Electronic Supplementary Information

Revealing a new fluorescence peak of the Enhanced Green Fluorescent Protein using three-dimensional fluorescence spectroscopy

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S.1. SDS-PAGE electrophoresis

A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to confirm the equivalency of the EGFP standard from the Biovision® quantification kit and the EGFP produced and purified in this study, following the protocol detailed in dos Santos *et. al* 2018[1] with small modifications: 12% resolving gel (wt/v%) instead of 10%; MW BluEye Sigma® as the molecular weight standard; concentration of samples of 0.1 g.L^{-1} of EGFP and 10 µL of sample per well. The SDS-PAGE electrophoresis gel is presented in **Figure S1**.

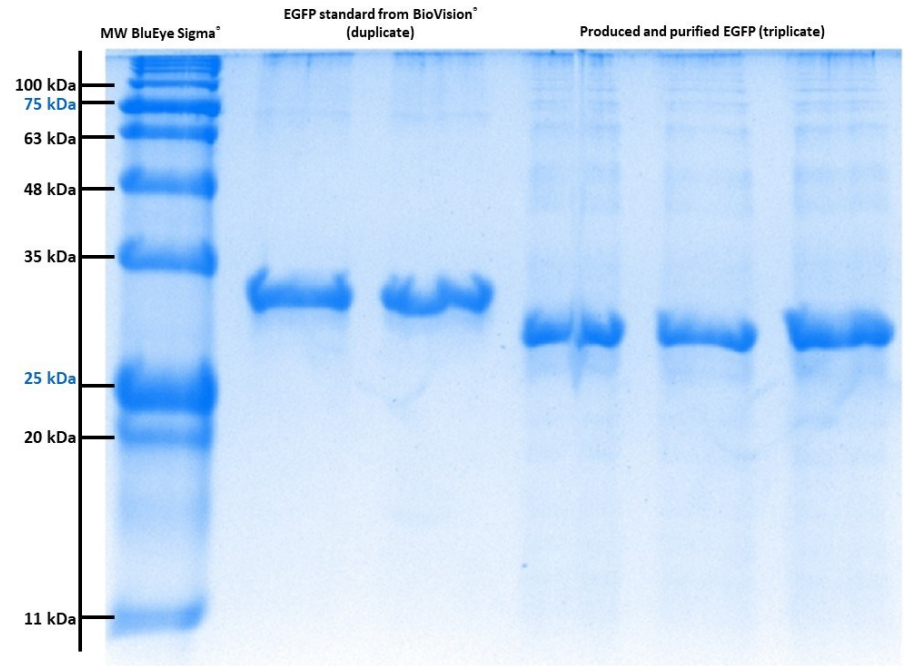

Fig. S1. SDS-PAGE electrophoresis gel of the molecular weight standard MW BluEye Sigma® (1st well), duplicate of the EGFP standard from the Biovision® quantification kit (2nd and 3rd well) and triplicate from the EGFP produced and purified in this study (4th, 5th and 6th well).

As can be seen in **Fig. S1**, both the EGFP standard and the produced EGFP presented a single strong band between 25 and 35 kDa, with an extremely light band around 75 kDa for the standard and very faint bands for the purified EGFP above 35 kDa. As indicated in the EGFP quantification kit, EGFP presents a molecular weight of 32.7 kDa, in accordance with the SDS-PAGE electrophoresis gel. The similarity of the protein profile depicted in the SDS-PAGE electrophoresis gel and the 3D fluorescence spectra presented in **Fig. 1** and **Fig. 2** (where the highest fluorescence peaks for EGFP standard and produced EGFP are the same – λ_{ex} 488 nm / λ_{em} 510 nm) allow to confirm the equivalency of both EGFP in study.

