2008 Sunrise Free Radical School
Presentation by:

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Evaluating Superoxide/Hydrogen Peroxide Detection Methods in Cells and Tissues – A Chemist's View

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“Being able to do any chemistry was like being the one-eyed man in the kingdom of the blind.”

- Roger Tsien

Acknowledgements

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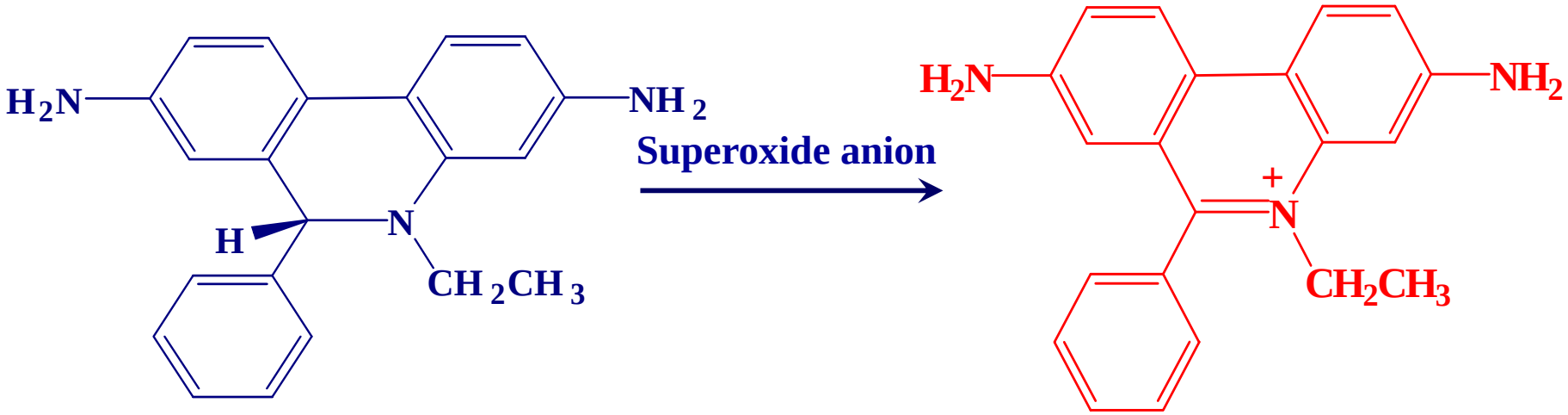
- Hongtao Zhao (MCW, NIH)
- Jacek Zielonka (MCW)

Intracellular detection of oxidants using fluorescent probes

- The chemical reaction between the fluorescence probes and oxidants
- Redox reactions of fluorescent probe-derived radicals
- The cellular uptake and transport of probes
- Intracellular levels of reductants
- Translocation of fluorescent products in cells
- Understanding the chemistry and redox signaling mechanisms of inhibitors (apocynin, MnTBAP, N-acetylcysteine, etc.) that are frequently used to determine the nature of oxidants responsible for intracelluar fluorescence

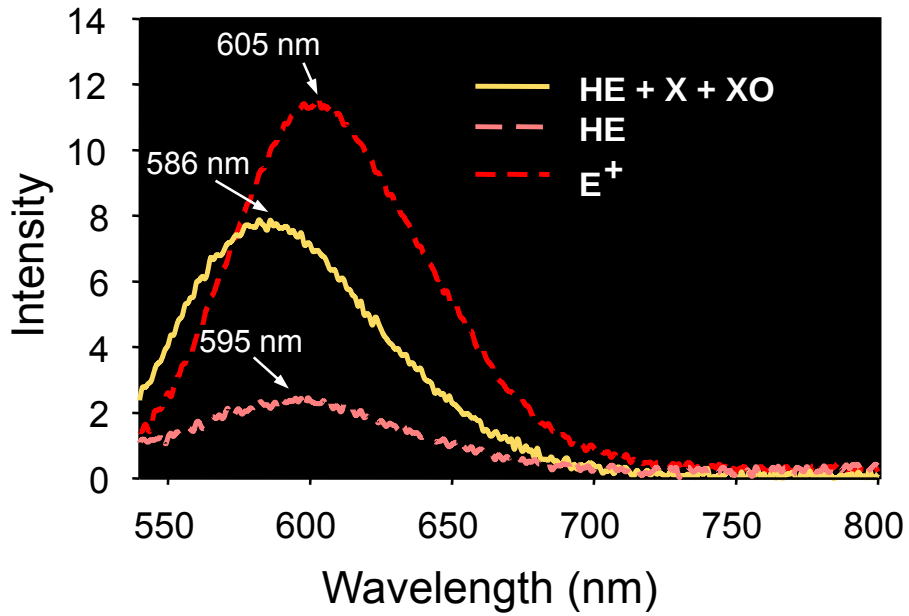
Reaction between hydroethidine and superoxide



Hydroethidine (HE)

Ethidium (E^+)
(red fluorescence)

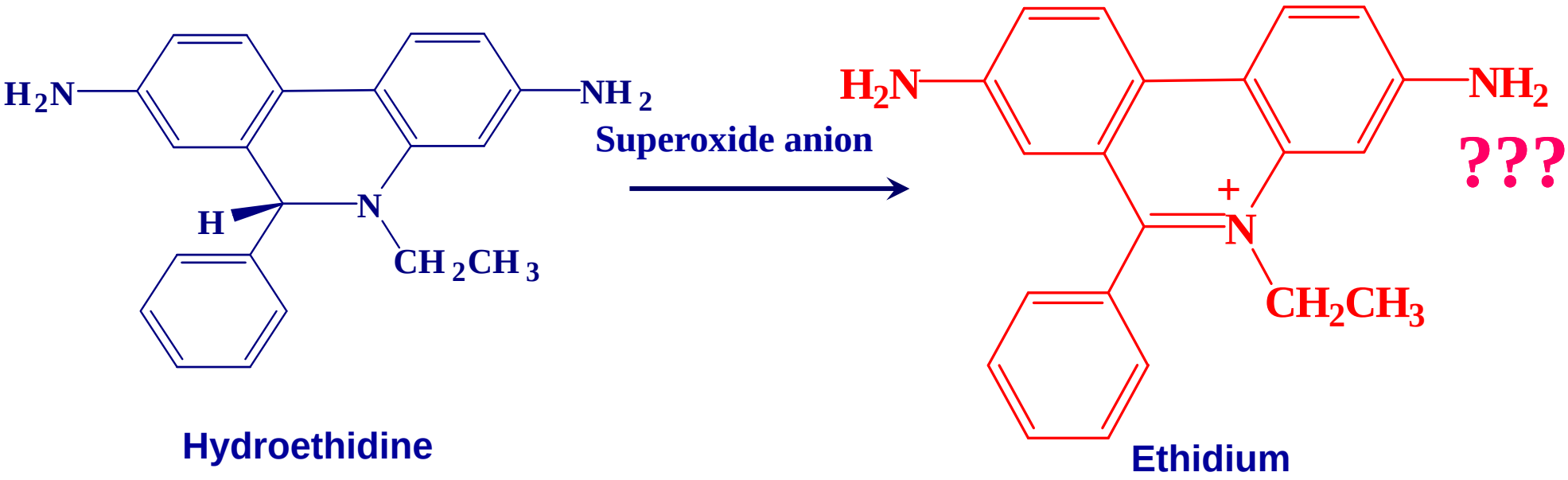
Fluorescence spectra of the product formed during oxidation of HE by X/XO



Incubation mixtures consisted of HE (50 μM), xanthine (1 mM), DTPA (100 μM), and XO (0.05 U ml^{-1}) in aerated phosphate buffer (100 mM, pH 7.4). Reaction was initiated by the addition of XO and the fluorescence spectrum recorded after 30 min. Shown for comparison is the spectrum obtained from E^+ (50 μM) obtained under identical experimental conditions.

Zhao H, et al. *FRBM* 34:1359-1368, 2003

Does superoxide react with hydroethidine to form ethidium?



Hydroethidine

Ethidium

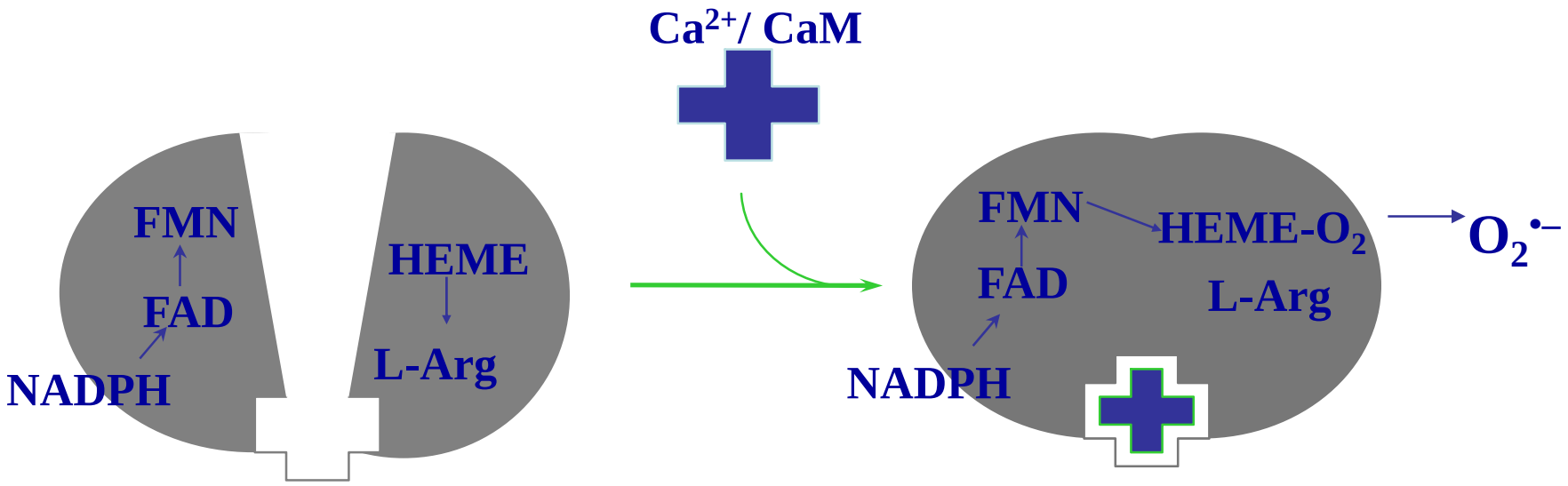
Superoxide generating systems

1. Xanthine/xanthine oxidase systems

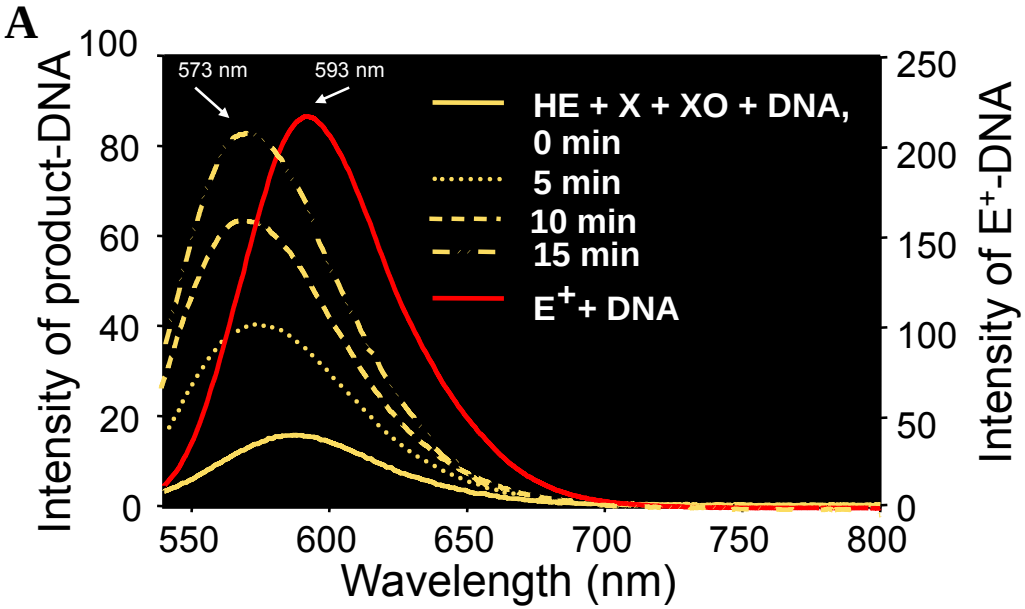
$$\text{Xanthine (1 mM)} + O_2 \xrightarrow{\text{Xanthine Oxidase (0.05U/ml)}} \text{Uric acid} + O_2^{\bullet -}$$

