Going Above and Beyond: A Tenfold Gain in the Performance of Luminescence Thermometers Joining Multiparametric Sensing and Multiple Regression

Fernando E. Maturi, Carlos D. S. Brites, Erving C. Ximendes, Carolyn Mills, Bradley Olsen, Daniel Jaque, Sidney J. L. Ribeiro, and Luis D. Carlos*

Luminescence thermometry has substantially progressed in the last decade, rapidly approaching the performance of concurrent technologies. Performance is usually assessed through the relative thermal sensitivity, $S_r$, and temperature uncertainty, $\delta T$. Until now, the state-of-the-art values at ambient conditions do not exceed maximum $S_r$ of 12.5% K$^{-1}$ and minimum $\delta T$ of 0.1 K. Although these numbers are satisfactory for most applications, they are insufficient for fields that require lower thermal uncertainties, such as biomedicine. This has motivated the development of materials with an improved thermal response, many of them responding to the temperature through distinct photophysical properties. This paper demonstrates how the performance of multiparametric luminescent thermometers can be further improved by simply applying new analysis routes. The synergy between multiparametric readouts and multiple linear regression makes possible a tenfold improvement in $S_r$ and $\delta T$, reaching a world record of 50% K$^{-1}$ and 0.05 K, respectively. This is achieved without requiring the development of new materials or upgrading the detection system as illustrated by using the green fluorescent protein and Ag$_2$S nanoparticles. These results open a new era in biomedicine thanks to the development of new diagnosis tools based on the detection of super-small temperature fluctuations in living specimens.

1. Introduction

Temperature is a physical quantity that measures the thermal energy of a body, and temperature fluctuations play a central role in a myriad of natural and man-made processes. Since the time-response of a thermometer is limited by its size, the real-time measurement of temperature at the microscopic scale is hindered by the dimensions of the thermal probe. The size effect motivates the development of novel solutions for temperature determination at the sub-micrometer scale, in particular, luminescent nanothermometry is based on the emission properties of luminescent nanomaterials and thus allows a remote temperature detection, improving spatial and temporal resolutions in comparison with the macroscopic counterparts.

There are plenty of luminescent materials used as nanothermometers, e.g., polymers, organic dyes, proteins, quantum dots (QDs), and lanthanide-doped particles. Different classes of nanothermometers exploit distinct photophysical properties of the emitting center, such as the integrated emission intensity of a single transition, the intensity ratio of two emission bands, the energy shift or the bandwidth of an emission line, the polarization state of the emission, or the emission lifetime of an excited state. Irrespective of the property analyzed, all these examples are based on a thermometric parameter (usually denoted by $\Delta$) that expresses the relationship between the luminescent property to be analyzed and the temperature.

The different thermometric parameters reported motivated the community to adopt a figure of merit to compare the performance of distinct luminescent nanomaterials regardless of...
the thermometric parameter used.\cite{10,31} This figure of merit is the so-called relative thermal sensitivity, \(S_r = \frac{1}{\Delta T} \frac{\partial \Delta}{\partial T}\), where \(\Delta T\) is the change of \(\Delta\) with respect to the temperature (also known as absolute sensitivity, \(S_a\), with \(S_a\) values commonly presented in units of percentage change per degree of temperature change (\%/K)).\cite{11} Currently, the most sensitive luminescent nanothermometer operating at ambient conditions reaches a maximum value of \(S_r\) represented by \(S_r = \frac{1.25}{0.1\%}\).\cite{33}

In the past years, luminescence nanothermometry has been used in both applied and fundamental science. In nanomedicine, for example, the accurate determination of the temperature can yield the development of new thermal diagnosis and therapy methods,\cite{8,11,34} whereas in micro or nanoelectronics tracking the thermal exchanges at submicrometer length scales can afford a detailed understanding of the thermal properties in spatial domains for which the macroscopic transfer laws are not valid anymore.\cite{35} In fact, real-world applications of luminescence thermometry are hindered by the accuracy of the nanothermometers, which is given by the temperature uncertainty, \(\delta T = \frac{1}{\Delta T} \frac{\partial \Delta}{\partial T}\) where \(\Delta \Delta / \Delta\) is the relative uncertainty in \(\Delta\), determined by the detection system used. The best \(\delta T\) value reported by now was achieved by using lanthanide-bearing nanomaterials, with \(\delta T\) ranging between 0.1 and 0.3 K.\cite{4,20}

