# Direct Conversion of Cytochrome *c* Spectral shifts to Fluorescence Using Photochromic FRET

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## SUPPORTING INFORMATION

### Reagents and Preparation

Potassium ferrocyanide, potassium ferricyanide, N,N,N',N'-tetramethyl-1,4-benzenediamine (TMPD) were obtained from Sigma-Aldrich (St. Louis, MO). Dithiothreitol (DTT), sodiumhydrosulfide, sodiumazide were obtained from VWR (West Chester, PA). CM sephadex C-50 was purchased from Sigma-Aldrich (St. Louis, MO). Chemicals were used as received without any further purification. BL21 (DE3) *E.coli* cells were purchased from Invitrogen (Carlsbad, CA). The pBTR-1 ($Amp^r$) plasmid containing the iso-Cyt *c* gene and the yeast Cyt *c* heme lyase gene was kindly provided by Bruce E. Bowler of the University of Montana, Missoula, and Montana. It was used as a scaffold to construct Cyt *c*-Venus and apoCyt *c*-Venus fusion. The plasmid encoding Venus was kindly provided by Adam E. Cohen of Harvard University, Cambridge, and Massachusetts. After PCR amplification using the forward and reverse primers (Table S1) information), Venus and template had two identical restriction sites (NotI-HF and BamHI-HF). After digestion, a Cyt *c*-Venus construct was prepared by ligating Venus into the corresponding restriction sites of digested pBTR via standard subcloning method. To prepare a template for apoCyt *c*-Venus, fragment encoding Cyt *c* heme lyase was removed and religated after filling recessed 3' of the Cyt *c*-Venus (Figure S1 (a). The plasmid was verified by sequencing at Macrogen Inc (Table S2). All samples were prepared in 50 mM phosphate buffer at pH 7.0 which is obtained by mixing 50 mM sodium dibasic and sodium monobasic solution in a 1.6:1 ratio unless otherwise specified. The concentration of Venus, reduced-Cyt *c* and oxidized Cyt *c* were determined by their extinction coefficients ($\varepsilon_{Cyt\ c\ (Fe^{+3})}$ = 9.8 $mM^{-1}$ $cm^{-1}$ at 550nm; $\varepsilon_{Cyt\ c\ (Fe^{+2})}$ = 29.4 $mM^{-1}$ $cm^{-1}$ at 550 nm; $\varepsilon_{Venus}$ = 92.2 $mM^{-1}$ $cm^{-1}$ at 518 nm).

### Expression and Purification

The Cyt *c*-Venus, apoCyt *c*-Venus and Cyt *c* were expressed separately in *E. coli* BL21(DE3) and purified according to previous methods. The expression level of each construct is similar to that of the unlabeled Cyt *c* as indicated by 10 % SDS page stained with Coomassie blue. Briefly, for expression of proteins, a 5 ml overnight culture in Luria-Bertani (LB) broth was grown at 37 $^oC$. Then 100 µl were transferred into 100 ml LB broth medium containing heme and 50 µg $ml^{-1}$ ampicillin. Cells were grown at an overnight culture and harvested by centrifugation. After resuspension in lysed solution, they were sonicated for 5 minutes (30 sec. on-10 sec. off) and centrifuged at 18.000 rpm for 20 minutes. The supernatant was collected and concentrated using 30 kDa centrifugal filter (Millipore) to remove impurities. It was washed with 20 mM phosphate buffer at pH 8.0 before loading into CM column. The sample were purified by using CM cation exchange column. Briefly, 1.0 gr. of CM was added to phosphate buffer to

prepare slurry solution and poor into 10x1 cm flex column (Thomas scientific). Then, the sample was loaded into the column and washed several times with the phosphate buffer (Fig. S5). An orange color solution was retained the column.

The Cyt *c*-Venus was eluted by varying the ionic strength from 50 mM to 500 mM NaCl in the phosphate buffer and collected in small tubes. The purified samples were characterized by SDS gel electrophoresis (Fig. S5). We observed a single broad band that was an indication of Cyt *c*-Venus which was purified at high concentration. From the absorption spectrum, the Cyt *c*-Venus oxidation state was determined at the reduced form. The oxidized Cyt *c* -Venus was prepared by adding 500 μM of $[Fe(CN)_6]^{+3}$. Then excess $[Fe(CN)_6]^{+3}$ was removed by using centrifugal 30 kDa cutoff filter.