S.2. 3D fluorescence spectra analysis

The 3D fluorescence spectra (also called total fluorescence or fluorescence excitation-emission matrix) are depicted in 2D view, where the excitation wavelength (λ_{em}) is shown on the Y axis, the emission wavelength (λ_{em}) on the X-axis, and the fluorescence intensities are given by the scale from blue to red in units of fluorescence (UF). **Fig. S2** demonstrates the relationship between the 3D fluorescence spectra and their respective excitation and emission spectra. In **Fig. S2A**, the red horizontal line across the 3D spectra represents an emission spectrum at a specific excitation (in this case, λ_{ex} 488 nm) and the purple vertical line is an excitation spectrum at certain emission (in this example, λ_{em} 510 nm). The respective excitation and emission spectra for the red and purple lines of the 3D analysis in **Fig. S2A** are presented in **Fig. S2B** and **S2C**. The first and second Rayleigh light scattering (noted as S1 and S2, respectively)[2, 3] are also presented in **Fig. S2** and **Fig. 1**. S1 occurs as result of the elastic scattering of the light that strikes small particles, and because there is no loss or gain of energy in the interaction between matter and the Rayleigh light, λ_{ex} and λ_{em} are the same in S1 ($\lambda_{ex} = \lambda_{em}$) and present a linear aspect. S2 is an artifact generated by grating monochromators, where selecting light at a specific wavelength (λ_{ex}) also allows the transmission of light with half the wavelength ($\lambda_{ex}/2$), and again causes Rayleigh scattering from small particles when $2\lambda_{ex} = \lambda_{em}$.[2] For example, S1 is observed at λ_{ex} 500 nm / λ_{em} 500 nm and S2 at λ_{ex} 500 nm / λ_{em} 250 nm in the 3D spectrum presented in **Fig S2A**. Fluorophores present a more spherical or oval shape, with highest fluorescence intensity at their central region and decreasing intensity in their in their extremities, as can be seen in **Figure S2A** for EGFP (5.4 $\mu g.mL^{-1}$).

