



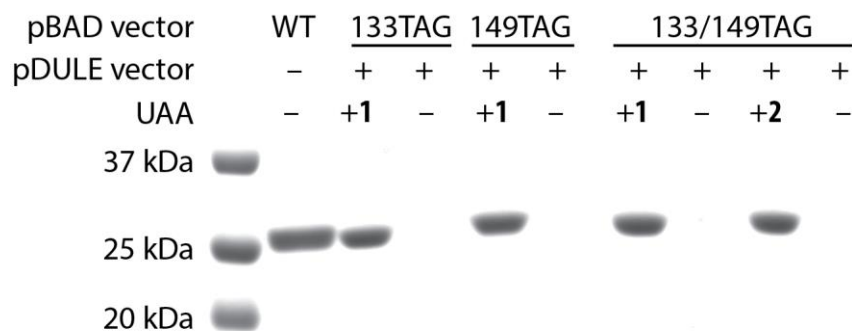
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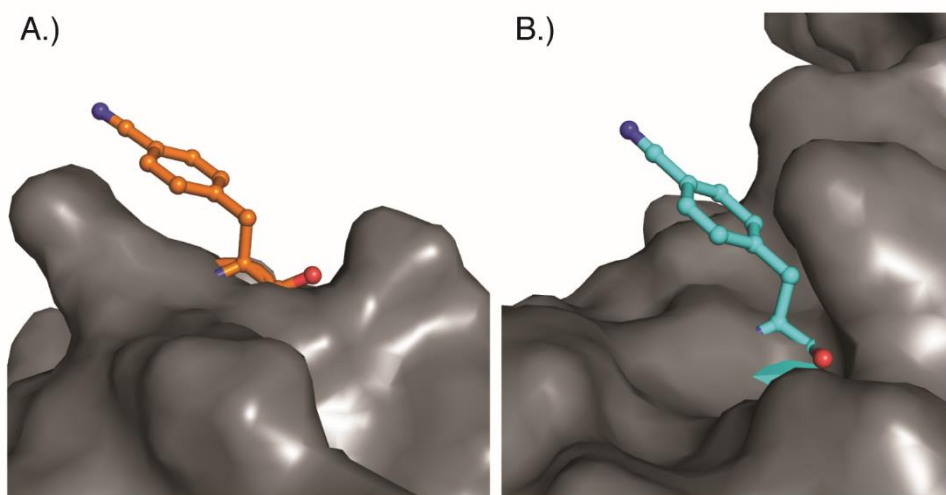
**Supporting information for article:**