2. Potassium superoxide (KO_2) in DMSO with crown ether

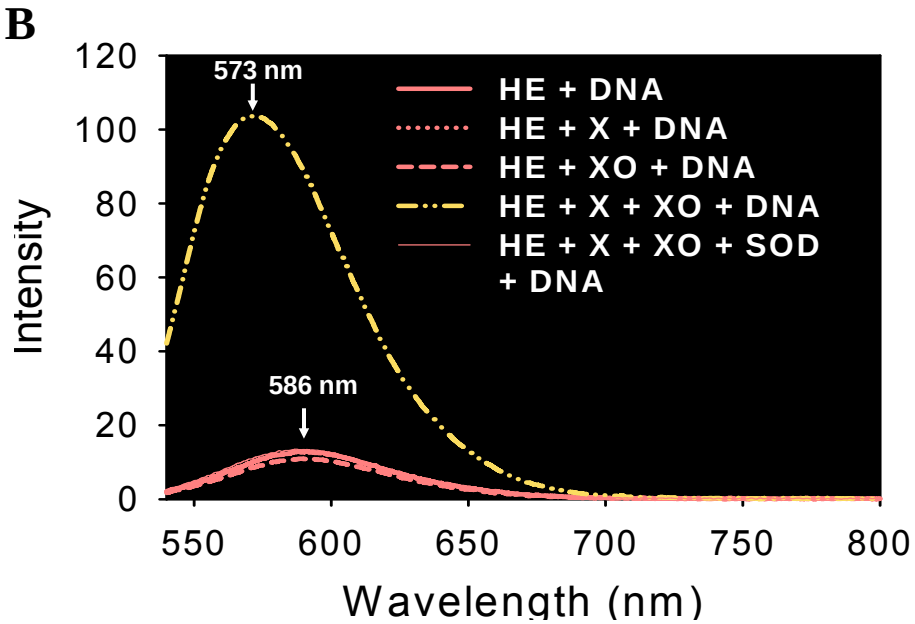
3. BH_4-depleted eNOS systems

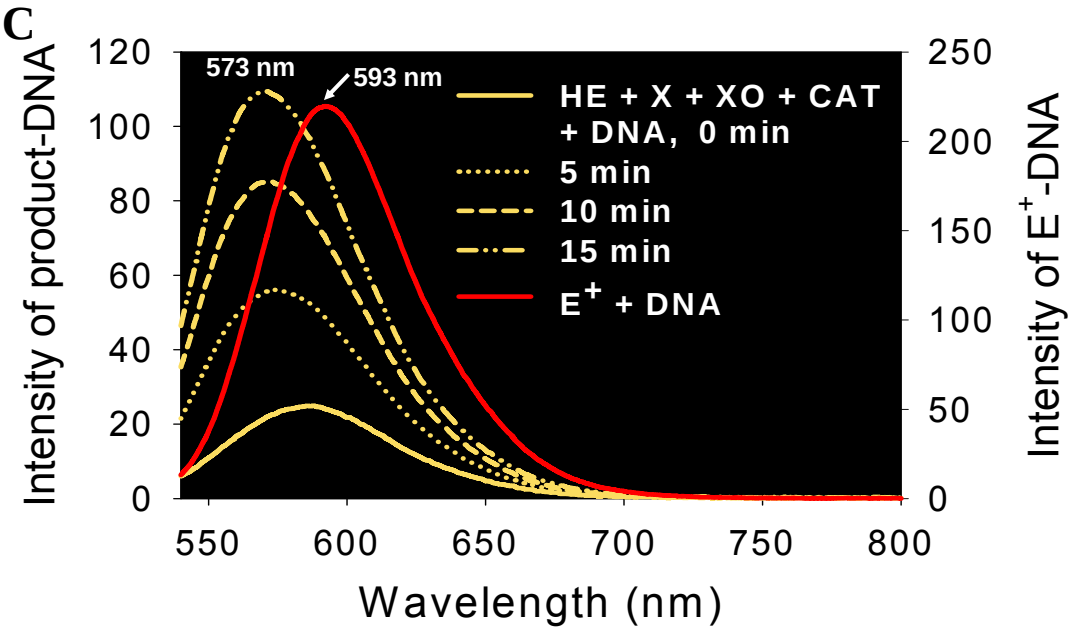


Fluorescence spectra of HE/X/XO-DNA and EB-DNA

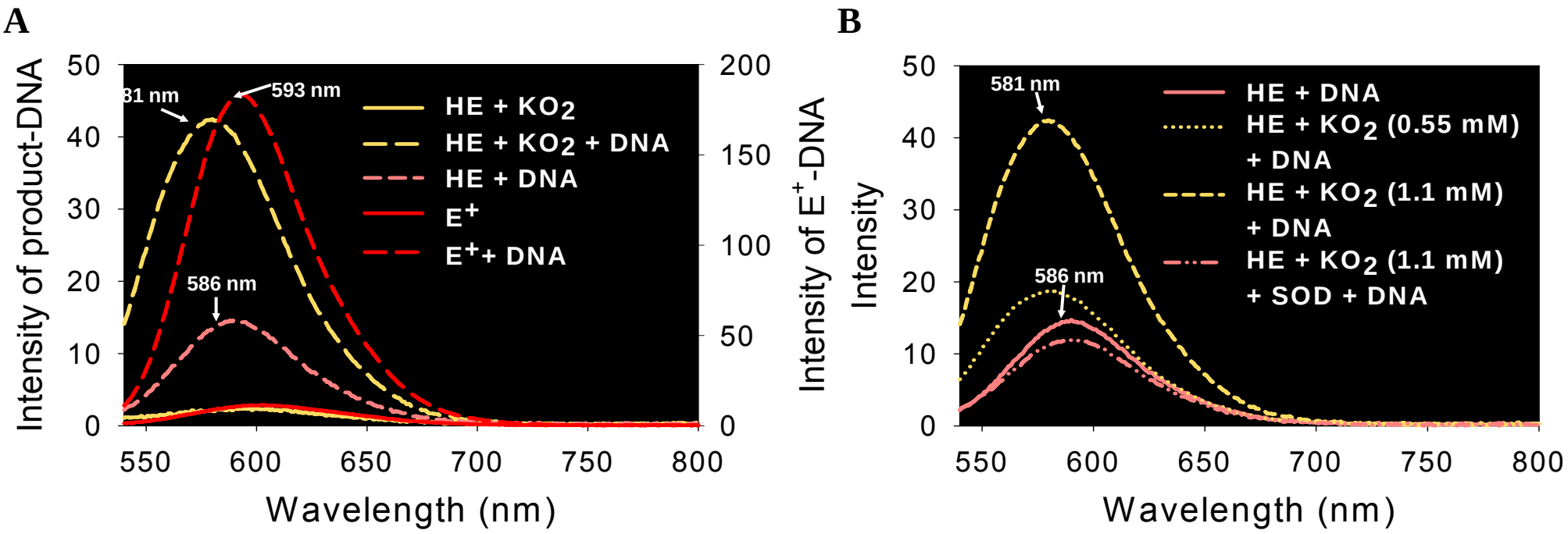


Incubation mixtures consisted of HE (50 μM), xanthine (1 mM), DTPA (100 μM), and XO (0.05 U ml^{-1}) in aerated phosphate buffer (100 mM, pH 7.4). Reaction was initiated by the addition of XO and the fluorescence spectrum recorded after 30 min. Shown for comparison is the spectrum obtained from E^+ (50 μM) obtained under identical experimental conditions. (A) in the presence of DNA (250 μg ml^{-1}). (B) Incubations contained HE (50 μM), DTPA (100 μM), and DNA (250 μg ml^{-1}) and xanthine (1 mM) or XO (0.05 U ml^{-1}), both in the presence and absence of SOD (20 μg ml^{-1}). (C) Same as (B) but in the presence of catalase (1000 U ml^{-1}).



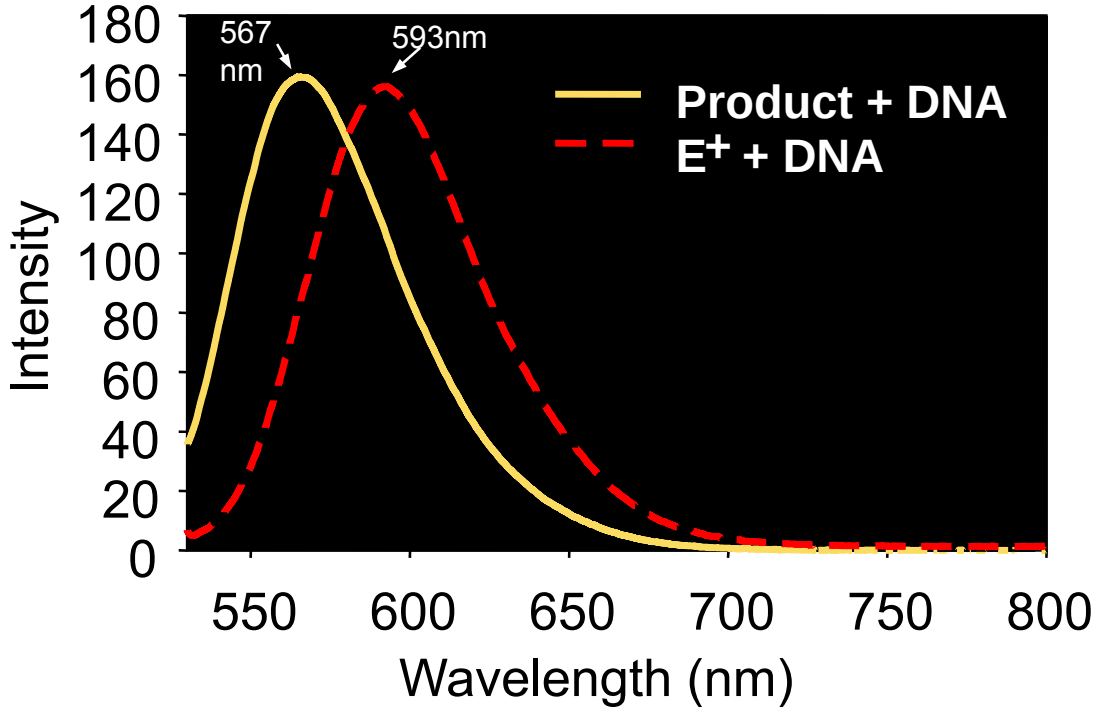


Fluorescence spectra of HE/KO_2-DNA and EB-DNA



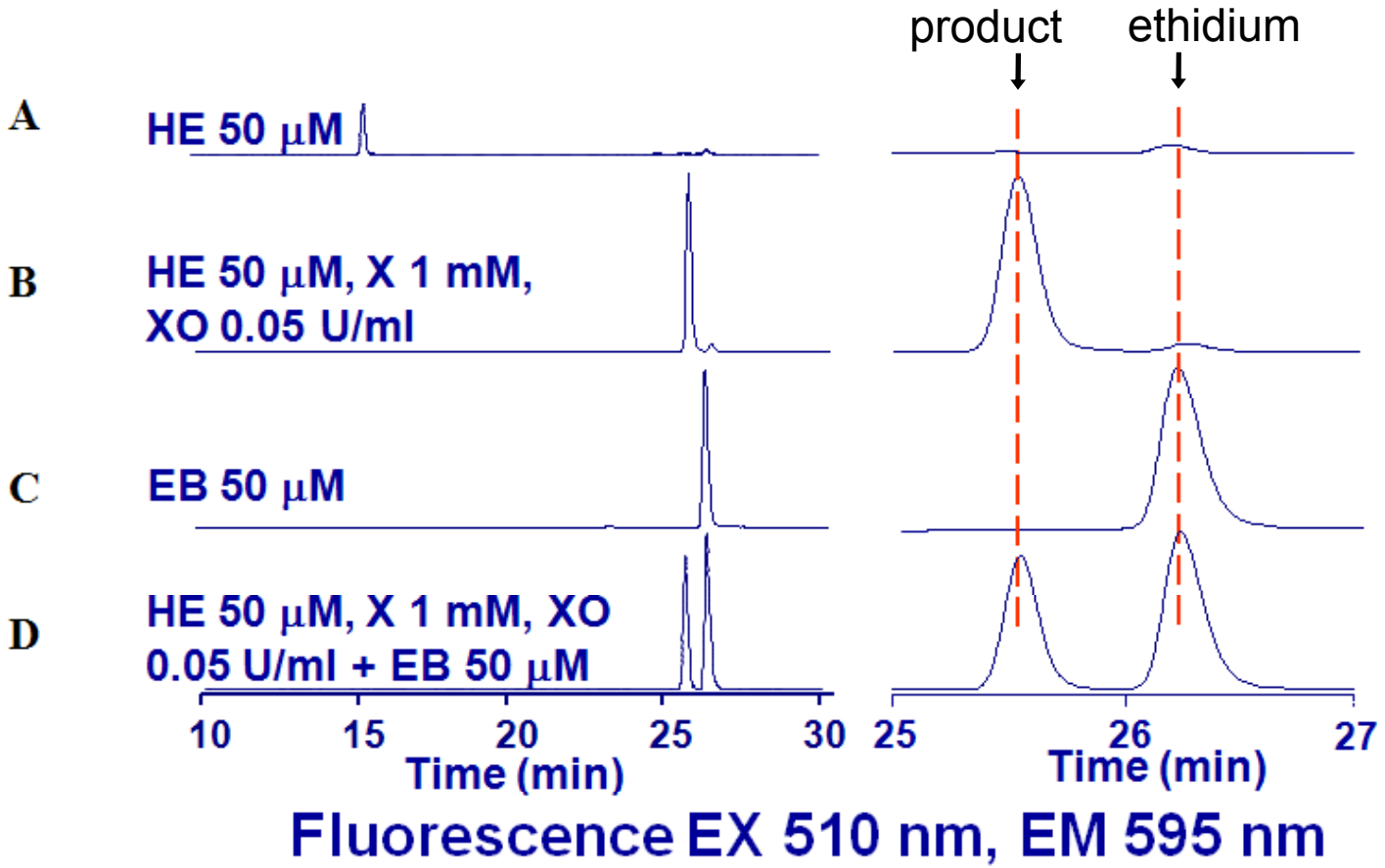
Fluorescence spectra of the product formed during oxidation of HE by potassium superoxide. (A) Incubations consisted of HE (50 μM), DTPA (100 μM), and KO_2 in the presence and absence of DNA (250 μg ml^{-1}) in phosphate buffer (100 mM, pH 7.4). Superoxide solution was prepared by adding 1 mg of KO_2 in 1 ml of dry DMSO and vortexed vigorously for 10 min. The reaction was initiated by adding 40 μl of freshly prepared KO_2 in DMSO to 0.46 ml of the above buffer (final concentration of KO_2 was 1.1 mM). (B) Same as (A) but also containing SOD (20 μg ml^{-1}). The extent of oxidation of HE under these conditions is much less than that observed in X/XO.

