Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.


# Supporting Information

## Successive Energy Transfer within Multiple Photosensitizers Assembled in a Hexameric Hemoprotein Scaffold

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Contents:

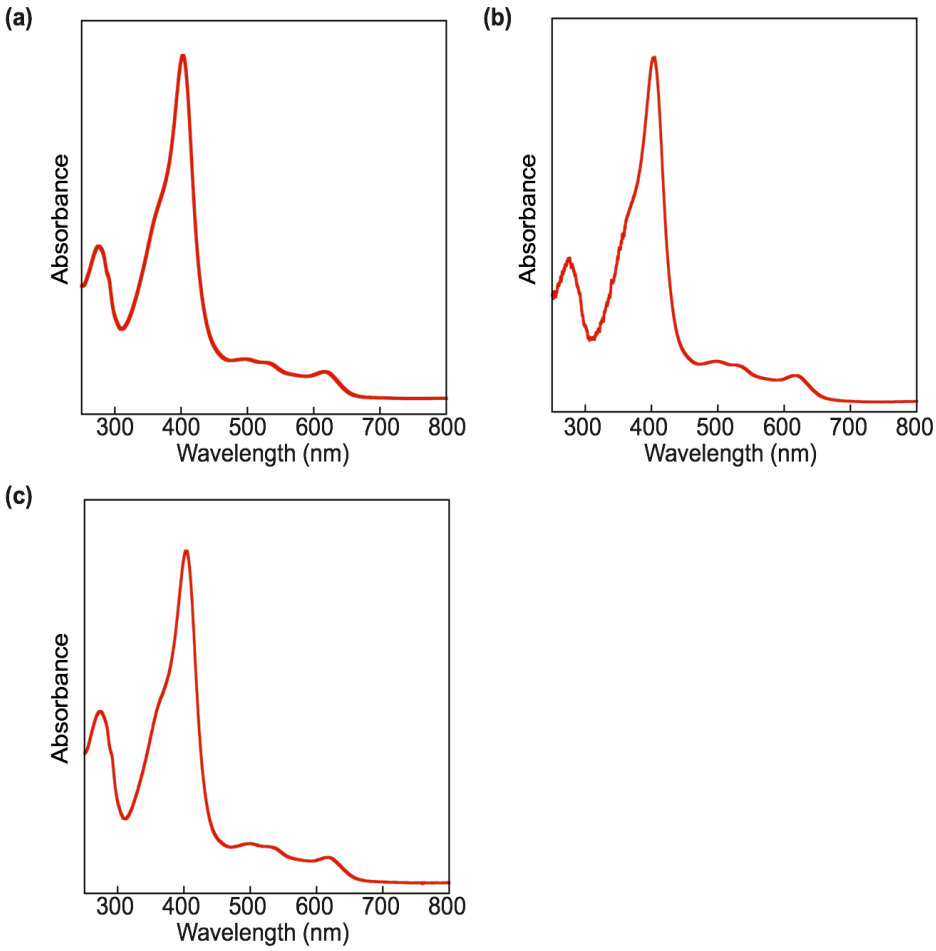


**Fig. S1** UV-vis absorption spectra of (a) $HTHP^{T12C}$, (b) $HTHP^{D18C}$, and (c) $HTHP^{K29C}$ in 100 mM potassium phosphate buffer at pH 7.0 and 25 ˚C.

