Electronic Supplementary Material (ESI) for ChemComm.


# Supporting Information

# Assessing the potential of photosensitizing flavoproteins as tags for correlative microscopy

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## Materials and methods

### Materials

Ampicillin sodium salt (Panreac), L-arabinose (Amresco), protease inhibitor cocktail (Amresco) and glycine (98%, Prolabo) were used as received. Lysozyme, imidazole (99%), 3,3'-diaminobenzidine (DAB, 99%), dihydroethidium (HEt, 95%), acrolein (90%) and sodium cacodylate trihydrate (98%) were purchased from Sigma-Aldrich and used without further purification. Solutions and buffers were always prepared using doubly deionized water (18 MΩ, Wasserlab ultramatic). When required, buffer solutions were prepared with an appropriate proportion of deuterium oxide ($D_2O$, 99.9%, Sigma-Aldrich).

### Site-directed mutagenesis

Quick-change site-directed mutagenesis was performed to induce W81F, Q103L or Q103V mutations into pBAD/Myc-His A plasmid encoding miniSOG. The digested PCR products were transformed into *E. coli* DH10β competent cells, extracted by miniPREP (Promega) and confirmed by sequencing. Bacteria were stored at −80 °C in 20% glycerol (v/v).

### Protein expression and purification

Recombinant fluorescent proteins were expressed and purified as described previously, with minor modifications.[1] Fresh *E. coli* DH10β cultures were growth in LB broth containing 0.1 mg $mL^{-1}$ ampicillin and 0.1% arabinose as inducing agent, with gentle shaking at 220 rpm, at 37 °C for 48 hours. Bacteria were then harvested by centrifugation during 15 min at 4700 g and resuspended in lysis buffer (50 mM phosphate, pH = 7.4, 300 mM NaCl). Subsequently, cells were lysed by sonication after adding lysozyme supplemented with an aliquot of protease inhibitor cocktail and DNAse. The lysate was cleared by centrifugation during 45 min at 4 °C and 10700 g. His-tagged fluorescent proteins were then purified from the supernatant using a nickel-agarose affinity column (Jena BioScience) and eluting with Tris buffer containing imidazole (50 mM Tris/HCl, pH = 8.0, 150 mM NaCl, 300 mM imidazole). Samples were dialyzed (Spectra/Por dialysis membrane, 6-8 kDa cutoff, Spectrumlabs) with PBS buffer (50 mM phosphate, pH = 7.4, 150 mM NaCl) at 4 °C overnight. Protein concentration was finally determined by measuring the absorbance at 448 nm (miniSOG and W81F mutant), 440 nm (Q103L and Q103V mutants) or 488 nm (EGFP) on a Cary 50 UV-vis spectrophotometer (Varian). MiniSOG and EGFP molar extinction coefficients (16700 and 55000 $M^{-1} cm^{-1}$, respectively) were used for the calculations. Aliquots of purified proteins were stored at −20 °C. We found some variability in the photophysical properties of some of the mutants upon aging of the samples (even if frozen), so when possible, experiments were performed with freshly produced protein.

### Monitoring of DAB polymerization by photosensitizing proteins in solution

Fresh DAB stock solutions were always prepared one hour before being used to avoid self-polymerization. Purified proteins were transferred to a 96 well plate and diluted with PBS to 0.5, 1.0, 3.3, 6.6, 10.0 and 20.0 µM. Appropriate volumes of fresh 1.6 mM DAB stock solution in PBS were then added to protein solution to reach a concentration of 0.32 mM. Subsequently, samples were illuminated with 1.8 mW $cm^{-2}$ blue LED light (M420L3, Thorlabs)

for 90 min. The irradiation was carried out at ~5 °C to minimize spontaneous self-polymerization of DAB and thus assuring that most polymerization was induced by photosensitized singlet oxygen. Increase of optical density at 440 nm by DAB polymerization was recorded before and upon irradiation at different irradiation times in a Synergy H4 hybrid plate reader (Biotek). The same protocol was also followed for 1:2 (v/v) mixtures of PBS and dPBS (50 mM phosphate, pH = 7.4, 150 mM NaCl, in $D_2O$). Measurements were repeated at least four times for each protein concentration.

