Structural and Spectral Response of the *Aequoria victoria* Green Fluorescent Proteins to the Chromophore Fluorination

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SUPPLEMENTARY DATA

[A] Fluorescence profiles of native ECFP, EYFP and EGFP and their fluorinated variants at pH 7 and pH 10

[B] Absorption maxima of chromophores in denatured proteins at low and high pH values

[C] Crystallizations X-ray data collections and structure elucidation of (2-F)Tyr-EGFP

[D] Microenvironment description of (2F)Tyr-, and (3-F)Tyr-residues in the related crystal structures

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[A] Fluorescence profiles of native ECFP, EYFP and EGFP and their fluorinated variants at pH 7 and pH 10

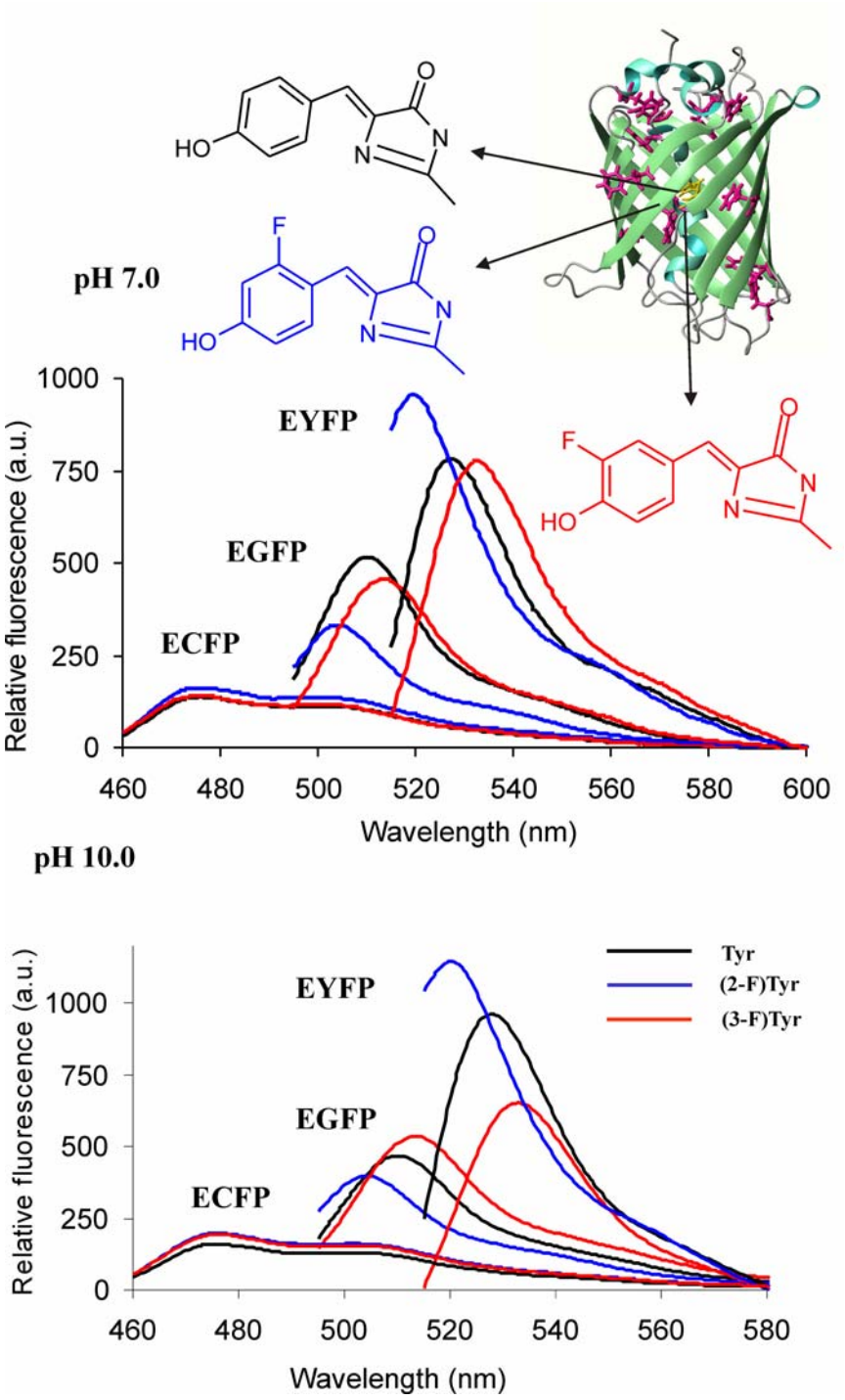

Figure S1. Fluorescence emission profiles generated upon excitation in the chromophore as described in Materials and Methods. Note that there are no shifts in the spectral maxima in ECFPs upon fluorination.

[B] Absorption maxima of chromophores in denatured proteins at low and high pH values

To check to which extent C-H → C-F exchange in the chromophore and in the rest of Tyr residues influences protonated/deprotonated (**AH**/**A**$^-$) chromophore the "enhanced cyan fluorescent protein" (ECFP, Ser65Thr/Tyr66Trp) was used as a control system for comparison. Since the ECFP contains 10 Tyr residues with the 11th one replaced with Trp at position 66 (chromophore), global fluorination should not affect its chromophore. Indeed, no difference in the absorption maxima between parent protein and either 2-, or 3-fluorotyrosyl-ECFP was observed (Table S1). These results indicate that the observed blue shifts in EGFP and EYFP variants principally originate from fluorine-containing chromophores.