Fluorescence spectra of the product of HE reaction with $O_2^{\cdot-}$ and EB-DNA



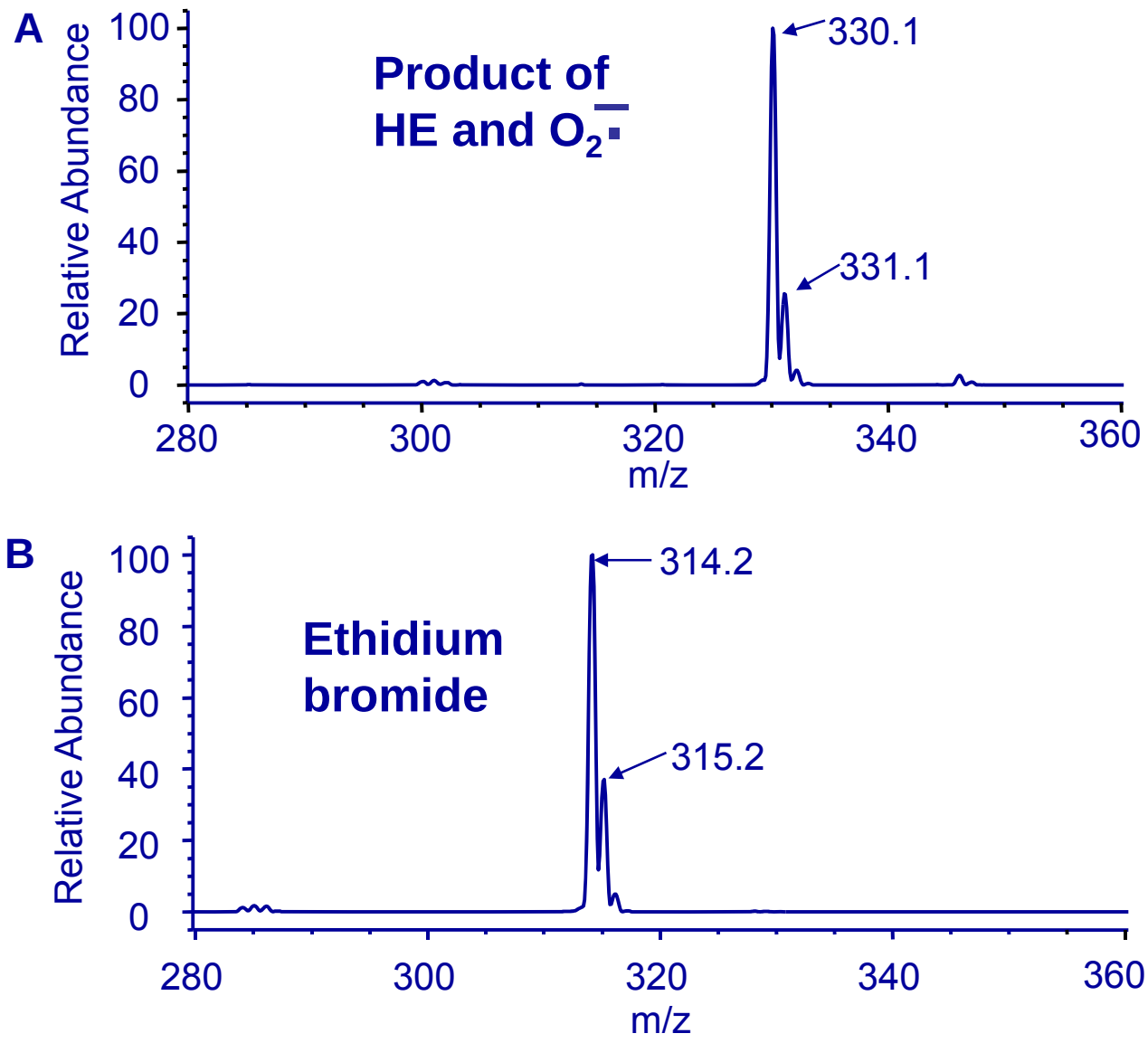
Fluorescence spectra of the purified product formed from oxidation of HE by X/XO and E^+ in the presence of DNA. Incubations contained 50 μM of the pure product, 50 μM E^+ and DNA (250 μg ml^{-1}) in phosphate buffer (100 mM, pH 7.4).

HPLC analysis



HPLC chromatograms of HE, E^+, and the product formed from oxidation of HE by the X/XO system. (A) Incubations contained xanthine (1 mM), HE (50 μM), and DTPA (100 μM). (B) Same as above but in the presence of XO (0.05 U ml^{-1}). HPLC traces in (A) and (B) were obtained 30 min after starting the incubation. (C) HPLC trace of authentic E^+ (50 μM) in phosphate buffer (100 mM, pH 7.4). (D) Same incubation conditions as in (B) but spiked with authentic E^+ (50 μM). Right trace (A–D), HPLC chromatograms recorded on an expanded scale. Fluorescence detection at 510 nm (excitation) and 595 nm (emission) was used to monitor HE, E^+, and the oxidation product of HE.

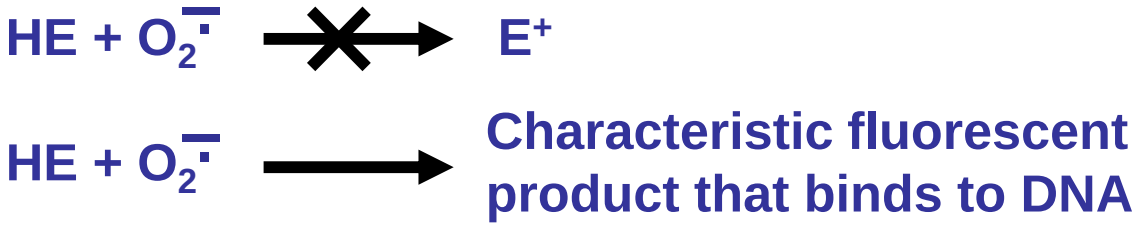
Mass spectrometry



HPLC-mass spectrometry. (A) The mass spectrum of new product taken from the LC peak at retention time of 34.30 min; incubations contained xanthine (1 mM), HE (50 μM), and DTPA (100 μM) and XO (0.05 U ml^{-1}) in phosphate buffer (100 mM, pH 7.4). (B) The mass spectrum of E^+ taken from the LC peak at retention time of 33.97 min; incubations contained E^+ (50 μM) in phosphate buffer (100 mM, pH 7.4).

Summary

1. Hydroethidine reacts with superoxide to form a fluorescent compound, which is different from ethidium bromide. This compound is a specific product for hydroethidine reaction with superoxide. The fluorescence of the new compound is also be enhanced by DNA.

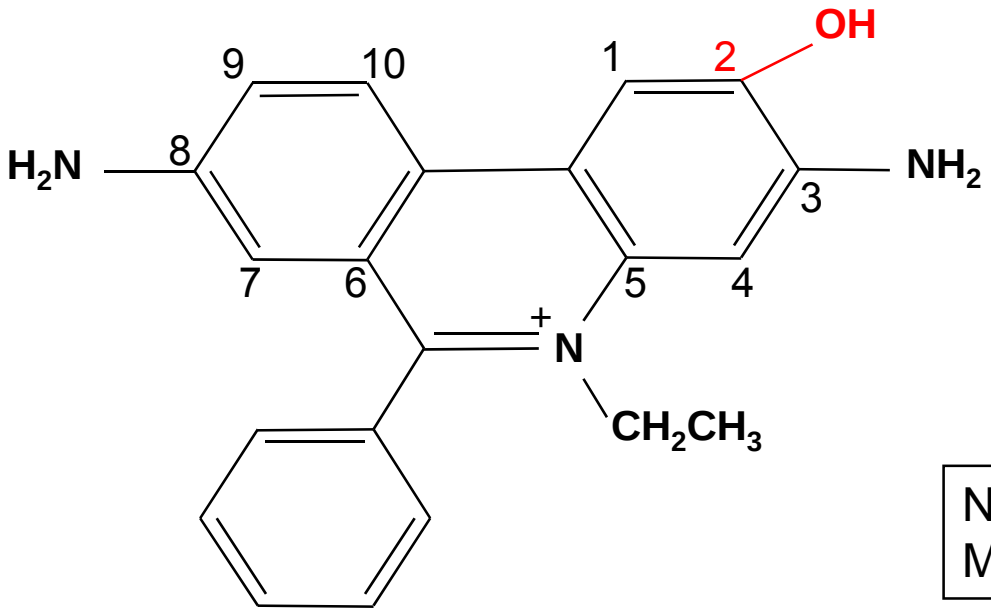


2. Other oxidants (peroxynitrite, HOCl, H_2O_2, hydroxyl radical and peroxyl radicals) react with HE but do not form this particular product.

3. The new compound has a different retention time and molecular weight compared to ethidium cation.

4. The fluorescence properties of the new compound are different from that of ethidium bromide.

Structure of the HE/$O_2^{\cdot-}$ reaction product

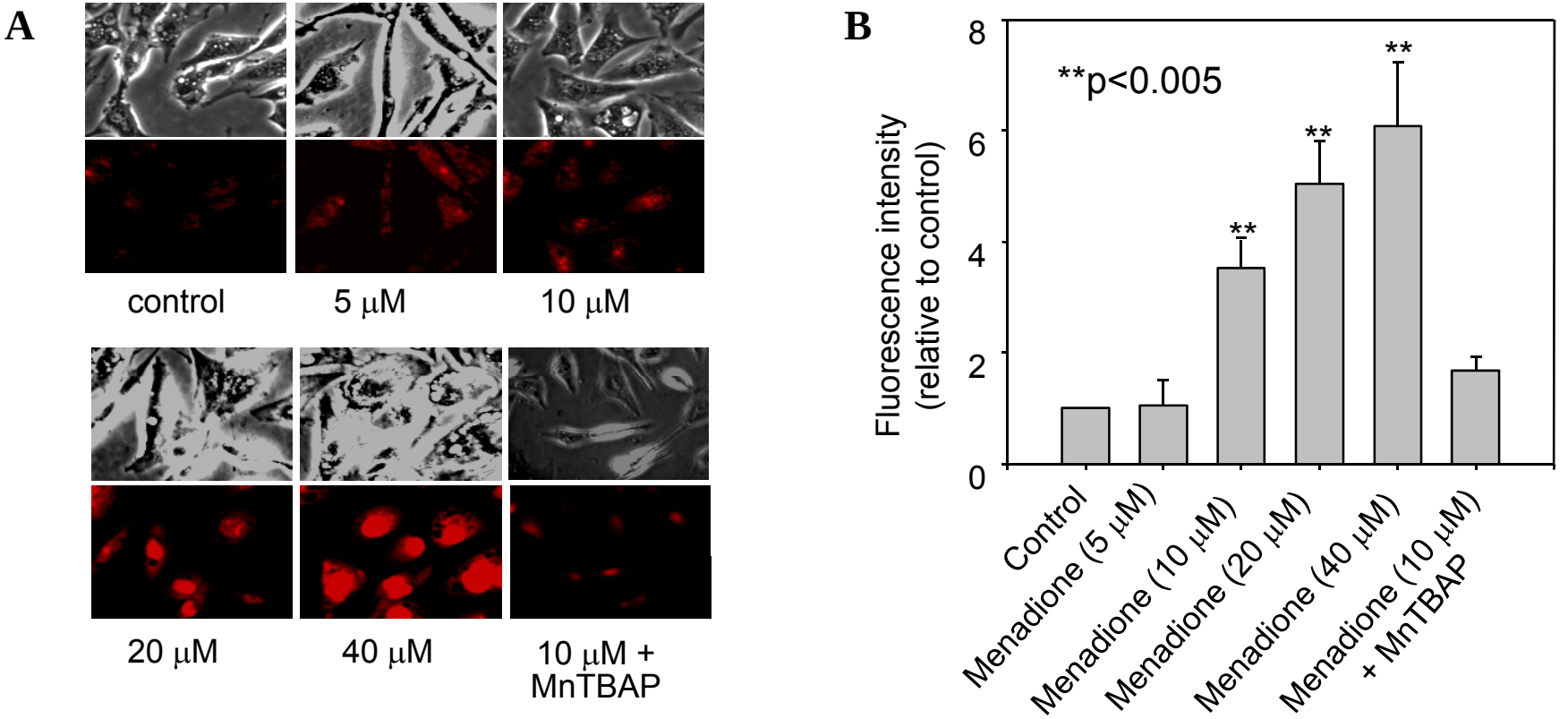


NMR
MS/MS analysis

2-Hydroxyethidium (2-OH-E^+)