**Characterization and Fluorescence Spectroscopy**

The fusion proteins was washed twice with phosphate buffer and concentrated with a centrifugal filter. All redox reagents were prepared at 500 mM in a small vial. 10 μM of Cyt *c*-Venus was transferred in a small plastic cuvette. The absorption spectrum of proteins was measured at 25 $^{o}$C by UV-3600 UV-Vis-NR spectrophotometer (Shimadzu Instruments). The same procedure was repeated for titrating samples with other redox reagents. Small volume of redox reagent (2 μl) was added to the protein solution and the spectra were recorded.

The fluorescence of each sample was measured separately at 25 $^{o}$C by FluoroMax-3 spectrophotometer. DTT, sodiumhydrosulfide, ferrocyanide, sodium azide and TMPD were used as a reducing agent. Ferricyanide was the only oxidizing agent in pcFRET experiments. apoCyt *c*-Venus and Cyt *c* were used as a control sample in all experiments.

**Fluorescence lifetime measurements**

ApoCyt *c*-Venus and Cyt *c*-Venus at different oxidation states were prepared and characterized before the measurement. 100 μl of sample was dropped on to the coverslip. Nikon TE 2000 inverted fluorescence microscope with a 60X oil immersion objective, numerical aperture 1.49 were used for lifetime measurements using time-correlated single photon counting (TCSPC). A pulsed 488 nm laser beam obtained by frequency-doubling the 976 nm output of an ultrafast Ti:Sa laser (pulse width 140 fs, repetition rate 80 MHz - Chameleon Ultra II; Coherent) was sent to a single photon counting module (SPCM) serving as the start detector. Fluorescence was collected using the same microscope objective, transmitted through the dichroic mirror FEL500 (Thorlabs) and emission filter HQ530/50 (Chroma) and detected by another SPCM serving as the stop detector. A time to amplitude converter (TAC) and a multipurpose data acquisition card serving as a multi-channel analyzer (MCA) were used to generate the histogram of coincidence detection events as a function of the delay time between start and stop pulses. The data was recorded using custom data acquisition software and analyzed with MATLAB (R2011a, The MathWorks, Natick, Massachusetts). Fluorescence lifetime was calculated by using a single exponential curve fitting. The least-squares method was used in obtaining the fit parameters.

**Epifluorescence microscopy**

Two holes were drilled on a glass microscope slide by Dremel 4000 equipped with diamond bits. Silica cover slips and slides were cleaned in piranha solution (a highly corrosive 3:1 mixture of concentrated $H_2SO_4$ and 30% aqueous $H_2O_2$) and assembled into a flow-cell using a 0.3 cm thick spacer (McMaster-Carr Corp., Princeton, NJ). The flow cell was filled with polylysine (Sigma Aldrich Co. LLC, St Louis, MO) and stand for 10 minutes and wait . After washing three times with buffer, the 30 μl sample is loaded to the flow cell. Then it was washed two times to remove unbounded *E. coli*.

The samples were excited by a diode pumped solid-state 473 nm laser (DragonLaser), which is controlled with a custom software (Arduino v1.0.5) and microcontroller board (ATmega328, Arduino Uno, Italy). Nikon eclipse inverted fluorescence microscope with a 60X oil immersion objective, numerical aperture 1.40 were used for epifluorescence measurement. The fluorescence images of Cyt *c*-Venus and apoCyt *c*-Venus expressing cells were recorded by an Andor Luca S EMCCD. Images were analyzed with MATLAB and ImageJ (1.6.0v, Bethesda, Maryland). All experiments were performed at room temperature.

**Förster radius ($R_0$) of Cyt c-Venus**

The overlap integral of Cyt *c*-Venus was calculated using[1]:

$$J(\lambda) = \int_{\lambda_i}^{\lambda_f} F_v(\lambda)\varepsilon_{Cy}(\lambda)\lambda^4 d\lambda \qquad \text{(Eq. S1)}$$

Where $F_v$ is the normalized Venus emission spectrum, $\varepsilon_{Cy}$ is the Cyt *c* molar extinction coefficient. In the spectrum, a region from 520 nm to 600 nm was selected to calculate the overlap integral. We consider $\varepsilon_{Cyt\,c\,(Fe^{+2})} = 29400\ M^{-1}\ cm^{-1}$ at 550 nm as the reference to calculate molar extinction coefficients at all wavelengths. $J(\lambda)$ was calculated as $1.12 \times 10^{-13}\ M^{-1}cm^3$.