Table S1. The pH-dependence of the absorption of denatured EGFP, EYFP, ECFP and their fluorinated variants in 100 mM NaCl, 50 mM buffer solutions (KCl/HCl for pH 1.0 and Na-borate/NaOH for pH 11.0) (denaturant 6 M Guanidine Chloride)[*].

Protein	λ_{max}/nm (pH 1.0)	λ_{max}/nm (pH 11.0)
EGFP	380	447
(2-F)Tyr-EGFP	380	444
(3-F)Tyr-EGFP	380	443
EYFP	378	447
(2-F)Tyr-EYFP	378	444
(3-F)Tyr-EYFP	378	443
ECFP	420	418
(2-F)Tyr-ECFP	420	418
(3-F)Tyr-ECFP	420	418

[*]There is no change in the absorption maxima upon addition of 5 mM DTT or use of other denaturant (8 M urea with or without DTT). Proteins with final concentration ~ 0.5 mg/ml were first incubated in denaturing solution for 1 hour and subsequently heated (5-10 min) at 95° C, cooled down and absorbance spectra measured at room temperature as described in Materials and methods. Each value is an average of at least two measurements.

[C] Crystallizations, X-ray data collections and structure elucidation of (2-F)Tyr-EGFP.

The (2-F)Tyr-EGFP was crystallized in 0.05 M NaOAc, 0.1 M Tris/HCl buffer pH 8.5, and 14% (w/v) Polyethyleneglycol (PEG) 4000 within 2 days. For the crystallization, hanging drops were made of 1 μl of protein solution (ca. 30 μg/μl in deionized water) and 1 μl of precipitant solution at 20°C. Drops were equilibrated against 0.2 ml of the precipitant solution. The structure of (2-F)Tyr-EGFP was solved by the molecular replacement technique using native EGFP structure (EGFP, 1EMG) as the platform for the molecular replacement. Crystal space group was primitive orthorhombic, $P2_12_12_1$, the same as previously solved structures. The data set was collected (resolution: 2.2 Å) on an X-ray image plate system (Mar Research, Hamburg, Germany) using CuKα-radiation generated by a Rigaku rotating anode at 5.4kW in cryo-protected condition at 100°K. Crystals were transferred to their mother solutions containing 20% (w/v) meso-Erythritol as a cryo-protectant and shock-frozen in a nitrogen stream.