Zhao H, et al. *PNAS* 102:5727-32, 2005

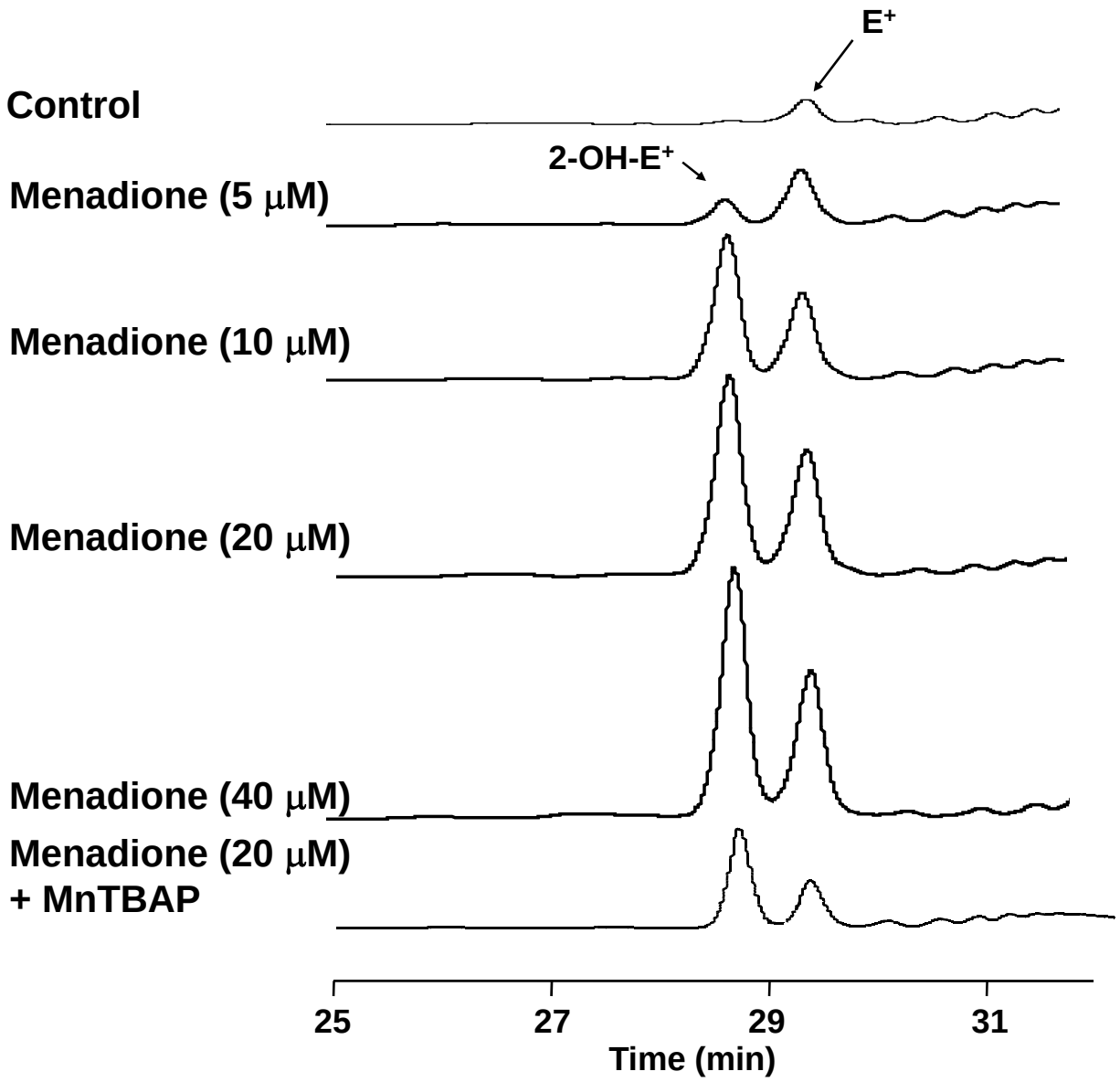
Intracellular oxidation of HE: Menadione-induced HE fluorescence in BAEC



Menadione-induced HE fluorescence in BAECs. (*A*) Phase-contrast and fluorescence images of BAECs treated with different concentrations of menadione for 30 min. After menadione treatment, BAECs were washed with DPBS and incubated with HE (10 μM) for 20 min. Cells were washed twice with DPBS and kept in the culture medium. The red fluorescence generated from HE was monitored by using fluorescence microscopy. BAECs were preincubated with MnTBAP (150 μM) for 2 h. After washing the cells free of extracellular MnTBAP, they were treated with menadione (10 μM) for 30 min, and HE-derived fluorescence was measured. (*B*) Densitometric analysis of data shown in *A*.

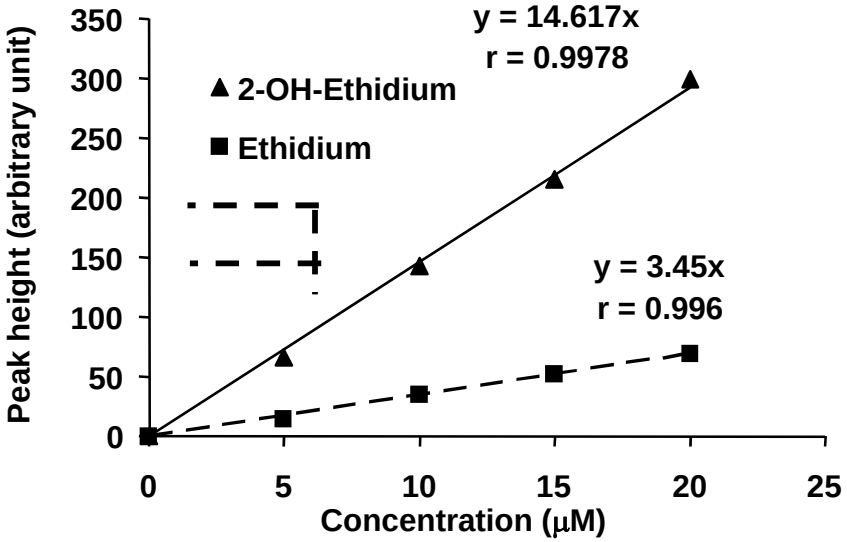
Zhao H, et al. *PNAS* 102:5727-32, 2005

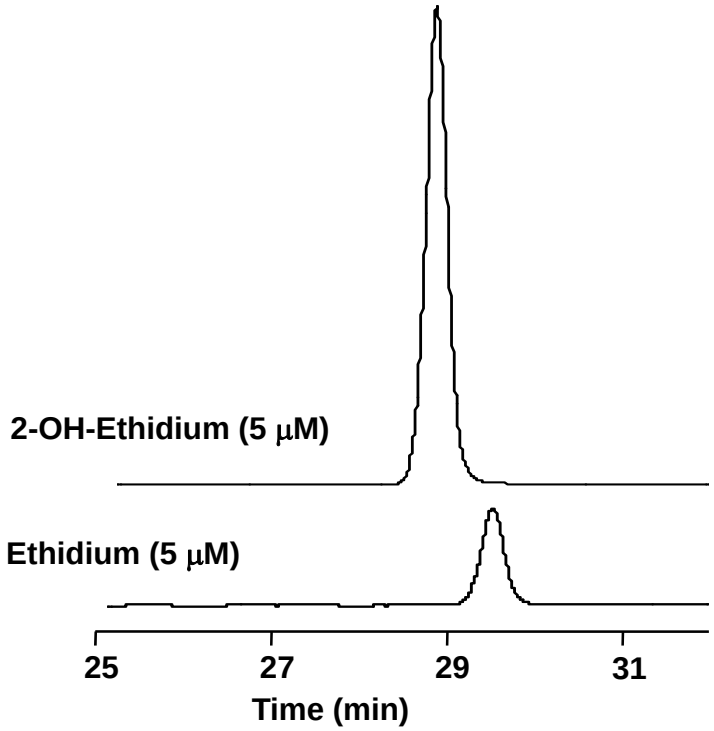
HPLC identification of 2-OH-E$^+$ from the $O_2^{\cdot-}$/HE reaction in BAECs



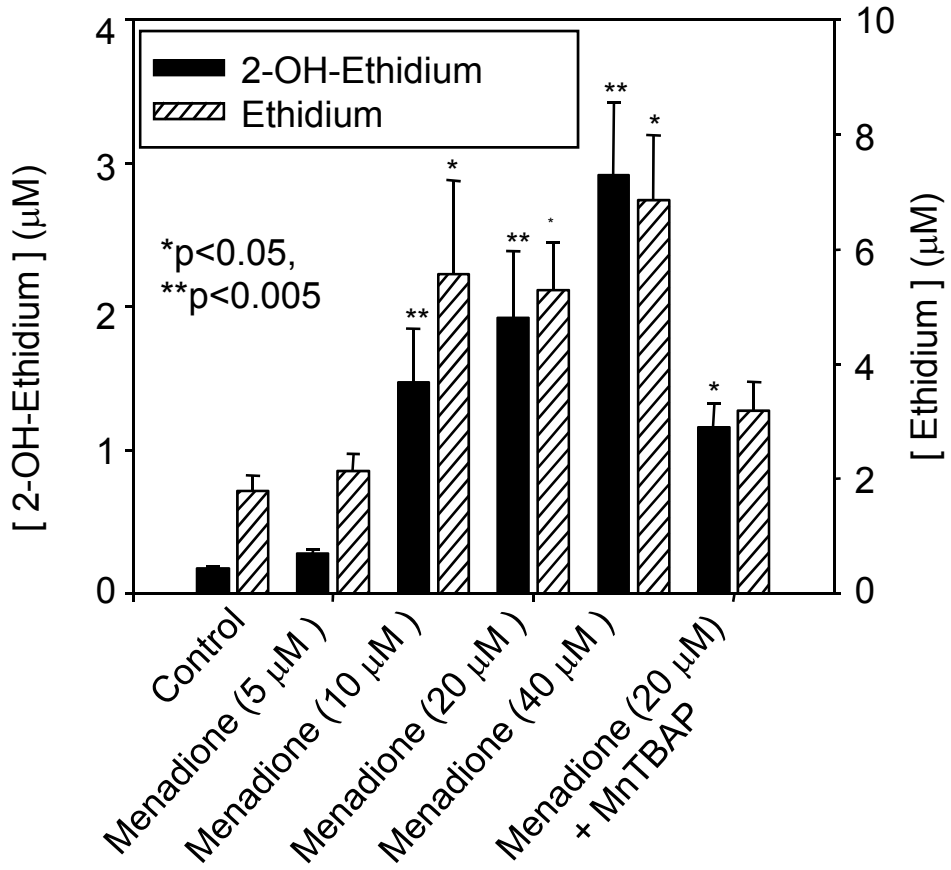
HPLC/fluorescence chromatograms of authentic 2-OH-E$^+$ (labeled 1) and E$^+$ (labeled 2). (*Inset*) HPLC peak intensity of 2-OH-E$^+$ and E$^+$ at different concentrations. BAECs were treated with various concentrations of menadione

