Engineering 'Golden' Fluorescence by Selective Pressure Incorporation of Non-canonical Amino Acids and Protein Analysis by Mass Spectrometry and Fluorescence

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Abstract

Fluorescent proteins are fundamental tools for the life sciences, in particular for fluorescence microscopy of living cells. While wild-type and engineered variants of the green fluorescent protein from Aequorea victoria (avGFP) as well as homologs from other species already cover large parts of the optical spectrum, a spectral gap remains in the near-infrared region, for which avGFP-based fluorophores are not available. Red-shifted fluorescent protein (FP) variants would substantially expand the toolkit for spectral unmixing of multiple molecular species, but the naturally occurring red-shifted FPs derived from corals or sea anemones have lower fluorescence quantum yield and inferior photo-stability compared to the avGFP variants. Further manipulation and possible expansion of the chromophore's conjugated system towards the far-red spectral region is also limited by the repertoire of 20 canonical amino acids prescribed by the genetic code. To overcome these limitations, synthetic biology can achieve further spectral red-shifting via insertion of non-canonical amino acids into the chromophore triad. We describe the application of SPI to engineer avGFP variants with novel spectral properties. Protein expression is performed in a tryptophan-auxotrophic E. coli strain and by supplementing growth media with suitable indole precursors. Inside the cells, these precursors are converted to the corresponding tryptophan analogs and incorporated into proteins by the ribosomal machinery in response to UGG codons. The replacement of Trp-66 in the enhanced "cyan" variant of avGFP (ECFP) by an electron-donating 4-aminotryptophan results in GdFP featuring a 108 nm Stokes shift and a strongly red-shifted emission maximum (574 nm), while being thermodynamically more stable than its predecessor ECFP. Residue-specific incorporation of the non-canonical amino acid is analyzed by mass spectrometry. The spectroscopic properties of GdFP are characterized by time-resolved fluorescence spectroscopy as one of the valuable applications of genetically encoded FPs in life sciences.

Video Article

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Introduction

Since the discovery of the green fluorescent protein in the jellyfish Aequorea victoria (avGFP) in 1962 and the first heterologous expression in 1994 in other eukaryotic cells, fluorescent proteins of the GFP family have become highly valuable tools and targets in life sciences. Extensive genetic and molecular engineering included the adjustment of species-specific codon usage, acceleration of folding, improved maturation, increased brightness, prevention of oligomerization and tailoring of spectral and photochemical properties including the ability to reversibly photoswitch⁴,⁵,⁶. GFP owes its fluorescence from its 4-(p-hydroxybenzylidene)imidazolidin-5-one (HBDI) chromophore. The latter is autocatalytically formed from the so-called chromophore triad of amino acids (Ser-65/Tyr-66/Gly-67 in avGFP) after the formation of an additional covalent bond within the peptide backbone under the influence of molecular oxygen⁷. The resonantly stabilized conjugated system interacts dynamically with its molecular environment, allowing for the absorption in the visible range and characteristic green fluorescence of these proteins. Within the chromophore triad, the presence of an aromatic amino acid is mandatory. However, the standard amino acid repertoire comprises only four aromatic residues (His, Phe, Trp and Tyr). This limits conventional mutagenesis approaches to achieve substantially more red-shifted avGFP variants relative to the most red-shifted natural FPs such as DsRed⁸ from Discosoma striata coralimorphs or mKate/mNeptune⁹ from the sea anemone Entacmaea quadricolor. Therefore, the far-red and near-infrared portion of the optical spectrum above 600 nm is sparsely covered by GFP variants. This is, of course, a severe limitation for fluorescence microscopic approaches that require spectral demultiplexing of several
fluorophore species at the same time. For example, long-wavelength markers are also necessary to make use of the low absorption regime of skin tissue between 700-1,000 nm in settings for deep tissue imaging10.

