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Supporting Information

Disulphide bond-mediated hetero-dimer of a hemoprotein and a fluorescent protein exhibiting efficient energy transfer

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1. General Information

1.1 Materials and Methods

Instruments: MALDI-TOF MS analyses were performed with an Autoflex III mass spectrometer. UV-Vis spectra were measured with a Shimadzu BioSpec-nano or Shimadzu UV-3600 plus double-beam spectrometer. Luminescence spectra were measured with a JASCO FP-8600 fluorescence spectrometer. Size exclusion chromatographic (SEC) analyses were performed with an ÄKTA Purifier System (GE Healthcare) at 4 °C. Fluorescence lifetimes were measured by a C10196 Hamamatsu picosecond light pulser equipped with a C9300 Hamamatsu digital camera and laser excitation by a Hamamatsu laser beam (M10306-33 model): peak wavelength = 464 nm, laser power = 119 mW, typical pulse width = 70 ns. The pH measurements were carried out with an F-25 Horiba pH meter.

Materials: NEBuilder HiFi DNA Assembly kit, ampicillin sodium salt, isopropyl-β-D-1-thiogalactopyranoside (IPTG), Trizma base, ethylenediaminetetraacetic acid (EDTA), desthiobiotin, Strep-Tactin Superflow resins, dithiothreitol (DTT), dimethyl sulfoxide (DMSO), 2,2'-dithiodipyridine, bromophenol blue, acrylamide, glycerol, tetramethylethylenediamine (TEMED), ammonium persulfate (APS), Tricine, Coomassie Brilliant blue G-250, sodium dodecyl sulfate (SDS), and Novex Sharp prestained protein standard were purchased and used as received. Unless mentioned otherwise, all protein solutions were dissolved in a 100 mM potassium phosphate buffer (pH 7.0). Deionized water was prepared using a Millipore Integral apparatus.

1.2 Protein Sequence of Cytochrome b_{562} mutants

Cyt b_{562}^{wt}

ADLEDNMETLNDNLKVIEKADNAAQVKDALTKMRAAALDAQKATPPKLEDKSPDSPEMKDFR
HGFDILVGQIDDALKLANEGKVKEAQAAAEQLKTTRNAYHQKYR

Cyt b_{562}^{K15C}

ADLEDNMETLNDNLCVIEKADNAAQVKDALTKMRAAALDAQKATPPKLEDKSPDSPEMKDFR
HGFDILVGQIDDALKLANEGKVKEAQAAAEQLKTTRNAYHQKYR

Cyt b_{562}^{H63C}

ADLEDNMETLNDNLKVIEKADNAAQVKDALTKMRAAALDAQKATPPKLEDKSPDSPEMKDFR
CGFDILVGQIDDALKLANEGKVKEAQAAAEQLKTTRNAYHQKYR

Cyt b_{562}^{N80C}

ADLEDNMETLNDNLKVIEKADNAAQVKDALTKMRAAALDAQKATPPKLEDKSPDSPEMKDFR
HGFDILVGQIDDALKLACEGKVKEAQAAAEQLKTTRNAYHQKYR

Cyt b_{562}^{A100C}

ADLEDNMETLNDNLKVIEKADNAAQVKDALTKMRAAALDAQKATPPKLEDKSPDSPEMKDFR
HGFDILVGQIDDALKLANEGKVKEAQAAAEQLKTTRNCYHQKYR

1.3 Protein Sequence of GFP mutants

GFP^{wt}

SKGEELFTRVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVT
TLSYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNR
IELKGVDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKANFKVRHNIEDGSVQLADHY
QQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK

GFP^{K25C}

SKGEELFTRVVPILVELDGDVNGHCFSVSGEGEGDATYGKLTLKFIATTGKLPVPWPTLVT
TLSYGVQAFSRYPDHMKRHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNR
IELKGVDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKANFKVRHNIEDGSVQLADHY
QQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGIT HGMDELYK

GFP^{S174C}

SKGEELFTRVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFIATTGKLPVPWPTLVT
TLSYGVQAFSRYPDHMKRHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNR
IELKGVDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKANFKVRHNIEDGCVQLADHY
QQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK

2. General Procedures

2.1 Preparation of Cyt b_{562} and GFP mutants

The pUC118 gene expression systems and purification used to obtain Cyt b_{562} mutants were reported in our previous works.[1] The expression of GFP mutants were carried out using the pET-21b(+) expression system containing a Strep-tag II gene for the purification step. First, a gene for GFP was inserted into a pET-21b(+) vector containing the Step-tactin sequence. An insert encoding the GFP gene was amplified by PCR using a pEX-A2J2 plasmid (Eurofin Genomics Co., Ltd) as a template. The PCR products were then treated with *Dpn*1 restriction enzymes (New England Biolabs Japan), purified by agarose gel electrophoresis, and assembled with a linearized pET-21b(+) vector using NEBuilder HiFi DNA Assembly. The assembled products were transformed into chemically competent *E. coli* DH5α cells to afford a plasmid encoding GFP. DNA sequencing of purified plasmids verified the correct insertion of the gene sequence into the expression vector. The resulting expression plasmid was transformed into *E. coli* BL21(DE3) competent cells. A LB medium (1 L) containing ampicillin (100 mg) was inoculated with 10 mL of the culture (OD = 0.5) of the relevant transformed cells. After the cells were grown aerobically with vigorous shaking at 37 °C until the OD600 reached 0.5–0.7, IPTG was added to a final concentration of 0.5 mM to induce the protein expression. The incubation was continued at 37 °C overnight. The cells were harvested by centrifugation at 8000 ×g for 10 min at 4 °C and re-suspended in a 20 mL of a 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and lysed by freeze-thaw cycles with subsequent sonication for 20 sec × 10 times at 4 °C. The lysate was centrifuged at 10000 rpm for 10 mins and the supernatant was

applied to a Strep-tag column. The elution of purified recombinant protein was performed by addition of 2.5 mM desthiobiotin prepared in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA.

2.2 SEC Analysis

The analyses were performed using a Superdex 75 Increase 10/300 GL (GE Healthcare) column with a flow rate of 0.5 mL/min at 4 °C with monitoring of the absorbance at 418 nm, 395 nm, and/or 280 nm for detection. The 100 mM potassium phosphate buffer containing 300 mM NaCl pH 7.0 was used as an elution buffer.

2.3 Non-reducing SDS-PAGE Analysis

Equal volumes of purified samples were mixed with 2X SDS-PAGE sample buffer containing 10% sucrose, 4% SDS, 125 mM Tris-HCl, and 0.005% Bromophenol blue and incubated at 90 °C for 5 min. After incubation, samples were cooled to room temperature and 10 µL of each aliquot was loaded into wells of pre-cast with separating gel consisting of 14% (v/v) acrylamide, 33% (v/v) gel buffer, 13% (v/v) glycerol, 0.07% (v/v) APS, and 0.2% (v/v) TEMED and a stacking gel consisting of 4% (v/v) acrylamide, 25% (v/v) gel buffer, 0.25% (v/v) APS, and 0.3% (v/v) TEMED. Gel buffer stock used for gel preparations composition; 0.01 M SDS, and 3 M Trizma base in 1 L.

The buffer solutions stock contained 100 mM Tricine, 100 mM Trizma base in 1 L (upper cassette) and 200 mM Trizma base in 1 L, pH 8.9 (lower cassette). The electrophoresis was run for 60 min, 150 V, 120 mA, 18W. The gel was stained with a staining solution containing 0.06% Coomassie Brilliant Blue G-250, 10% (v/v) acetic acid in 1 L for 12 h and destained by a decolorizing solution containing 10% (v/v) acetic acid in 1 L for 6 h.