Nowadays, cutting-edge reports on luminescence nanothermometry are reaching the boundary of the accuracy of the nanothermometers.\cite{11,36} Therefore, the development of new approaches to obtain low-uncertainty luminescence thermal probes is needed to push the field forward, mainly for in vitro and in vivo thermal sensing.\cite{37,38} Two strategies are envisaged to decrease \(\delta T\). The first one is the design of high sensitivity light detectors and brighter materials to achieve a higher signal-to-noise ratio, consequently decreasing \(\delta \Delta / \Delta\). The second one relies on the improvement of \(S_r\), which can be attained either through the fabrication of new materials or the design of new strategies to define the thermometric parameter. In this work, we address the latter approach.

Recently, the reliability of luminescent nanothermometers has been improved using the combination of distinct thermometric parameters.\cite{39,41} This strategy is based on the use of so-called multiparametric nanothermometers, in which temperature impacts, simultaneously, different luminescence properties or different emitting centers.\cite{42–45} The use of different thermal readouts improves the reliability of temperature measurements by providing self-calibrated nanothermometers, increasing the precision of temperature measurement.\cite{33,40,50} Multiparametric nanothermometers are gaining special attention in biomedicine: Ag\(_2\)S nanocrystals have demonstrated their potential for reliable thermal sensing in small animal models,\cite{46,31} whereas the multifaceted changes in the band-shape of green fluorescent protein (GFP) can be used for temperature sensing and imaging in cell biology and physiology.\cite{28,32–35} Despite it helps to improve the reliability, the so-called multiparameter sensing is still unable to improve significantly the relative thermal sensitivity of the nanothermometers and the reported values are far below the most sensitive luminescent thermometers reported so far.\cite{33,56,57}

Multiple linear regression (MLR) is an ingenious method to fully expand the potential of multiparameter temperature sensing, which may raise luminescence nanothermometry to a whole new level. In its easier form, MLR is a powerful tool that evaluates the impact of multiple independent variables on a single experimental outcome.\cite{8} This technique is widely used in economics to forecast the price of oil\cite{99} or cryptocurrencies,\cite{60} in social sciences to identify fake news\cite{61} and political trends,\cite{62} in medicine to predict blood glucose\cite{63} and cholesterol\cite{64} in overweight patients, and chemistry to quantify metabolites\cite{65} and proteins\cite{66} by mass spectrometry. By analogy, if a luminescent nanothermometer presents different thermometric parameters displaying the same temperature-dependent linear trend, the application of MLR to its multiple thermal reading would lead to a relevant improvement in its performance as a temperature sensor. This possibility has never been proposed neither demonstrated.

Therefore, we herein provide experimental evidence of how the synergy between MLR and multiparametric thermal sensing leads to a tenfold improvement in the performance of multiparametric nanothermometers establishing world-record values for \(S_r\) and \(\delta T\). This is demonstrated by selecting enhanced GFP (EGFP) and Ag\(_2\)S nanocrystals as paradigmatic multiparametric thermographic phosphors displaying distinct (and independent) temperature-dependent parameters. In vivo experiments involving Ag\(_2\)S nanocrystals were revisited illustrating the impact of this new methodology on the potential application of luminescent nanothermometry in biomedicine.