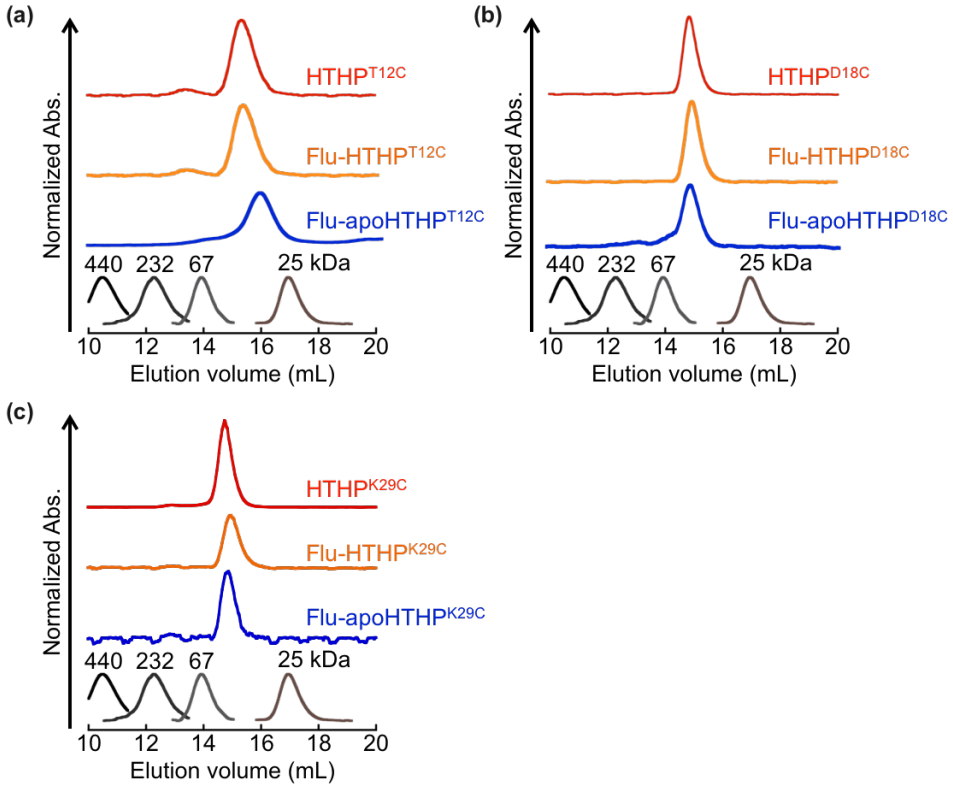


**Fig. S2** Analytical SEC traces of (a) HTHP$^{T12C}$ (red), Flu-HTHP$^{T12C}$ (orange) and Flu-apoHTHP$^{T12C}$ (blue); (b) HTHP$^{D18C}$ (red), Flu-HTHP$^{D18C}$ (orange) and Flu-apoHTHP$^{D18C}$ (blue); and (c) HTHP$^{K29C}$ (red), Flu-HTHP$^{K29C}$ (orange) and Flu-apoHTHP$^{K29C}$ (blue). The black profiles were obtained by various protein standards. Red, orange, blue, and black profiles were detected by absorptions at 402, 495, 495, and 280 nm, respectively. Eluent is 100 mM potassium phosphate buffer at pH 7.0 and 4 ˚C at a flow rate of 0.5 mL·min$^{-1}$.

