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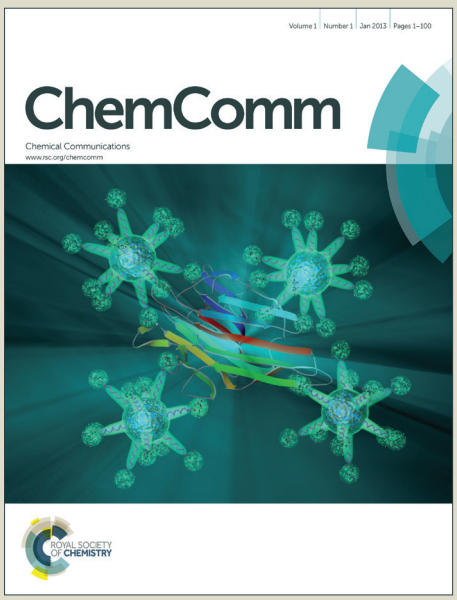

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Direct conversion of Cytochrome *c* spectral shifts to fluorescence using photochromic FRET

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Photochromic fluorescence resonance energy transfer (pcFRET) was used to monitor the redox activity of non-fluorescent heme protein. Venus fluorescent protein was used as a donor where its emission intensity was reversibly modulated by the absorption change of Cytochrome *c*.

Redox activity of c-type Cytochromes regulates an energy metabolism and apoptosis in cells.[1] For example, Cytochrome *c* (Cyt *c*) is a key electron carrier protein in the mitochondrion. Its main function is to transport charges between the Cytochrome *c* reductase and the Cytochrome *c* oxidase in the respiratory chain. Many spectroscopic methods were used to study the electron transfer mechanism of Cyt *c*.[2] Overexpression of Cyt *c* was needed to determine its oxidation state.[3] Dye labelling was used to measure heme dynamics.[4] In living cells, the release of GFP labelled Cyt *c* was used to study the oxidation events but showed that it was not sensitive to the redox state of Cyt *c*.[5] Alternatively, fluorescent indicators such as dihydrorhodamine 123 and mitotracker-red were indirectly used to measure a single oxidation event in living cells. The direct and continuous measurement of Cyt *c*'s redox state still remains elusive.[6] New molecular probes and methods are needed to resolve multiple oxidation events for heme proteins.[7]

Ideally, Cyt *c* labelled with a fluorescent probe would be used not only to determine the protein expression but also measure the redox activity. A key challenge is to relate the Cyt *c* oxidation state by the fluorescent intensity change of the reporter protein. New methods that indicate the redox state of Cyt *c* can provide invaluable information on electron transport process. The fluorescence emission from Cyt *c* is very low due to the endogenous heme cofactor therefore has no detectable intrinsic signal change. Alternatively, fluorescence reporters would be used to monitor Cyt *c*'s redox events at the spatiotemporal resolution.

We demonstrate that photochromic fluorescence energy transfer (pcFRET) can be used to measure the changes in redox state of Cyt *c* reversibly. pcFRET is an important tool for studying the dynamics of macromolecules.[8] We recently used the pcFRET to resolve the protein dynamics of microbial rhodopsins and determine the intermediate states of the photocycle at a single molecule level.[9] Briefly, FRET is generally used as a fluorescence method to determine the change in distance between donor and acceptor fluorophores.[10] In pcFRET, the probe is used to measure spectral overlap (J(λ), Eq. S1) of fluorescent donor with a non-fluorescent acceptor. The magnitude of quenching depends on the spectral separation between the donor emission and the acceptor absorption. Therefore, the spectral separation can be measured quantitatively even though the distance between the proteins remains the same.

Cyt *c* contains a heme (protoporphyrin IX) chromophore that is covalently linked by two thioether bonds to a Cys-X-X-Cys-His (X=amino acid) motif of the backbone. The insertion of heme and the folding of Cyt *c* were catalysed by Cyt *c* Heme Lyase. The oxidized Cyt *c* has a soret peak at 407 nm that shifts to 414 nm at the reduced medium. The absorption intensity of distinguished α and β peaks at 520 and 550 nm that represent the electronic transitions in heme, also arise at the reduced Cyt *c*. The redox reaction results in spectra change whose direction determines the oxidation state of the Cyt *c*. If it is conjugated to a fluorescence reporter, it may quench its fluorescence selectively at a specific region of the emission spectrum. The magnitude of the FRET signal gets stronger as the degree of overlap increases between the emission of donor and the absorption of the acceptor. The broad emission peak of Venus fluorescence protein overlaps with the absorption of both α and β bands that would selectively reduce the emission. Therefore, we selected Venus as a donor to measure the redox activity of Cyt *c*. Venus attached to c-terminus of Cyt *c* by a short linker to increase the FRET efficiency (Fig S1). The distance (r) from Cyt *c* heme to Venus chromophore was estimated as 4.9 nm by using the crystal structure of both proteins (Fig S2). For Cyt *c*-Venus expression, heme cofactor was covalently attached by Cyt *c* heme lyase that was co-expressed at the same cells (Fig. 1A). We expected that Cyt *c*-Venus expressing cells were highly distinguishable