Reflections were integrated with the program DENZO, scaled and reduced using SCALE (Otwinowski, Z.; Minor, W. *Method Enzymol*. **1997**, *276*, 307-326). Relevant statistics are listed in Table S1. Model building and refinement were performed with CNS. (Brünger, A. T. *et al*., *Acta Cryst.* **1998**, *D54*, 905-921) The initial model was refined alternating automatic minimization protocols performed with CNS inspecting visual electron density map and manually adjusted using the program O (Jones, T. A.; Zou, J. Y.; Cowan, S. W.; Kjeldgaard, M. T. *Acta Cryst*. **1991**, *A47*, 110-119).

Table S2. The structure determination of 2F-Tyr-EGFPs and its refinement

Crystal	2F-Tyr-GFP
Source	CuKα
Wavelength, Å	1.54178
Resolution Range, Å	50-2.2
Completeness, %	99.2/95.5(2.28-2.20)
R_{sym}[1], %	12.3/33.1
No. Of protein atoms/waters	1805/59
R_{factor}[2](%)/R_{free}[3](%)	19.7/25.1
rms bond lengths, Å	0.007015
rms angles, Å	1.35279
Refleection used(I/Sigma > 0)	12029

[D] Microenvironment description of (2-F)Tyr-, and (3-F)Tyr-residues in the related crystal structures

Out of the eleven residues in EGFP, Tyr237 is not crystallographically defined, while Tyr182 is fully exposed and no electron density is observed for the fluorine atom due to the high B-factor. Diversity of the novel local microenvironments introduced by fluorine atoms either in the *meta*-, or *ortho*- positions in the tyrosyl moieties are demonstrated by the examples described below. For example, partially exposed Tyr143 has both conformers but has no detectable distances that might indicate its involvement in a particular interaction (Figure S5). Buried residues Tyr74 (Figure S2), 106 (Figure S4) and 145 (Figure S5) are tightly packed in the protein interior and their fluorine has only one conformation. Fluorine electron densities of (2-F)Tyr-residues are found to be exclusively in one conformeric state. A similar situation is observed in some (3-F)Tyr-residues as well. However, most of the electron densities of (3-F)Tyr-residues appear to be distributed (usually in a ratio of 40:60) between two conformeric states (see e. g. Figure S6) as described below.

The **(2-F)Tyr39** is solvent exposed and has contact with Thr38. The F atom is forming H-bond with the main chain oxygen of Thr38. The electron density of the fluorine atom in surface exposed residue of **(3-F)Tyr39** is distributed among two conformeric states. Fluorine atom in the minor conformation is involved in contacts with the terminal oxygen atoms of neighboring Asp36 (distances 3.28 Å and 3.27 Å).

The **(2-F)Tyr74** is buried inside the molecule and has extensive interactions with nearby residues. Ala226, Asp82 and His81 forms hydrophobic interactions with (2-F)Tyr74 whereas the hydroxyl group of the phenolate is in a H-bond distance with the imidazole group of His199. This in turn forms another H-bond with main chain of Asp197. F-atom of (2-F)Tyr74 forms a H-bond with the main chain oxygen of Met78. The difference electron density map of **(3-F)Tyr74** indicates a single conformeric state of the fluorine in crystal structure (Figure S2). Since this residue is buried inside the protein core, it has hydrophobic interactions with neighboring Leu201 and Ala226. Side chain atoms of Asp82, His81, and His199 are also in contacts. Hydroxyl group is forming a H-bond with the imidizole N of His199 which in turn forms a salt-bridge with the Asp197 carboxylate.

The **(2-F)Tyr92** is completely buried inside the EGFP core (Figure S3). It has hydrophobic interactions with Met88, Ile188, Ala87, and Phe84. Nonetheless, its fluorination at *meta*-position results in electron density distribution among two conformeric states. The fluorine of **(3-F)Tyr92** in 'minor' conformation is at the interaction distance of 2.79 Å with an amide oxygen of Phe84, and is probably in contact with the ring carbon of Phe84 through cation-π interactions (3.71 Å). Other ('major') **(3-F)Tyr92** has extensive interactions with Met88 and Ile188 while its F atom is at hydrogen bond distance with the main chain of Phe84.