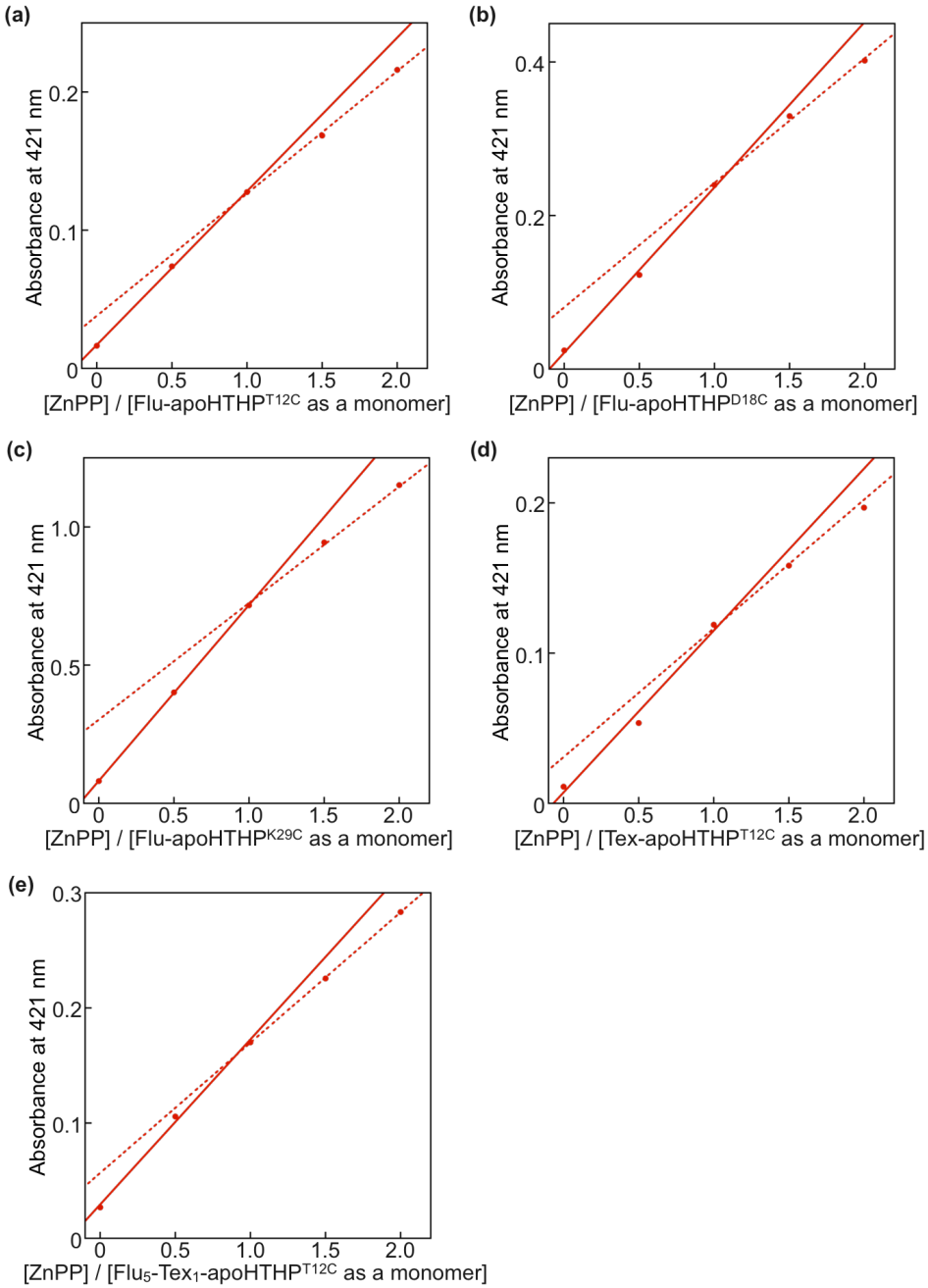


**Fig. S3** ZnPP titration experiments of (a) Flu-apoHTHP$^{T12C}$, (b) Flu-apoHTHP$^{D18C}$, (c) Flu-apoHTHP$^{K29C}$, (d) Tex-apoHTHP$^{T12C}$, and (e) Flu$_5$-Tex$_1$-apoHTHP$^{T12C}$. The protein concentrations are 1.3 μM, 1.0 μM, 2.8 μM, 0.5 μM, and 0.7 μM as a monomer, respectively, in 100 mM potassium phosphate buffer at pH 7.0 and 25 ˚C.