Supplementary Figures

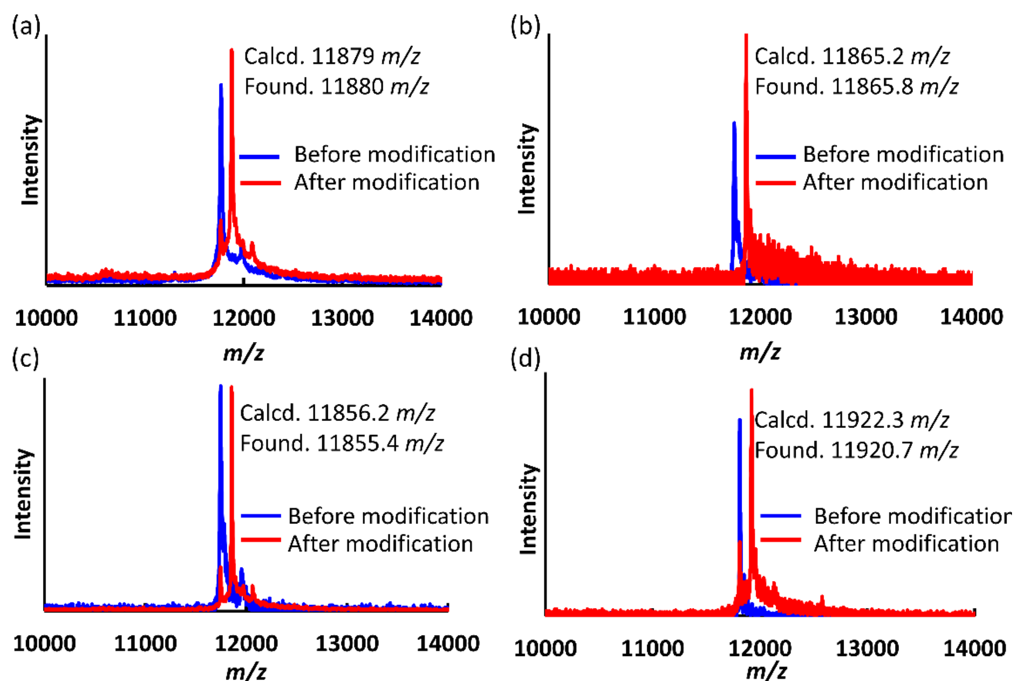

Fig. S1 MALDI-TOF mass spectra of Cyt b_{562} mutants before and after modification by 2,2'-dithiodipyridine. (a) Cyt b_{562}^{N80C}, (b) Cyt b_{562}^{K15C}, (c) Cyt b_{562}^{H63C}, and (d) Cyt b_{562}^{A100C}.

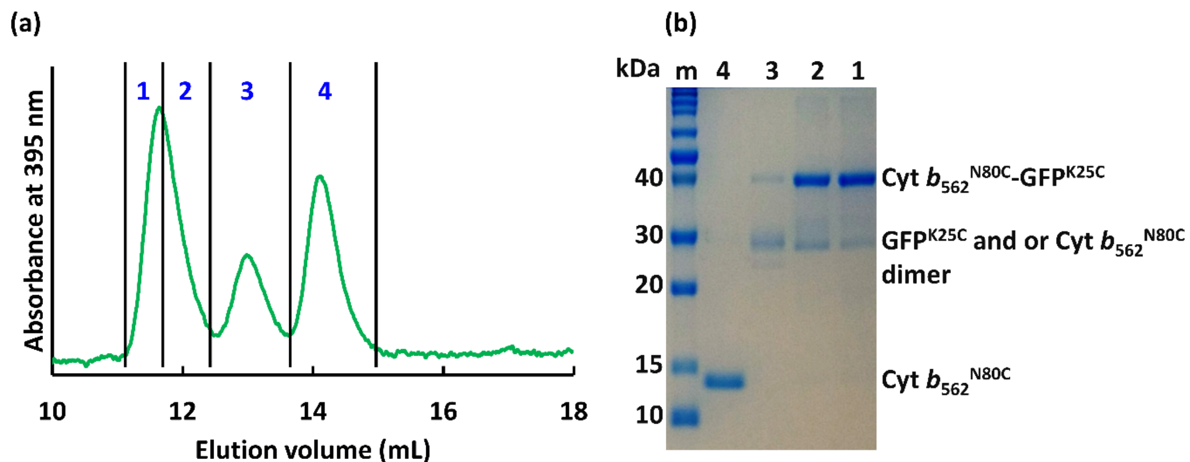

Fig. S2 (a) Fractionation of the Cyt b_{562}^{N80C} conjugated with GFP^{K25C} hetero-dimer by Superdex 75 Increase 10/300 GL column resulting in four fractions. (b) Non-reducing SDS-PAGE analysis of fractions 1–4 corresponding to the collected fractions labeled in (a).