**Time-resolved detection of singlet oxygen phosphorescence**

Time-resolved near-infrared phosphorescence signals at 1275 nm were measured using a customized PicoQuant Fluotime 200 lifetime system. Briefly, an AO-Z-473 solid state AOM Q-switched laser (Changchun New Industries Optoelectronics Technology Co., China) was used for excitation, working at 1.5 kHz repetition rate at 473 nm (<1.5 mW average power). An uncoated SKG-5 filter (CVI Laser Corporation, Albuquerque, U.S.A.) was placed at the exit port of the laser to remove any NIR component. The luminescence exiting from the sample was filtered by a 1100 nm long-pass filter (Edmund Optics, York, U.K.) and a narrow bandpass filter at 1275 nm (bk-1270-70-B, bk Interfernzoptik, Germany) to remove any scattered laser radiation and isolate the singlet oxygen emission. A TE-cooled near-IR sensitive photo multiplier tube assembly (H9170−45, Hamamatsu Photonics Hamamatsu City, Japan) in combination with a multichannel scaler (NanoHarp 250, PicoQuant Gmbh, Germany) was used as photon-counting detector. The time-resolved singlet oxygen emission decays were analyzed by fitting eqn (S1) to the data using GraphPad Prism 5.

$$S(t) = S_0 \frac{\tau_\Delta}{\tau_\Delta - \tau_T} \left( e^{-\frac{t}{\tau_\Delta}} - e^{-\frac{t}{\tau_T}} \right) \quad \text{(S1)}$$

The quantum yield of singlet oxygen production, $\Phi_\Delta$, was calculated by comparing the fitted $S_0$ values for the protein and for optically-matched solutions of the reference photosensitizer flavin mononucleotide (FMN), for which the value of $\Phi_\Delta$ = 0.57 in deuterated water was used (which in turn was determined using Rose Bengal as reference) (eqn (S2)):

$$\Phi_\Delta = \Phi_{\Delta_{FMN}} \frac{S_0}{S_{0_{FMN}}} \quad \text{(S2)}$$

The absorbance of the sample and reference solutions at the excitation wavelength was kept below 0.1 to prevent inner-filter effects. All measurements were performed in air equilibrated dPBS solutions at room temperature, using quartz cuvettes (Hellma) and magnetic stirring.

**Determination of other ROS with dihydroethidium**

A 16 mM dihydroethidium (HEt) stock solution in anhydrous MeOH was prepared and stored under $N_2$ at -80 °C until use to avoid fast oxidation. Purified proteins were transferred to a 96

well plate and diluted with PBS to obtain identical absorbance at 420 nm (A = 0.30 ± 0.01). An aliquot of fresh 2 mM HEt was then added to each well to reach a concentration of 20 µM. Shortly afterwards, samples were continuously irradiated with 0.45 mW $cm^{-2}$ blue LED light (M420L3, Thorlabs) for 5 min in order to generate ROS. Fluorescence spectra of oxidized HEt ($\lambda_{exc}$ = 525 nm, $\lambda_{emi}$ = 545-800 nm) were recorded and integrated before and upon irradiation at different times in a Synergy H4 hybrid plate reader (Biotek). Photoconversion of HEt at similar concentration in absence of photosensitizing protein was carried out as control. Measurements were repeated five times for each protein solution.

**Fluorescence photobleaching**

Fluorescent protein solutions (1.5 µM in PBS) were transferred to a 96 well plate and illuminated with a halogen lamp (24.7 mW $cm^{-2}$). A glass bandpass filter (315–700 nm) was used to avoid IR absorption and thus temperature increase in the solutions. Fluorescence spectra in the 420–700 nm region were recorded with excitation at 400 nm at 5 min intervals during 45 min of illumination by using the plate reader described above. Relative fluorescence intensities were obtained as an average over 4 independent measurements taken at similar concentration.

**DAB polymerization in *E. coli***

Samples were prepared as described previously in literature[2] with minor modifications. *E. coli* DH10β cultures expressing recombinant fluorescent proteins in the cytosol were grown as detailed above. Cells were centrifuged during 15 min. at 4700 g, washed twice with 10 mM phosphate, pH 7.4, 100 mM NaCl and resuspended in the same buffer. A fraction of this suspension was then diluted to $OD_{600}$ 0.14 and kept at 4 °C until use. Bacteria were fixed by adding 2% acrolein to the cell suspension at a ratio 1:2 (v/v) for 20 min at room temperature. Afterwards the cells were centrifuged during 12 min at 4500 g, washed twice with cold cacodylate buffer (100 mM cacodylate, pH = 7.2), resuspended in the same buffer containing 100 mM glycine to react with residual acrolein and maintained at room temperature for 20 min. Subsequently, fresh cacodylate buffer containing DAB was added to cells to reach a final concentration of 0.9 mM DAB. Samples were then placed on a chambered glass coverslip and irradiated through a microscope objective (60x, 1.49 NA, Nikon) with a blue laser (488 nm, 39.0 W $cm^{-2}$) to polymerize DAB. The extent of DAB polymerization in *E. coli* upon irradiation was observed by brightfield microscopy with a Nikon Eclipse Ti inverted microscope.