Fluorescent proteins derived from avGFP are divided into several classes based on the spectroscopic properties and chemical nature of their chromophores11. With its triad Ser-65/Tyr-66/Gly-67, the wild-type chromophore exists as an equilibrated mixture between the neutral, phenolic form (λ<sub>max</sub> = 395 nm, ε = 21,000 M<sup>-1</sup> cm<sup>-1</sup>) and the anionic phenolate form (λ<sub>max</sub> = 475 nm, ε = 7,100 M<sup>-1</sup> cm<sup>-1</sup>), and the emission spectrum exhibits a single peak at 508 nm. The hydroxyl group of Ser-65 is of critical importance, as it donates an H-bond to Glu-222 in the chromophore vicinity (distance: 3.7 Å), which promotes the ionization of this carboxylate. Class I is characterized by an anionic phenolate chromophore, as in EGFP (Phe-64-Leu/Ser-65-Thr, λ<sub>max</sub> = 488 nm, ε = 35,600 M<sup>-1</sup> cm<sup>-1</sup>). Due to the Ser-65-Thr(Ala,Gly) substitution, the 395 nm excitation peak of the neutral phenol form is suppressed and the 470-475 nm peak of the anionic phenolate is five- to six-fold enhanced and shifted to 490 nm. Class II comprises proteins with a neutral phenolic chromophore, as in sapphire-GFP. Here, the Thr-203-Ile substitution almost completely suppresses the 475 nm excitation, leaving only the peak at 399 nm. Since the anionic chromophore cannot be properly solvated, its neutral form is favored. Class III comprises the "yellow" fluorescent variants (EYFP: Ser-65-Gly/Val-66-Leu/Ser-72-Ala-Thr-203-Tyr; λ<sub>max</sub> = 514 nm, ε = 84,600 M<sup>-1</sup> cm<sup>-1</sup>, λ<sub>em</sub> = 527 nm) with π-stacking interaction of an aromatic side chain and the phenolate, as brought about by the Thr-203-His(Trp,Phe,Tyr) substitutions, which lead to up to 20 nm red-shifted emission maxima (Thr-203-Tyr). Further substitution (Gln-69-Lys) results in another 1-2 nm red shift to 529 nm, the most red-shifted avGFP variant known11. The exchange of the phenol for an indole (Tyr-66-Trp) creates class IV, as in the cyan-fluorescent ECFP (Ser-65-Thr/Tyr-66-Trp; λ<sub>max</sub> = 434 nm, ε = 24,800 M<sup>-1</sup> cm<sup>-1</sup>; λ<sub>max</sub> = 452 nm, ε = 23,600 M<sup>-1</sup> cm<sup>-1</sup>; λ<sub>em</sub> = 477 nm, λ<sub>em</sub> = 504 nm). Due to the Ser-65-Thr(Ala,Gly) substitution, the 395 nm excitation peak of the neutral form is suppressed and the 470-475 nm peak of the anionic phenolate is five- to six-fold enhanced and shifted to 490 nm. The accommodation of the bulky indole is probably enabled by other, compensatory mutations. The ECFP excitation and emission maxima fall in between those of proteins with neutral or anionic chromophores. Class V proteins harbor an imidazole in place of the phenol (Tyr-66-His), e.g., blue-fluorescent proteins like EBFP. Class VI is produced by a phenol-to-phenyl exchange favoring the neutral chromophore form exclusively, which consequently leads to the most blue-shifted excitation and emission peak positions (360 nm and 442 nm, respectively).

Classical site-directed mutagenesis is especially suitable for the production of novel avGFP chromophore variants, by the permutation of the 65-67 tripeptide and interacting residues in the frame of the 20 canonical amino acids. These possibilities can be further expanded when non-canonical variants of aromatic amino acids are introduced during ribosomal protein synthesis11. In principle, there are two ways to accomplish this. The first strategy relies on the substrate tolerance of the protein translation machinery, especially of aminoacyl-tRNA synthetases (aaRSs) towards related amino acid analogs. To achieve this with high efficiency, auxotrophic E. coli expression strains are employed that are unable to synthesize the corresponding natural amino acid. This allows the replacement of the latter by adding suitable non-canonical amino acids (ncAAs) or precursors thereof to the culture medium. This strategy, also known as Selective Pressure Incorporation (SPI), enables residue-specific replacements, which result in global incorporation of the ncAA. The second strategy uses stop codon suppressor tRNAs which are charged with the ncAA by engineered aaRS enzymes. This results in the readthrough of in-frame stop codons and allows site-specific ncAA incorporation. Consequently, this method of stop codon suppression (SCS) leads to the expansion of the genetic code15. Via mutagenesis, a stop codon is placed into the target gene at the desired site. In principle, SPCI can also be used to create recombinant peptides and proteins bearing a unique ncAA installation, given that rare canonical amino acids such as Met or Trp are chosen for substitution. With Trp, SPCI approaches have been shown to work with a large variety of analogs including 4-F-, 5-F- and 6-F-Trp, 7-aza-Trp, 4-OH- and 5-OH-Trp, as well as 4- and 5-NH<sub>2</sub>-Trp or even β(thienopyrrolyl)alanine derivatives16-18. Thus, SPCI could be highly advantageous for replacing aromatic amino acids of GFP chromophores by non-canonical variants to explore the possibility to further tailor spectra and Stokes shift of these FPs. As for all protein sequence modifications, the compatibility with FP folding and chromophore maturation must be tested experimentally.