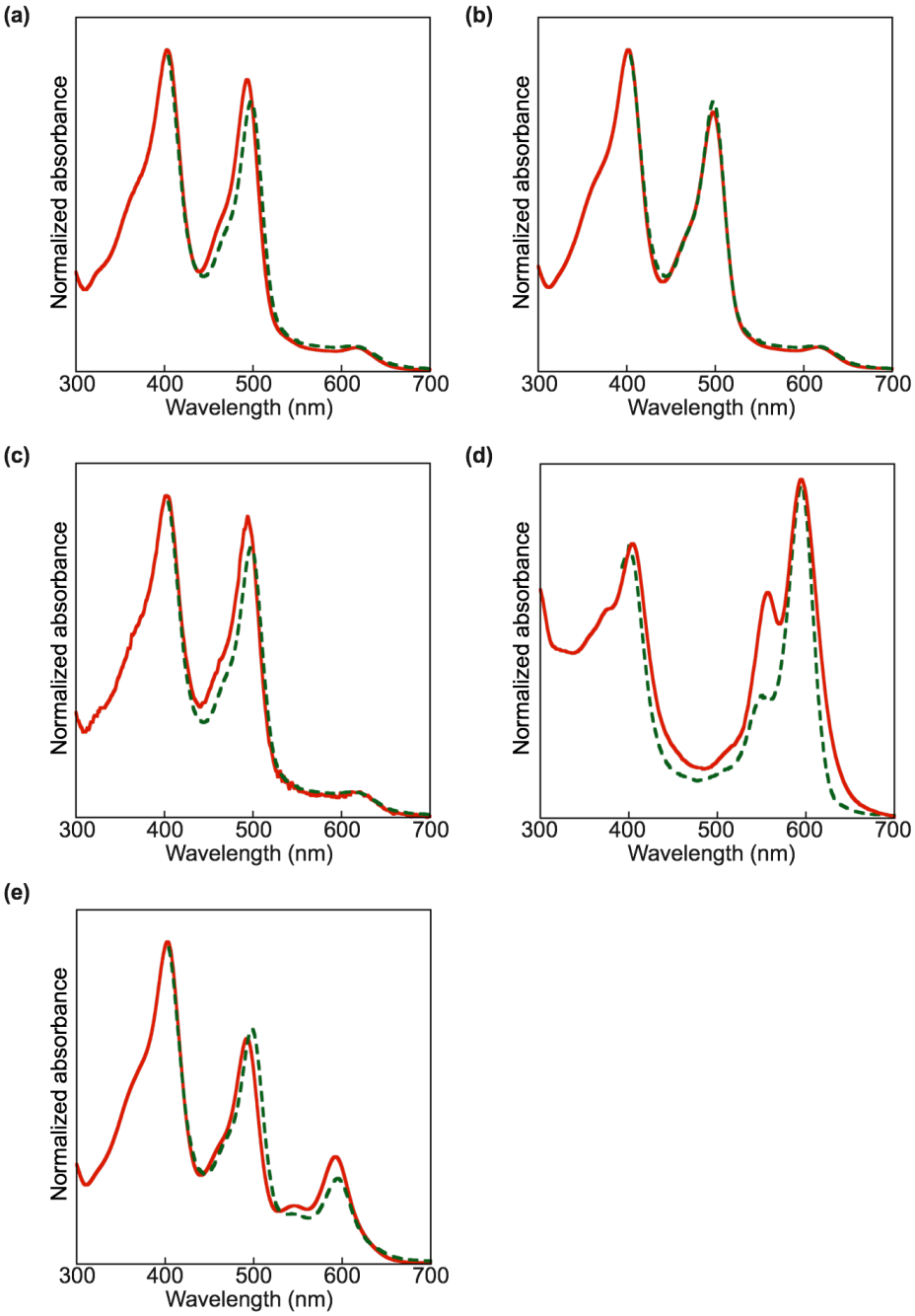


**Fig. S4** UV-vis spectra of (a) Flu-HTHP$^{T12C}$, (b) Flu-HTHP$^{D18C}$, (c) Flu-HTHP$^{K29C}$, (d) Tex-HTHP$^{T12C}$, and (e) $Flu_5$-$Tex_1$-HTHP$^{T12C}$ in 100 mM potassium phosphate buffer at pH 7.0 and 25 ˚C. Solid lines were obtained by experimental spectra and dotted lines are simulated spectra by the sum of HTHP and photosensitizer spectra with the extinction coefficients. (Heme: 95000 $M^{-1}$•$cm^{-1}$ at 402 nm, fluorescein: 68000 $M^{-1}$•$cm^{-1}$ at 497 nm, and Texas Red: 112000 $M^{-1}$•$cm^{-1}$ at 593 nm).

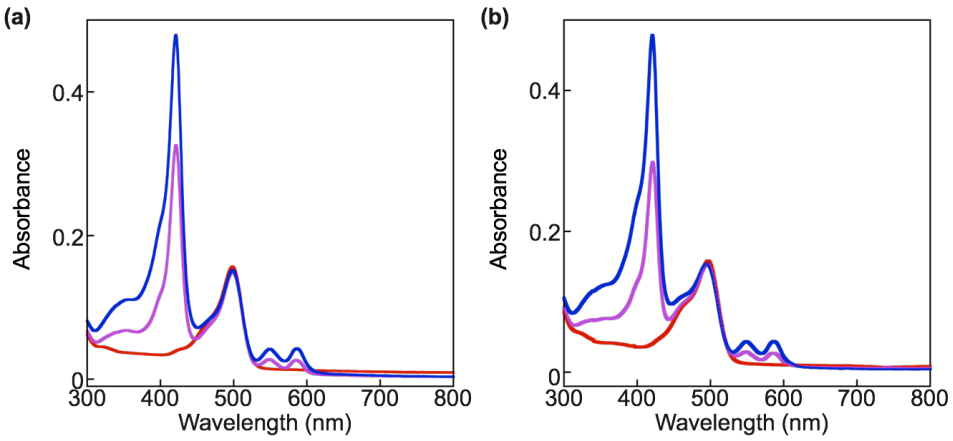


**Fig. S5** UV-vis spectral changes of (a) Flu-apoHTHP$^{D18C}$, and (b) Flu-apoHTHP$^{K29C}$ ([Protein as a monomer] = ca. 2 μM) occurring upon adding ZnPP at 0 (red), 0.5 (purple), and 1.0 equivalent (blue) in 100 mM potassium phosphate buffer at pH 7.0 and 25 ˚C under a nitrogen atmosphere.