The Forster radius of Cyt *c*-Venus was calculated using[1]:

$$R_0 = [8.8 \times 10^{-25} \kappa^2 \Phi_D n^{-4} J(\lambda)]^{1/6} \qquad \text{(Eq. S2)}$$

where $\Phi_D$ is the fluorescent quantum yield of the Venus, $\kappa$ is an orientation factor, n is the refractive index of the buffer, and J(λ) is the overlap integral. n and $\Phi_D$ were taken as 1.33 and 0.57[2] respectively. Since we used a flexible linker, Venus chromophore and the heme had a rapid and unrestricted rotation, so transition dipole of Cyt *c*-Venus can be regarded as isotropically degenerate that yields a value of 0.66 for the orientation factor. Moreover, Cyt *c* had a negligible structural change at different oxidation states. It includes small coordination change of axial ligand but no effect on heme position in the Cyt *c*.[3] By considering all factors, the Förster radius ($R_0$) was determined as 4.8 nm.

**Förster radius ($R_0$) from lifetime measurements**

$$\frac{1}{\tau_{re}} = \frac{1}{\tau_{ox}} + k_{pcFRET} \qquad \text{(Eq. S3)}$$

Where $k_{pcFRET}$ is the rate constant of nonradiative energy transfer due to pcFRET. $\tau_{ox}$ and $\tau_{re}$ represents the fluorescence lifetime of the oxidized and reduced Cyt *c*-Venus respectively.

$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} = 1 - \frac{\tau_{re}}{\tau_{ox}} \qquad \text{(Eq. S4)}$$

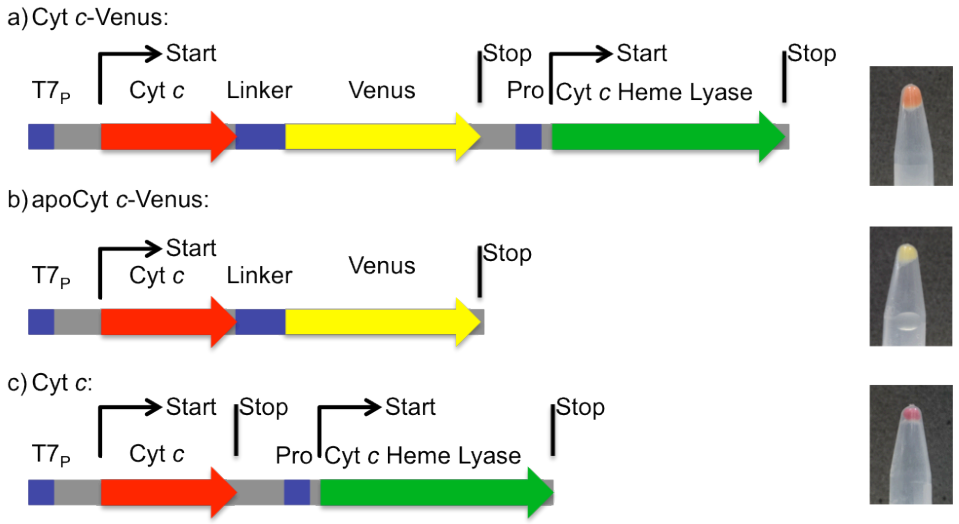
Where E is energy transfer from Venus to Cyt *c*. r and $R_0$ represent the distance from the Cyt *c* heme to Venus chromophore and Förster radius respectively. The Cyt *c*-Venus separation was calculated as 6.6 nm by using the $\tau_{ox}$ and $\tau_{re}$ that were measured from fluorescence lifetime measurements. All data were analyzed with MATLAB.