In this work, we utilize class IV ECFP<sup>21</sup>, which carries instead of the wild-type avGFP Tyr, a Trp residue within its chromophore triad. Using this Trp-66 (and Trp-57, the only other Trp residue in ECFP) is substituted by 4-amino-Trp. The presence of the electron-donating amino group has been shown to work with a large variety of analogs including 4-F-, 5-F- and 6-F-Trp, 7-aza-Trp, 4-OH- and 5-OH-Trp, as well as 4- and 5-NH<sub>2</sub>-Trp or even β(thienopyrrolyl)alanine derivatives16-18. Thus, SPCI could be highly advantageous for replacing aromatic amino acids of GFP chromophores by non-canonical variants to explore the possibility to further tailor spectra and Stokes shift of these FPs. As for all protein sequence modifications, the compatibility with FP folding and chromophore maturation must be tested experimentally.

1. **Transformation of Trp-auxotrophic E. coli**

1. Transform chemically or electrocompetent cells (50 µL) of a Trp-auxotrophic strain, e.g. ATCC 49980 (WP2, mutant derived from E. coli strain B/R<sup>22</sup>), with 1 µL of a 1 ng/µL aqueous solution of the pQE-80L His6-ECFP plasmid using heat shock or electroporation, respectively. Please refer to the JoVE Science Education Database3,24 for details.

**NOTE:** The expression vector pQE-80L His6-ECFP encodes an N-terminally 6x His-tagged ECFP strain, e.g. ATCC 49980 (WP2, mutant derived from E. coli strain B/R<sup>22</sup>), with 1 µL of a 1 ng/µL aqueous solution of the pQE-80L His6-ECFP plasmid using heat shock or electroporation, respectively. Please refer to the JoVE Science Education Database for details.

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**Protocol**

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**NOTE:** The expression vector pQE-80L His6-ECFP encodes an N-terminally 6x His-tagged ECFP<sup>21</sup> driven by a bacterial T5 promoter with lac operator. It further carries an Amp<sup>+</sup> selection marker and a colE1 origin of replication (the pQE-80L vector backbone sequence can be found on: https://www.qiagen.com/mx/resources/resourcedetail?id=3cb7f1572-4d82-4671-a79b-96357fe926d1&lang=en&autoSuggest=true). The theoretical molecular weight of the His6-ECFP wild-type protein (after chromophore maturation<sup>23</sup>) is 28303.52 Da. The translated target protein sequence is as follows (His-tag underlined, vector-derived sequences in bold):

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MRGSHHHHHHHGSMVSKGEEELFTGVPILVELDGDVNHGKFSVQEGEGDATYGLTKLFCTTGGKLVPWPTLVTTLTWGVOCSFRYPDHMKQHDFKKSAMPEGYQVETTFFKDFDGKTYRAEKFEGDFTLRLEKIDGFKEDGDNILGBKLEYNiHSNHYITADKQKGGNKANFKHJNIEDGSVOLADHYQQNTPGGPP/LLPDNHYLSTOSALSKDNPENKRDMVWILLEVFTAG1TLGMEDLYK.
```

1. Plate transformed cells on LB-agar plates (Table 1) supplemented with 10 g/L glucose, 100 µg/mL ampicillin and incubate the plates at 37 °C overnight.