1. R. Ruiz-Gonzalez, A. L. Cortajarena, S. H. Mejias, M. Agut, S. Nonell and C. Flors, *J Am Chem Soc*, 2013, **135**, 9564-9567.
2. G. A. Johnson, E. A. Ellis, H. Kim, N. Muthukrishnan, T. Snavely and J. P. Pellois, *PLoS One*, 2014, **9**, e91220.

**Figure S1**

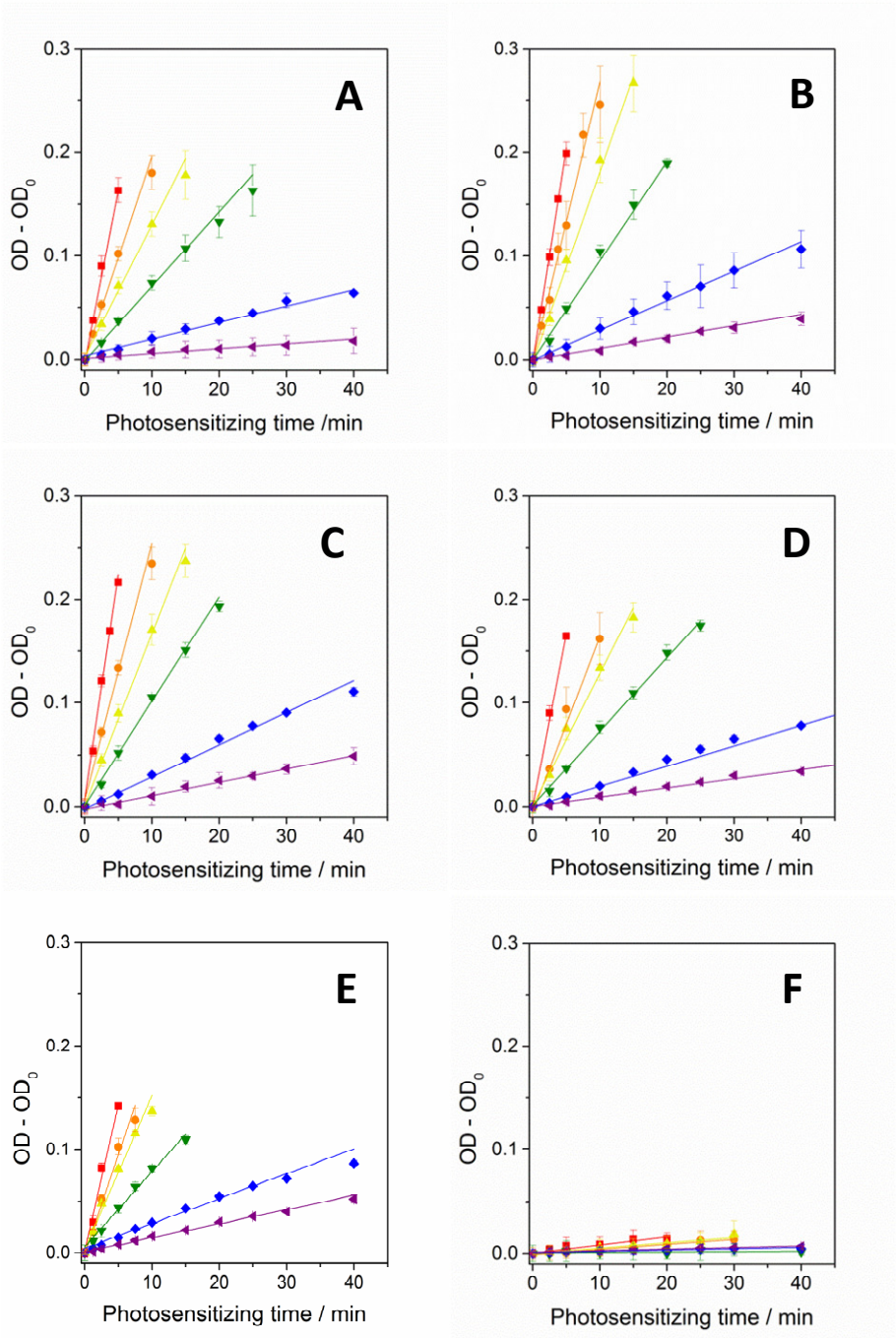


**Figure S1.** Linear parts of the plots in Figure 1 (main text), from which the slopes in Figure 2 and Table 1 are calculated. A) MiniSOG, B) Q103L, C) Q103V, D) W81F, E) FMN, F) EGFP, at different concentrations: 20.0 (red), 10.0 (orange), 6.6 (yellow), 3.3 (green), 1.0 (blue) and 0.5 µM (purple)

