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Supplemental information

Deciphering the Role of Positions 145 and 165 in Fluorescence Lifetime Shortening in the EGFP Variants

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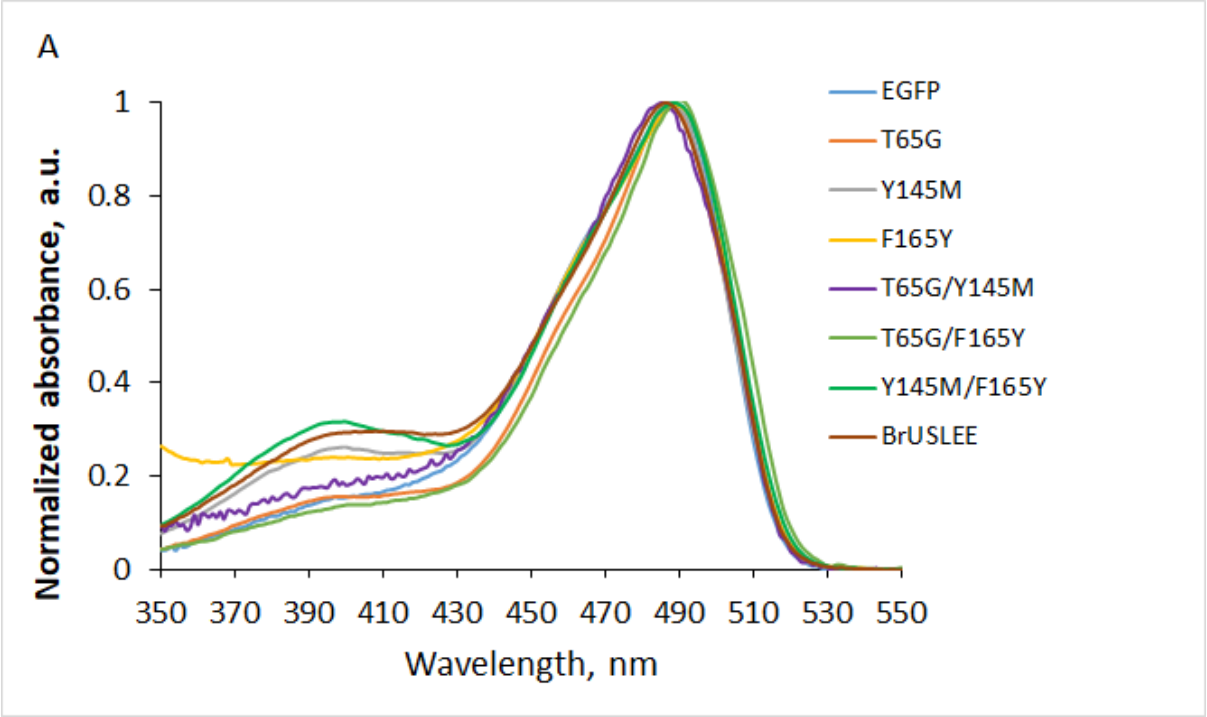