**Probing the effectiveness of spectroscopic reporter unnatural amino acids: a structural study**

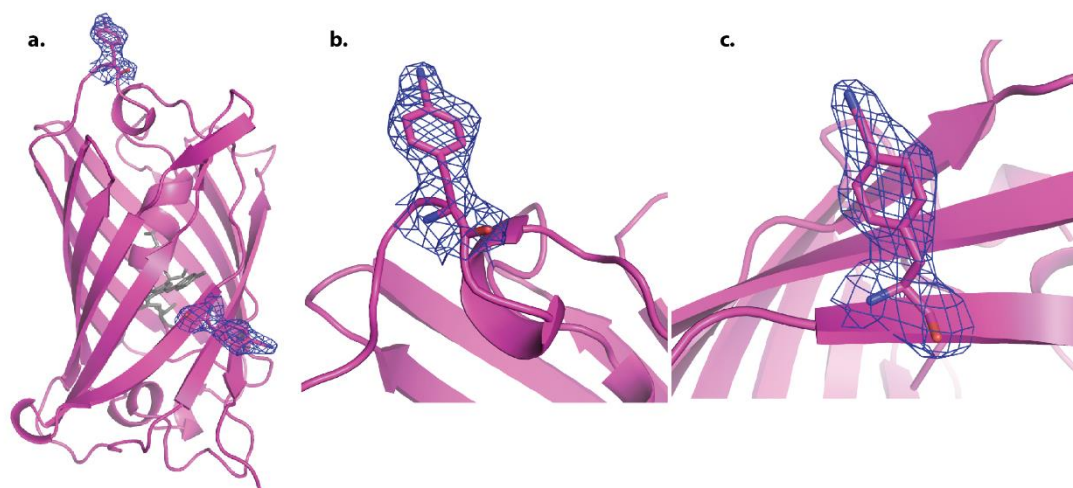
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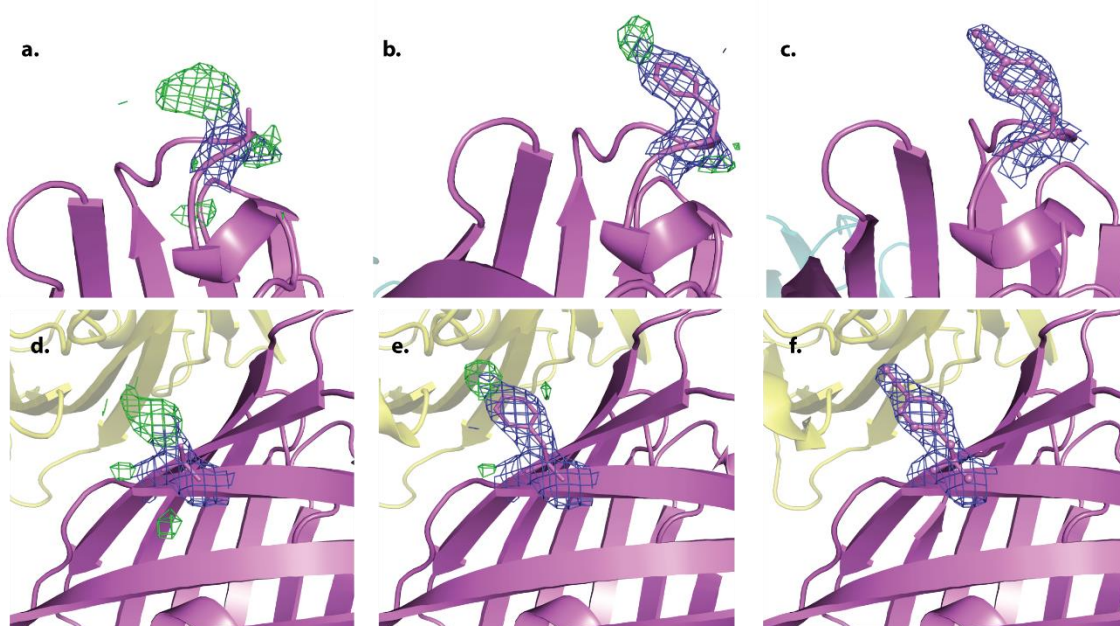
**Figure S1** Coomassie stained tris-glycine SDS-PAGE of purified proteins post Ni-NTA affinity chromatography illustrating efficient, site-specific incorporation of **1** and **2** with high fidelity at either the 133 or 149 sites. The protein constructs were expressed from *pBAD-sfGFP* (WT in lane 2); *pBAD-sfGFP-133TAG* and *pDULE-pCNF/pCCF* in the presence (lane 3) or absence (lane 4) of **1**; *pBAD-sfGFP-149TAG* and *pDULE-pCNF/pCCF* in the presence (lane 5) or absence (lane 6) of **1**; *pBAD-sfGFP-133/149TAG* and *pDULE-pCNF/pCCF* in the presence (lane 7) or absence (lane 8) of **1** and presence (lane 9) or absence (lane 10) of **2**.



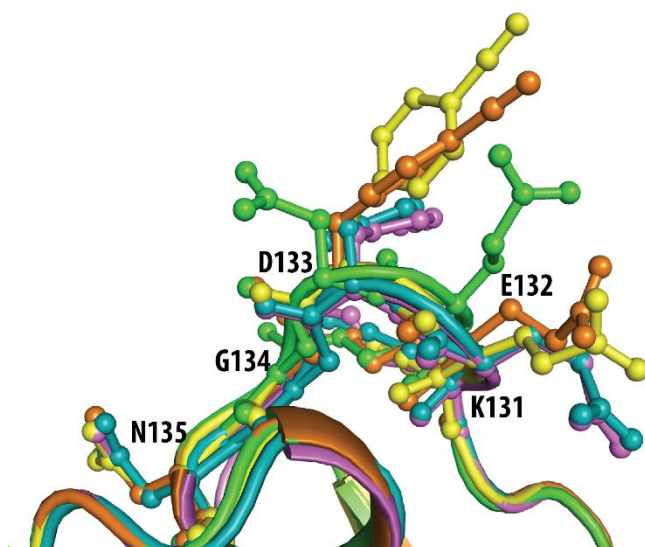
**Figure S2** Surface representations in the vicinity of the 133 (A) or 149 (B) sites in the sfGFP-133-pCNF or sfGFP-149-pCNF crystal structures, respectively. The 133pCNF residue (orange) is solvent accessible with a solvent accessibility of 89% and the 149pCNF residue (cyan) is partially buried with a solvent accessibility of 58%. Surface representations of surrounding residues were made in PyMol.