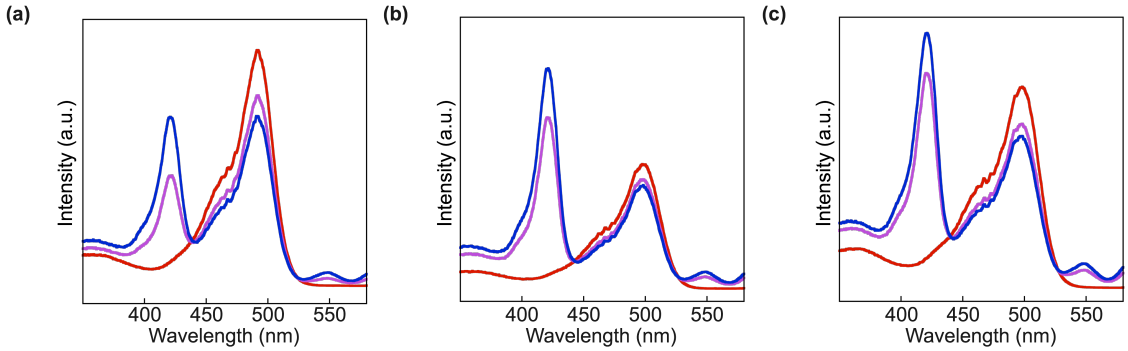


**Fig. S6** Fluorescence excitation spectra of (a) Flu-apoHTHP$^{T12C}$, (b) Flu-apoHTHP$^{D18C}$, and (c) Flu-apoHTHP$^{K29C}$ upon addition of ZnPP ([ZnPP] / [Flu-apoHTHP$^{Cys}$ mutants as monomers] = 0 (red), 0.5 (purple), 1.0 (blue)). Conditions: [Flu-apoHTHP$^{Cys}$ mutants as monomers] = ca. 1 μM in 100 mM potassium phosphate buffer at pH 7.0 and 25 ˚C under a nitrogen atmosphere. $\lambda_{em}$ = 590 nm.

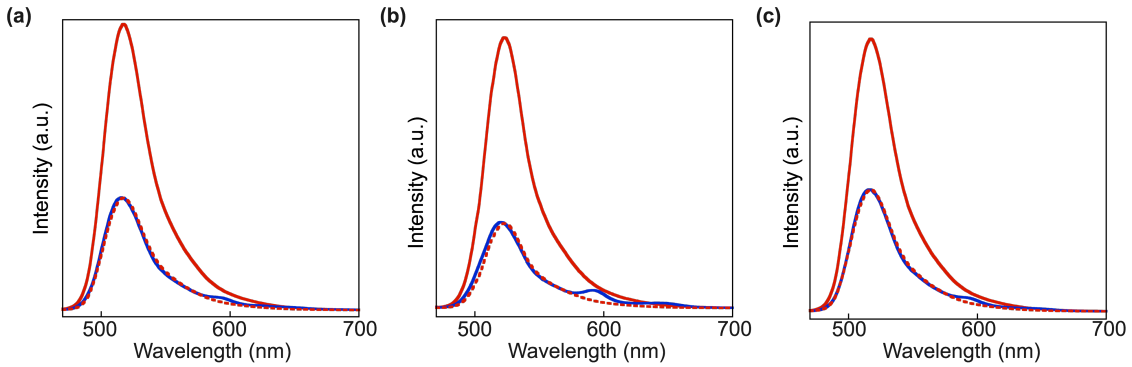


**Fig. S7** Fluorescence spectra of (a) Flu-apoHTHP$^{T12C}$ (red), Flu-rHTHP$^{T12C}$ (blue) and normalized Flu-apoHTHP$^{T12C}$ (red, dotted line) (b) Flu-apoHTHP $^{D18C}$ (red), Flu-rHTHP$^{D18C}$ (blue) and normalized Flu-apoHTHP$^{D18C}$ (red, dotted line) (c) Flu-apoHTHP$^{K29C}$ (red), Flu-rHTHP$^{K29C}$ (blue) and normalized Flu-apoHTHP$^{K29C}$ (red, dotted line). The spectra were normalized at the fluorescence maximum of the Flu-apoHTHP$^{Cys}$ mutants. Conditions: [Flu-apoHTHP$^{Cys}$ mutants as monomers] = ca. 1 $\mu$M in 100 mM potassium phosphate buffer at pH 7.0 and 25 °C under a nitrogen atmosphere. $\lambda_{ex}$ = 460 nm.