2. Recombinant Protein Expression

1. **Overnight culture of *E. coli* ATCC 49980 pQE-80L His6-ECFP**
   1. Prepare 5 mL of LB medium (Table 1; supplemented with 10 g/L glucose, 100 µg/mL ampicillin) in a sterile 14 mL polystyrene culture tube for aerobic growth and inoculate with a singly colony from an agar plate using a sterile pipette tip or inoculation loop. 
   
   **NOTE:** Using colonies from freshly transformed cells is recommended. The plates with bacterial colonies (from step 1.2.) can be stored at 4 °C for several days.

   2. Incubate the cells at 37 °C in an orbital shaker at 200-250 rpm overnight.

2. **Expression of wild-type ECFP**
   1. Inoculate 10 mL fresh LB medium (Table 1; supplemented with 10 g/L glucose, 100 µg/mL ampicillin) with 100 µL of the overnight culture in a 100 mL Erlenmeyer flask. Incubate the flask at 37 °C in an orbital shaker at 200 rpm.
   
   **NOTE:** Optionally, this step can be performed in 10 mL NMM19 medium (Table 1) supplemented with 100 µg/L ampicillin and 0.5 mM L-tryptophan (alternatively, indole can be used).

   2. Measure the optical density at 600 nm (OD<sub>600</sub>) every 20 min. Preferentially measure cell density by determining the extinction at 600 nm (OD<sub>600</sub>) in a spectrophotometer using a cuvette with a path length of 1 cm. Always perform a reference measurement using the corresponding culture medium. Dilute the samples and mix the samples well to obtain a measurement value of 0.1-0.8, then calculate OD<sub>600</sub> using the dilution factor. For details, please refer to previous publication.26

   3. Upon reaching an OD<sub>600</sub> value of 0.5-0.8 (approximately 2-3 h after the inoculation), take sample "before induction" for SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis, step 4).

   4. Induce target protein expression by adjusting the liquid culture to 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside, from 1 M stock solution) and incubate it at 30 °C in an orbital shaker at 200 rpm for 4-8 h.

   **NOTE:** Cyan fluorescent proteins are commonly expressed at temperatures below 37 °C.27

   5. Take sample "after expression" for SDS-PAGE (step 4).

   6. Harvest the bacterial cells by centrifugation for 10 min at 5,000 x g and 4 °C.

   7. Discard the supernatant by decanting and freeze the cell pellets at -20 °C or -80 °C until target protein purification.

3. **SPI for producing GdFP**
   1. Inoculate 10 mL of NMM19 medium (Table 1) supplemented with 100 µg/mL ampicillin, 15 µM tryptophan and 10 µL of overnight culture in a 100 mL Erlenmeyer flask. Incubate the culture flask overnight at 30 °C in an orbital shaker at 200 rpm.

   **NOTE:** A variety of chemically defined media for cultivation of *E. coli* and SPI is available. In addition to NMM used herein, MOPS medium<sup>28</sup>, glucose-mineral salts medium<sup>29</sup>, Davis minimal medium<sup>30</sup>, M9 minimal medium<sup>31</sup>, or GMML<sup>32</sup> can be used.

   2. The next day, measure OD<sub>600</sub> every 30 min until the value only changes by less than 0.05 over 30 min. The plateau value should be approximately 1.

   **NOTE:** Deviations by ± 0.3 units are acceptable. Depending on the bacterial strain and medium used, the initial tryptophan concentration (step 2.3.1) may need adjustment.

   3. Take sample “before induction” for SDS-PAGE (step 4.).

   4. Harvest the bacterial cells by centrifugation for 10 min at 5,000 x g and 4 °C. Discard the supernatant by decanting.

   5. Resuspend the cells in 10 mL of NMM19 medium with 100 µg/mL ampicillin into a 100 mL Erlenmeyer flask and add 4-amino-indole to a final concentration of 1 mM using 50 mM stock solution. Continue the incubation for 30 min at 30 °C in an orbital shaker at 200 rpm.

   **NOTE:** This step is recommended because of the low chemical stability of ampicillin and ensures cellular uptake of 4-amino-indole.

   6. Induce target protein expression by adding IPTG to a final concentration of 0.5 mM using 1 M stock and incubate the sample overnight at 30 °C in an orbital shaker at 200 rpm.

