

Temperature Sensing Using Red Fluorescent Protein

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Abstract Genetically encoded fluorescent proteins are extensively utilized for labeling and imaging proteins, organelles, cell tissues, and whole organisms. In this study, we explored the feasibility of mRFP1 and its variants for measuring intracellular temperature. A linear relationship was observed between the temperature and fluorescence intensity of mRFP1 and its variants. Temperature sensitivities of *E. coli* expressing mRFP1, mRFP-P63A and mRFP-P63A[(4R)-FP] were -1.27%, -1.26% and -0.77%/°C, respectively. Finally, we demonstrated the potentiality of mRFP1 and its variants as an *in vivo* temperature sensor.

Keywords: temperature sensor, mRFP1, 4-fluoroproline, non-canonical amino acid incorporation, thermal stability

1. Introduction

Temperature is the most frequently measured physical parameter governing every cellular event, such as gene expression, enzyme reaction, cell division and metabolism of a living cell. In a diseased state, abnormal cells display elevated cellular temperature compared to normal cells due to increased cellular metabolism. Therefore, developing a temperature sensor may allow differentiation of healthy

cells from abnormal cells [1,2]. Further, the accurate determination of temperature can be utilized to explain intricate biological processes from gene expression to energy metabolism [3-5]. Given the significance of *in vivo* temperature sensing, a number of techniques have been developed. Most of the techniques are based on thermal quenching of fluorescence intensity of fluorescent nanomaterials such as organic fluorescent dyes, quantum dots, nanoparticles, nanogels, and semi-conducting polymer dots [5-12]. While these techniques are successful in measuring intracellular temperature, they require the introduction of physical objects or artificial probes through the plasma membrane or endocytotic pathway, which affects cell integrity or behavior and is also incompatible with cell imaging for long-term studies [13-17]. To avoid external introduction of a probe into cells, we aimed to develop a novel temperature sensing method using *in vivo* expressing protein that would serve as a better tool for measuring intracellular temperature.

In general, genetically encoded fluorescent proteins are extensively utilized in the field of targeted labeling and imaging of proteins, organelles, cell tissues, and whole organisms [18]. Fluorescent proteins are autocatalytic in nature and do not require additional enzymes and cofactors to form a mature chromophore. Moreover, fluorescent proteins are easily expressing inside cells; this superior qualifying distinction enriches the fluorescent proteins over fluorescent nanomaterials. Recently, green fluorescent protein (GFP) was utilized to measure the intracellular temperature by using fluorescence polarization anisotropy [13,14]. The main drawback of this methodology is the requirement of expensive instruments for measuring the intracellular temperature. Compared to GFP, red fluorescent proteins are highly advantageous due to their longer wavelength which helps to penetrate out from the target and exhibit less autofluorescence or background signals

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during imaging [19,20]. Therefore, we chose mRFP1 as a temperature sensor to measure intracellular temperature based on change in the fluorescence intensity relative to temperature.

2. Materials and Methods

2.1. Materials

PCR reagents, T4 DNA ligase and restriction endonucleases were purchased from Promega (Madison, WI, USA) and isopropyl-D-thiogalactopyranoside (IPTG) was purchased from Sigma Chemicals (St. Louis, MO, USA). Host bacterium *Escherichia coli* KC1325 (*E. coli*) strain XL1-blue (Stratagene, CA, USA) was used for plasmid DNA preparation. *E. coli* cells transformed with plasmids were grown aerobically in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, MI, USA) or on LB agar plates, supplemented with appropriate antibiotics for selection of transformants. *E. coli* KC1325 proline auxotroph was kindly provided by Prof. Laszlo N. Csonka. Natural amino acids were purchased from Sigma Chemicals (St. Louis, MO, USA), proline surrogate (2*S*, 4*R*)-4-fluoroproline [(4*R*)-FP] was purchased from Bachem (Bubendorf, Switzerland) and pQE-80L plasmid and nickelnitrilotriacetic acid (Ni-NTA) affinity column were purchased from GE Healthcare Bio-Sciences (Sweden).