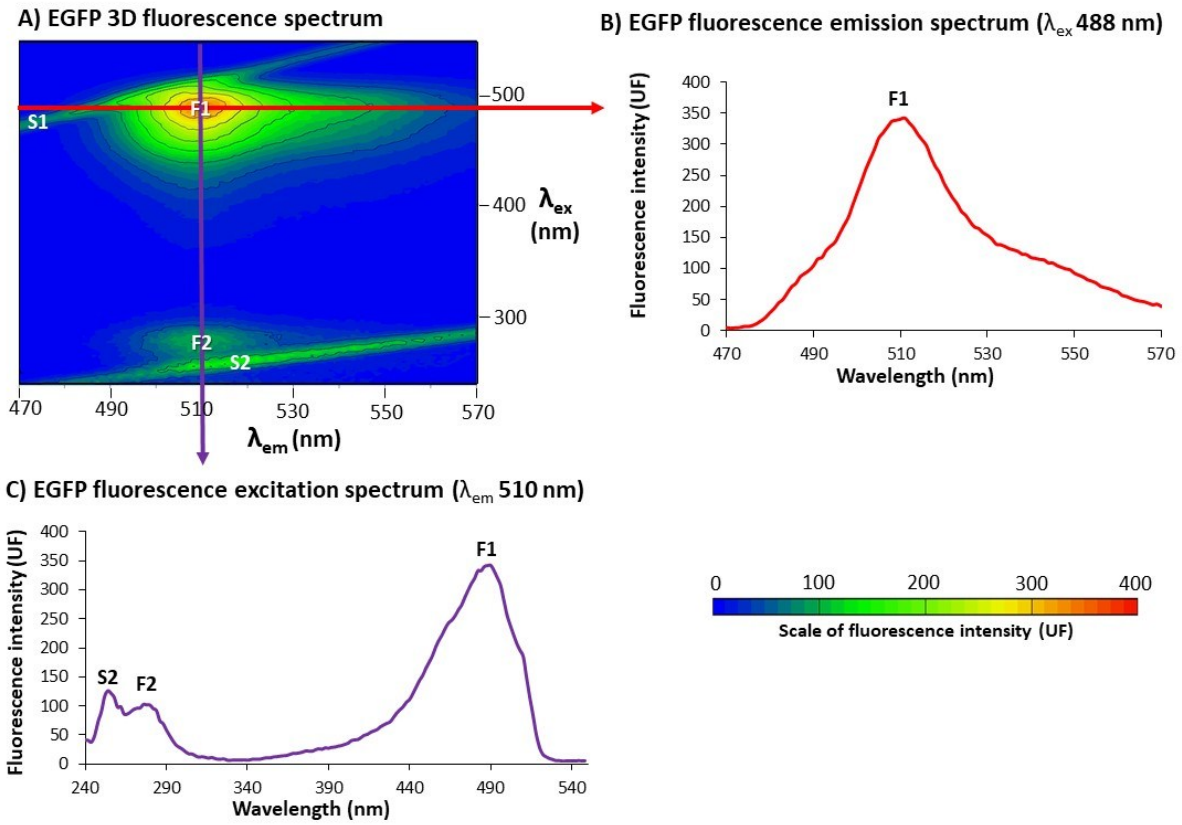

Fig. S2. (**A**) 3D fluorescence spectrum, (**B**) emission spectrum (λ_{ex} 488 nm) and (**C**) excitation spectrum (λ_{ex} 510 nm) of EGFP 5.4 µg.mL^{-1} 25°C in phosphate buffer (PB) pH 7.4. The emission spectrum in (**B**) is equivalent as the red horizontal line in the 3D fluorescence spectrum in (**A**), and the excitation spectrum in (**C**) comprises the purple line in (**A**). 3D fluorescence spectra with the excitation wavelengths in the Y axis (λ_{ex}), the emission wavelengths in the X axis (λ_{em}) and the scale of fluorescence intensity (unit of fluorescence, UF) according the color scale from blue to red. F1 (around λ_{ex} 488 nm / λ_{em} 510 nm) and F2 (around λ_{ex} 278 nm / λ_{em} 510 nm) are the two fluorescence points with highest intensity; S1 and S2 are first and second order light scattering, respectively.

S.3. Calibration curves and analytical parameters

To compare the range and precision of the calibration curves of EGFP for both peaks F1 and F2, calibration curves for each peak were produced (as presented in **Fig. 3B**, **Fig. S3** and **Fig. S4**) and different analytical parameters were evaluated and are presented at **Table 1**. The coefficient of determination (R^2) was obtained for the linear part of the calibration curves and the upper limit of the curve was defined as the concentration of EGFP at the last point in the linear part of the curve. To determine the minimal concentrations for detection and quantification for each curve, the limit of detection (LOD) and limit of quantification (LOQ) were calculated for confidence levels of 3 and 9 standard deviations (SD) respectively, as suggested by Mocak *et al.*[4] in a review of chemistry analytical procedures from American Chemistry Society (ACS) and International Union of Pure and Applied Chemistry (IUPAC), following **Eq. 1**:

Eq. (1): $y = \mu_b + \kappa\sigma_b$

where y is the fluorescence intensity at LOD or LOQ, μ is the average of the blank signal, κ is the confidence level (3 for LOD and 9 for LOQ) and σ is the standard deviation of the blank. LOD and LOQ were calculated as the concentration (x) of the y determined with **Eq. (1)**, following the calibration curves equations presented in **Fig. 3B** and **Fig. S4B**.

The relative standard deviation (RSD) was calculated for each point of the curve (RSD_n), following **Eq. (2)**:[5]

Eq. (2): $RSD_n = \frac{\sigma}{\mu} \times 100$

where σ is the standard deviation of the point of the curve and μ is the average of the points of the curve and *n*. The RSD for each curve was calculates as the average of RSD_n for all the points of the curve.