   **NOTE:** Cyan fluorescent proteins are commonly expressed at temperatures below 37 °C.27

   7. The next day, measure OD<sub>600</sub>.

   8. Take sample "after expression" for SDS-PAGE (step 4.).

   9. Harvest the bacterial cells by centrifugation for 10 min at 5,000 x g and 4 °C and discard supernatant by decanting.

   10. In case such a vessel was not used for centrifugation, transfer the cell pellet into a 50 mL conical polystyrene tube using a spatula. Freeze the cell pellet at -20 °C or -80 °C until target protein purification.

3. **Target Protein Purification via Immobilized Metal Ion Affinity Chromatography (IMAC)**

1. **Bacterial cell lysis**
   1. Thaw the cell pellet on ice for 10-20 min.

   2. Resuspend the cell pellet in a 50 mL conical polystyrene tube using 5 mL of ice-cold binding buffer (Table 1) on ice.

   3. Add 20 µL of 50 mg/mL lysozyme, 20 µL of 1 mg/mL DNase I, and 20 µL of 1 mg/mL RNase A. Close the tube, mix gently by inverting 5 times, and keep it on ice for 30 min.

   **NOTE:** Partial cell disruption occurs as catalyzed by lysozyme.

   4. Lyse the cells by sonification using an ultrasound homogenizer tip using three cycles of 3 min in a 15 mL polystyrene tube cooled by slush ice with 2 s of pulse, 4 s of pause and 45% amplitude.

   **NOTE:** Alternatively, high pressure homogenization can be used, e.g., 20 cycles at 14,000 psi. If necessary, dilute using binding buffer to reach the minimal instrument volume. Moreover, protein extraction reagents can be used for cell disruption. See Materials table for examples.

   5. Centrifuge the sample for 30 min at 15,000-18,000 x g, 4 °C.

   6. Transfer the supernatant into a fresh tube and note down the liquid volume.

   7. Filter the solution through a 0.45 µm syringe filter using a 5 mL plastic Luer lock syringe and a polyvinylidene fluoride (PVDF) syringe filter.

   8. Take sample "lysate" for SDS-PAGE (step 4.).
9. Resuspend cell debris pellet in ddH₂O (equal volume as former lysate).
10. Take sample "pellet" for SDS-PAGE (step 4.).

2. IMAC purification
   1. Use a 1 mL prepacked or self-packed IMAC FPLC (fast protein liquid chromatography) column according to manufacturer’s instructions. Use binding buffer (Table 1) for column equilibration as well as for the wash step which follows after the cell lysate has been applied to the column.
2. Collect and pool eluate fractions with GdFP which can be identified by visible light golden color.
   NOTE: Optionally, the target protein can be eluted using a linear imidazole gradient (0-250 mM) using an automated FPLC system.
3. Determine the protein concentration using the literature value for the extinction coefficient at 486 nm (ε₄₈₆nm = 23,700 M⁻¹ cm⁻¹) with elution buffer as reference. For details on the procedure, please refer to previous publication.
4. Take sample "eluate" for SDS-PAGE, and use 1-10 µg of protein per lane in case of Coomassie-staining.
   NOTE: SDS sample amounts can vary depending on the staining method and dye sensitivity.
5. Dialyze an aliquot of the eluate fractions against dialysis buffer or MS buffer using a membrane with a molecular weight cutoff (MWCO) of 5,000-10,000. Prepare dialysis membrane according to manufacturer’s instructions. Dialyze a 1 mL sample three times against 100 mL buffer for at least 2 h. For details on this procedure, please refer to previous publication.
6. For storage, freeze protein sample in dialysis buffer at -80 °C.
   NOTE: Aliquots should be stable for at least 6 months.

4. SDS-PAGE Sample Preparation of *E. coli* Whole Cell Extract
   1. Transfer a cell suspension equivalent to 1 mL of OD₆₀₀ = 1 suspension (e.g., 500 µL of OD₆₀₀=2) into a 1.5 mL microcentrifuge tube.
2. Harvest the cells by centrifugation for 10 min at 5000 x g, room temperature. Discard the supernatant by pipetting.
3. Add 80 µL of ddH₂O and 20 µL of 5x SDS loading dye buffer (Table 1) to the cell pellet and mix by pipetting.
4. Denature the cells by heating to 95 °C for 5 min in a water bath or heat block. Subsequently, cool the samples to room temperature.