2.2. Expression and purification of mRFP1, mRFP1-P63A, and mRFP1-P63A[(4*R*)-FP]

The construct pQE-80L-mRFP1 and pQE-80L-mRFP1-P63A was transformed into *E. coli* BL21 (DE3)pLysS (KC1325) Pro auxotroph. mRFP1 and mRFP1-P63A were expressed in LB media and purified as described earlier [21]. mRFP-P63A[(4*R*)-FP] was expressed in minimal media and purified as described earlier [21]. Briefly, the limiting concentration of Pro (0.05 mM) allowed cells to attain an OD₆₀₀ value of 0.6 ~ 0.8; and the target proteins were induced with 1 mM IPTG with simultaneous addition of (4*R*)-FP (0.5 mM) and allowed expression for 7 h. Harvested cells were subjected to centrifugation and stored at -70°C until further use. Collected cell pellet was suspended in lysis buffer (5 mM imidazole and 50 mM sodium-Phosphate buffer pH 7.5 containing protease inhibitor), followed by sonication and centrifuged at 16,000 rpm, 4°C for 20 min. The supernatant was saved as a soluble protein fraction and further soluble protein was purified by Ni-NTA column chromatography using standard protocol. Elution fractions were analyzed by SDS-PAGE, and those enriched in the desired mRFP1 and its variants were pooled and dialyzed against 1X phosphate buffered saline. Protein concentration was quantified using Bradford assay.

2.3. Fluorescence Analysis of mRFP1, mRFP1-P63A, and mRFP1-P63A[(4*R*)-FP] at Different Temperature

For examining the time dependency of fluorescence intensity of mRFP1, mRFP1-P63A, and mRFP1-P63A[(4*R*)-FP] with rapid change of temperature from 4 to 40°C. Aliquot (10 µL) of protein solution (1 mg/mL) was added to 990 µL of water in a cuvette pre-incubated at 40°C, and fluorescence intensity was measured every 5 sec in a time drive mode using a PerkinElmer LS55 fluorescence spectrophotometer.

For measuring the fluorescence emission spectra of purified mRFP1, mRFP1-P63A, and mRFP1-P63A[(4*R*)-FP] at different temperature, each sample of protein (100 µg/mL) was prepared in aqueous solution and incubated at temperatures ranging from 10 to 80°C for 10 min. Fluorescence emission scans of protein samples were measured.

For determining the robustness of temperature probe, the mRFP1, mRFP1-P63A, and mRFP1-P63A[(4*R*)-FP] were examined with the temperature gradient experiment. The mRFP1, mRFP1-P63A, and mRFP1-P63A[(4*R*)-FP] (100 µg/mL) were prepared in aqueous solution. The temperature gradient experiment was carried out at 10 to 50°C and 50 to 10°C with a scan speed of 1°C/min, and every 5 min fluorescence emission scan were performed using fluorescence spectrophotometer.

Fluorescence emission spectra of *E. coli* expressing mRFP1, mRFP1-P63A, or mRFP1-P63A[(4*R*)-FP] was measured at different temperatures. *E. coli* expressing mRFP1, mRFP1-P63A, or mRFP1-P63A[(4*R*)-FP] was harvested, washed twice with distilled water and prepared in aqueous solution to attain an OD₆₀₀ value of 0.1 and incubated at temperatures ranging from 10 to 80°C for 10 min. Samples were diluted to 1,000 times, and fluorescence emission scan was performed.

2.4. Circular dichroism of mRFP1, mRFP1-P63A, and mRFP1-P63A[(4*R*)-FP] at different temperature

A far UV circular dichroism (CD) spectrum was recorded for mRFP1, mRFP1-P63A, and mRFP1-P63A[(4*R*)-FP] on a JASCO J-715 spectrometer. For analysis, 250 µL of 3 µM protein was prepared in 1 × PBS buffer (pH 7.5) and placed in a 0.2 cm cuvette, and then CD spectrum was obtained at different temperatures. Twenty scans were accumulated per spectrum and raw data processed using Jasco software. Finally, the results were analyzed and a graph drawn using Origin software.