2. Results and Discussion

2.1. Multiparametric Nanothermometry Using EGFP

Figure 1 shows the temperature-dependent emission spectra of EGFP displaying a significant thermal quenching due to the enhancement of nonradiative decays upon heating.\cite{67,68} The denaturation of EGFP was not observed as the measurement was performed at temperatures below the temperature threshold,\cite{69} as shown by the high repeatability of the emission intensity during different heating and cooling cycles (Figure S1, Supporting Information). The detailed analysis of the band-shape shows that measurable changes are seen after the spectral deconvolution (Section S1.3, Supporting Information), revealing that temperature impacts several parameters such as the intensity ratio of the integrated areas of peaks 1 and 2 (\(E_1 / E_2\)), peak energy of both peaks (\(E_1\) and \(E_2\)), and their respective full width at half maximum (\(W_1\) and \(W_2\)). Each of these parameters can be used as independent \(\Delta\) values for multiparametric thermal reading (Figure 1d–h), and as their temperature dependencies are described by a single linear fit with a positive correlation, there are five reliable independent pathways for determining the temperature from EGFP in a single experiment. At ambient conditions, \(I_{n}, E_1, E_2, W_1,\) and \(W_2\) present \(S_{tot}\) values of 0.17, 1.6 × 10⁻², 2.1 × 10⁻², 0.33, and 0.10% K⁻¹, respectively. These values are within the same thermal sensitivity range that the previously reported nanothermometry data of other EGFP-like proteins (Table 1).

We can argue that larger \(S_r\) values can be obtained by choosing the energy shift of each peak as thermometric parameters instead of the corresponding peak energies (since \(S_r\) depends on \(\Delta \)). However, although the energy shift has already been used in both Raman\cite{70,71} and luminescence\cite{72,73} thermometry, we adopt here the peak energy as a thermometric parameter because it is still
Figure 1. a) 3D view of the EGFP structure (based on Protein Data Bank ID 2y0g). b) Neutral and anionic forms of EGFP chromophore. The amino acid residues are shown close to the anionic form and the optically active part of the chromophore is depicted in a green blur for both forms. c) Emission spectra of EGFP under excitation at 408 nm at different temperatures. Temperature dependence of the distinct thermometric parameters: d) $E_1$, e) $E_2$, f) $w_1$, g) $w_2$, and h) $I_R$. The lines are the best linear fits of the data to straight lines ($r^2 > 0.99$ in all cases). The fit parameters are shown in Table S1 (Supporting Information).

Table 1. Temperature calibration range, maximum relative thermal sensitivity ($S_m$) and the temperature at which it occurs ($T_m$) for different GFP-based proteins using multi- and single-parametric analysis.

| Protein | Temperature range [K] | $S_m$ [% K$^{-1}$] | $T_m$ [K] | Method | Reference |
|---------|-----------------------|-------------------|-----------|--------|-----------|
| EGFP    | 283–323               | 3.0               | 283.0     | MLR    | This work |
|         | 0.17                  | 283.0             | $I_R$     |        |           |
|         | $1.6 \times 10^{-2}$  | 283.0             | $E$       |        |           |
|         | $2.1 \times 10^{-2}$  | 283.0             | $W$       |        |           |
|         | 0.33                  | 283.0             |           |        |           |
|         | 0.10                  | 283.0             |           |        |           |
| emGFP-Mito | 296–312            | 2.2               | 296.0     | Peak fraction$^e$ | [15]  |
|         | 4.4                  | 312.0             |           | Single intensity$^f$ |        |
| GFP     | 293–333               | 2.1               | 333.0     | Single intensity$^f$ | [78]  |
| actin-GFP$^g$ | 288–343       | 0.2               | 302.0     | Lifetime$^g$ | [53]  |
| tsGFP1$^b,c$ | 307–314        | 3.0               | 314.0     | $I_R$  | [55]  |
| tsGFP2$^b,c$ | 311–319        | 3.1               | 319.0     |        |           |
| gTEMP$^d$ | 278–323            | 2.6               | 278.0     | $I_R$  | [79]  |