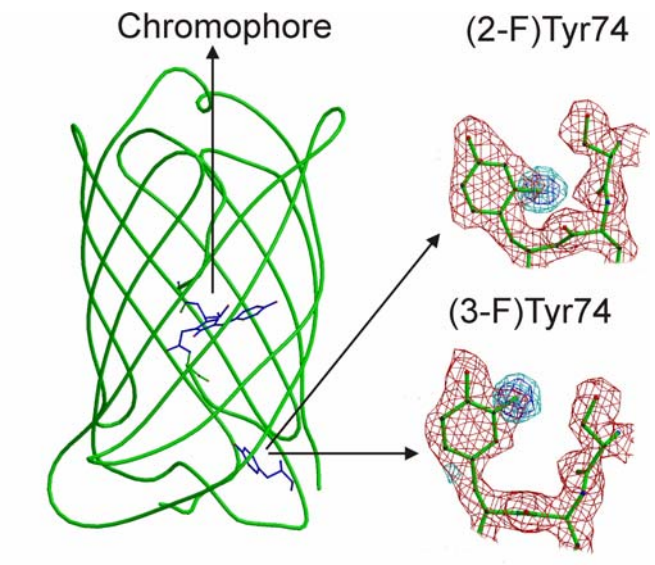

Figure S2. The position of Tyr74 in the EGFP structure and its experimental electron densities for both substitutions. The difference Fourier maps (blue, Fo-Fc; contouring levels: 3.0 σ) are superimposed on its continuous electron density (red, 2Fo-Fc; contouring levels 1 σ).

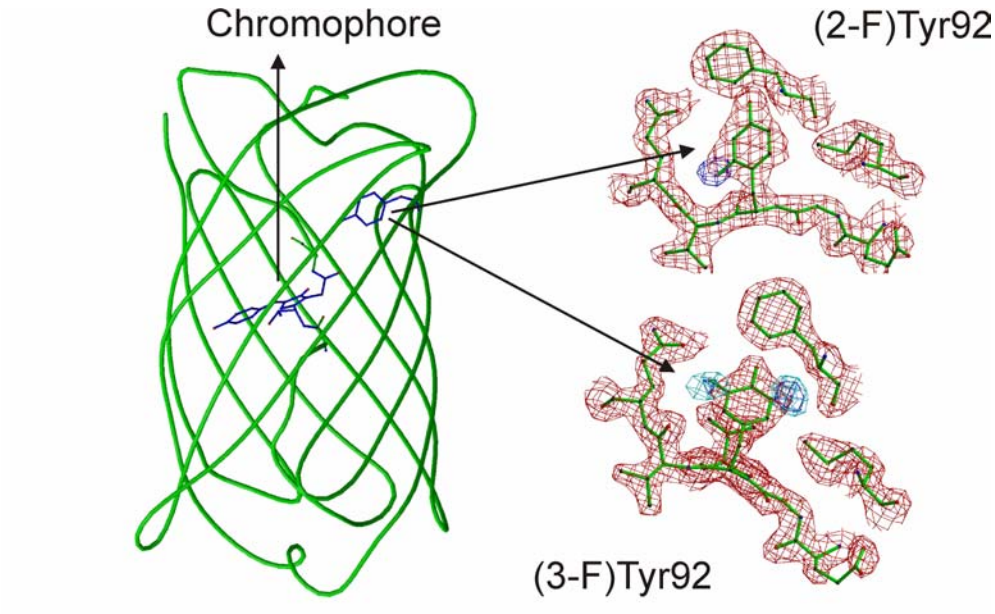

Figure S3. The Tyr92 is buried in the interior of the EGFP structure. Despite its involvement in numerous interaction contacts with its microenvironment of (3-F)Tyr92, the experimental densities of the fluorine atoms are distributed between two ('major' and 'minor') conformations. The difference Fourier maps and continuous electron densities are countered as described before (see Figure S2).