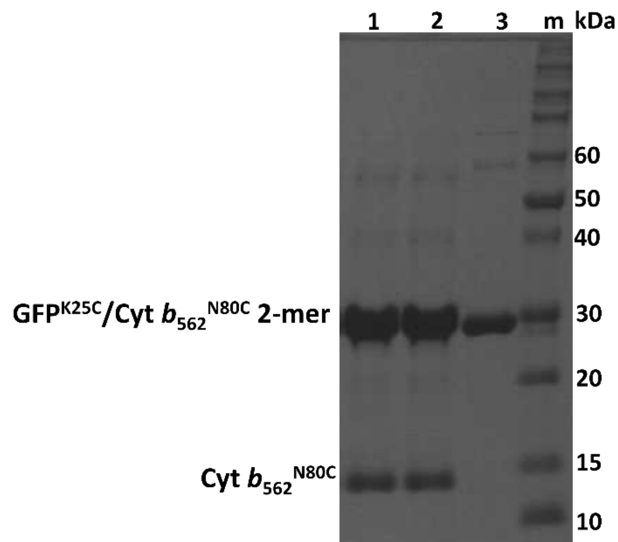

Fig. S3 Non-reducing SDS-PAGE for samples obtained by conjugation of reduced Cyt b_{562}^{N80C} and reduced GFP^{K25C} in Lane 1 = 2 : 1 (Cyt b_{562}^{N80C} : GFP^{K25C}), Lane 2 = 3 : 1 (Cyt b_{562}^{N80C} : GFP^{K25C}), control GFP^{K25C} in Lane 3.

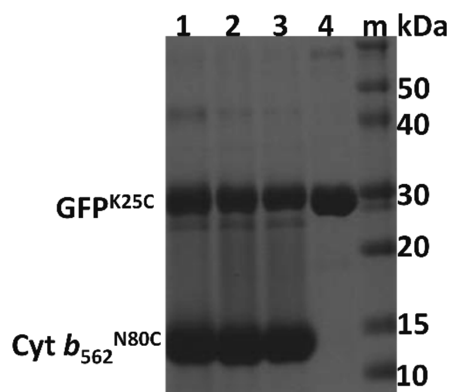

Fig. S4 Reducing SDS-PAGE of hetero-dimers. Samples were prepared by DTT-containing SDS buffer. Lane 1: Cyt b_{562}^{N80C}–GFP^{K25C}, Lane 2: Cyt b_{562}^{H63C}–GFP^{K25C}, Lane 3: Cyt b_{562}^{K15C}–GFP^{K25C}, and GFP^{K25C} control in Lane 4.

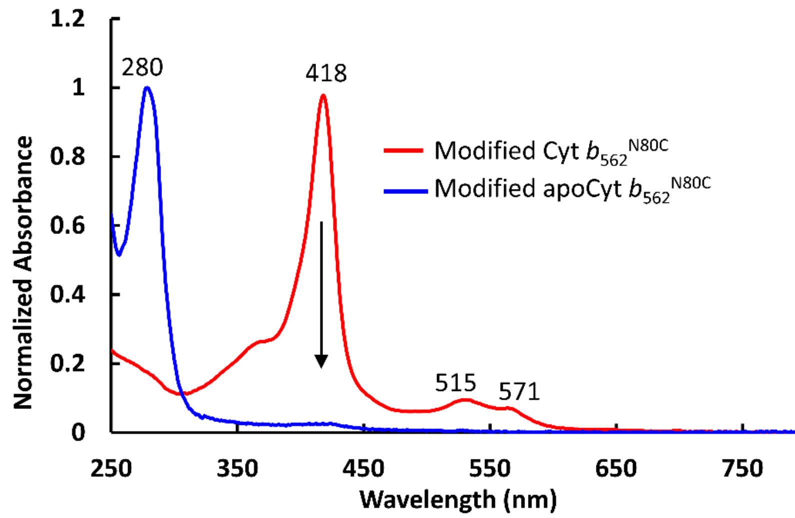

Fig. S5 UV-Vis spectra of modified Cyt b_{562}^{N80C} (red) and modified apoCyt b_{562}^{N80C} after heme removal (blue).