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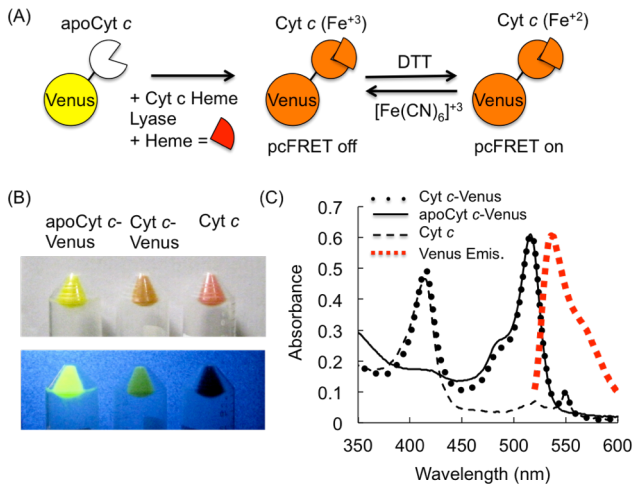

Fig. 1 (A) Schematic depiction of Cyt *c*-Venus formation. Cyt c Heme Lyase catalysed the insertion of heme into apoCyt *c*-Venus. pcFRET from Venus donor to non-fluorescent Cyt c acceptor after reduction by DTT or oxidation by $[Fe(CN)_6]^{+3}$. (B) ApoCyt *c*-Venus, Cyt *c*-Venus and Cyt *c* expressing cells. The images are collected under white light (top) and 365 nm UV (bottom). (C) The absorption spectra of Cyt *c*-Venus (dotted line), apoCyt *c*-Venus (solid line), Cyt *c* (dashed line). Emission spectrum of Venus (red, square line) was demonstrated for spectral overlap.

by its color that was dependent on the strong absorption of both Venus and Cyt *c*. After collecting cells, an intense orange color paste was a strong indication of Cyt *c*-Venus expression (Fig. 1B, top). The color of apoCyt *c*-Venus and Cyt *c* expressing cells were yellow and red respectively. The fluorescence emission was checked by using a UV light at 365 nm (Fig. 1B, bottom). We observed that the fluorescence intensity of the Cyt *c*-Venus was 30% lower than the apoCyt *c*-Venus (Fig S3). As expected, Cyt *c* expressing cells were dark indicating that fluorescence from heme was unobservable at the same illumination. The reduced fluorescence of Cyt *c*-Venus and the color shift resulted from the strong absorption of heme. The decrease of fluorescence emission from Cyt *c*-Venus was also observed in individual cells that were quantified by the fluorescence microscope (Fig. S4). We also concluded that the Venus labelling did not alter the insertion of heme to Cyt *c*. Moreover, Cyt *c* and Venus had a strong interaction and spectral overlap for pcFRET.

For pcFRET measurements, the purified Cyt *c*-Venus and apoCyt *c*-Venus were prepared in 50 mM phosphate at pH 7.0 (Fig S5). The visible absorption of the Cyt *c*-Venus and apoCyt *c*-Venus were recorded by a UV-Vis spectrophotometer (Fig. 1C). Three sharp peaks from Cyt *c*-Venus were clearly resolved in the visible region. Cyt *c*-Venus had a 414 nm Soret band and a β-band at 550 nm indicating that Cyt *c* was isolated in the ferrous state. These absorption peaks of Cyt *c*-Venus were at identical positions to the Cyt *c* control sample that was expressed without Venus. We concluded that Venus attachment neither changed the coordination of heme nor affected the Cyt *c* folding. The intense peak at 514 nm was an indication of Venus absorption. Small α-band of the Cyt *c* at 520 nm was unresolved due to significant spectral overlap of the Venus while the β-band was unaffected due to the reduced absorption of Venus around 550 nm. A single peak at 514 nm was observed for apoCyt *c*-Venus. By using the emission spectrum of Venus and Cyt *c* absorption, the Förster radius (R_0) of Cyt *c*-Venus was estimated as 4.8 nm (Eq. S2). It was close to our initial estimation for distance.