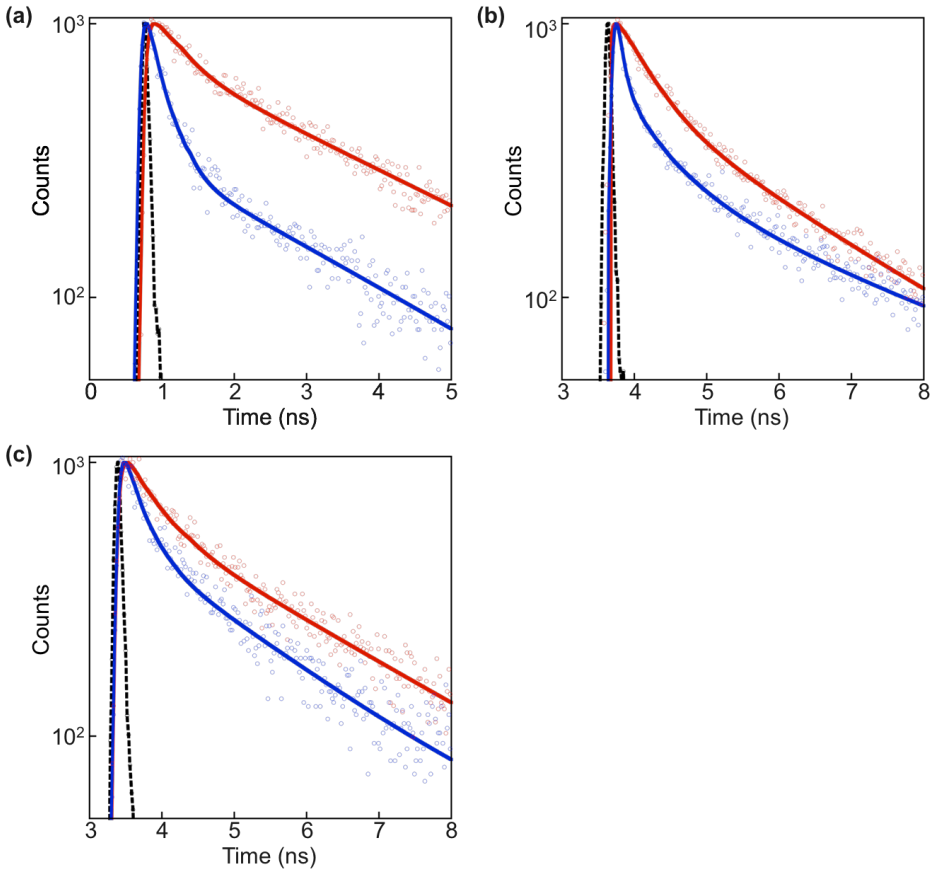


**Fig. S8** Time-resolved fluorescence decay profiles of (a) Flu-apoHTHP$^{T12C}$ (red) and Flu-rHTHP$^{T12C}$ (blue); (b) Flu-apoHTHP$^{D18C}$ (red) and Flu-rHTHP$^{D18C}$ (blue); and (c) Flu-apoHTHP$^{K29C}$ (red) and Flu-rHTHP$^{K29C}$ (blue). The instrument responses are shown by the black broken lines. Decay plots were analyzed by double- or triple-exponentially fitted curves (solid lines): $y = y_0 + A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2)$, or $y = y_0 + A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2) + A_3\exp(-t/\tau_3)$, where $A$ and $\tau$ are population and lifetime of the decay, respectively (Table 2). Conditions: The samples are dissolved in 100 mM potassium phosphate buffer at pH 7.0 and 25 ˚C under a nitrogen atmosphere. $\lambda_{ex}$ = 509 nm and $\lambda_{em}$ = 550 nm.