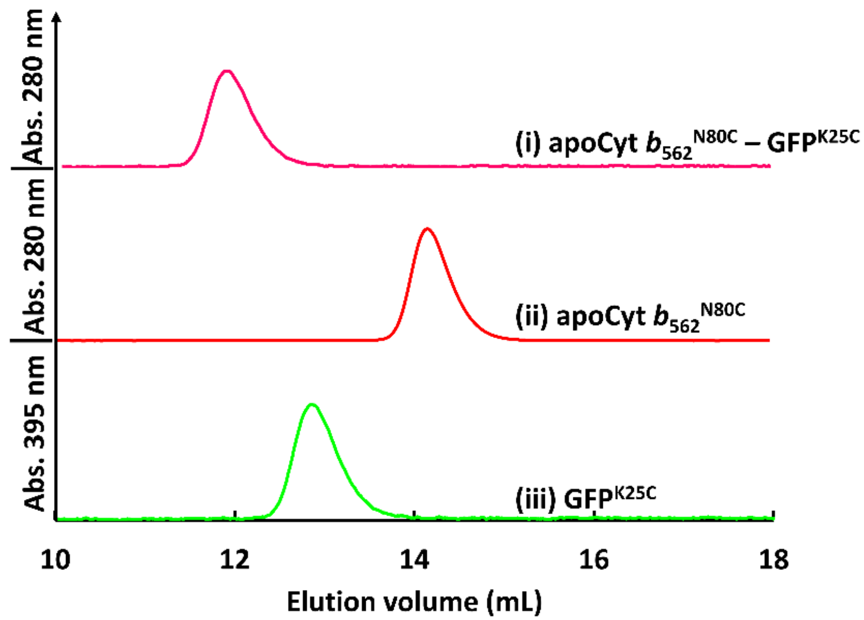

Fig. S6 SEC traces of apoCyt b_{562}^{N80C}–GFP^{K25C}, apoCyt b_{562}^{N80C}, and GFP^{K25C} using Superdex 75 Increase 10/300 GL column.

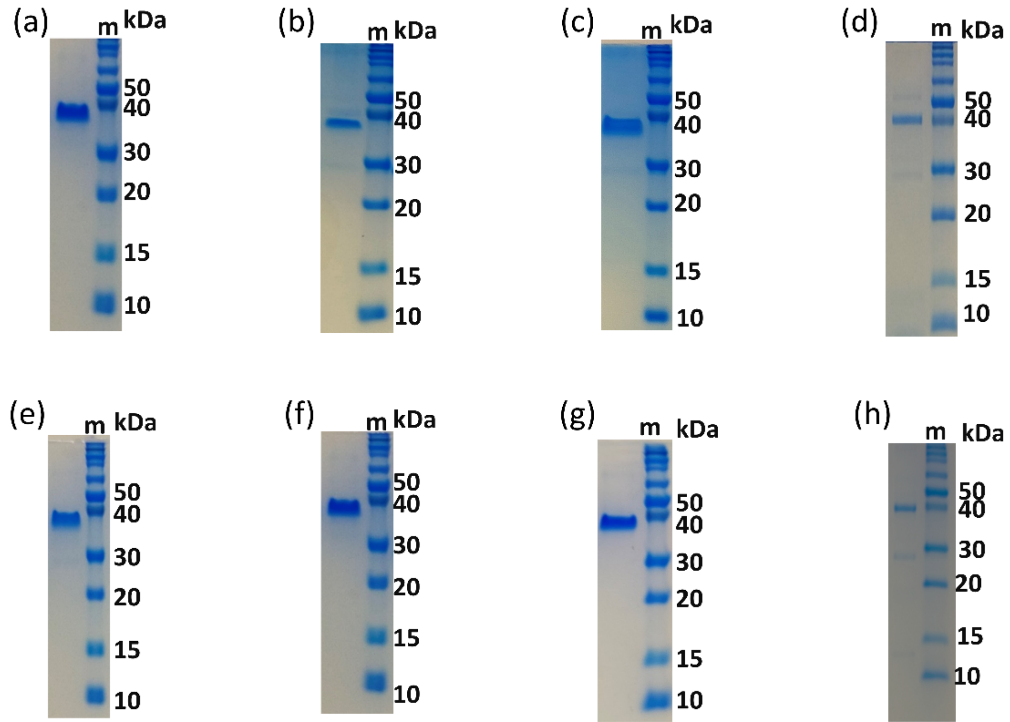

Fig. S7 Non-reducing SDS PAGE of purified hetero-dimers of (a) Cyt b_{562}^{A100C}–GFP^{K25C}, (b) Cyt b_{562}^{H63C}–GFP^{K25C}, (c) Cyt b_{562}^{K15C}–GFP^{K25C}, (d) Cyt b_{562}^{N80C}–GFP^{K25C}, (e) Cyt b_{562}^{A100C}–GFP^{S174C}, (f) Cyt b_{562}^{H63C}–GFP^{S174C}, (g) Cyt b_{562}^{K15C}–GFP^{S174C}, and (h) Cyt b_{562}^{N80C}–GFP^{S174C}.