HPLC calibration using authentic compounds



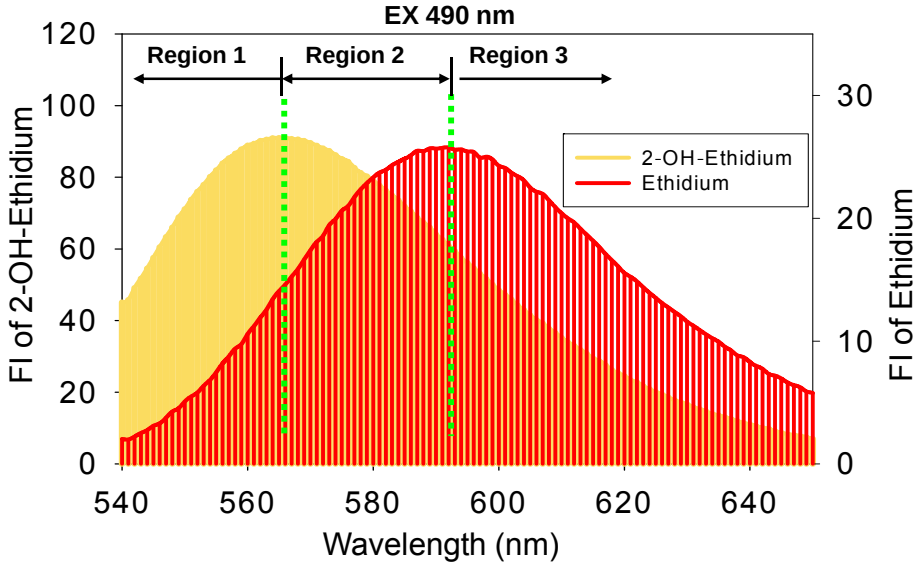


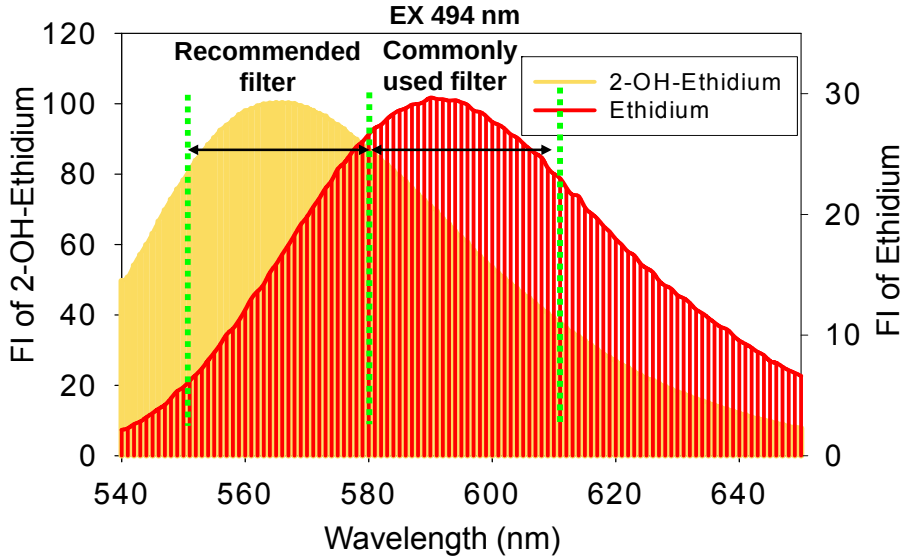
Intracellular concentration of 2-OH-E^+ and E^+

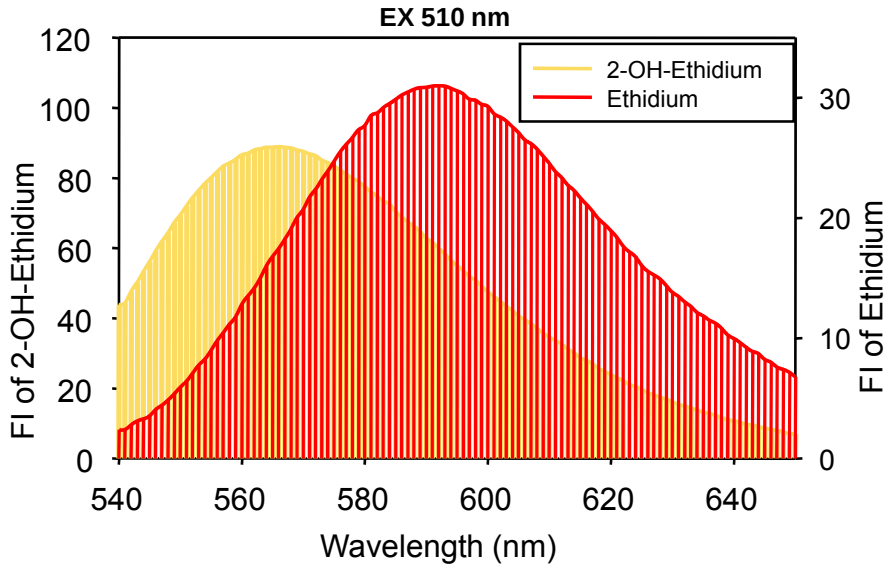


The actual concentrations of 2-OH-E^+ and E^+ in cell lysates generated under the conditions described above were calculated by using the calibration data shown

Optimal conditions for detection by fluorescence



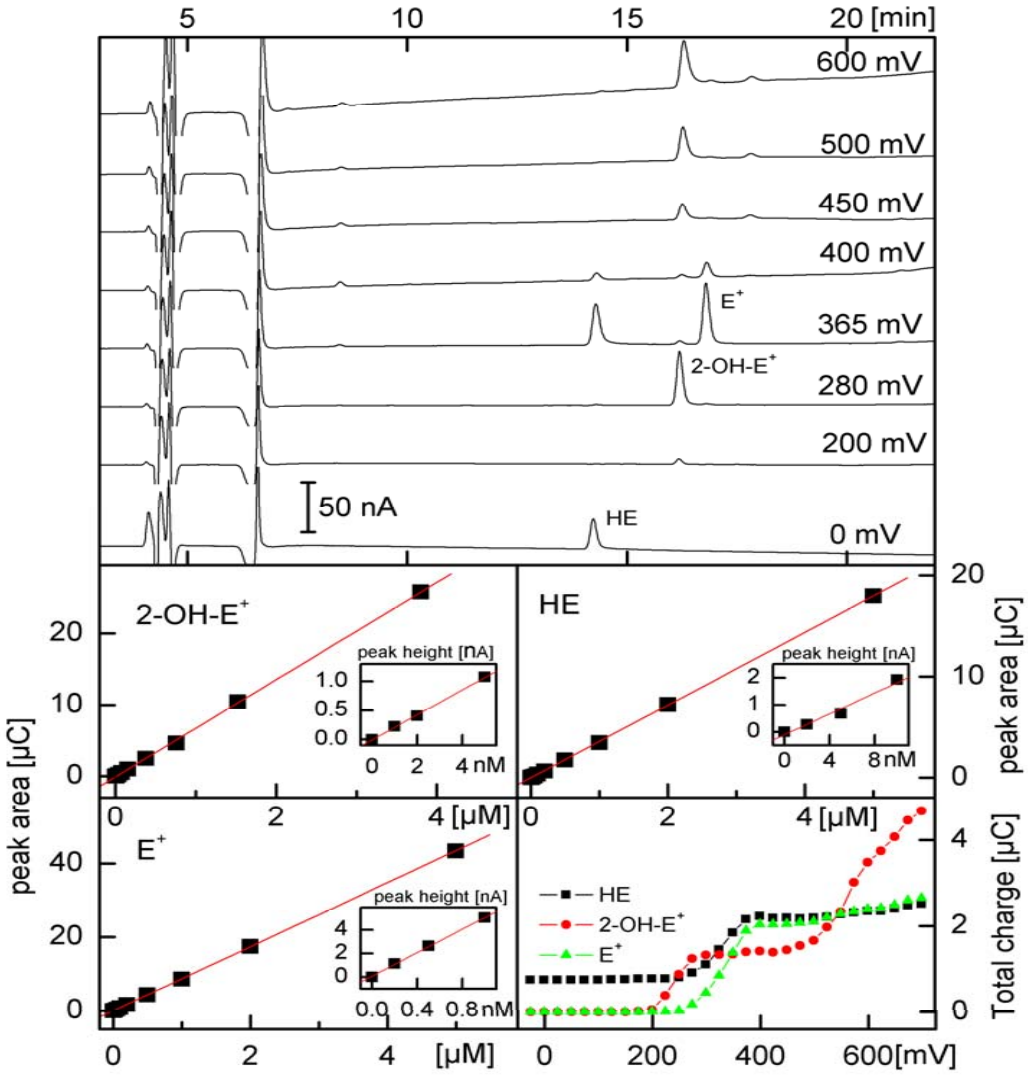




Conclusions

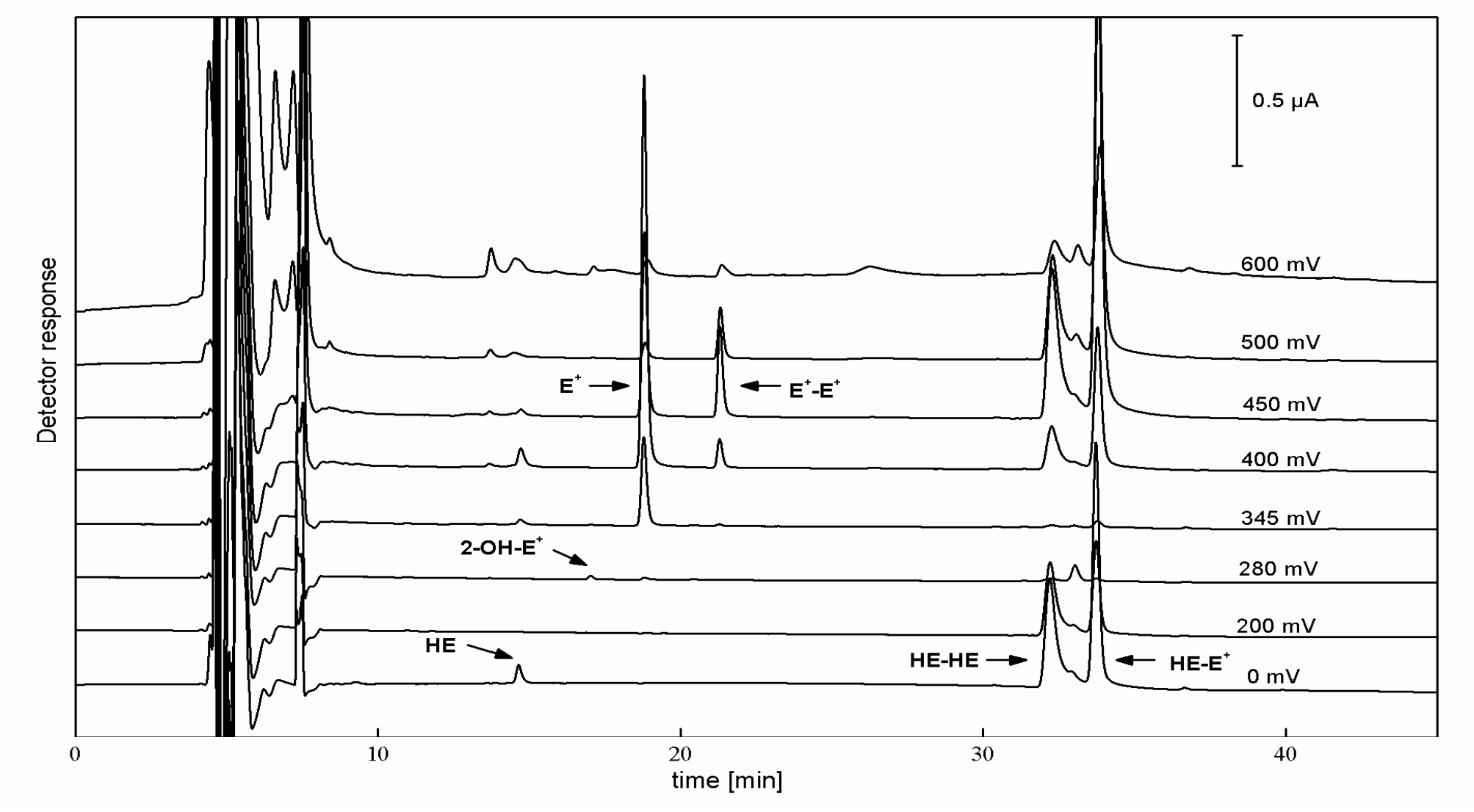
- Because of the overlapping fluorescence spectra from 2-OH-E^+ and E^+, the “red fluorescence” formed from HE cannot be used to quantitate intracellular $O_2\cdot^-$ formation.
- The new HPLC/fluorescence assay using HE as a probe is more suitable for quantifying intracellular $O_2\cdot^-$.
- Intracellular formation of 2-OH-E^+ is a diagnostic marker product of $O_2\cdot^-$.