**Preparation of Cyt *c*-Venus c and apoCyt *c*-Venus constructs:**

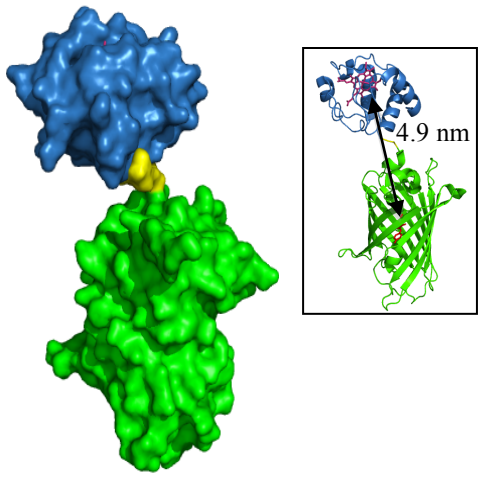
For the expression of Cyt *c*-Venus, we attached the Venus gene to the C terminus of the Cyt *c*. Cyt *c* heme lyase that catalyze the heme insertion is coexpressed at the same cell. Cyt *c* heme lyase was not fused to the Cyt *c*-Venus. The yellow color formation from apoCyt *c*-Venus indicates that the heme inserted Cyt *c* cannot be obtained without the Cyt *c* heme lyase gene. Cyt *c* expressing pBTR-1 plasmid was previously prepared[4] and used to study the interactions of Cyt *c* and Cyt *c* heme lyase.[5]

| Primer Sequence | Description |
|---|---|
| 5' – AAA AGC GGC CGC ACAGGC CCC TTT TCC T – 3' | Forward primer for Cyt *c* amplification |
| 5' – TTT TGC GGC CGC CTC ACT GGC TTT TTT CAA GTA GGT – 3' | Reverse primer for Cyt *c* amplification |
| 5' – AAA AGC GGC CGC AAT GGT GAG CAA GGG CGA G – 3' | Forward primer for Venus amplification |
| 5' – CCC CGG ATC CTC ACT TGT ACA GCT CGT CCA TGC CGA – 3' | Reverse primer for Venus amplification |

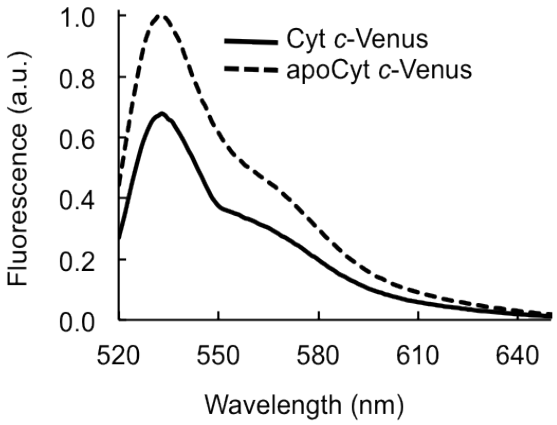
**Supplementary Table S1**. The sequence of the primers used for the cloning of Venus region into pBTR-1 encoding Cyt *c* and Cyt *c* Heme Lyase. pBTR-1 was reversibly amplified to add NotI restriction site.