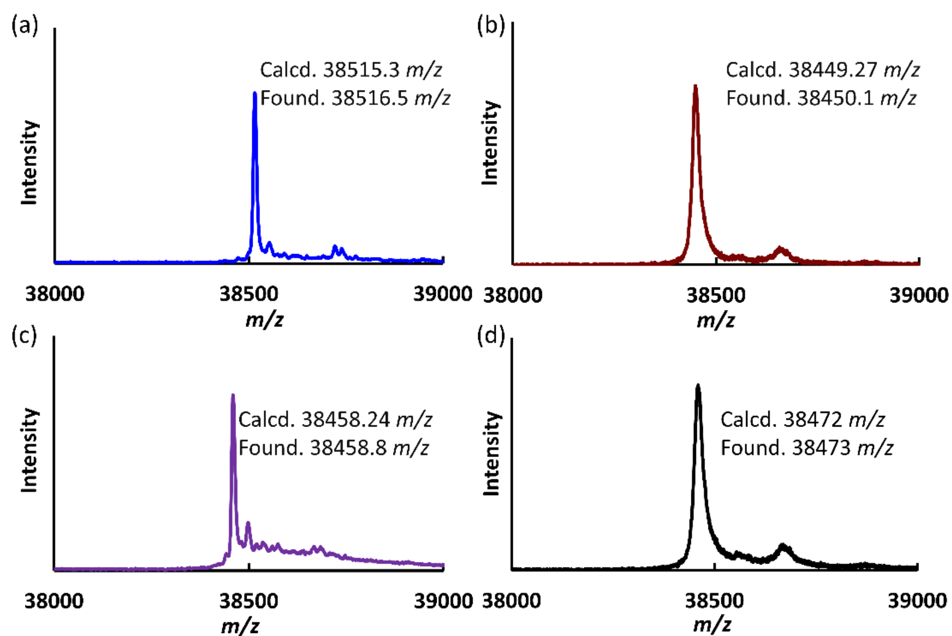

Fig. S8 MALDI-TOF mass spectra of purified hetero-dimers of (a) Cyt b_{562}^{A100C}–GFP^{K25C}, (b) Cyt b_{562}^{H63C}–GFP^{K25C}, (c) Cyt b_{562}^{K15C}–GFP^{K25C} and (d) Cyt b_{562}^{N80C}–GFP^{K25C}.

