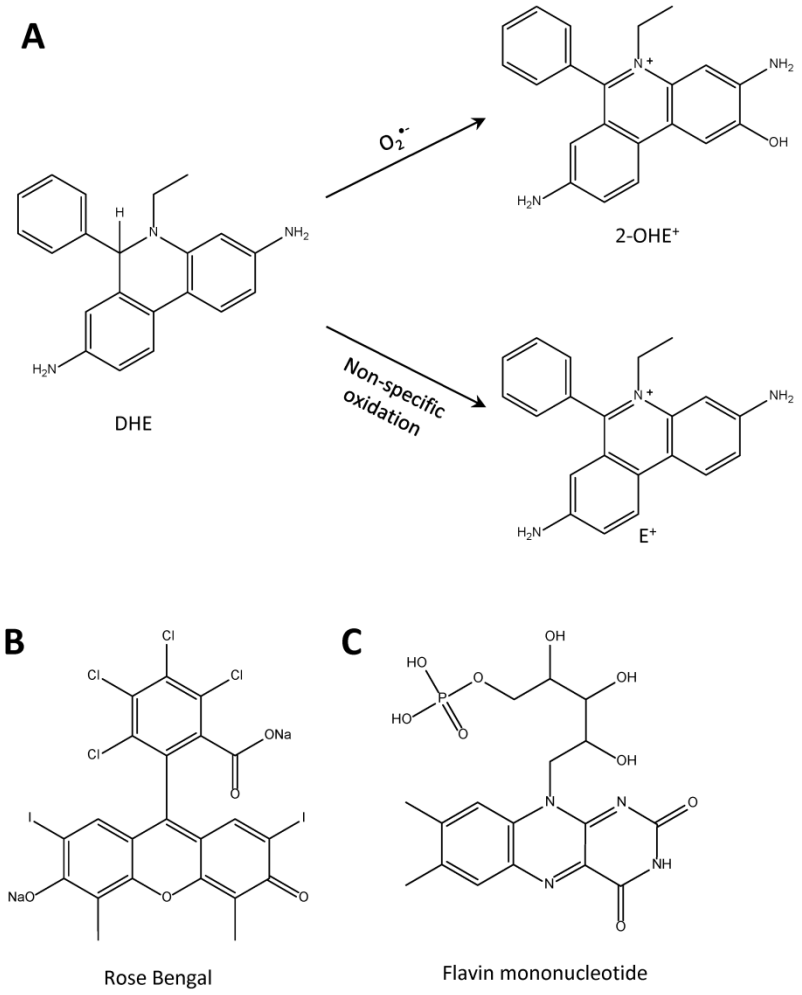
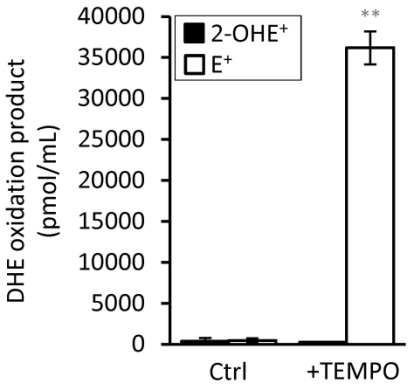
Quantification of light-induced miniSOG superoxide production using the selective marker, 2-hydroxyethidium