2.5. Live cell imaging of *E. coli* expressing mRFP1, mRFP1-P63A, or mRFP1-P63A[(4*R*)-FP] at different temperatures

E. coli expressing mRFP1, mRFP1-P63A, or mRFP1-P63A[(4*R*)-FP] was prepared in aqueous solution to attain an OD₆₀₀ value of 0.1 and incubated at temperatures ranging

from 10 to 80°C for 10 min. A small aliquot of sample was taken and spread on a glass slide, which was pre-incubated at the desired temperature. Images were captured with a magnification of 1.00X and an exposure of 100 ms, using a fluorescence microscope.

3. Results and Discussion

3.1. Effect of temperature on fluorescence intensity of mRFP1

In temperature sensing methods based on fluorescent nanomaterials, the fluorescence intensity of nanomaterials decreased with an increase in temperature by thermal quenching of fluorescence. Similarly, we observed a change in fluorescence intensity of mRFP1 with a rapid change in the temperature from 4 to 40°C. Fluorescence intensity of mRFP1 decreased 40.5% of original fluorescence intensity and reached plateau after ~5 min (Fig. 1A). Generally,

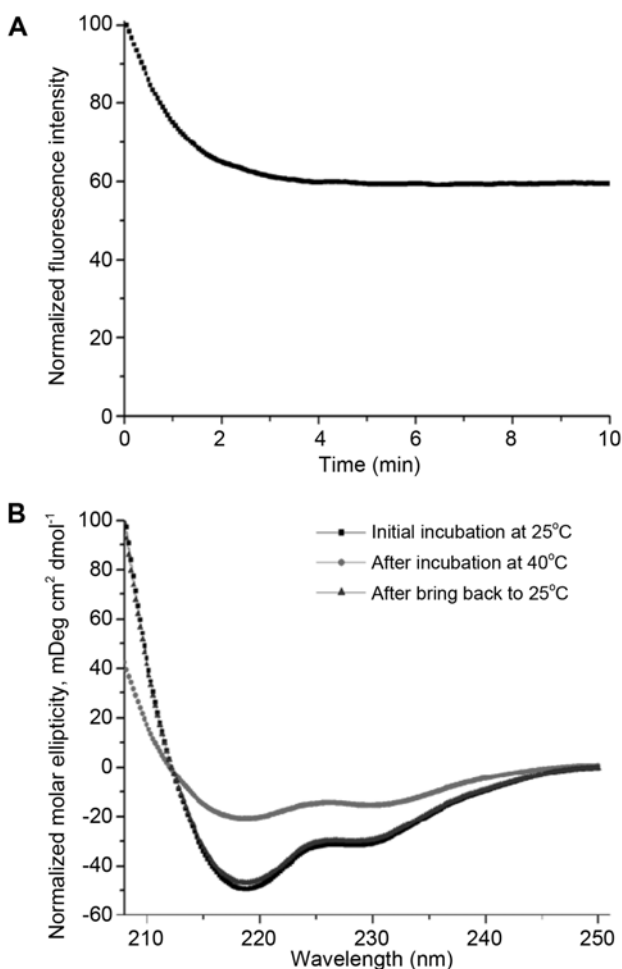


Fig. 1. (A) Time dependence of fluorescence intensity of mRFP1 with a rapid change from 4 to 40°C. (B) Circular dichroic profiles of mRFP1 with a temperature shift from 25 to 40°C.

proteins undergo conformational changes with changing temperatures [22,23]. It is likely that mRFP1 takes ~5 min to reach equilibrium state of structure after conformational change and maintains constant fluorescence (Fig. 1A). It is also possible that a decrease in fluorescence intensity is caused by simple thermal denaturation of mRFP1. To understand whether the change in fluorescence intensity of mRFP1 was influenced by thermal denaturation of proteins, circular dichroism (CD) studies were carried out. CD profiles of mRFP1 (3 μ M) were initially analyzed by incubating in a cuvette for 10 min at room temperature (25°C). Then temperature was quickly raised to 40°C and CD analysis was carried out after 10 min incubation (Fig. 1B). Since mRFP1 comprises 11 beta sheets, the CD profiles of mRFP1 at room temperature showed sharp negative major deflection at 218 ~ 220 nm and minor deflection at approximately 230 nm. CD profiles at 40°C showed that molar ellipticity of mRFP1 was reduced, but overall secondary structure of mRFP1 was unchanged based on the deflection pattern (Fig. 1B). Finally, the temperature was bringing back to 25°C and CD analysis was carried out after 10 min incubation and the initial molar ellipticity of mRFP1 was recovered. Results confirmed that the decrease in fluorescence intensity of mRFP1 is caused by thermal quenching of fluorescence, not thermal denaturation of protein.