5. Use 10 µL for Coomassie-stained SDS-PAGE according to previous publication.
   NOTE: SDS sample amounts can vary depending on the staining method and dye sensitivity.

5. Intact Protein Mass Analysis by High-performance Liquid Chromatography (HPLC) Coupled to Electrospray Ionization Time-of-flight Mass Spectrometry (LC-ESI-TOF-MS)

   NOTE: HPLC gradient, settings and buffers may vary depending on the separation column and instrument used. See Materials table for exemplary equipment.

1. Determine the protein concentration from a sample dialyzed against MS buffer as described above (step 3.2.3.) using MS buffer (see Materials table) as reference.
2. Dilute the protein sample to 0.1 mg/mL using MS buffer for a final volume of 80 µL, mix by careful pipetting, transfer the solution into a MS autosampler vial with glass insert and close it with a cap. Remove air bubbles by flicking the vial.
3. Fill a second autosampler vial without glass insert (buffer blank) with 1 mL of MS buffer.
4. Allow the instrument to warm up. Perform instrument calibration. Make sure that sufficient amounts of liquid chromatography-grade solvents are available (>100 mL).
5. Program a linear 20 min HPLC gradient from 5% to 80% buffer A (0.1 % formic acid in ddH₂O), combined with buffer B (0.1 % formic acid in acetonitrile).
6. Start HPLC at a flow of 0.3 mL/min and wait until the column pressure is stable.
7. Set an autosampler injection volume of 5 µL for the LC-ESI-TOF-MS method, create a worklist for a blank run followed by a sample run and assign the corresponding autosampler vial positions. Run the worklist.
8. After worklist completion, open the generated sample data file. Select a range in the total ion current (TIC) plot for deconvolution and deconvolute the MS spectrum using the maximum entropy deconvolution algorithm.
   NOTE: Depending on experimental conditions, additional species can occur from non-matured FP or buffer ion adducts.

6. Fluorescence Lifetime Measurements and Decay-associated Spectra (DAS) of GdFP

   NOTE: For the instrumentation of time-resolved fluorescence spectroscopy, please refer to Table of Materials for exemplary equipment.

1. Wavelength-resolved fluorescence lifetime measurement of GdFP
   1. Prepare 2 mL of a 1 µM solution of GdFP by dilution into PBS buffer (Table 1) at pH 7. Fill the solution into a 1 cm quartz cuvette.
2. Install ps-pulsed 470 nm laser for sample excitation and the 488 nm long-pass emission filter, and adjust 600 L/mm grating of the time- and wavelength-correlated single photon counting (TWCSPC) detector for acquisition of the wavelength regime 500-700 nm.
3. Acquire fluorescence emission at a count rate of about 200 x 10³ photons/s until about 10⁴ counts are accumulated in the acquisition maximum of the fluorescence decay curves with single photon counting software.

2. Measurement of the instrumental response function (IRF)
   1. Replace the sample cuvette with a 1 cm quartz cuvette filled with 1 g/L colloidal silica (~220 m²/g) in PBS buffer at pH 7.
   NOTE: The silica suspension is prepared using a 400 g/L aqueous suspension.
Representative Results

Using the technique of selective pressure incorporation, Trp-66 in the chromophore triad of ECFP (and Trp-57, the only other Trp residue in ECFP) can be replaced by 4-amino-Trp, thereby generating the red-shifted GdFP with distinct spectral properties. Mass spectrometry must be used to demonstrate the desired stoichiometric integration of the non-canonical amino acid into the protein, with results shown in Figure 1. Afterwards, we provide data from microscopy, UV-Vis absorption spectroscopy as well as steady-state and time- and wavelength-resolved fluorescence spectroscopy to characterize the properties of the GdFP fluorophore with a focus on the pH dependence of the spectra.

To confirm the exchange of the two Trp residues in ECFP by 4-amino-Trp, mass spectrometric analysis is carried out. Figure 1 shows a representative deconvoluted ESI-MS spectrum of GdFP. While wild-type ECFP has a calculated protein mass of 28,283.9 Da after the chromophore maturation, the corresponding mass of GdFP is 28,313.9 Da. The deconvoluted ESI-MS spectrum of GdFP exhibits a main mass peak at 28,314.1 ± 0.1 Da, which deviates from the theoretical value by less than 10 ppm. Being within the typical accuracy range for this type of analysis, this confirms the incorporation of the ncAA via SPI (experimental value for wild-type ECFP: 28,283.7 Da).