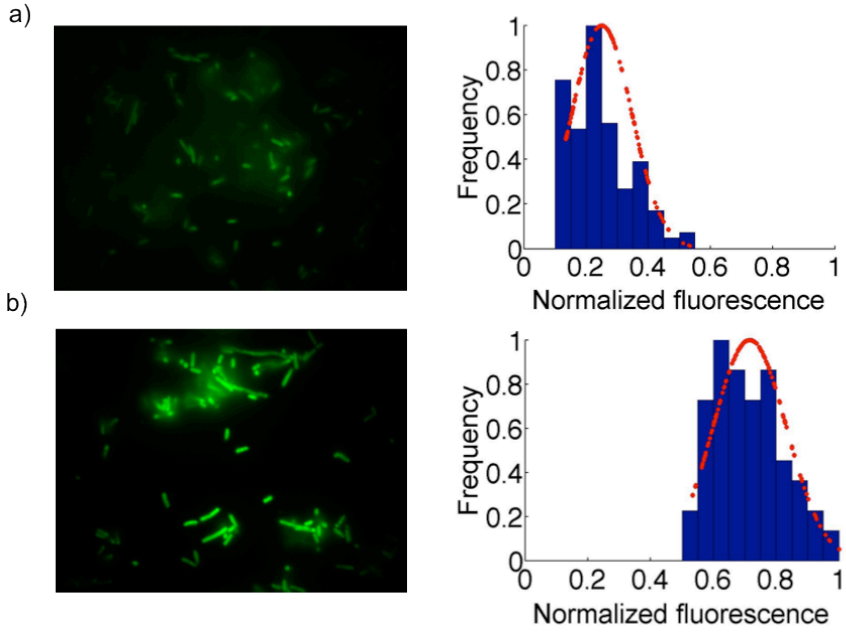

**Supplementary Figure S1**. Schematic representation of the constructs that were used to express Cyt *c*-Venus, apoCyt *c*-Venus and Cyt *c* respectively. The corresponding proteins expressed using these plasmids were also demonstrated on the pictures (right). a) Cyt *c*-Venus encoding part of the construct. b) ApoCyt *c*-Venus plasmid construct. Cyt *c* Heme Lyase was removed from the plasmid. c) Cyt *c* encoding part of the construct. The sequence of each plasmid was verified at the sequencing center of Macrogen Inc (Amsterdam, The Netherlands).



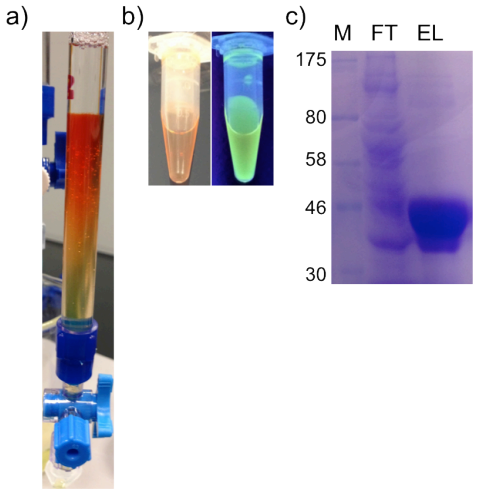

**Supplementary Figure S2**. The surface map of Cyt *c*-Venus complex. The crystal structures of Cyt *c* (blue, PDB ID-3CYT) and Venus (green, PDB ID-3V3D) were downloaded from Protein Data Bank. A linker composed of three alanine residues (yellow) was added to the N terminus of the Venus. To avoid any the steric effect that may arise from Cyt *c* and Venus crowding, the surface map of the complex was calculated by using PyMol (Schrödinger LLC.) software. Energy minimization was also performed by using Swiss PDB-viewer. Identical surface maps were obtained. Then, C terminus of Cyt *c* was virtually combined to the N terminus of the Venus. The average measure from Cyt *c* heme center to Venus chromophore was 4.9 nm. (Inset, cartoon structure of the Cyt *c*-Venus complex, the heme and Venus chromophore were shown in red.)



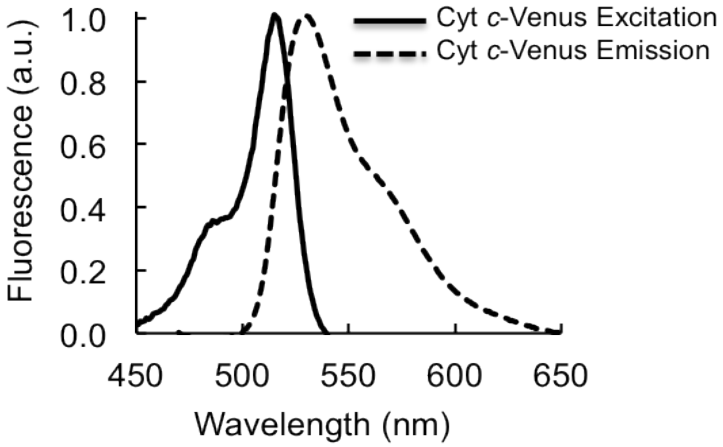

**Supplementary Figure S3**. Normalized fluorescence emission of Cyt *c*-Venus (solid line) and apoCyt *c*-Venus (dashed line) in BL21(DE3) cells. The fluorescence intensity from Cyt *c*-Venus was reduced due to the energy transfer from Venus to Cyt *c*.