$^a$GFP coupled to actin filaments; $^b$Fluorophore-forming region of GFP inserted between tandem repeats of the coiled-coil region of TlpA and the full-length TlpA, respectively; $^c$Genetically encoded ratiometric fluorescent temperature indicator from Sirius and mT-Sapphire GFP-derived proteins linked by a Thosso aspiga virus 2A peptide; $^d$[I$\text{I}_1$–I$\text{I}_2$]/[I$\text{I}_1$+I$\text{I}_2$], where $I_1$ and $I_2$ are the integrated emission areas between 495 and 504 nm and 505 and 600 nm, respectively (nonlinear dependence); $^e$Fluorescence intensity of the peak maximum at around 510 nm under excitation at 488 nm (nonlinear dependence); $^f$Fluorescence intensity of the peak maximum at around 510 nm under excitation at 473 nm; $^g$Emission lifetime under excitation at 467 nm monitoring emission at 510 nm.
the largest reported thermometric parameter. Nevertheless, the pros and cons of using peak energy and energy shift as thermometric parameters are discussed in detail in Section S1.3.3.1 (Supporting Information).

As all the five thermometric parameters defined for EGFP display the same linear temperature dependence, it is possible to further improve $S_r$ and $\delta T$ by treating the data through the MLR approach. If a nanothermometer displays distinct thermometric parameters that vary linearly with the temperature, i.e., $\Delta_1, \Delta_2, \ldots, \Delta_n$, then the temperature can be expressed as a function of each $\Delta_i$, i.e., $T = f(\Delta_1, \Delta_2, \ldots, \Delta_n)$

$$T = \beta_0 + \beta_1 \Delta_1 + \cdots + \beta_n \Delta_n + \epsilon = \beta_0 + \sum_{i=1}^{n} \beta_i \Delta_i + \epsilon$$  (1)

where $\beta_0$ is the intercept, $\beta_i$ ($i = 1, \ldots, n$) is the slope of each thermometric parameter $\Delta_i$ (explanatory variable $i$), and $\epsilon$ is the residual.\(^{[74]}\) Henceforth, one can rewrite $S_i$ (details in Section S2.2, Supporting Information)

$$S_i = \sqrt{\sum_{i=1}^{n} \left( \frac{1}{\Delta_i} \left| \frac{\partial \Delta_i}{\partial T} \right| \right)^2} = \sqrt{\sum_{i=1}^{n} \left( \Delta_i \left| \frac{\partial T}{\partial \Delta_i} \right| \right)^{-2}}$$  (2)

Because the model is linear, the relative thermal sensitivity depends on each thermometric parameter taken into account ($\Delta_i$) and its respective slope ($\beta_i$), and thus

$$S_m = \sqrt{\sum_{i=1}^{n} (\Delta_i \beta_i)^{-2}}$$  (3)

The MLR was applied to the EGFP data considering the five distinct $\Delta_i$ parameters previously defined and the correlation between the temperature measured with a K-type thermocouple and the temperature obtained from MLR is presented in Figure 2 (see Section S2.4, Supporting Information, for further information). By combining all the parameters, $S_m$ reaches 3.0% K$^{-1}$, which represents a tenfold increase compared to the highest $S_m$ obtained in single parametric sensing of EGFP (0.33% K$^{-1}$ for $W_i$). An improved sensitivity obtained through MLR is observed as the model considers the weighted contribution of each temperature-dependent light emission of EGFP (i.e., each $\Delta_i$), therefore reducing the uncertainties provided by measurements of the temperature based on individual $\Delta_i$. This is well demonstrated by the measurement of consecutive heating and cooling cycles (Figure 3), where the thermal transient curves obtained by luminescence thermometry in Figure 3a–e tend to
deviate from the curve measured by a K-type reference thermocouple (this is much more evident for $W_2$ and $I_R$). The histograms of the temperature deviation ($\Delta T$) are presented in Figure S11 (Supporting Information), and the results show that MLR provides a lower temperature deviation in Figure 3f because the uncertainties from each $\Delta_i$ were already reduced in the model, validating the improved performance of MLR in multiparametric nanothermometry.

Beyond the giant improvement in the relative thermal sensitivity, this novel approach allows achieving more reliable and accurate thermal readouts without requiring the development of new materials, the upgrade of the detection system, or further time-consuming measurements over long integration times. This last makes, for instance, possible to perform time-resolved measurements in vitro and in vivo.