HPLC/EC detection of HE, E^+, and 2-OH-E^+: A more sensitive methodology

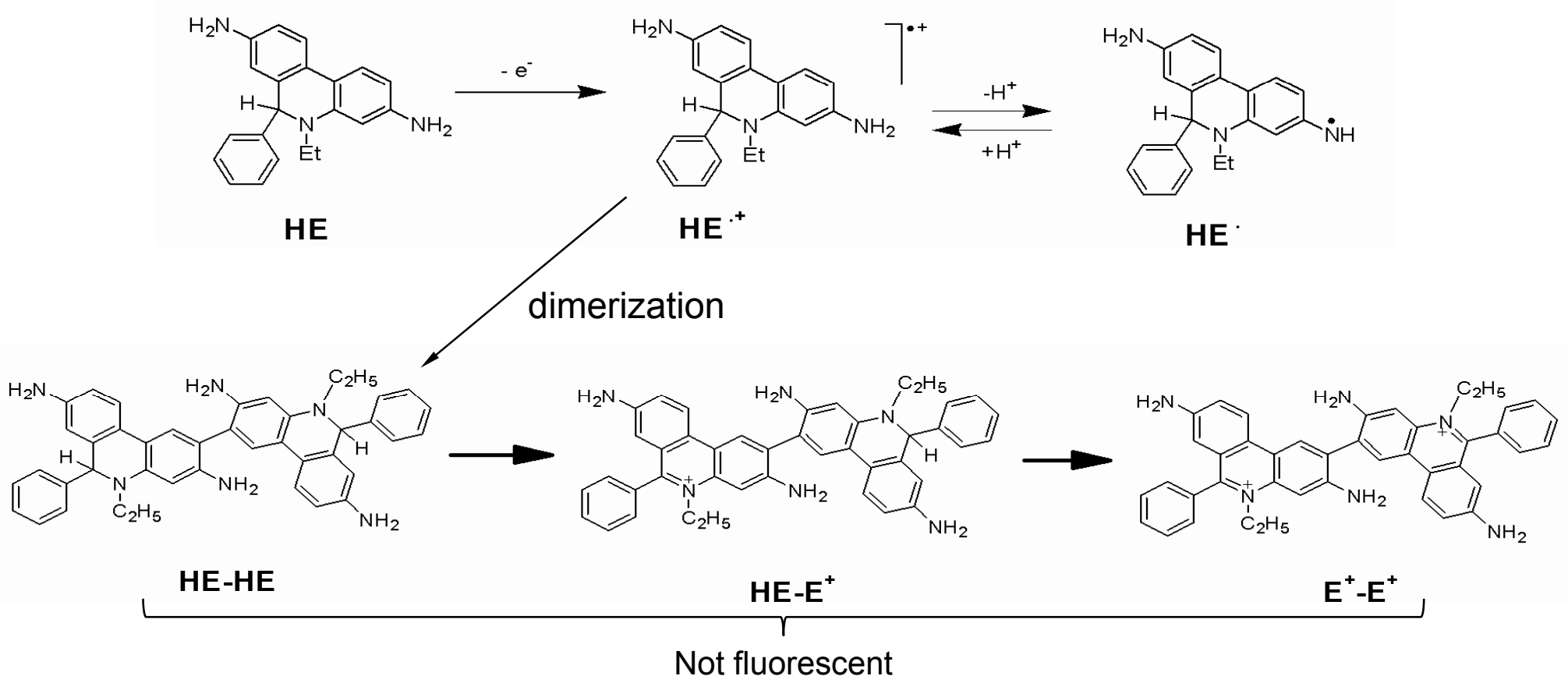


Zielonka J, et al. *FRBM* 41:1050-7, 2006

HPLC-EC identification of dimers

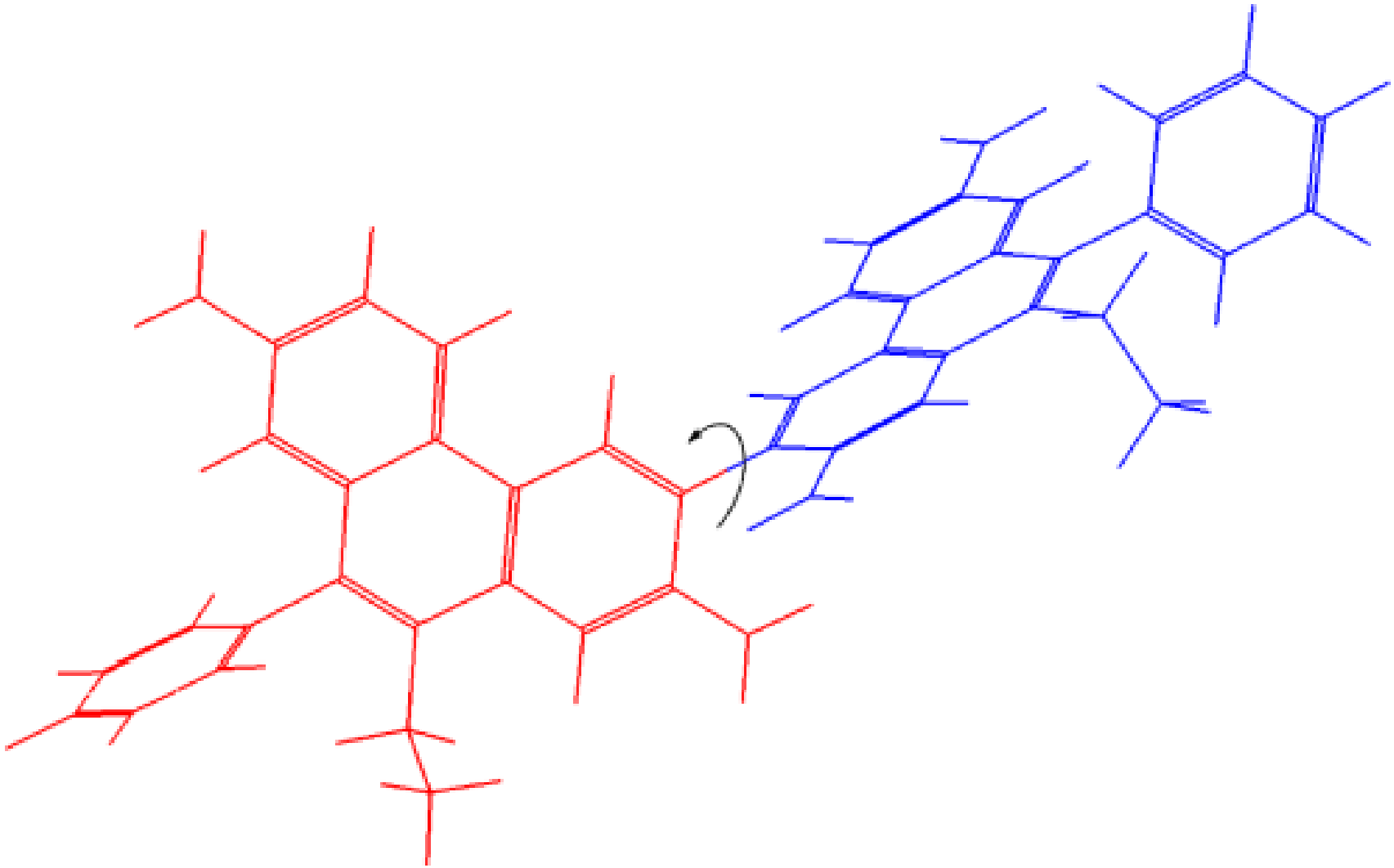


Radical-mediated oxidation of HE



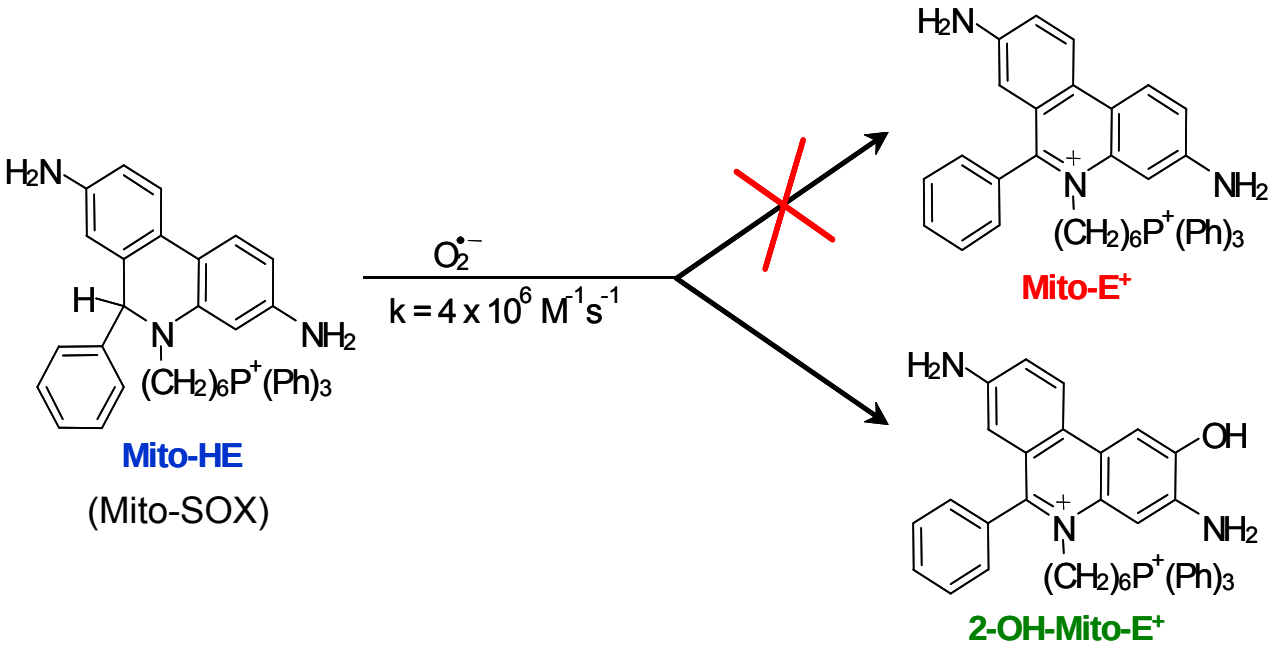
Zielonka J, et al. *FRBM* 44:835-46, 2008

Geometry of the dimer molecule



Zielonka J, et al. *FRBM* 44:835-46, 2008

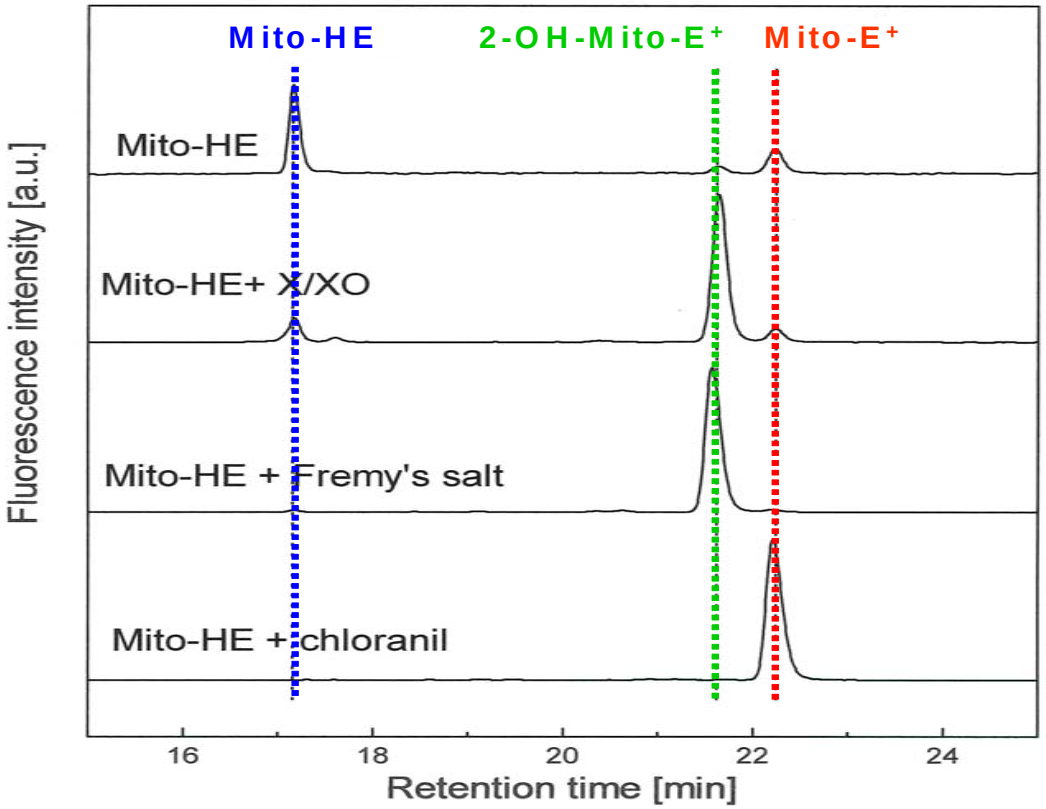
Superoxide reaction with Mito-HE leads to formation of a specific product, 2-OH-Mito-E+