To determine the pcFRET, the reaction of Cyt *c*-Venus with DTT was recorded by the fluorometer. The Cyt *c*-Venus was prepared at the oxidized state and its emission spectra was measured. The oxidized Cyt *c*-Venus had a broad peak with a maximum at 537 nm that was indistinguishable from apoCyt *c*-Venus (Fig S6). Then, 5.0 μM of Cyt *c*-Venus in the cuvette was mixed with DTT until the Cyt *c* was fully reduced in the solution. The intensity of Venus emission decreased after the addition of 0.25 mM DTT to the cuvette. A small decrease at 550 and 537 nm were observed at the spectra (Fig. 2A). Addition of more DTT resulted in subsequent decrease in Venus emission that was expected due to the increasing overlap between Venus emission and Cyt *c* absorption. The change around 537 nm was relatively small compared to the decrease at 550 nm at which the largest fluorescence decrease was measured. The fluorescence emission ratio of 550/537 nm ($F_{550/537}$) was calculated as 0.49 for the reduced Cyt c. Upon addition of 0.5 mM of $[Fe(CN)_6]^{+3}$, the heme in Cyt *c*-Venus was reoxidized and rapid increase in the fluorescence emission was observed. $F_{550/537}$ emission ratio was determined as 0.79 at the oxidized state. In parallel, the absorption of Cyt *c*-Venus was measured by absorption spectrophotometer in order to determine if the signal aroused from the overlap of absorption spectra (Fig. 2B). The increased signal at 550 nm was apparent at the reduced state. A spectral shift in soret band from 407 to 414 nm was an indication of the ferrous heme formation for Cyt *c*-Venus. The absorption of Venus at 514 nm remained unchanged indicating that reduced environment did not affect the chromophore of the Venus (Fig S7). The absorption increase of β peak was consistent with the fluorescence decrease of the Venus and an opposite direction to the Cyt *c* absorption (Fig. 2C). The fluorescence lifetime of Cyt *c*-Venus at reduced and oxidized state was measured to determine the effect of spectral overlap on pcFRET. The data was fitted to a single exponential decay function with least-squares method (Fig. 2D). The lifetime of Cyt *c*-Venus was 2.81 ns and 2.45 ns at the oxidized and the reduced states, respectively. The shorter signal resulted from higher spectral overlap in pcFRET. Then, distance (r) from Cyt *c* to Venus was determined as 6.6 nm by using the fluorescence lifetime measurement (Eq. S3-S4). The length was longer than our initial prediction that explained the lower energy transfer from Venus to Cyt *c*.

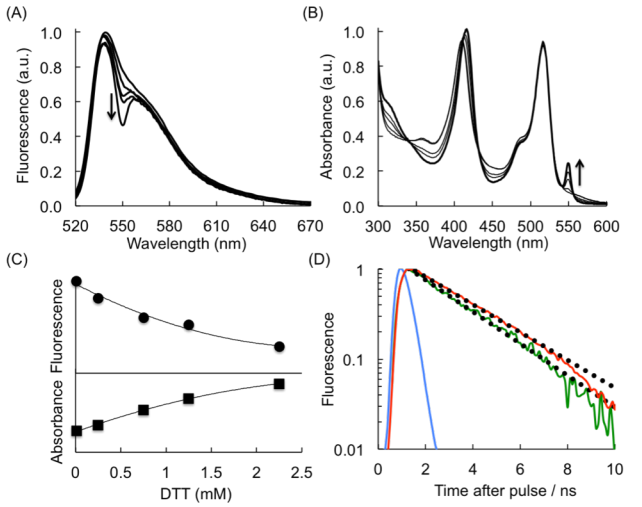

Fig. 2 pcFRET of Cyt *c*-Venus at varied DTT concentrations. (A) The emission spectra of Cyt *c*-Venus. (B) The absorption spectra of Cyt *c*-Venus. (C) The Venus fluorescence was probed at 550 nm (top). The Cyt *c* absorption was probed at 550 nm (bottom) (D) Fluorescence lifetime of Cyt *c*-Venus at reduced (green), at oxidized state (red) and internal response of the laser (blue) at log scale. The lifetime was predicted by fitting the data to a single exponential decay function (circle).

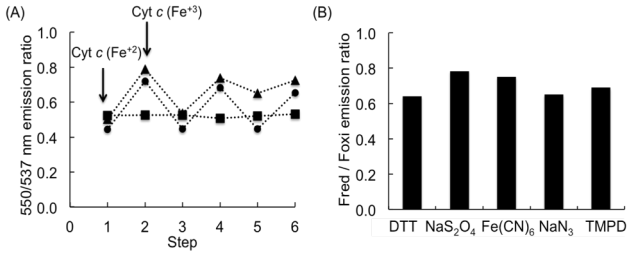

Fig. 3 Monitoring Cyt *c* redox states by pcFRET (A) The odd and even steps indicate the Cyt *c* at reduced and oxidized states respectively. The cycle with DTT/$[Fe(CN)_6]^{+3}$ (circle), $[Fe(CN)_6]^{+2}/[Fe(CN)_6]^{+3}$ (triangle) and apoCyt *c*-Venus with DTT/$[Fe(CN)_6]^{+3}$ as a control sample (square). (B) The ratiometric pcFRET measurement of Cyt *c*-Venus in the presence of different redox reagents.