### 2.2. Revisiting In Vivo Measurements Using Ag$_2$S Nanoparticles

Despite the utility of fluorescent proteins, the novelty of MLR is not limited to luminescent organic compounds, but it also can be applied to inorganic nanoparticles with multi-parametric thermal sensing capabilities. We here, indeed, explore the

Table 2. Temperature calibration range, maximum relative thermal sensitivity ($S_m$), and the temperature at which it occurs ($T_m$) for different Ag$_2$S nanoparticles, using distinct methods.

| Nanoparticle       | Temperature range [K] | $S_m$ [% K$^{-1}$] | $T_m$ [K] | Method                  | Reference |
|--------------------|-----------------------|--------------------|-----------|-------------------------|-----------|
| Ag$_2$S-PEG$^{a}$  | 295–353               | 50                 | 295.0     | MLR                     | This work$^{[44]}$ |
|                    |                       | $9.5 \times 10^{-2}$ | 344.0     | E                       |           |
| Ag$_2$S-PEG        | 293–353               | 5.0                | 295.0     | Single intensity$^{b}$  | $^{[44]}$ |
|                    |                       | 0.10               | 295.0     | Peak wavelength$^{c}$   |           |
|                    |                       | 2.0                | 295.0     | $I_R$                   |           |
| Ag/Ag$_2$S         | 288–323               | 8.5                | 323.0     | Single intensity$^{d}$  | $^{[80]}$ |
|                    |                       | 2.0                | 323.0     | $I_R$                   |           |
| Ag$_2$S-PEG        | 295–323               | 3.0                | 295.0     | Lifetime$^{e}$          | $^{[81]}$ |
| Ag$_2$S-DDT$^{f}$  | 4.0                   | 295.0              |           |                         |           |
| Ag$_2$S-PEG        | 299–313               | 3.0                | 299.0     | Single intensity$^{f}$  | $^{[77]}$ |
| Ag$_2$S-PEG        | 293–318               | 3.0                | 293.0     | Single intensity$^{g}$  | $^{[82]}$ |

$^{a}$ Nanoparticles functionalized with polyethylene glycol (PEG); $^{b}$ Total integrated emission area (nonlinear dependence); $^{c}$ Wavelength at the maximum intensity of the peak; $^{d}$ Emission lifetime under excitation at 450 nm monitoring emission at the maximum intensity of the peak; $^{e}$ Nanoparticles functionalized with 1-dodecane-thiol (DDT).
application of MLR to Ag₂S nanoparticles. Ag₂S nanoparticles possess a unique combination of properties that makes them exceptional for in vivo thermal sensing. They operate in the second biological window (infrared spectral range from 1000 to 1400 nm where tissues become partially transparent[75]) so that they allow for real sub-tissue thermal sensing. Ag₂S nanoparticles show excellent in vivo biocompatibility thanks to their good physical-chemical stability[44,76,77]. The emission band of Ag₂S nanoparticles centered at 1200 nm shows a strong temperature dependence and it has been widely reported how a reduced temperature...
change around room temperature induces relevant changes in the band shape. These temperature-induced changes have been largely used for thermal reading by analyzing either the peak wavelength or the ratio between the emitted intensities at 1175 and 1260 nm. The use of these two thermometric parameters has made possible reliable thermal reading with modest thermal sensitivities at 310 K ranging from $9.5 \times 10^{-2}$ to $5.0$% K$^{-1}$ (Table 2). As a consequence of these "modest" thermal sensitivities, the thermal uncertainty achieved by Ag$_2$S nanothermometers during in vivo experiments is not better than $0.5\%$. The temperature dependence of both intensity ratio and peak energy of emission band corresponding to Ag$_2$S nanoparticles is presented in Figure 4, leading to relative thermal sensitivities of 2.0 and 0.10% K$^{-1}$, respectively. Hereafter, we will focus our attention on these two thermoresponse parameters as they both display a quasi-linear trend, making them amenable to MLR analysis.