## Figure S2

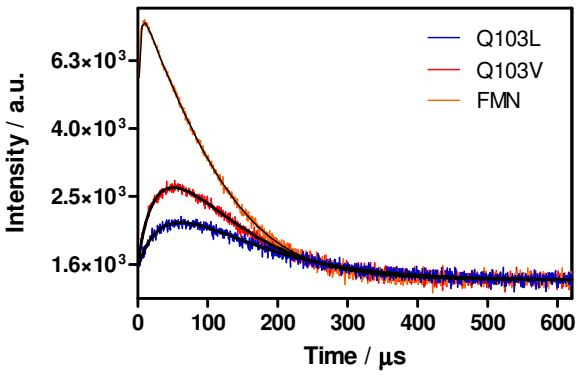


**Figure S2.** Time-resolved $^1O_2$ phosphorescence decays for miniSOG variants Q103V, Q103V and FMN, in air- saturated dPBS solution, upon excitation at 473 nm.

**Figure S3**

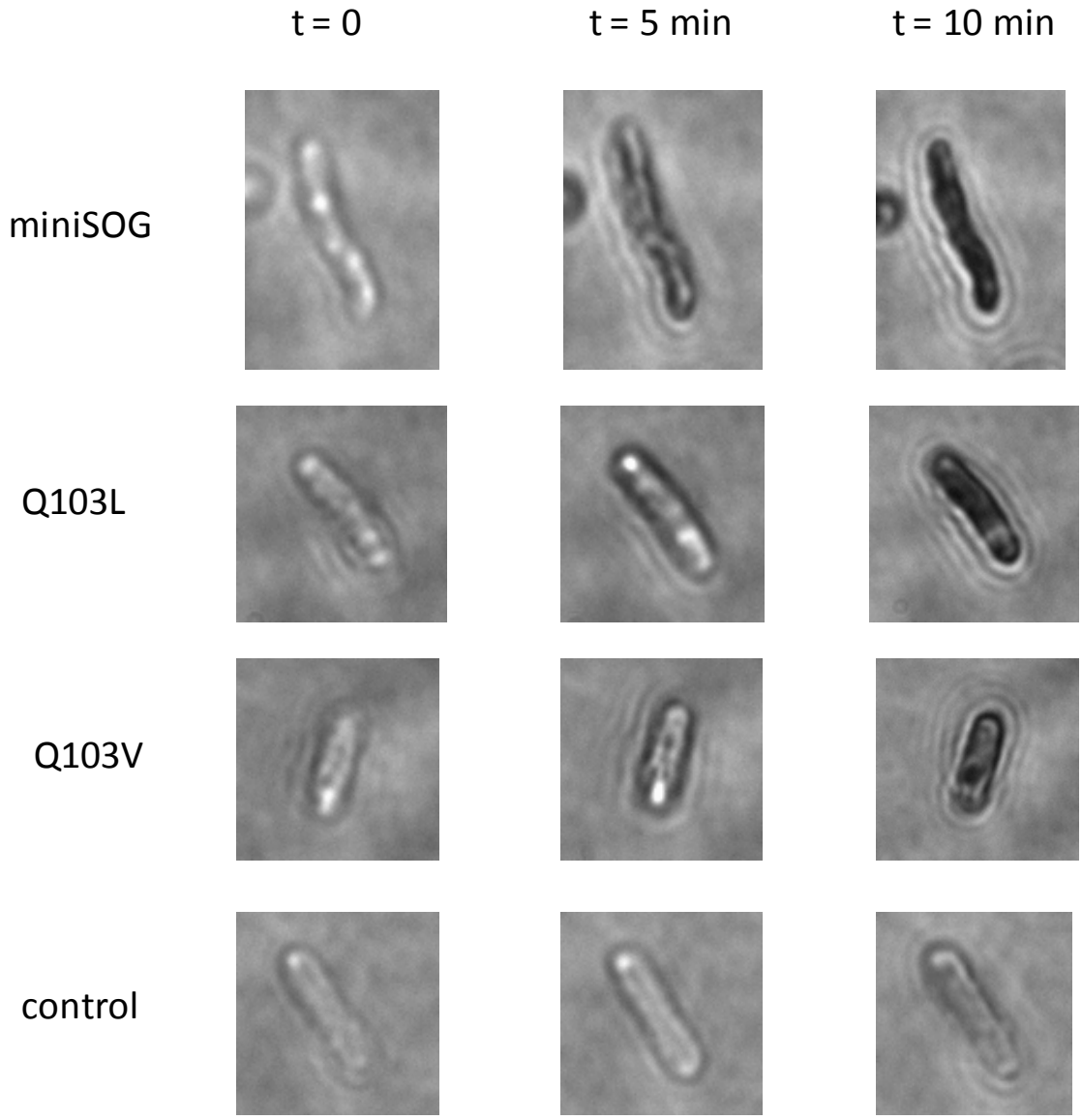


**Figure S3.** Polymerization of DAB in bacteria expressing genetically-encoded photosensitizers in their cytosol, followed by brightfield microscopy at different irradiation