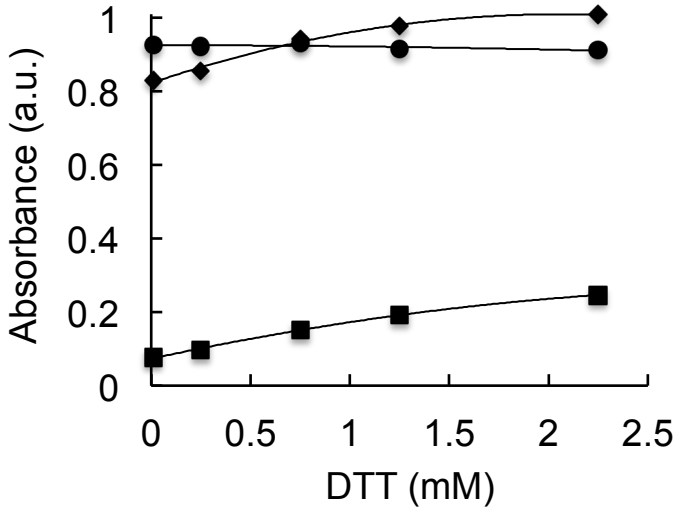

**Supplementary Figure S4**. The quantification of protein expression in BL21(DE3) cells. a) Cyt *c*-Venus expression. b) ApoCyt *c*-Venus expression. The images of the cells were analyzed with MATLAB (The MathWorks, Natick, MA). The histogram (Cyt *c*-Venus, top and Apo Cyt *c*-Venus, bottom) represents the distribution of Venus fluorescence in the cells. The fluorescence intensities were normalized by using the maximum fluorescence from apoCyt *c*-Venus cells. Briefly, the fluorescence intensity of each pixel above a threshold was determined from images. Then, the values were counted to calculate the fluorescence distribution. All data was normalized before plotting. From a Gaussian fit (red dotted line), the average intensity and errors of Cyt *c*-Venus and apoCyt *c*-Venus were calculated as 0.25±0.09 and 0.71±0.11 respectively.




**Supplementary Figure S5**. Purification of Cyt *c*-Venus. a) The cell lysate was loaded on the CM sephadex column. After washing several times, orange color Cyt *c*-Venus binding to CM at low ionic strength was observed. b) Eluted Cyt *c*-Venus under white light (left) and UV illumination (right). c) The purification and expression of Cyt *c*-Venus was verified by SDS gel electrophoresis. Both the flow through and elution fractions were loaded on a SDS gel. A distinct band can be seen in the third lane. (FL, flow through fraction, EL, elution fraction, M, Marker (kDa)).




**Supplementary Figure S6**. Fluorescence excitation and emission spectra of Cyt *c*-Venus in 50 mM phosphate buffer at pH 7.0. The emission maximum was identical to the free Venus protein used as a control sample. An excitation spectrum was collected at an emission of 530 nm. An emission spectrum was collected at 488 nm excitation. Both spectrums were measured by FluoroMax-3 spectroflurometer. (Scanned at 1 nm/min)




**Supplementary Figure S7**. The titration of Cyt *c*-Venus with DTT. The normalized absorption changes at 414 nm (diamond), 515 nm (circles) and 550 nm (squares) were shown. The change of Venus absorption was undetectable while an increase was observed at 550 and 414 nm that was an indication of reduced Cyt *c* ($Fe^{+2}$) formation.

ATGACTGAATTCAAGGCCGGTTCTGCTAAGAAAGGTGCTACACTTTTCAAGACTAGATGTCTA
CAATGCCACACCGTGGAAAAGGGTGGCCCACATAAGGTTGGTCCAAACTTGCATGGTATCTTT
GGCAGACACTCTGGTCAAGCTGAAGGGTATTCGTACACAGATGCCAATATCAAGAAAAACGTG
TTGTGGGACGAAAATAACATGTCAGAGTACTTGACTAACCCAGCCAAATATATTCCTGGTACC
AAGATGGCCTTTGGTGGGTTGAAGAAGGAAAAAGACAGAAACGACTTAATTACCTACTTGAAA
AAAGCCAGTGAGGCGGCCGCAATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCC
ATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAG
GGCGATGCCACCTACGGCAAGCTGACCCTGAAGCTGATCTGCACCACCGGCAAGCTGCCCGTG
CCCTGGCCCACCCTCGTGACCACCCTGGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGAC
CACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACC
ATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACC
CTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCAC
AAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCACCGCCGACAAGCAGAAGAACGGC
ATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCGGCGTGCAGCTCGCCGACCAC
TACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGC
TACCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTC
GTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTGA

**Supplementary Table S2**. Nucleotide sequence of Cyt *c*-Venus encoding region. The sequence of the plasmid was verified at the sequencing center of Macrogen Inc. (Amsterdam, The Netherlands).

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