To test whether photochromic FRET can be used to probe the redox change continually, the Cyt *c*-Venus was titrated reversibly by DTT and $[Fe(CN)_6]^{+3}$. The 10.0 μM of reduced Cyt *c* (Fe^{+2})-Venus was transferred to the cuvette and pcFRET was characterized by the concomitant decrease at 550 nm. The redox state of Cyt *c* was identified by ratiometric analysis. The $F_{550/537}$ was calculated as 0.44 (Fig. 3A, circles). Then, addition of 3.0 μl of $[Fe(CN)_6]^{+3}$ at 1.5 mM final concentration oxidized the Cyt *c* that lead to immediate absorbance decrease at 550 nm, shifting of soret peak to 407 nm and finally broadened and smaller peak at 530 nm, consequently pcFRET did not occur. The fluorescence signal was higher at the oxidized state. $F_{550/537}$ was increased to 0.72. In the third step, the addition of DTT returned the $F_{550/537}$ back to 0.45. The decrease in pcFRET ratio indicates that the Cyt *c* was in the reduced state. We repeated changing the medium recursively and observed a similar identical fluorescence ratio for different oxidation states of Cyt *c*. The average of $F_{550/537}$ was 0.44±0.01 and 0.69±0.03 for the reduced and oxidized Cyt *c*-Venus respectively. The pcFRET measurements were repeated with $[Fe(CN)_6]^{+2}$ used as a reducing agent. The fluorescence decreased in the presence of $[Fe(CN)_6]^{+2}$ and reversed as $[Fe(CN)_6]^{+3}$ was added into the Cyt *c*-Venus (Fig. 3A, triangles). The average of $F_{550/537}$ was 0.56±0.08 and 0.75±0.04 for the reduced and oxidized Cyt *c*-Venus respectively. The change of DTT and $[Fe(CN)_6]^{+2}$ were comparable and at the same magnitude that was opposite to Cyt *c* absorption at all steps. apoCyt *c*-Venus alone did not demonstrate any pcFRET at the same concentrations of DTT and $[Fe(CN)_6]^{+3}$ (Fig. 3A, squares). The change in $F_{550/537}$ was negligible, means that the Venus fluorescence emission was unaffected by the addition of redox reagents and cannot be explained by the changes around the Venus chromophore. pcFRET was repeated with other redox reagents such as NaS_2O_4, NaN_3, TMPD. The redox activity of Cyt *c* with different reagents was quantified by the ratio of pcFRET at the reduced and oxidized state (Fig. 3B). The ratio varied from 0.61 to 0.78. The smallest change was observed for DTT, which had the largest pcFRET magnitude.

Our results demonstrate that pcFRET can be used to convert Cyt *c* absorbance changes into fluorescence. The reduction of Cyt *c* resulted in higher spectral overlap with Venus. It quenched the emission of Venus fluorescence more than the oxidized Cyt *c*. The increase in $F_{550/537}$ was an indication of oxidized Cyt *c* formation and used to determine the redox state of Cyt *c*. FRET efficiency during the redox reaction was smaller than our initial predictions. On the basis of lifetime measurements, the change could be explained by a 6.6 nm separation between Cyt *c* and Venus that was 1.8 nm longer than Förster radius. A small Cyt *c* absorption and spectral overlap with Venus may result in less efficient energy transfer during the redox reaction. Radiative transfer and the protein orientation may explain the initial brightness change after the Cyt *c*-Venus formation in cells.[10b] However, the reduction of Venus lifetime supported pcFRET mechanism, and concluded that heme quenched the fluorescence emission of the Venus. The method could expand the scope of photochromic fluorescent method to monitor heme protein dynamics. For example, the oxidation state of the Cyt *c* embedded in lipid bilayer may be measured fluorescently without isolating the Cyt *c*. The pcFRET could also be used to study the redox activity of other heme proteins such as Cyt *c* oxidase or CytP450 that metabolize drug compounds in cells. We are currently working to understand if the Cyt *c* dynamics can be observed directly in biological samples. pcFRET method can be used to study the enzymes that directly bind to the Cyt *c* and change its redox activity. For example, the interaction of IP3R protein to Cyt *c* was known to initiate Ca^{2+} release from ER, but its role of Cyt *c* oxidation state remains unclear. The sensitivity of Cyt *c* redox cycle to mitochondrial membrane potential may be measured simultaneously by using voltage sensitive fluorescent reporters. Cyt *c*-Venus could be used as a probe to measure the redox events in the presence of small molecules and peptides if they inhibit the oxidation or reduction events in cells.

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