Applying the MLR to the Ag$_2$S data (giving the relative contributions indicated in Figure 4c), we obtained a calibration curve that depends linearly on the peak energy and intensity ratio parameters with a relative thermal sensitivity up to 50% K$^{-1}$ (Figure 4d). This constitutes a tenfold improvement when compared to that previously reported for Ag$_2$S nanoparticles and 4 times greater than the greatest $S_{\text{m}}$ value reported so far (12.5% K$^{-1}$ at 293 K, from a lanthanide metal-organic network, using the intensity ratio approach).

The performance, in terms of $S_r$ and $\delta T$, achieved by combining linear regression to the multiparametric reading of Ag$_2$S nanothermometers is shown in Figure 4e,f. It is evidenced how the use of linear regression procedures makes it possible to drive the thermal uncertainties provided by Ag$_2$S nanothermometers well below 1.0 K. It is worth pointing out that since the MLR deals with linear models, only the thermometric parameters presenting linear response upon heating and cooling can be considered for multiparametric temperature sensing through MLR, which is the main disadvantage of this newly proposed method. For that reason, the temperature-dependent emission intensity of the Ag$_2$S nanoparticles was intentionally left out of the calculations when performing the MLR analysis due to its nonlinear trend, as seen in ref. [44].

The MLR-induced thermal sensitivity enhancement of Ag$_2$S nanothermometers opens the possibility of improving the thermal resolution of in vivo measurements. In recent work, intratumoral thermal transients were measured by analyzing the time evolution of the infrared emission generated by Ag$_2$S nanoparticles allocated inside a melanoma tumor during laser irradiation. The different intratumoral thermal readings provided by the different thermometric parameters (emission intensity, peak wavelength, and intensity ratio) were used to assess the reliability of measurements but not to improve the accuracy of the final intratumoral readout. The intratumoral emission spectra generated by Ag$_2$S nanoparticles were re-analyzed by MLR and the new intratumoral thermal transients during photothermal treatment are shown in Figure 5 (the thermal transients obtained by using the individual calibration curves are also included for comparison). We identify slight differences in the temperature profiles during the transient regime that are explained by the distinct thermal sensitivity provided by each parameter and are within the temperature uncertainty of each thermometric parameter. Moreover, the intratumoral temperature values calculated from MLR and the new intratumoral thermal transients during photothermal treatment are almost identical to those provided by the sole analysis of peak energy which, at first glance does not justify the effort of using MLR.

The improved performance of MLR is, however, reflected in both $S_r$ and $\delta T$ values calculated from each thermometric parameter (Figure 5b and Table 2). The intensity ratio allows a temperature determination with uncertainty values ranging from 1.1 to 2.1 K (the lower the sensitivity the higher the temperature uncertainty), and the peak energy improves this value roughly by one order of magnitude ($\delta T \approx 0.15$ K). Finally, the MLR approach renders temperature uncertainties between 0.05 and 0.10 K, which represents an improvement of more than 20 times with respect to the obtained with the intensity ratio approach. Note that recent perspective articles pointed out that the real use of luminescence thermometry at the clinical level would require achieving thermal resolutions during in vivo experiments better than 0.1$\%$, which is reached in this work. As a matter of fact, we demonstrate how MLR converts Ag$_2$S
nanoparticles into ultra-sensitive nanothermometers making them a unique system for in vivo detection, for instance, of brain diseases through precise and remote thermal sensing. Once again, it should be highlighted that such improvement for in vivo thermal uncertainty has been achieved without requiring any change in the experimental setup or sample preparation.

3. Conclusions

Several strategies have been used to improve the reliability and accuracy of luminescent nanothermometers, namely, the combination of different emitting centers or the simultaneous assessment of the temperature via several thermometric parameters. This work demonstrates that any luminescent thermometer presenting a linear correlation of the distinct thermometric parameters with the temperature can be used to achieve higher sensitivity and lower temperature uncertainty through MLR. We demonstrate the potential of the synergy between MLR and luminescent nanothermometry in two of the most promising systems for thermal sensing in biomedicine: GFPs and Ag2S nanoparticles. The combination of MLR and the multiparametric thermal readout of EGFP leads to a significant increase of its thermal sensitivity, with a tenfold improvement. MLR has been also found to improve the relative thermal sensitivity of intratumoral Ag2S multiparametric nanothermometers by one order of magnitude, reaching a world record of 50% K−1.