The **(2-F)Tyr106** side chain is buried and has hydrophobic interactions with Phe100, Phe130 and Leu125. The hydroxyl group of (2-F)Tyr106 forms a hydrogen bond with the side chain of Thr59. On the other hand, the difference electron density for the fluorine atom of the **3-F)Tyr-106** is presented in a single conformation state. This residue, buried inside the core, has hydrophobic contacts with Phe130, Phe100, Val22 and Leu125. Its hydroxyl group forms hydrogen bond with the Thr59 side chain. Interestingly the fluorine atom itself does not exhibit any interaction distances with the surrounding atoms. The fluorine of Tyr106 is 2.95 Å distant from the ring carbons of Phe130 (Figure S4).

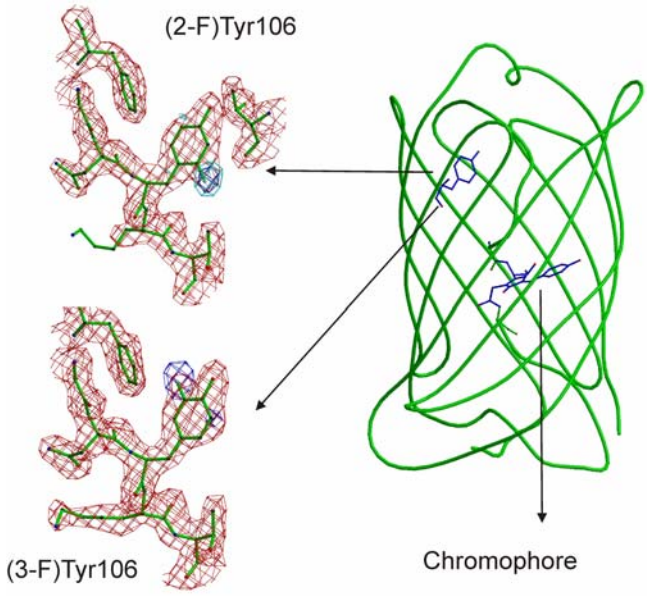

Figure S4. The fluorine atoms in both *ortho*-, or *meta*-position in Tyr106, having their experimental electron densities located at single conformations. The difference Fourier maps, and continuous electron densities are countered as described before (see Figure S2).

The **(2-F)Tyr143** is partially solvent exposed; however the hydroxyl group is surrounded by Lys209 and Leu207. Its fluorine atom does not show contact distances with its molecular neighborhood. Curiously, the **(3-F)Tyr143** in spite of its partial solvent exposure has both conformers in its X-ray structure (Figure S5). In both 'minor' and 'major' conformations fluorine atoms show no detectable distances that might indicate its involvement in a particular interaction(s). In the major conformation the fluorine atom is completely solvent exposed.

The **(2-F)Tyr145** (Figure S5) side chain is buried inside the molecule with a network of interactions; forming hydrophobic Van der Waals contacts with Val61, His148, His169 and Ser205. The fluorine atom is in a hydrogen bond distance with the main chain of Asn144. Similarly, the side chain of **(3-F)Tyr145** (Figure S5) is completely buried and has

hydrophobic contacts with Ser205, His169 and Val61. For example, the fluorine of (3-F)Tyr145 is within the contact range of the amide oxygen of Pro58 (2.54 Å). Ring nitrogen of His169 is also quite close to fluorine (3.27 Å) of (3-F)Tyr145 although the angle between these residues is not favorable for hydrogen bonding. Furthermore, the water molecule S14 (2.99 Å) is also in the hydrogen bond distance to fluorine as well as to a hydroxyl group (2.76 Å) of (3-F)Tyr145.