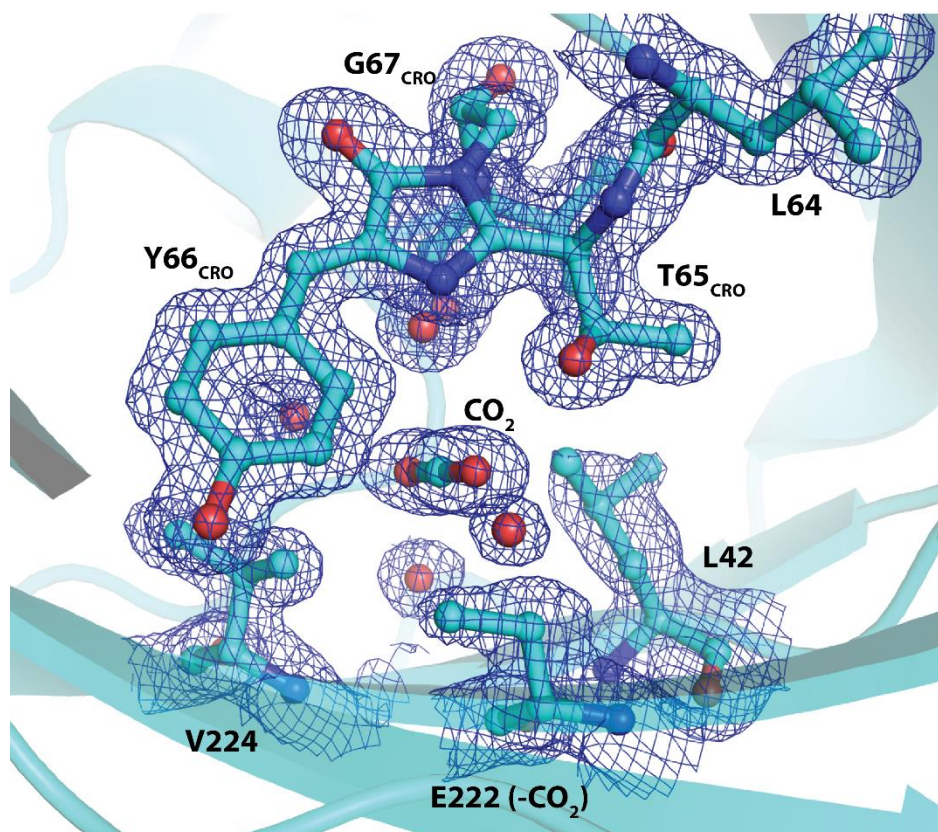
**Figure S3** Final refined structure of sfGFP-133/149-pCNF. The overall structure of one of the molecules in the asu is shown in (a.) with  $2F_o-F_c$  map at  $1\sigma$  (blue mesh). Focused views including  $2F_o-F_c$  map around the 133 (b.) and 149 (c.) sites.



**Figure S4** Refinement progression for sfGFP-133/149-pCCF. Electron density  $2F_o - F_c$  map at  $1\sigma$  (blue mesh) and difference density  $F_o - F_c$  map at  $+3\sigma$  (green mesh) are shown for the 133 (a. – c.) and 149 (d. – f.) sites throughout refinement. Initial structure from molecular replacement with alanine modeled at the 133 (a.) and 149 (d.) sites, refinement following incorporation of phenylalanine at the 133 (b.) and (e.) sites, and final maps at the 133 (c.) and 149 (f.) sites.



**Figure S5** Structural flexibility of the loop region containing pCCF at the 133 site. Alignment of each of the four chains in the asymmetric unit of sfGFP-133/149-pCCF with wild type sfGFP (in green, PDB ID 2B3P) in the loop around the 133 site. The four unique chains of sfGFP-133/149-pCNF are shown in magenta, orange, blue, and yellow and WT sfGFP is shown in green. All atoms for residues 131 – 135 are shown in ball and stick.



**Figure S6** Decarboxylation of E222 in the sfGFP-149-pCNF structure. The GFP chromophore (CRO) and residues near E222 are shown in stick with atoms colored by atom type (carbon in cyan, nitrogen in blue, oxygen in red).  $2F_o - F_c$  electron density contoured at  $1\sigma$  is shown in blue mesh. The E222 residue has been decarboxylated and truncated at the  $\gamma$ -C and the dissociated linear  $\text{CO}_2$  molecule is shown in clear electron density above the  $\gamma$ -C. The distance between the  $\gamma$ -C of E222 and the carbon in  $\text{CO}_2$  is 3.2 Å.