Moreover, the re-analysis of in vivo results by using MLR have also demonstrated how is possible to drive the thermal accuracy of its thermal sensitivity, with a tenfold improvement. MLR has been also found to improve the relative thermal sensitivity of intratumoral Ag2S multiparametric nanothermometers by one order of magnitude, reaching a world record of 50% K−1. Moreover, the re-analysis of in vivo results by using MLR have also demonstrated how is possible to drive the thermal accuracy of in vivo measurements well below 0.1 K, starting a new era of luminescent nanothermometry at the preclinical level.

These results support that MLR is a valid and easily implementable strategy, paving the way for reaching a temperature resolution below 0.1 K without further upgrade of the detection systems or materials design, an important step forward for the spread of luminescent nanothermometers as a tool for broader diverse scientific proposes.

4. Experimental Section

Photoluminescence Spectroscopy: The emission spectra of EGFP were recorded in the right-angle experimental setup shown in Figure S2 (Supporting Information). The excitation of the sample was carried out at 408 ± 7 nm with a power density of 0.01 W cm−2 by using a CW multichannel LED light source (MCLS, Sandhouse Design). The excitation source was collimated twice by attaching an adjustable collimator to the tip of the optical fiber and placing a plano-convex lens (LA1145-AB, Thorlabs) between the optical fiber and the sample. The light emission was registered by a USB-portable spectrometer (Maya 2000 Pro, Ocean Optics) coupled to an optical fiber (Q450-1-XSR, Ocean Optics) using an edge pass filter (FES010750, Thorlabs) to cut off the excitation signal during the spectral acquisition. A high-precision quartz cuvette (QS, 114F-10-40, Hellma Analytics) was filled with 0.50 mL of the EGFP aqueous suspension to perform the measurements.

Thermal Calibration: The temperature-dependent measurements were performed using the setup described above and a temperature-controlled cuvette holder (Luma 40, Quantum Northwest) coupled to a temperature controller (TC, Quantum Northwest) using a water circulator (Bath 10, Quantum Northwest) for heating and cooling the sample. The calibration temperature of the sample was performed with a K-type thermocouple with a temperature uncertainty of 0.1 K (KAA1-3, TME Thermometers) coupled to a thermocouple data logger (TC-08, Pico Technology).

Cloning of EGFP: The gene of EGFP containing BamHI and HindIII restriction sites at 5′- and 3′-ends of the sequence, respectively, was prepared by polymerase chain reaction (PCR). The pQE9 vector containing the BamHI/HindIII restriction sites and the gene encoding for EGFP was purified through a spin column purification and ligated at 3:1 insert to vector molar ratio.

Protein Expression and Purification: The EGFP plasmid was transformed into Escherichia coli strain Tuner (DE3), and the expression of the protein was carried out in Luria broth (LB), where the cells were grown to OD600 = 0.8–1.0 at 310 K before induction. The protein expression was induced by the addition of isopropyl-β-1-thiogalactopyranoside (IPTG, 0.5 mmol L−1) and a temperature drop to 303 K. The cells were harvested by centrifugation 18–24 h after induction and resuspended in lysis buffer (Na2HPO4 = 50 mmol L−1, NaCl = 300 mmol L−1, imidazole = 10 mmol L−1 in water, pH = 8.0). The obtained lysates were stored at 193 K. After thawing, lysozyme (1 mg mL−1) was added to resuspended cells, and the cells were incubated at 277 K for 1 h. The resuspended cells were sonicated with a tip probe and clarified by centrifugation (12,400 rpm) at 277 K. The obtained EGFP aqueous suspension was purified by Ni-NTA affinity chromatography (Ni-NTA Agarose, Qiagen) under native conditions and fast protein liquid chromatography (FPLC) at pH = 8.0. FPLC purification was performed using an anion exchange chromatography column (HiTrap Q HP, GE Life Sciences) under 30 column volumes elution with an increasing linear gradient of NaCl concentration from 0 to 2 mol L−1. The suspension of the protein was dialyzed against ultrapure water and the purity of each protein was confirmed by denaturing gel electrophoresis (SDS-PAGE). The resulting EGFP aqueous suspension was diluted in tris buffer at pH = 8.0 and stored at 277 K. The confirmed amino acid sequence of the obtained EGFP is presented in Figure S6 (Supporting Information).