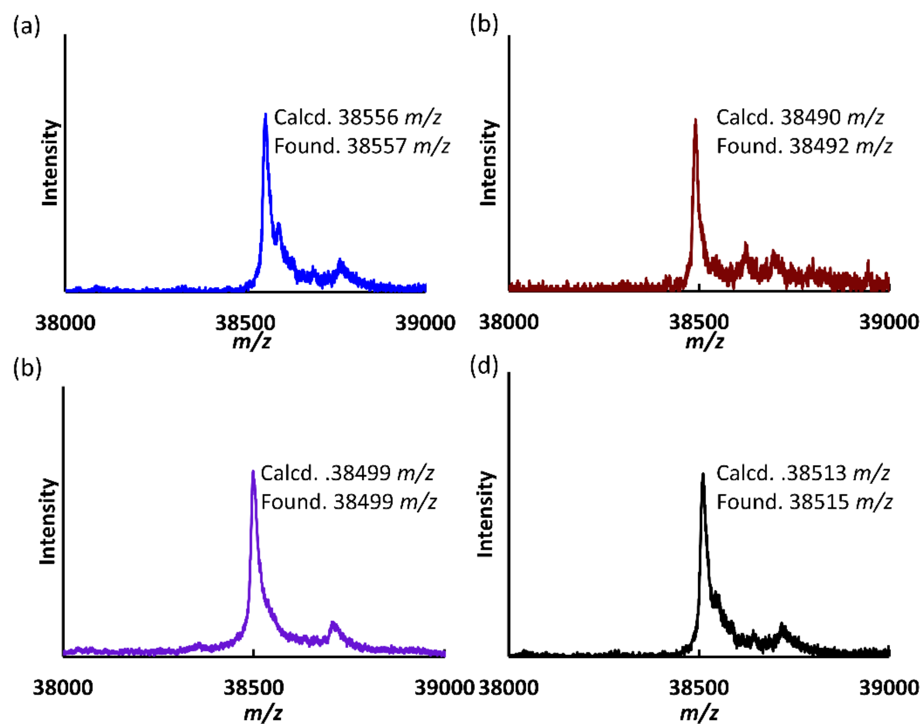

Fig. S9 MALDI-TOF mass spectra of purified hetero-dimers of (a) Cyt b_{562}^{A100C}–GFP^{S174C}, (b) Cyt b_{562}^{H63C}–GFP^{S174C}, (c) Cyt b_{562}^{K15C}–GFP^{S174C} and (d) Cyt b_{562}^{N80C}–GFP^{S174C}.

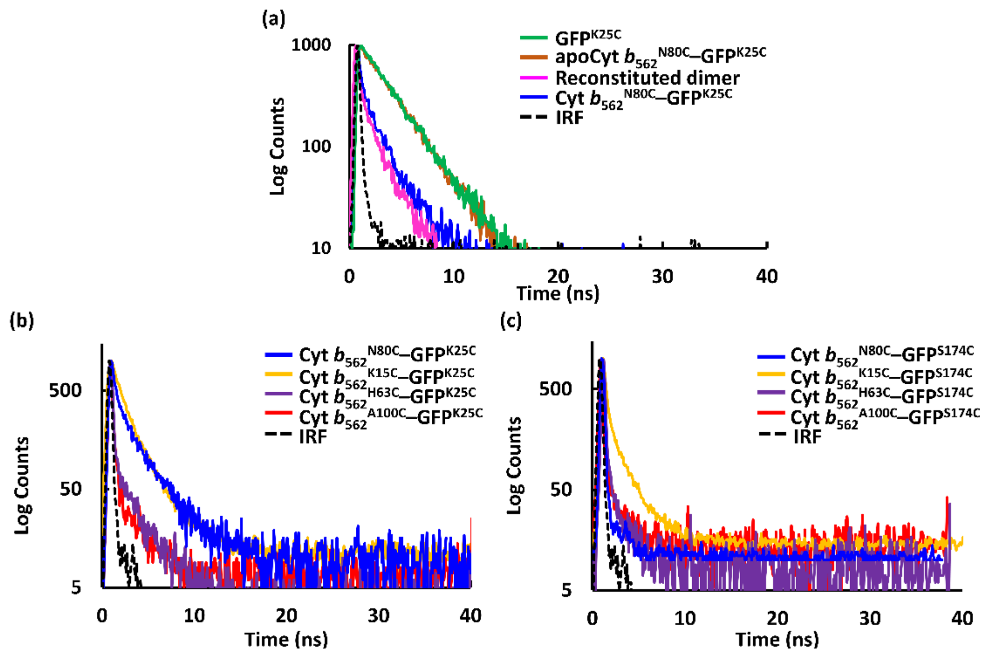

Fig. S10 Full scale of the fluorescence lifetime measurements (Fig. 4 in main text).