Fluorescence emission spectrum of mRFP1 was measured after 10 min incubation at different temperatures, as it takes ~5 min to reach plateau (Fig. 1A). Fluorescence intensity of mRFP1 decreased linearly with increase in temperature (Fig. 2A). Generally, a rise in temperature will give in greater absorptivity of mRFP1 for the exciting radiation, resulting in increased fluorescence intensity. In contrast, a rise in temperature will reduce fluorescence intensity by thermal quenching of the emitted fluorescence. Therefore, in the tested temperature range, thermal quenching of the emitted fluorescence is dominant [24]. Temperature sensitivity of mRFP1 is $-1.28\%/^{\circ}\text{C}$, which is comparable to Rhodamine B sensitivity of $-3.5\%/^{\circ}\text{C}$ [25].

To expand application of fluorescence protein as a temperature probe for monitoring temperature changes in thermophiles, a microfluidic system, chemical microreactors *etc.*, robustness was considered as one parameter [26,27]. The robustness of temperature probe was examined by temperature gradient experiment carried out at 10 to 50°C and 50 to 10°C, with a scan speed of 1°C/min. mRFP1 showed partial hysteresis during the heating and cooling cycle. In each cycle, mRFP1 lost ~6.0% of initial fluorescence intensity. After three cycles, mRFP1 recovered 82.1% of initial fluorescence intensity (Fig. 2B). Therefore, a more stable fluorescent protein would be useful in expanding its application as a temperature probe.

3.2. Effect of temperature on fluorescence intensity of mRFP1-P63A and mRFP1-P63A[(4R)-FP]

Conventional protein engineering aims at modifying the sequence of a protein with 20 canonical amino acids by altering DNA sequence to create proteins with improved functional properties such as stability, specific activity [28,29]. Alternative approaches were developed to improve the stability and folding efficiency of proteins through incorporation of non-canonical amino acids (NCAA) [30-37]. We generated mRFP1-P63A[(4R)-FP] through NCAA mutagenesis after mutating non-permissive site Pro63 into Ala (mRFP1-P63A) [21]. The half-life of mRFP1, mRFP1-P63A, and mRFP1-P63A[(4R)-FP] at 50°C were 5.01, 6.17, and 13.25 h, respectively. The half-life of mRFP1-P63A[(4R)-FP] showed ~2.6 fold enhancement at 50°C compared to mRFP1. As, we expected mRFP1-P63A[(4R)-FP] can be a better temperature sensor than mRFP1.

Fluorescence intensities of mRFP1-P63A and mRFP1-P63A[(4R)-FP] were examined with a rapid temperature

change from 4 to 40°C as described above. Fluorescence intensity of mRFP1-P63A and mRFP1-P63A[(4R)-FP] decreased 33.8 and 16.0% of original fluorescence intensity, respectively. Similarly with mRFP1, the fluorescence intensities of mRFP1-P63A and mRFP1-P63A[(4R)-FP] took ~5 min to reach plateau after changing temperature from 4 to 40°C. CD analysis of mRFP1-P63A and mRFP1-P63A[(4R)-FP] at different temperatures showed that the overall secondary structure was unchanged based on deflection pattern. Molar ellipticity of mRFP1-P63A and mRFP1-P63A[(4R)-FP] at 40°C was reduced similarly with mRFP1.