Colloidal Characterization: The hydrodynamic size (diameter, d) of EGFP was measured by dynamic light scattering (DLS) in a Malvern Zetasizer Nano series instrument (Nano-ZS Model ZEN3600, Red badge operating with a 632.8 nm laser) at 298 K. The measurement of the zeta potential (ζ) was carried out in the same equipment by using the Smoluchowski model to analyze the colloidal stability of EGFP in aqueous suspension (0.25 mg mL−1 in 20 mmol L−1 Tris-Cl, pH = 8.0 ± 0.1). The EGFP sample was measured in a folded capillary tube (DTS1070, Malvern Instruments) for both DLS and zeta potential measurements. Three measurements were performed with ten scans each, where the average values are reported in Figure S7 (Supporting Information).

UV–Vis Absorption Spectroscopy: The UV–vis absorption spectrum of the EGFP aqueous solution was recorded in a spectrophotometer (Cary 50, Varian) at 293 K with a spectral resolution of 0.5 nm using a 10 mm pathlength quartz cuvette (Figure S8, Supporting Information).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.
[54] G. G. Lambert, H. Depernet, G. Gotthard, D. T. Schultz, I. Navizet, T. Lambert, S. R. Adams, A. Torreblanca-Zanca, M. Chu, D. S. Bindels, V. Levesque, J. Nero Moffatt, A. Salih, A. Royant, N. C. Shaner, Proc. Natl Acad. Sci. 2020, 118, e000936.

[55] S. Kyonaka, T. Kajimoto, R. Sakaguchi, D. Shinmi, M. Omatsu-Kanbe, H. Matsuura, H. Imamura, T. Yoshizaki, I. Hamachi, T. Morii, Y. Mori, Nat. Methods 2013, 10, 1232.

[56] J. Abraham, D. Higdon, J. Nelson, Proc. Natl Acad. Sci. 2013, 110, 4980.

[57] S. Liang, Y. Wang, X. Wu, M. Chen, L. Mu, G. She, W. Shi, Chem. Commun. 2019, 55, 3509.

[58] C. M. Mi, J. Zhou, F. Wang, G. Lin, D. Jin, Chem. Mater. 2019, 31, 9480.

[59] L. L. Nathans, F. L. Oswald, K. Nimron, Pract. Assess., Res. Eval. 2012, 17, 9.

[60] J. A. Abraham, D. Higdon, J. Nelson, SMU Data Sci. Rev. 2018, 1, 22.

[61] C. Buntain, J. Golbeck in IEEE Int. Conf. Smart Cloud, IEEE, 2017, New York, NY, USA, pp. 208–215.

[62] J. D. Creek, A. Jankevics, R. Breitling, D. G. Watson, M. P. Barrett, K. E. V. Burgess, Anal. Chim. Acta. 2011, 83, 8703.

[63] M. Fernández-Fernández, P. Rodríguez-González, M. E. Añón Álvarez, F. Rodríguez, F. VÁ. Menéndez, J. I. C. Alonso, Anal. Chem. 2015, 87, 3755.

[64] A. M. dos Santos, J. Fluoresc. 2012, 22, 151.

[65] E. Jacchetti, E. Gabellieri, P. Cioni, R. Bizzarri, R. Nifosi, Phys. Chem. Chem. Phys. 2016, 18, 12828.

[66] H. F. Arata, F. Gillot, T. Nojima, T. Fujii, H. Fujita, Lab Chip 2008, 8, 1436.