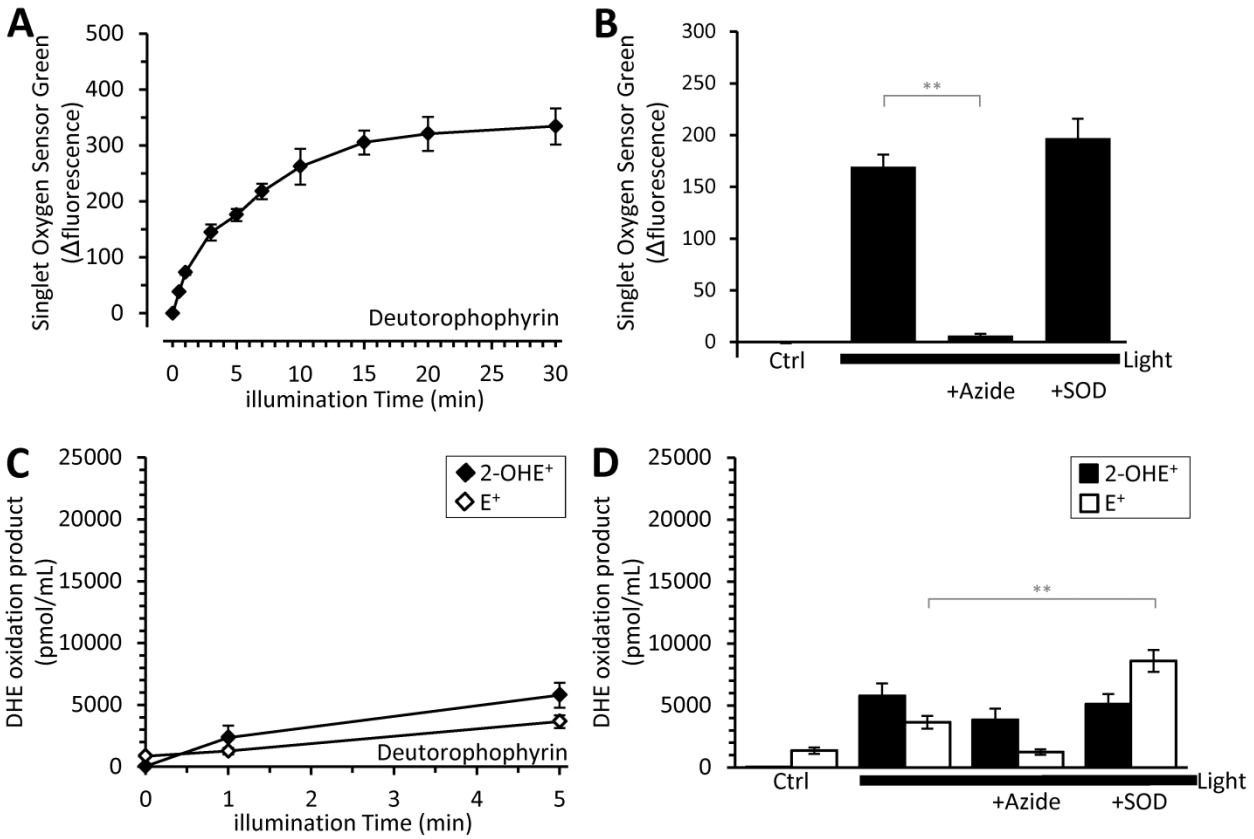
Supplemental Figures



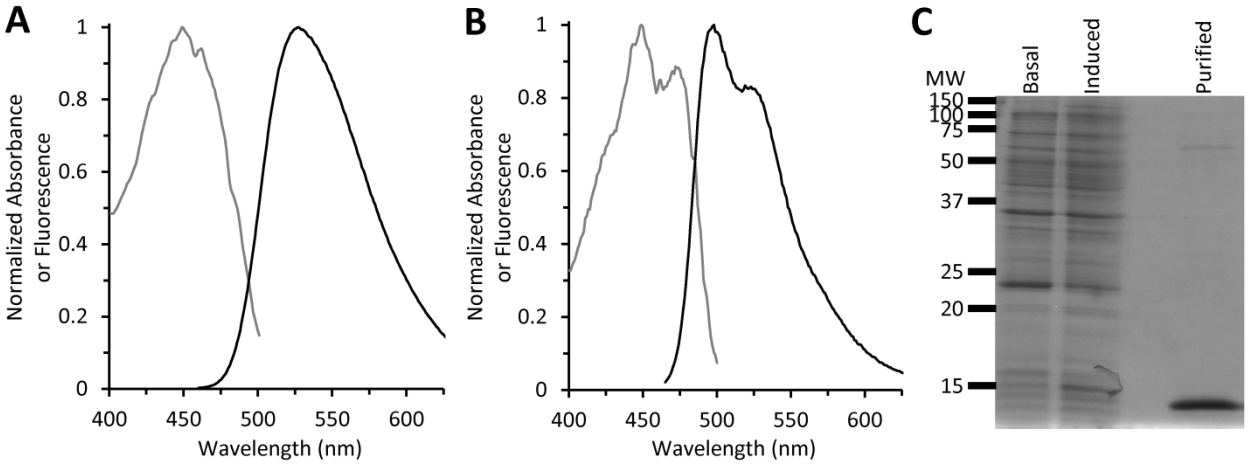
Supplemental Fig. 1. Structure of reagents and oxidant products. (A) Dihydroethidium (DHE) and the formation of ethidium (E^+) and the superoxide selective marker, 2-hydroethidium (2-OHE$^+$). DHE reacts with superoxide ($O_2^{\bullet-}$) to result in the formation of 2-OHE$^+$, while E^+ is generated via non-specific oxidation. Figure adapted from [16]. Structure of (B) Rose Bengal and (C) Flavin mononucleotide.