times (488 nm). The control sample corresponds to a bacterium with no photosensitizer.

## Figure S4

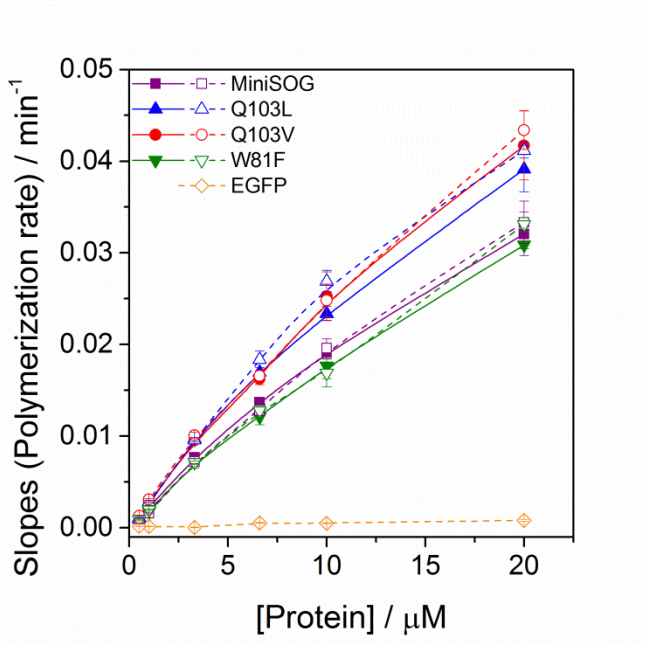


**Figure S4.** Comparison of photosensitized DAB polymerization rates in dPBS (solid lines) and PBS (dashed lines).

## Figure S5

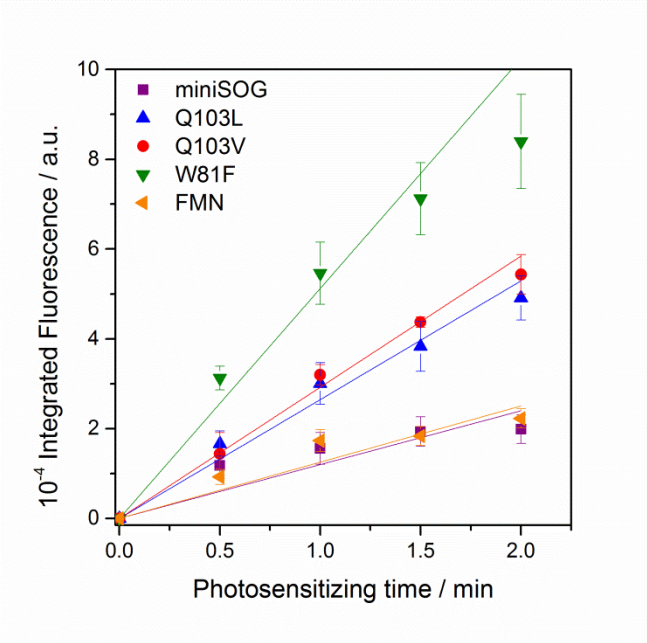


**Figure S5.** Determination of the photosensitization of ROS other than singlet oxygen using the fluorescent probe dihydroethidium.