To examine temperature sensitivity, fluorescence emission spectra of mRFP1-P63A and mRFP1-P63A[(4R)-FP] were determined in aqueous solution at different temperatures. An increase in temperature linearly reduced the fluorescence intensity of mRFP1-P63A and mRFP1-P63A[(4R)-FP] (Fig. 2A). Temperature sensitivity of mRFP1-P63A and mRFP1-P63A[(4R)-FP] was -1.26 and -0.77%/°C, respectively. mRFP1-P63A[(4R)-FP] showed less temperature sensitivity than mRFP1 and mRFP1-P63A. This suggests that thermal quenching of emitted fluorescence is affected by incorporating

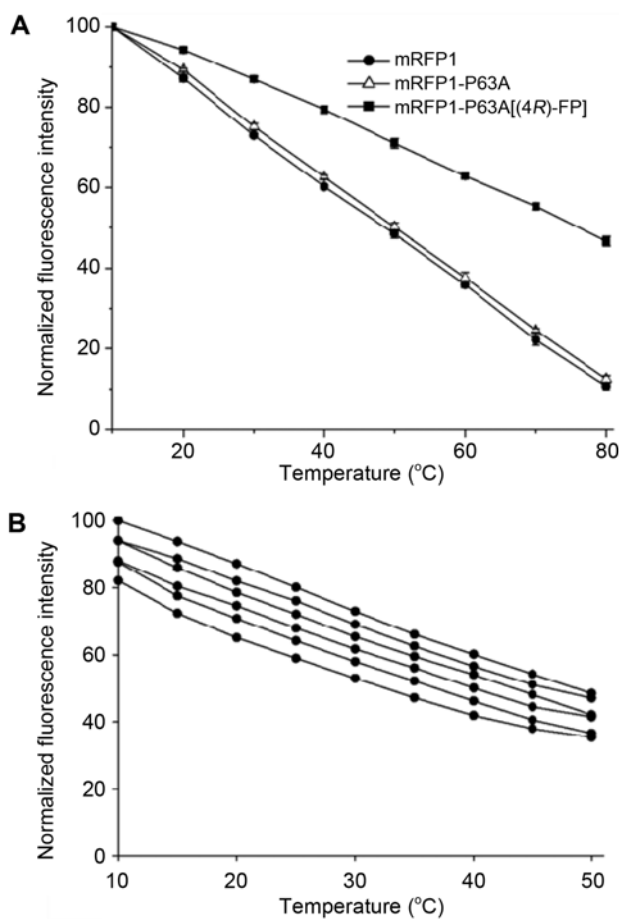


Fig. 2. (A) Normalized fluorescence intensities of purified mRFP1, mRFP1-P63A, and mRFP1-P63A[(4R)-FP] at different temperatures. (B) Fluorescence intensity of mRFP1 in temperature gradient cycle carried out at 10 to 50°C and 50 to 10°C with a scan speed of 1°C/min.

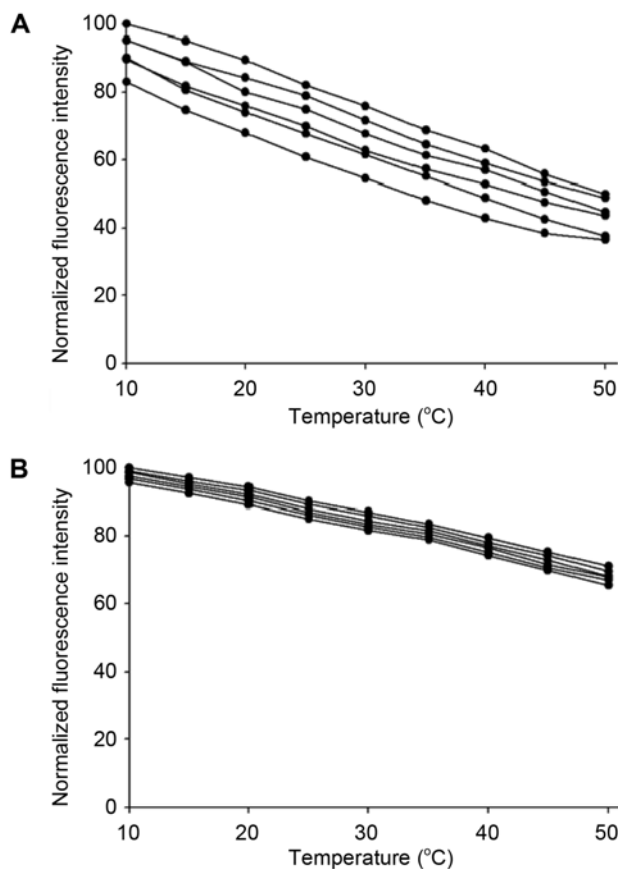


Fig. 3. Fluorescence intensity of mRFP1-P63A (A) and mRFP1-P63A[(4R)-FP] (B) in temperature gradient cycle carried out at 10 to 50°C and 50 to 10°C with a scan speed of 1°C/min.