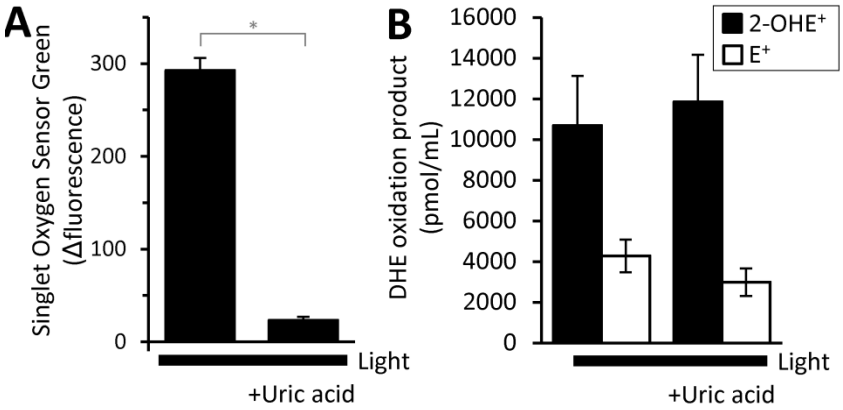
Supplemental Fig. 2. Interaction between DHE and TEMPO. Superoxide scavenger TEMPO reacts with DHE to form ethidium (E^+). DHE (50 μM) and 4-hydroxyl tempo (5 mM) were mixed and the samples were immediately processed. HPLC analysis showed that all of the DHE was converted to E^+ and thereby preventing any possible 2-OHE^+ formation. Since the E^+ product is superoxide ($O_2^{\bullet-}$)-nonspecific, this unintended reaction prevents any detection of $O_2^{\bullet-}$ by 2-OHE^+. This effect was also observed after a 5-minute incubation period (data not shown). All data are means ± SEM, N > 4, **p < 0.01 versus control (2-way ANOVA with Tukey correction).



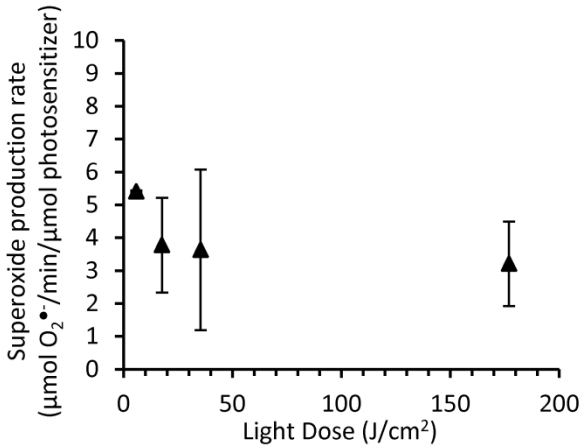
Supplemental Fig. 3. Singlet oxygen sensor green (SOSG) and dihydroethidium (DHE) detection of Deuteroporphyrin (DP) chemical photosensitizer-generated singlet oxygen and superoxide. Singlet oxygen sensor green (SOSG) and Dihydroethidium (DHE) detection of chemical photosensitizer-generated singlet oxygen ($^1O_2$) and superoxide ($O_2^{\bullet-}$), respectively. (A) DP was illuminated (560 ± 20 nm, 10.6 mW/mm^2) for the indicated time in the presence of SOSG. $^1O_2$ production was determined by the change in SOSG fluorescence intensity. (B) Where indicated the $^1O_2$ scavenger, azide (20 mM) or $O_2^{\bullet-}$ scavenger superoxide dismutase (SOD, 800 U/mL) was present when illuminated for 5 minutes. Azide or SOD in the presence of SOSG in the dark alone had no effect. SOSG illuminated for 5 minutes in the absence of photosensitizer yielded a change in fluorescence of -6.28 ± 1.4 a.u., as noted in Figure 2 (C) Detection of $O_2^{\bullet-}$ from DP (2.5 μM) using HPLC separation of DHE oxidized products. Samples were illuminated (560 ± 20 nm, 10.6 mW/mm^2) for increasing amounts of time in the presence of DHE (50 μM). Peaks were integrated and quantified using a standard curve. Superoxide production (2-OHE$^+$) and ethidium components (E$^+$) were detected by HPLC separation. (D) SOD (800 U/mL) did not reduce the $O_2^{\bullet-}$ component, 2-OHE$^+$ generated by DP. 2-OHE$^+$ and E$^+$ components were insensitive to the $^1O_2$ scavenger azide ($p = 0.37$; $p = 0.20$; 5 min light vs 5 min light + azide for 2-OHE$^+$ and E$^+$, respectively). DHE illuminated for 5 minutes resulted in 258 ± 186 pmol/mL 2-OHE$^+$ and 607 ± 423 pmol/mL E$^+$ as noted in Figure 2. All data are means ± SEM, N > 4. ** $p < 0.01$ versus 5-minute light (2-way ANOVA with Tukey correction).