Robinson KM, et al. *PNAS* 103:15038-43, 2006

Zielonka J, et al. *FRBM* 44:835-46, 2008

HPLC-fluorescence detection of products formed from oxidation of Mito-HE

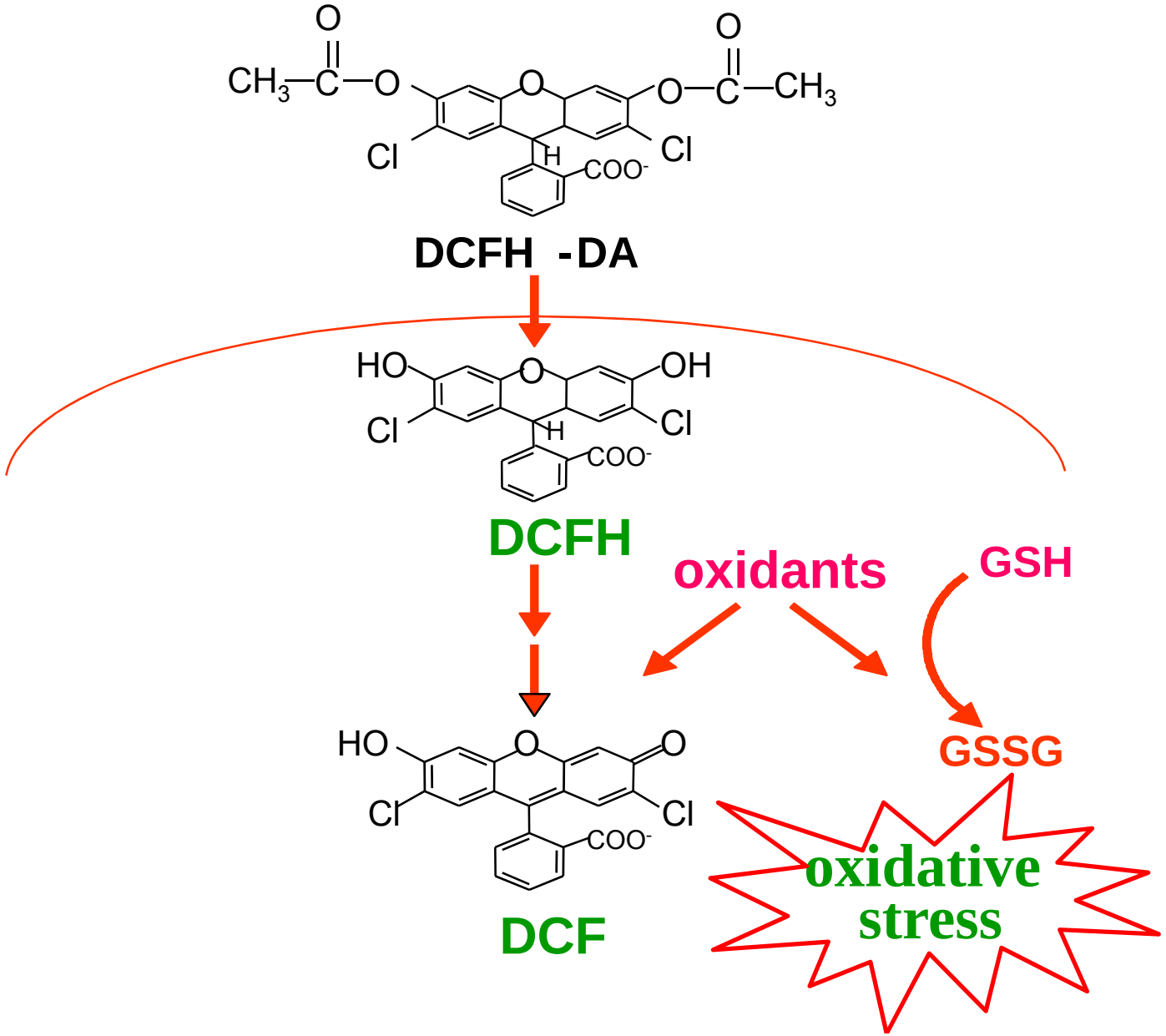


Zielonka J, et al. *FRBM* 44:835-46, 2008

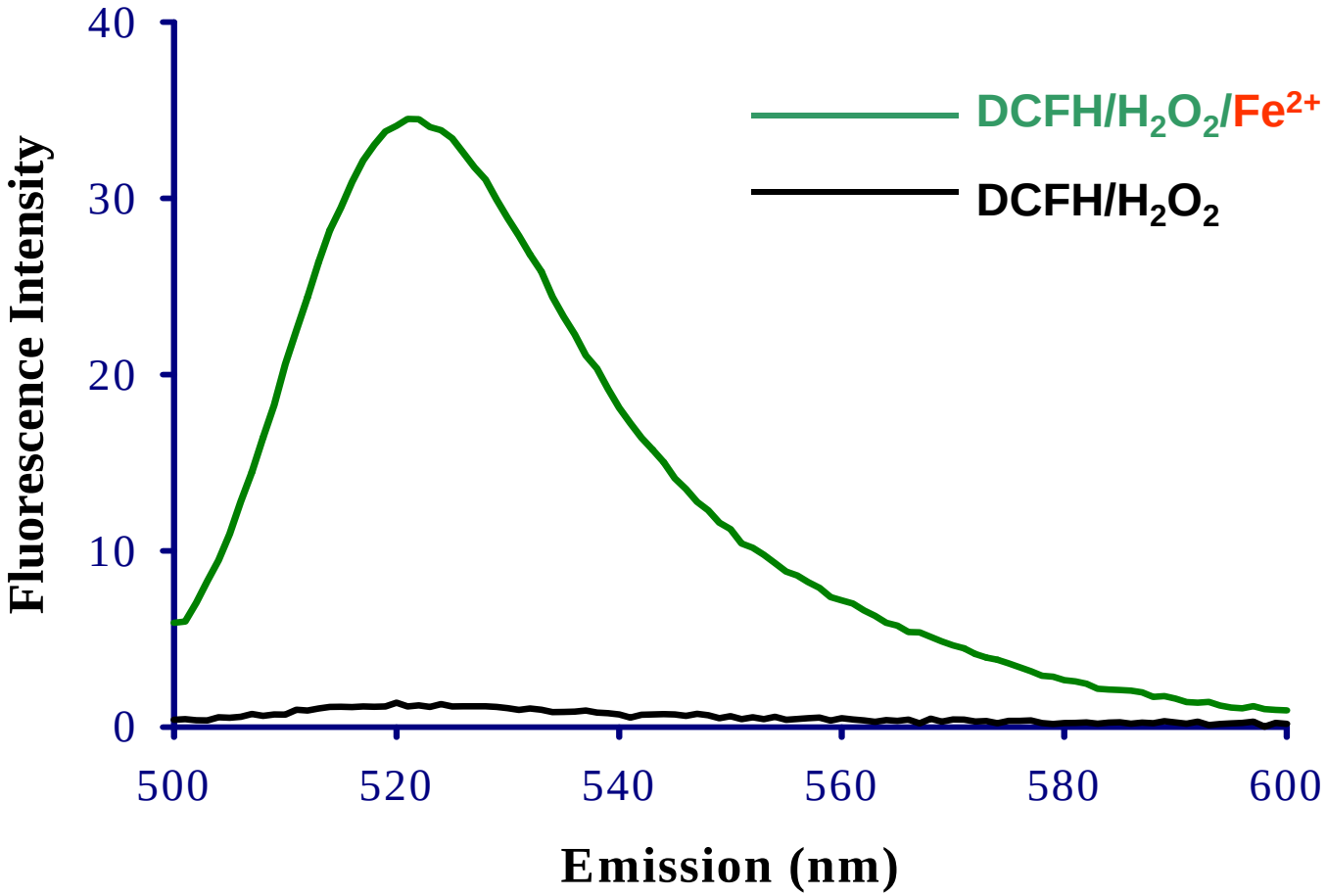
Overall conclusions

- HE and its mitochondria-targeted analogs react with superoxide to form hydroxylated products that can be easily identified using HPLC techniques.
- However, prospects for exclusive visualization of these products in cells using fluorescence are not very good because of confounding oxidation chemistry of HE and Mito-HE.