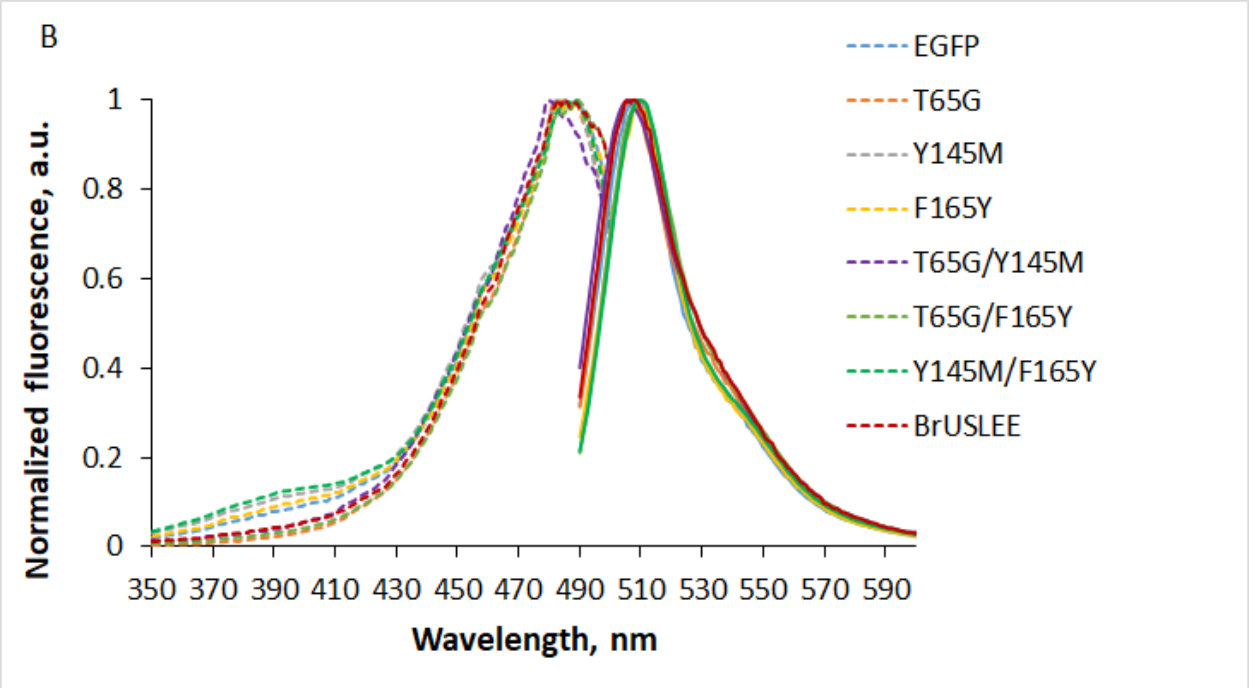

Figure 1. Absorption (**A**) and fluorescence (**B**) spectra of EGFP and mutants. In the fluorescence graph, dashed lines show fluorescence excitation, solid lines – fluorescence emission.

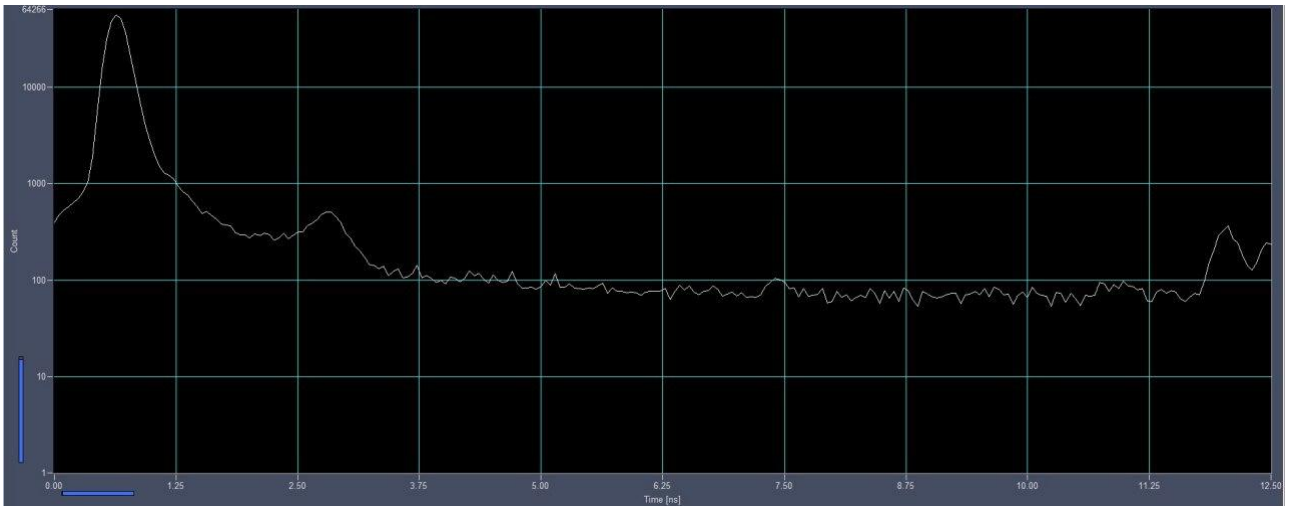

Figure 2. Instrument response function. IRF shape was recorded separately under the same excitation conditions (980 nm) and represented in a screenshot from Becker & Hickl SPCM v. 9.82 data acquisition software. .