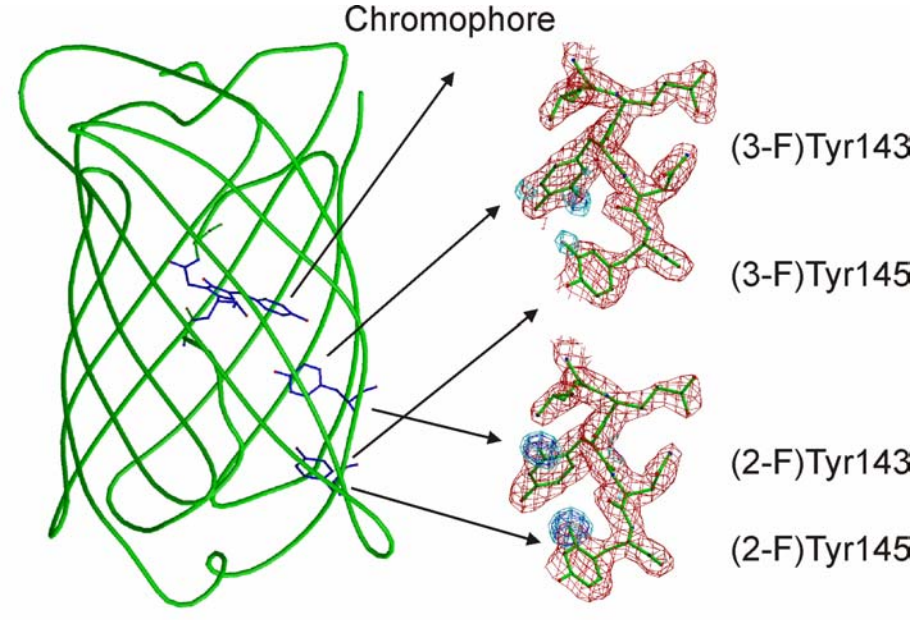

Figure S5. The fluorine atom of Tyr145 can be mapped in both *ortho*-, or *meta*-positions in only one conformation, which is surprising due to numerous interactions that this residue makes with its microenvironment. In contrast, the neighboring (3-F)Tyr143 has its fluorine atom in a single conformeric state. The difference Fourier maps and continuous electron densities are countered as described before (see Figure S2).

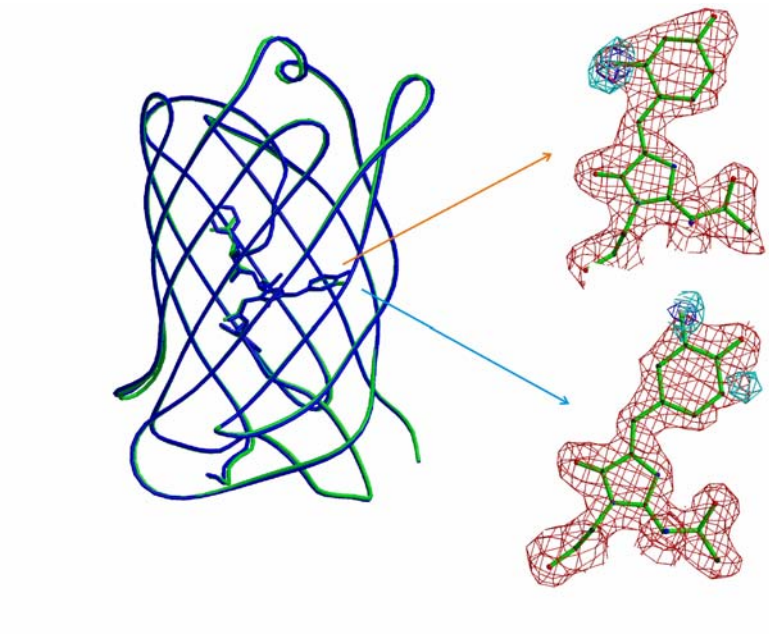

Figure S6. Difference Fourier maps (Fo-Fc; contouring levels: 2.0 and 3.0 σ) of (3-F)Tyr-chromophore in (3-F)Tyr-EGFP (green) superimposed on its continuous electron density (2Fo-Fc; contouring levels 1σ) revealed the existence of two conformers. The crystallographic occupancy (i.e. intensities of electron densities) is higher (about 60%) for one conformer (assigned as "major") than that for the other (40%, "minor"). In contrast, X-ray crystallographic structure analysis (with the same parameters as above) of (2-F)Tyr-EGFP (blue) mapped a single conformer state of (2-F)-Tyr-containing chromophore.