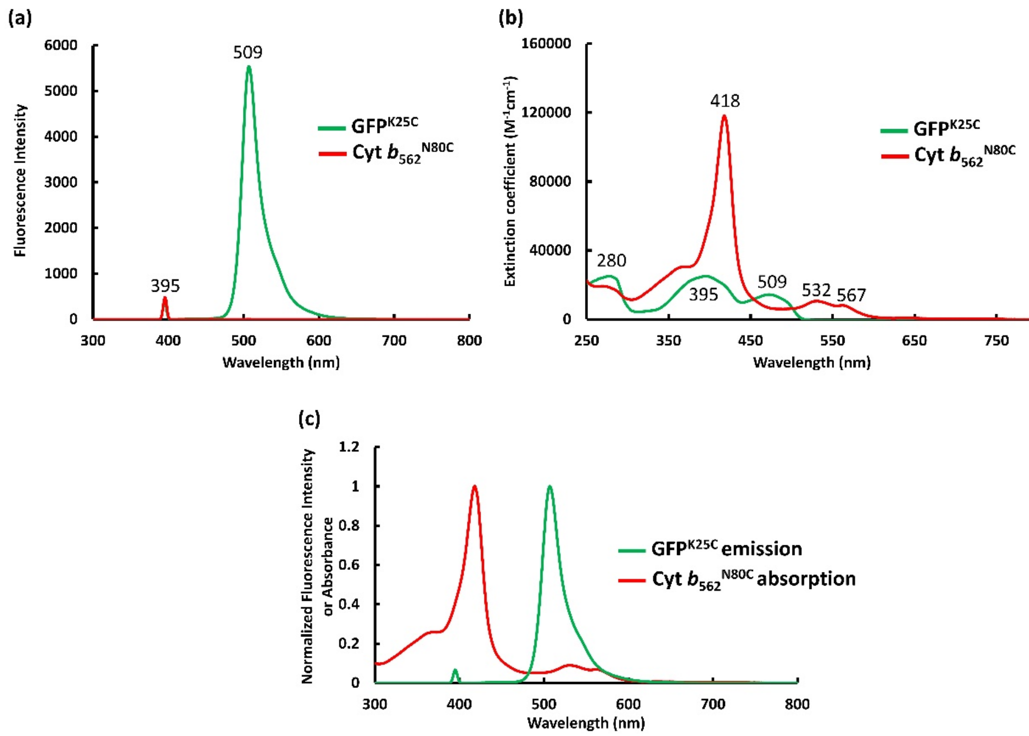

Fig. S11 (a) Fluorescence spectra of GFP^{K25C} and Cyt b_{562}^{N80C} λ_{ex} = 395 nm. (b) UV-vis spectra of GFP^{K25C} and Cyt b_{562}^{N80C}. (c) Emission spectrum of GFP^{K25C} and absorption spectrum of Cyt b_{562}^{N80C}.

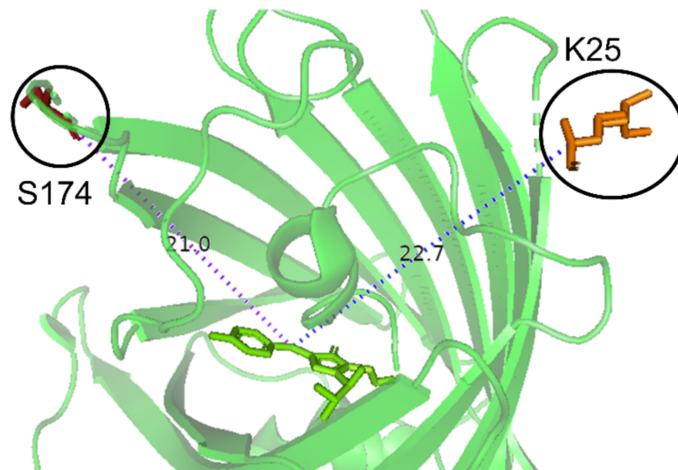

Fig. S12 Estimated distances of K25 = 22.7 Å, and S174 = 21.0 Å to GFP chromophore by PyMol.

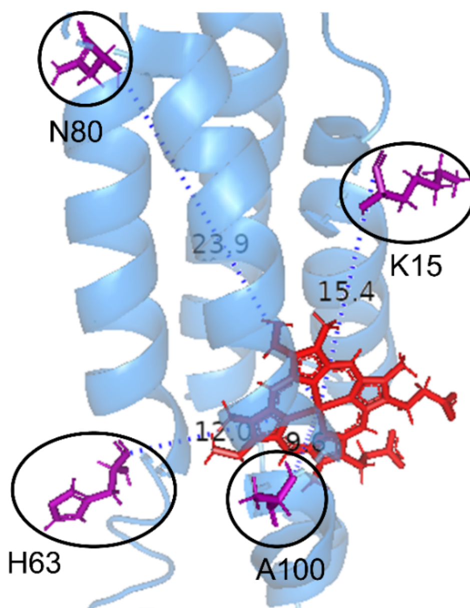

Fig. S13 Estimated distances of N80 = 23.9 Å, K15 = 15.4 Å, H63 = 12.0 Å, and A100 = 9.6 Å to the Cyt b_{562} heme centre by PyMol.

Reference

1. H. Kitagishi, K. Oohora, H. Yamaguchi, H. Sato, T. Matsuo, A. Harada, T. Hayashi, *J. Am. Chem. Soc.,* 2007, **129,** 10326–10327.