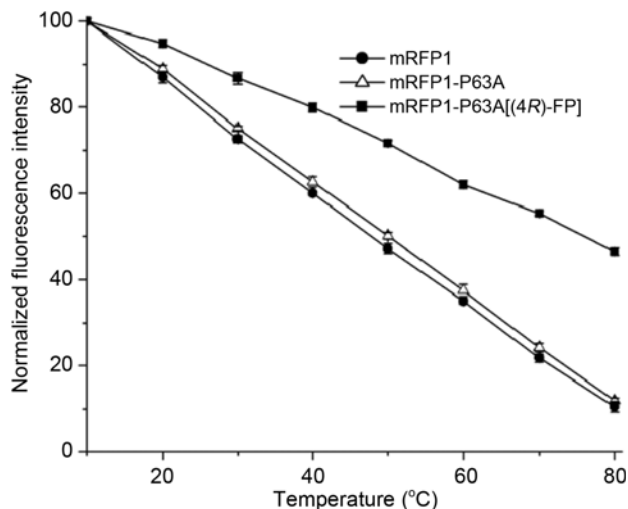


Fig. 4. Normalized fluorescence intensities of *E. coli* expressing mRFP1, mRFP1-P63A, or mRFP1-P63A[(4R)-FP] at different temperatures.

NCAA. The fusion protein with different NCAA containing variants of different sensitivity can create a new strategy in developing a ratiometric temperature sensor [10–12].

Further, the robustness of mRFP1-P63A and mRFP1-P63A[(4R)-FP] were examined through a temperature gradient experiment. In each cycle, mRFP1-P63A and mRFP1-P63A[(4R)-FP] lost 5.6 and 1.5% of initial fluorescence intensity, respectively. After three cycles, mRFP1-P63A and mRFP1-P63A[(4R)-FP] recovered 83.1 and 95.5% of initial fluorescence intensity, respectively (Figs. 3A and 3B). Recovered fluorescence intensity of mRFP1-P63A[(4R)-FP] (95.5%) was higher than mRFP1 (82.1%) and mRFP1-P63A (83.1%). mRFP1-P63A[(4R)-FP] showed less hysteresis during the heating and cooling cycle. mRFP1-P63A[(4R)-FP] has excellent stability and reversibility, so mRFP1-P63A[(4R)-FP] is suitable than mRFP1 for general usage such as monitoring of temperature changes in thermophiles.

3.3. Effect of temperature on fluorescence intensity of living cell

Fluorescence emission spectra of *E. coli* expressing mRFP1, mRFP1-P63A, or mRFP1-P63A[(4R)-FP] was measured at different temperatures (Fig. 4). Temperature sensitivities of *E. coli* expressing mRFP1, mRFP1-P63A, and mRFP1-P63A[(4R)-FP] were -1.27 , -1.26 , and $-0.77\%/^{\circ}\text{C}$, respectively (Fig. 4). Temperature sensitivities of *E. coli* expressing mRFP1, mRFP1-P63A, or mRFP1-P63A[(4R)-FP] were similar to corresponding purified proteins. This result indicated that other cellular factors may not affect temperature sensitivity of mRFP1, mRFP1-P63A, or mRFP1-P63A[(4R)-FP]. This result suggested that mRFP1 variants can be used as a probe to detect intracellular temperature.

4. Conclusion

In this study, we demonstrated the feasibility of mRFP1 and its variants in application to the measurement of intracellular temperature. Temperature sensitivity of *E. coli* expressing mRFP1, mRFP1-P63A, and mRFP1-P63A[(4R)-FP] was -1.27 , -1.26 , and $-0.77\%/^{\circ}\text{C}$, respectively. This approach is applicable in measuring the cellular or micro organelle temperature in real time by combining a targeting peptide with mRFP1 or its variants.

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