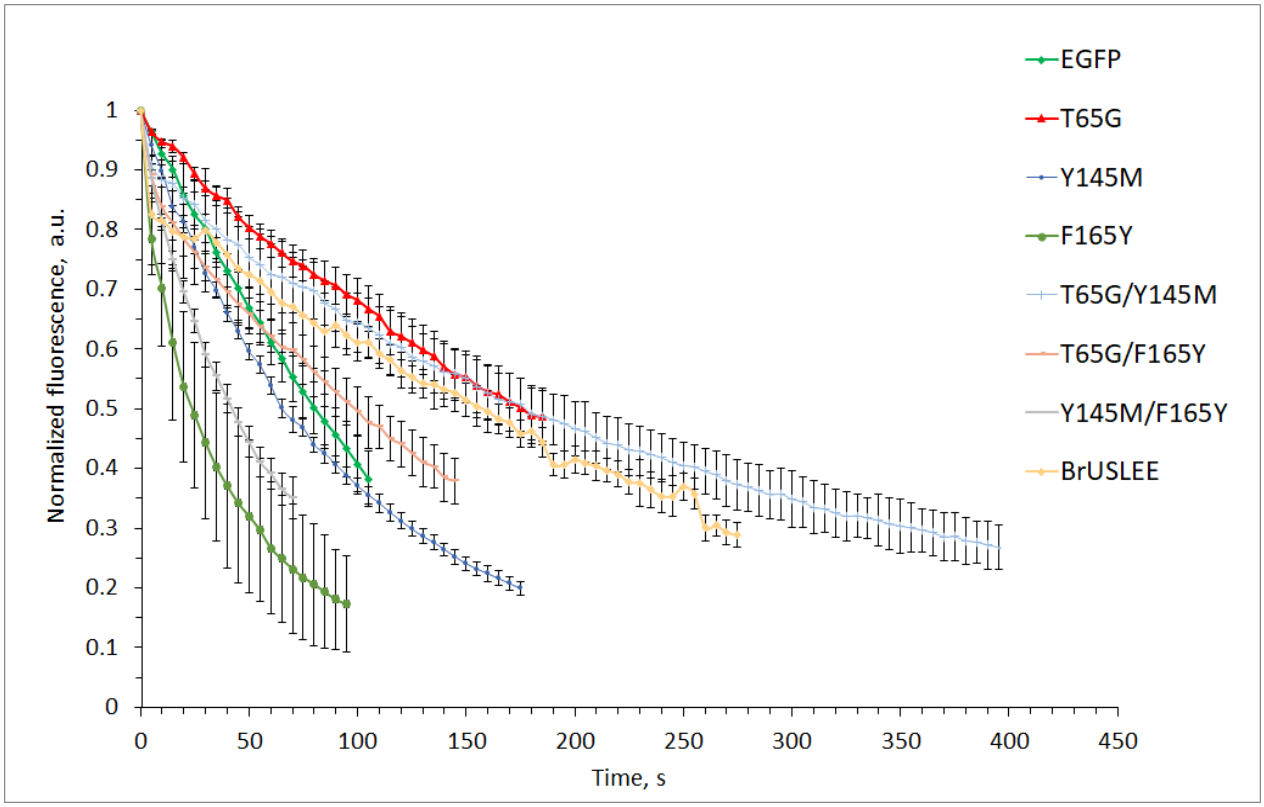

Figure 3. Photobleaching kinetics of the immobilized proteins, EGFP and its mutants, measured in vitro, in PBS. Green fluorescence intensities were background-subtracted and normalized to the maximum values. Standard deviation values (n = 15–20 measurements in a representative experiment out of five independent experiments) are shown.

Table 1. Photostability of EGFP and its mutants *in vitro*.

Fluorescent protein	Photobleaching, s*
EGFP	80±10
T65G	170±25
Y145M	51±5
F165Y	30±6
T65G/Y145M	140±15
T65G/F165Y	65±8
Y145M/F165Y	24±2
T65G/Y145M/F165Y (BrUSLEE)	142±17

*Photobleaching value is represented as the photobleaching half-time for each fluorescent protein, i.e., larger values correspond to the slower photobleaching rate and higher photostability.