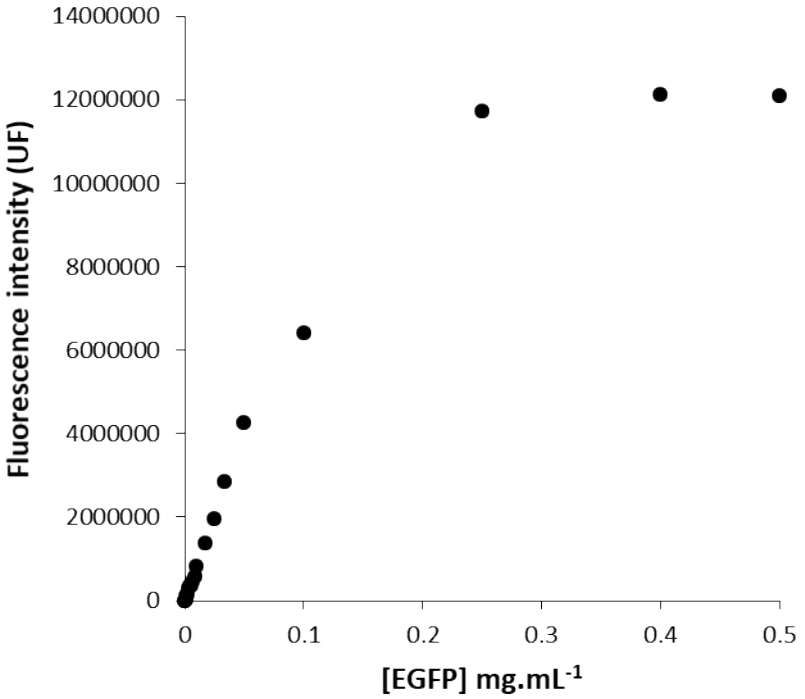

Fig. S3. Complete calibration curve for EGFP F2 peak (λ_{ex} 278 nm / λ_{em} 510 nm), concentration of EGFP [EGFP] (mg.mL^{-1}) in the X axis and fluorescence intensity (UF) in the Y axis.

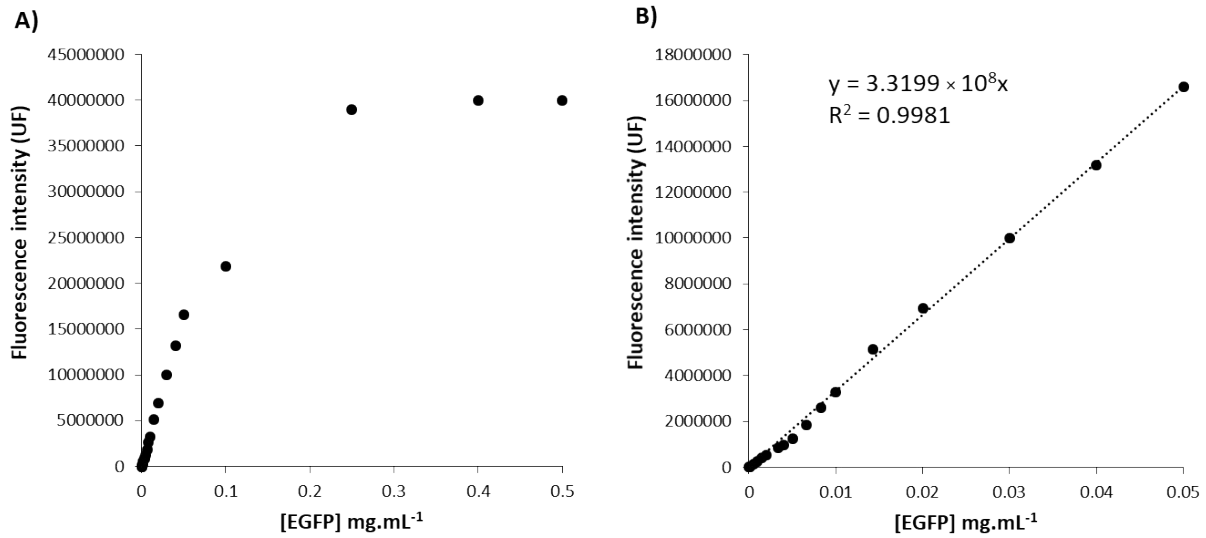

Fig. S4. Calibration curves for EGFP F1 peak (λ_{ex} 488 nm / λ_{em} 510 nm), concentration of EGFP [EGFP] (mg.mL^{-1}) in the X axis and fluorescence intensity (UF) in the Y axis at two different ranges. (**A**) [EGFP] from 0 to 0.5 mg.mL^{-1} and fluorescence intensity from 0 to 45000000 UF. (**B**) [EGFP] from 0 to 0.05 mg.mL^{-1} and fluorescence intensity from 0 to 18000000 UF).

Table S1. Comparison of range and precision of EGFP calibration curves at fluorescence peaks F1 (λ_{ex} 488 nm / λ_{em} 510 nm) and F2 (λ_{ex} 278 nm / λ_{em} 510 nm). Parameters presented are upper limit of the curve, coefficient of determination (R^2) of the curve, limit of detection (LOD), limit of quantification (LOQ) and relative standard deviation (RSD).

	F1 (λ_{ex} 488 nm / λ_{em} 510 nm)	F2 (λ_{ex} 278 nm / λ_{em} 510 nm)
Upper limit of the curve ($mg.mL^{-1}$)	0.05	0.05
R^2	0.9981	0.9978
LOD ($ng.mL^{-1}$)	25	462
LOQ ($ng.mL^{-1}$)	33	633
RSD (%)	2.1	2.8

References

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