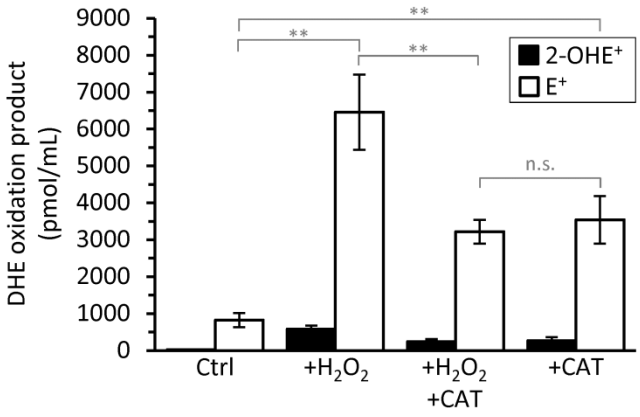
Supplemental Fig. 4. Properties of free FMN and purified miniSOG. To confirm the purified miniSOG, we performed a series of analysis in addition to fluorescent quantum yield and extinction coefficient reported in the main text. (A) The excitation and emission spectra of free FMN. (B) The excitation and emission spectra of purified miniSOG demonstrated an excitation and emission peak at 448 and 501, respectively [5]. (C) Coomassie stain of SDS-Page gel with uninduced (basal) and IPTG-induced BL21pLyS cells and purified miniSOG (50 µg of total protein loaded in each well). The purified His-tagged miniSOG has an expected molecular weight (MW) of 15.3kDa and runs consistent with the reported MW of 13.9 ± 0.4 kDa observed by light scattering [5].



Supplemental Fig. 5. Uric acid scavenges FMN-generated $^1O_2$ with no impact on $O_2^{\bullet-}$ detection. The $^1O_2$ scavenger azide interacted with the FMN triplet state and resulted in confounding results (Fig. 3), experiments were repeated with uric acid, a different $^1O_2$ scavenger. Free flavin mononucleotide (FMN, 10 µM) was illuminated (470 ± 20 nm; 5.9 mW/mm^2) for 5 minutes in the presence of SOSG (A), DHE (B) and uric acid (250 µM) where indicated. Uric acid significantly reduced fluorescence in the presence of SOSG (A) while it had no significant effect on either the 2-OHE$^+$ or E$^+$ component in the presence of DHE (B). Uric acid had no significant effect on singlet oxygen sensor green (SOSG) alone and SOSG illuminated for 5 minutes yielded a change in fluorescence of -1.53 ± 0.3 a.u as noted in Fig. 3. DHE illuminated for 5 minutes resulted in 50 ± 13 pmol/mL 2-OHE$^+$ and 673 ± 112 pmol/mL E$^+$ as noted in Fig. 4. All data are means ± SEM, N > 4. *p<0.05, ** p <0.01 versus 5-minute light (2-way ANOVA with Tukey correction).



Supplemental Fig. 6. Superoxide production rate of miniSOG is independent of light dose. miniSOG (10 µM) and DHE (50 µM) were illuminated (470 ± 20 nm; 5.9 mW/mm^2) for increasing amounts of time to determine the rate of 2-OHE^+ formation (data are from Fig. 4C). The rate the 2-OHE was calculated from the X/XO production with DHE (Data are from Fig. 1C) and expressed as a function of light dose. 5 min of light in the absence of miniSOG resulted in 1.0 ± 0.004 nm µmol $O_2^{\bullet-}$/min/µmol at 177 J/cm^2. All data are means ± SEM, N > 4.



Supplemental Fig. 7. Effect of H_2O_2 and catalase (CAT) on 2-OHE^+ detection of $O_2^{\cdot-}$. (A) DHE (50 μM) and H_2O_2 (300 μM) were incubated in the dark for 5 minutes and DHE oxidation products were analyze by HPLC. Where indicated catalase (1 mg/mL) was present. DHE in the dark for 5 minutes (no photosensitizer) resulted in 17 ± 5 pmol/mL 2-OHE^+ and 347 ± 37 pmol/mL E^+. H_2O_2 does not readily react with DHE [19]. Impurities in the H_2O_2 or catalase solution may facilitate the increased E^+ formation in the presence of H_2O_2 or catalase alone experiments. All data are means ± SEM, N > 4. *p<0.05, ** p <0.01 versus 5 minute no light (2-way ANOVA with Tukey correction).