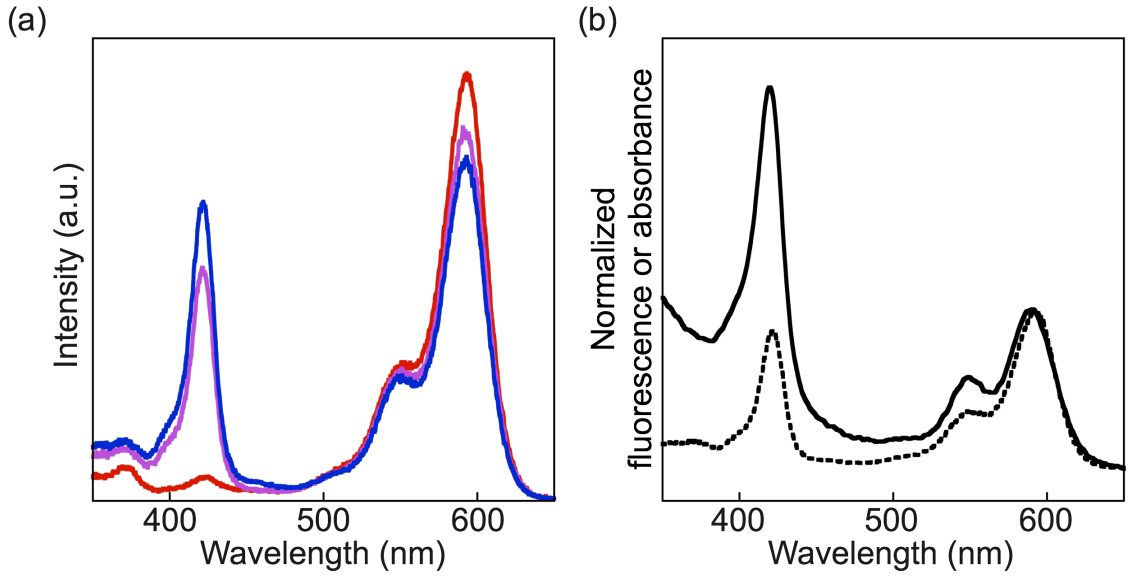


**Fig. S9** (a) Fluorescence excitation spectral changes of Tex-HTHP$^{T12C}$ occurring addition of ZnPP ([ZnPP] / [Tex-apoHTHP$^{T12C}$ as a monomer] = 0 (red), 0.5 (purple), 1 (blue)). (b) UV-vis spectra (solid line) and fluorescence excitation spectra (dotted line) of Tex-rHTHP$^{T12C}$ were normalized at the acceptor excitation at 593 nm. Conditions: [Tex-apoHTHP$^{T12C}$ as a monomer] = ca. 0.5 μM in 100 mM potassium phosphate buffer at pH 7.0 and 25 ℃ under a nitrogen atmosphere, $\lambda_{em}$ = 680 nm.

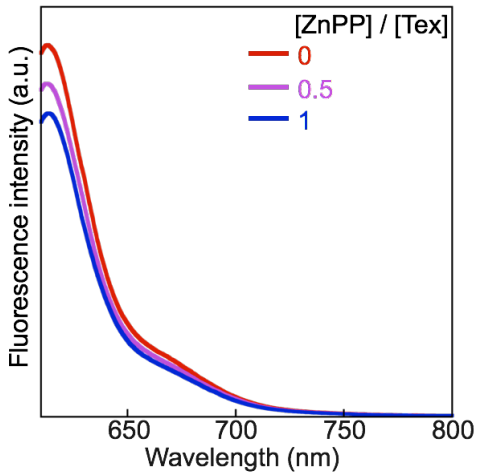


**Fig. S10** Fluorescence spectral changes of Tex-apoHTHP$^{T12C}$ occurring upon addition of ZnPP ([ZnPP] / [Tex-apoHTHP$^{T12C}$ as a monomer] = 0 (red), 0.5 (purple), 1 (blue)). Conditions: [Tex-apoHTHP$^{T12C}$ as a monomer] = 0.5 μM in 100 mM potassium phosphate buffer at pH 7.0 and 25 ˚C under a nitrogen atmosphere. $\lambda_{ex}$ = 593 nm.

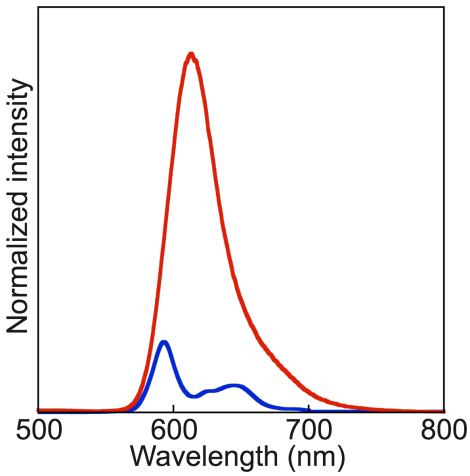


**Fig. S11** The normalized fluorescence spectra of Tex-apoHTHP$^{T12C}$ (red) and rHTHP$^{ZnPP}$ (blue). The areas of these spectra are normalized with the fluorescence quantum yields.

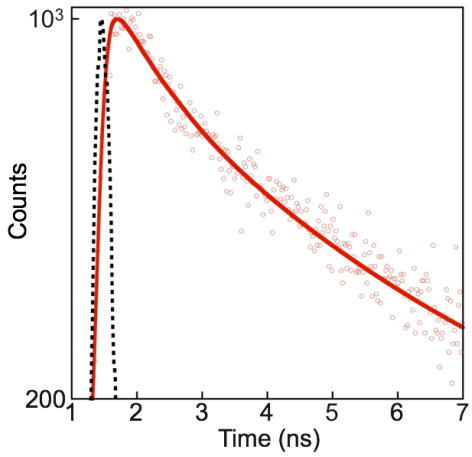


**Fig. S12** Time-resolved fluorescence decay profiles of Tex-apoHTHP$^{T12C}$. The instrument response is shown by the black broken line. Decay plots were analyzed by double-exponentially fitted curve (solid line): $y = y_0 + A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2)$, where $A$ and $\tau$ are population and lifetime of the decay, respectively (Table S2). Conditions: [Tex-apoHTHP$^{T12C}$ as a monomer] = 2 μM in 100 mM potassium phosphate buffer at pH 7.0 and 25 ˚C under a nitrogen atmosphere. $\lambda_{ex}$ = 509 nm and $\lambda_{em}$ = 613 nm.