Measurement of intracellular oxidative stress by DCF fluorescence

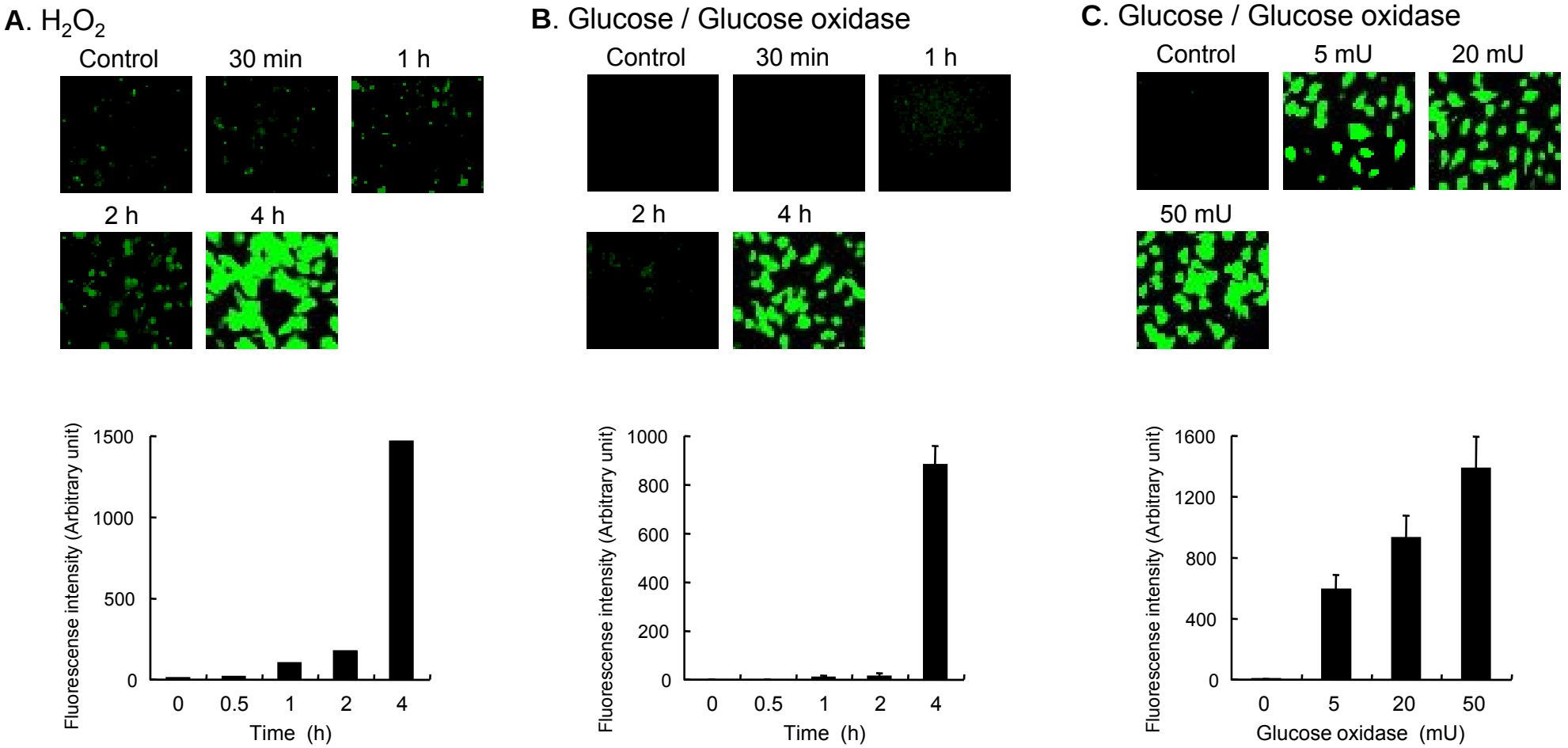


Oxidation of DCFH by H_2O_2 and iron



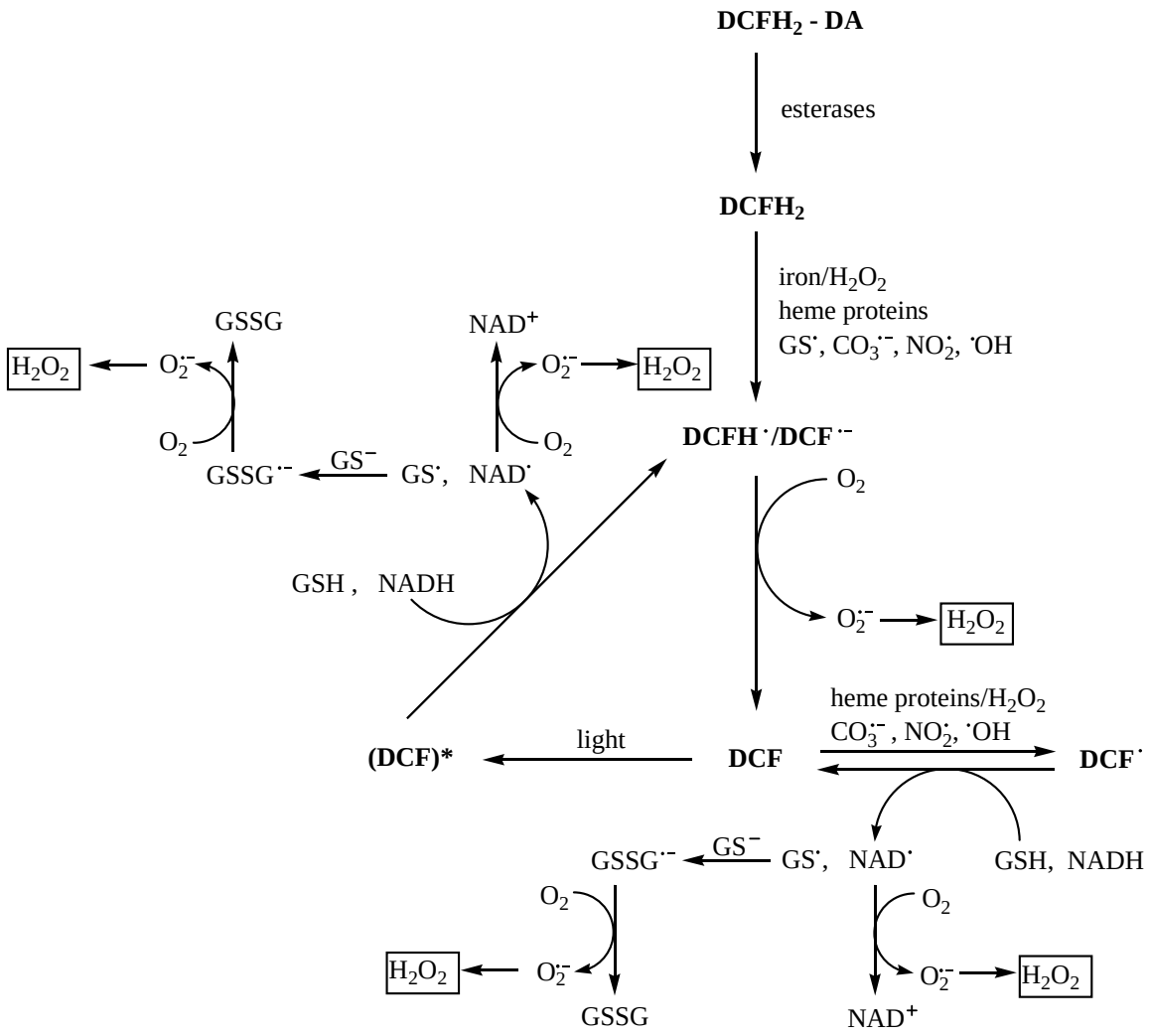
Thomas S, et al. *FRBM* 42:1049-61, 2007

Intracellular oxidation of DCFH to DCF



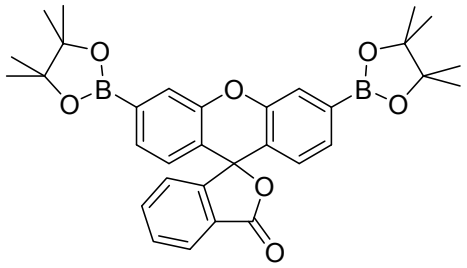
Tampo Y, et al. *Circ Res* 92:56-63, 2003

Redox reactions of DCF radical

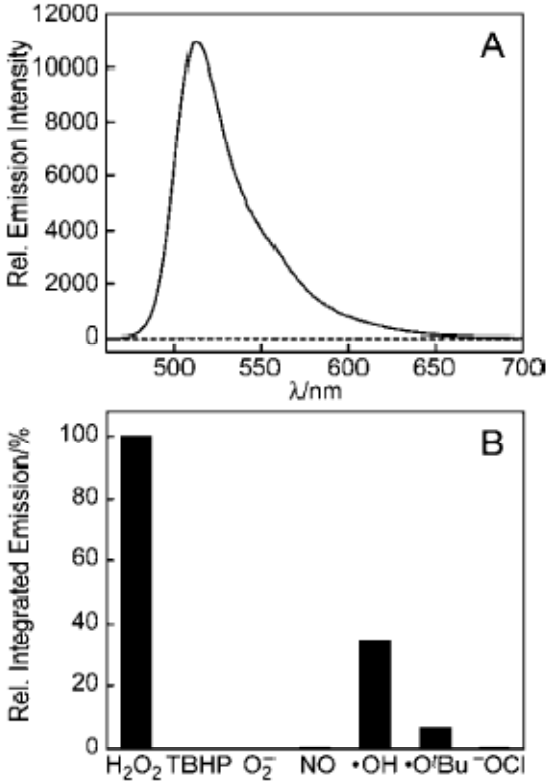


Zielonka J, et al. *FRBM* (in press, 2008) [doi:10.1016/j.freeradbiomed.2008.07.025]

Intracellular detection of H_2O_2 using the probe, PF1



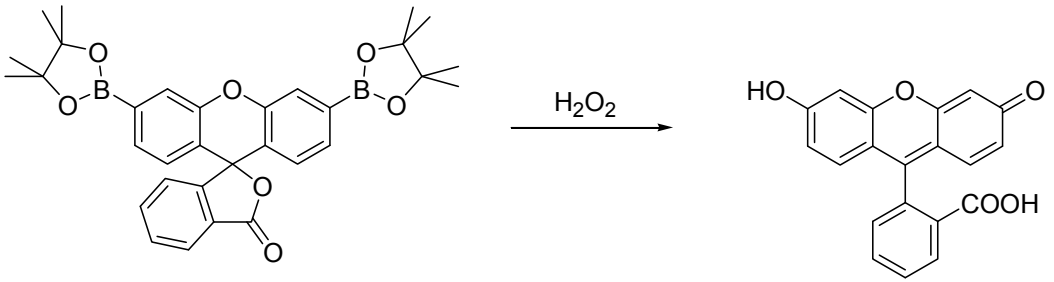
PF1



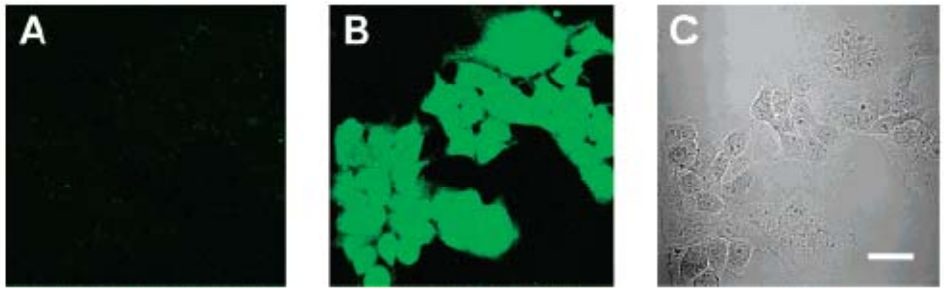
(A) Fluorescence response of 5 μM PF1 to 100 μM H_2O_2. The dotted and solid line spectra were recorded before and after H_2O_2 addition, respectively. Spectra were acquired in 20 mM HEPES, pH 7 (λ_{exc} = 450 nm). (B) Fluorescence responses of 5 μM PF1 to various ROS (10 mM O_2^-, 100 μM for all other ROS). $^{\bullet}$OH and $^{\bullet}$O^{t}Bu were generated by reaction of Fe^{2+} with H_2O_2 or *tert*-butyl hydroperoxide (TBHP), respectively. NO was delivered using *S*-nitrosocysteine (SNOC). Spectra were acquired in 20 mM HEPES, pH 7, and all data were obtained after incubation with the appropriate ROS at 25 °C for 1 h. Collected emission was integrated between 460 and 700 nm (λ_{exc} = 450 nm).

Chang MCY, et al. *J Am Chem Soc* 126:15392-15393, 2004

Intracellular detection of H_2O_2



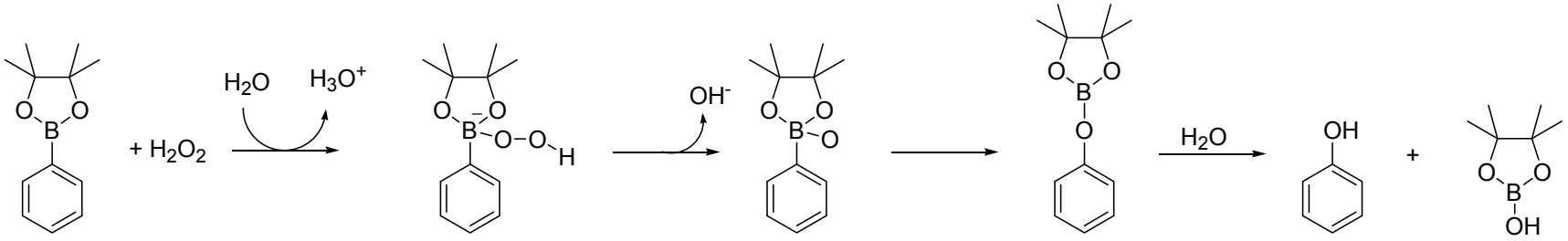
PF1 **Fluorescein**



Confocal fluorescence and phase contrast images of live HEK cells. (A) Fluorescence image of HEK cells incubated with 5 μM PF1 for 5 min at 25 °C. (B) Fluorescence image of PF1-stained HEK cells treated with 100 μM H_2O_2 for 5 min at 25 °C. (C) Brightfield image of live HEK cells after H_2O_2 addition to confirm viability. Scale bar = 30 μm.

Chang MCY, et al. *J Am Chem Soc* 126:15392-15393, 2004

Back to the drawing board!



Based on: Kuivila HG. *J. Am. Chem. Soc*. 76:870-874, 1954

Relevant publications

Zhao H, Kalivendi S, Zhang H, Joseph J, Nithipatikom K, Vasquez-Vivar J, Kalyanaraman B. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Radic Biol Med* 34:1359-1368, 2003.

Zhao H, Joseph J, Fales HM, Sokoloski EA, Levine RL, Vasquez-Vivar J, Kalyanaraman B. Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence. *Proc Natl Acad Sci USA* 102:5727-5732, 2005. [Erratum: *Proc Natl Acad Sci USA* 102:9086, 2005.]

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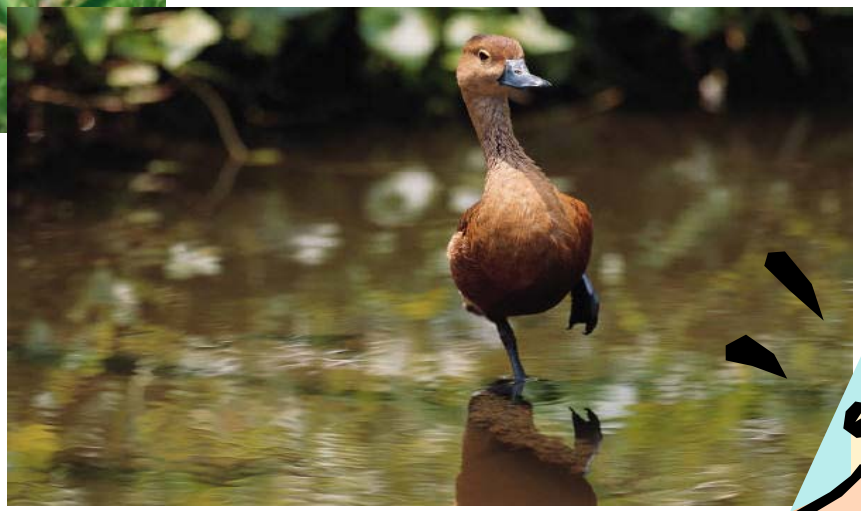

Happy Thanksgiving!