Figure 2 shows confocal fluorescence imaging microscopy (CFIM) images of bacterial cells expressing ECFP, EGFP, EYFP and GdFP upon resuspension of bacteria in PBS buffer. All images were acquired on a microscope equipped with a UV objective and laser excitation at about the same energy for each sample.

Figure 3A shows an overlay of CFIM images of E. coli bacteria expressing various FPs including GdFP, always monitored with very similar excitation energy (wavelengths as in Figure 2). Figure 3B shows the chromophore structures of the FP variants shown. Regarding the brightness of GdFP compared to ECFP (fluorescence quantum yield ϕ = 0.4), EGFP (ϕ = 0.6) and EYFP (ϕ = 0.6) it is important to note that for GdFP, a broader acquisition range of the fluorescence light (30 nm) was used in contrast to 20 nm used for all other species, in order to adjust the intensity of the images to similar values. With a slightly lower extinction coefficient and a reduced quantum yield as a consequence of unique photophysical properties, the brightness of GdFP is lower compared to the other FPs shown.

The absorption spectrum of ECFP (Figure 3C) has two characteristic maxima at 434 nm and 452 nm. In contrast, GdFP is characterized by one broad red-shifted absorption band with the maximum at 466 nm. The absorption of EGFP is further red-shifted to 488 nm. However, due to the much larger Stokes shift of GdFP (108 nm) compared to ECFP (41 nm) and EGFP (20 nm), the emission spectrum of GdFP is the most red-shifted of all three GFP derivatives investigated here (Figure 3D). While the fluorescence emission of ECFP shows two characteristic maxima at 475 nm and 505 nm, EGFP has one broad main emission band peaking at 508 nm (λmax) with a slight shoulder at 540 nm. The fluorescence of GdFP appears at about 565 nm (λmax) (Figure 3D). Its emission spectrum contains a small contribution of wild-type ECFP which is also visible as a small shoulder at 475 nm. This small ECFP fraction is synthesized before induction during the SPI procedure, as described.

Figure 3E shows the pH-dependent changes in the absorption spectrum of GdFP. For a change from 8 to 5, the emission maximum shifts slightly to the red and a slight broadening of the absorption band is observed. However, the reduction of the absorption amplitude is only about 10 % between pH 8 and pH 5, indicating that the ground state properties of the GdFP chromophore are very weakly modified by pH.

The time resolved fluorescence emission monitored by single photon counting is shown in Figure 4. The decay curves monitored in the spectral channels centered at 550 nm and 600 nm (Figure 4A) exhibit a slightly faster fluorescence decay at 600 nm compared to the decay at 550 nm. The results of a global fit of the fluorescence decay curves with two exponential components results in two spectrally distinguishable fluorescence decay components with time constants of 1.0 ns and 3.3 ns (Figure 4C and D).

The fluorescence emission of GdFP strongly depends on pH, as it is typical for many fluorescent protein variants of the GFP family. Figure 4B compares the fluorescence emission of GdFP between pH 5 and pH 8, which clearly shows a decrease in the fluorescence intensity at lower pH, while the spectral characteristics stay constant.

The decay-associated spectra (DAS) of GdFP (Figure 4C and D) are characterized by two distinct emission bands. The contribution of the slow 3.3 ns component is more pronounced in the short wavelength range around 550 nm (60 %) with minor contribution of the faster component (40 %). At 600 nm, both components have about the same amplitude. Upon a shift from pH 7 (Figure 4C) to pH 6 (Figure 4D), the spectral characteristics of the DAS hardly change and the time constants from the global fitting routine are also the same (the accuracy of the DAS time constants is about ± 0.15 ns). However, the difference in the absolute amplitudes of the two DAS components is clearly apparent, which fully accounts for the reduced fluorescence emission amplitude upon the same pH shift in Figure 4B.