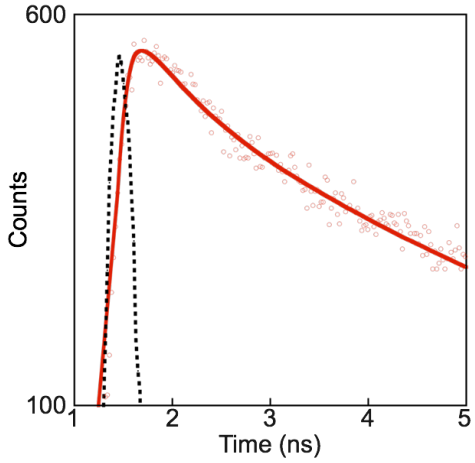


**Fig. S13** Time-resolved fluorescence decay profiles of wild type HTHP reconstituted with ZnPP (rHTHP). rHTHP is prepared by the previously reported method.[1] The instrument response is shown by the black broken line. Decay plots were analyzed by a double-exponentially fitted curve (solid line): $y = y_0 + A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2)$, where $A$ and $\tau$ are population and lifetime of the decay, respectively (Table S1). Conditions: [rHTHP as a monomer] = 4 μM in 100 mM potassium phosphate buffer at pH 7.0 and 25 ˚C under a nitrogen atmosphere. $\lambda_{ex}$ = 404 nm and $\lambda_{em}$ = 592 nm.

**Table S1** Decay parameters and fluorescence quantum yield ($\Phi_f$) of rHTHP

| Protein | $<\tau>$ (ns)[a] | $\tau_1$ (ns) | $\alpha_1$[b] | $\tau_2$ (ns) | $\alpha_2$[b] | $X^2$ | $\Phi_f$[c] |
|---|---|---|---|---|---|---|---|
| rHTHP | 0.95 | 0.839 | 0.94 | 2.774 | 0.06 | 1.100 | < 3% |

[a]Average fluorescence lifetime determined by the sum of the $\alpha_i\tau_i$ products. [b]$\alpha$ is the population for each component. [c]$\lambda_{ex}$ = 420 nm.

Calculation of the distance between donor and accepter within HTHP matrix with Förster theory

The expected distance ($r$) is estimated with the value of energy transfer efficiency $E$ derived from time-resolved fluorescence measurement and following equations: [2]

$$E = \frac{1}{1 + (r / R_0)^6}$$

$$R_0 = \left(\frac{9\, ln10}{128\, \pi^5\, N_A} \frac{\kappa^2 Q_\mathrm{D}}{n^4}\, J\right)^{1/6}$$

In the above equations, $R_0$ is the dynamically averaged Förster radius, in which energy transfer from donor to acceptor occurs with a 50% probability, $\kappa$ is the orientation factor between donor and acceptor transition dipole moments, $n$ is the refractive index, $Q_\mathrm{D}$ is the donor quantum yield, and $J$ is the integrated spectral overlap of normalized donor fluorescence and acceptor absorption coefficient. Since the photosensitizers are covalently attached onto the protein surface via flexible linker, $\kappa = 2/3$ is utilized to determine $R_0$.

**Table S2** Expected distances between donor and acceptor

| Protein | Expected distance (nm) | Maximum possible distance (nm)[a] | $R_0$ (nm) |
|---|---|---|---|
| Flu-rHTHP$^{T12C}$ | 3.3 | 3.0 | 3.6 |
| Flu-rHTHP$^{D18C}$ | 3.7 | 2.1 | 3.1 |
| Flu-rHTHP $^{K29C}$ | 3.1 | 1.7 | 3.2 |
| Tex-rHTHP$^{T12C}$ | 4.0 | 3.5 | 3.7 |

[a]The maximum possible distance was estimated as the sum of distances from the Fe center to the α carbon of the mutated residue in the crystal structure of wild type HTHP and distances from the α carbon to photosensitizers. The distance between the α carbon to photosensitizers was estimated with ChemBio3D® Ultra.

**Table S3** Decay parameters and fluorescence quantum yield ($\Phi_f$) of Tex-apoHTHP$^{T12C}$

| Protein | $\langle\tau\rangle$ (ns)[a] | $\tau_1$ (ns) | $\alpha_1$[b] | $\tau_2$ (ns) | $\alpha_2$[b] | $X^2$ | $\Phi_f$[c] |
|---|---|---|---|---|---|---|---|
| Tex-apoHTHP$^{T12C}$ | 2.33 | 0.628 | 0.378 | 3.36 | 0.622 | 1.004 | 19% |

[a]Average fluorescence lifetime determined by the sum of the $\alpha_i\tau_i$ products. [b]$\alpha$ is the population for each component. [c]$\lambda_{ex}$ = 570 nm.

References

1. K. Oohora, T. Mashima, K. Ohkubo, S. Fukuzumi and T. Hayashi, *Chem. Commun.*, 2015, **51**, 11138–11140.
2. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 2nd ed., Springer, New York, 1999.