare intracellular localization. While oxidized MitoSOX Red reagent produced distinct mitochondrial labeling, the oxidation product of DHE accumulated non-specifically throughout the cell.

Comparison of MitoSOX Red reagent and dihydroethidium localization in multicolor live cell fluorescence microscopy

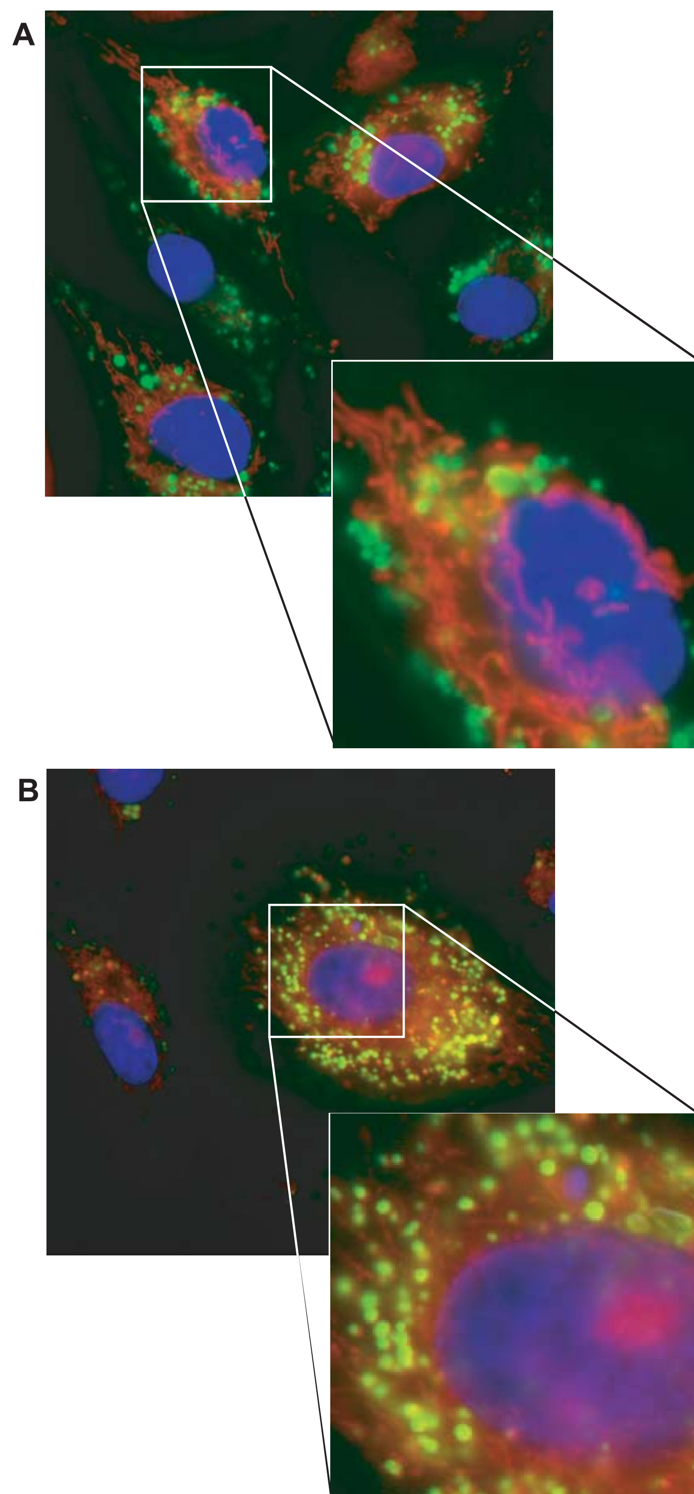


Figure 8. Live BPAE cells were loaded with 50 nM LysoTracker® Green reagent for 30 min before being labeled with 1 µM Hoechst 33342 and 5 µM (A) MitoSOX Red reagent or (B) dihydroethidium for 5 min at 37°C. While MitoSOX Red reagent distinctly labeled mitochondria only, dihydroethidium produced diffuse labeling throughout the cells including nucleolar and nuclear regions, mitochondria, lysosomes, and the cytosol.

Conclusion

Key features of this technology include:

- Highly selective oxidation by superoxide to produce red-fluorescent product readily detectable with standard filter sets (Ex 510 nm/Em 580 nm).
- Efficient competition with SOD for superoxide (approximate rate constant of $7x10^7 M^{-1}s^{-1}$) underscores the ability of MitoSOX Red reagent for superoxide detection in cell- and solution-based assays.
- More selective detection of superoxide than with dihydroethidium, the leading traditional probe for detection of intracellular superoxide.
- Preventable oxidation using SOD mimetics such as FeTCPP, MnTBAP, and Tiron.
- Permeability to living cells as well as rapid and selective targeting to the mitochondria.
- Unlike dihydroethidium, highly selective accumulation in mitochondria of live cells enables superoxide measurements in the major intracellular regulator of energy production and apoptosis.
- Amenability to solution-based fluorimetry, high content screening, and single-cell (or mitochondrion) analysis of populations by flow cytometry and multi-color fluorescence microscopy.

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