Figure 1: Representative deconvoluted ESI-MS spectrum of GdFP. The ESI-MS spectrum of GdFP (gold color, magnified plot shown as inset) shows a main peak at 28314.1 Da (calculated value 28313.9 Da). The spectrum for wild-type ECFP is shown in black. Please click here to view a larger version of this figure.

Figure 2: Confocal fluorescence microscopy images from bacterial populations expressing various FPs. The following wavelength settings were used for image acquisition: ECFP (λ<sub>ex</sub> = 457 nm, detection: 461-480 nm), EGFP (λ<sub>ex</sub> = 488 nm, detection: 495-515 nm), GDFP (λ<sub>ex</sub> = 476 nm, detection: 560-590 nm), EYFP (λ<sub>ex</sub> = 514 nm, detection: 520-530 nm). Please click here to view a larger version of this figure.
Figure 3: Spectral properties of GdFP. (A) CFIM image of a mixture of bacterial cells expressing ECFP, EGFP and GdFP after the resuspension of bacteria in PBS buffer. (B) Chromophore structures of GdFP (with 4-amino-Trp in place of residue 66), the parental ECFP (with Trp at position 66) and EFGP (with Tyr at position 66). (C) Comparison of the normalized absorption spectra of GdFP, ECFP and EGFP, whereas in (D), the normalized fluorescence emission spectrum of ECFP (excitation at 430 nm) is compared to the fluorescence emission spectra of EGFP and GdFP (both excited at 450 nm). (E) pH-dependence of the absorption spectra (normalized absorption at 280 nm). Please click here to view a larger version of this figure.
Figure 4: Time-resolved fluorescence of GdFP. (A) Fluorescence decay of GdFP monitored by time- and wavelength-resolved single photon counting in the spectral channels centered at 550 nm and 600 nm (± 12.5 nm) after excitation with 470 nm laser pulses. The instrumental response function (IRF) provides information about the time resolution of the used setup. (B) Variation of the emission spectrum of GdFP dependent on pH (excitation at 460 nm). (C, D) Decay-associated spectra (DAS) of GdFP at pH 7 (C) and pH 6 (D) determined after deconvolution of time- and wavelength-resolved fluorescence decays and global fitting of the decays in all channels by a global set of two exponential functions with linked time constants. Please click here to view a larger version of this figure.

Figure 5: Structures of the intramolecular charge transfer of ECFP (black) and GdFP (gold) chromophores. The increase in size of the chromophore system by the good electron donor of an amino group as part of the ncaa enables the formation of more mesomeric structures to achieve resonance stabilization of the excited state. The connection points to the FP scaffold are shown as semicircles. Please click here to view a larger version of this figure.
### Stock solution

| Stock solution       | concentration, solvent                                      | Note                                                                 |
|----------------------|-------------------------------------------------------------|----------------------------------------------------------------------|
| 20% D-glucose        | 200 g/L D-glucose in ddH₂O                                  | sterilize by filtration through a 0.45 μm pore size syringe filter   |
| indole               | 50 mM in isopropanol                                        |                                                                      |
| 4-amino-indole       | 50 mM in 20 % ethanol (20 mL ethanol in a final volume of 100 mL filled up with ddH₂O) |                                                                      |
| IPTG                 | 1 M in ddH₂O                                                |                                                                      |
| L-tryptophan         | 15 mM dissolved in ddH₂O using 1 M HCl (add HCl dropwise under stirring until powder is dissolved) |                                                                      |
| lysozyme             | 50 mg/mL in ddH₂O                                           |                                                                      |
| DNase I              | 1 mg/mL in ddH₂O                                            |                                                                      |
| RNase A              | 1 mg/mL in ddH₂O                                            |                                                                      |
| Amp100               | 100 mg/mL ampicillin in ddH₂O                               |                                                                      |
| sodium-dodecylsulfate (SDS) | 200 g/L in ddH₂O                                        |                                                                      |
| ammonium sulfate ((NH₄)₂SO₄) | 1 M in ddH₂O                                              | sterilize by autoclaving                                              |
| potassium dihydrogen phosphate (KH₂PO₄) | 1 M in ddH₂O                                              | sterilize by autoclaving                                              |
| di-potassium hydrogen phosphate (K₂HPO₄) | 1 M in ddH₂O                                              | sterilize by autoclaving                                              |
| magnesium sulfate (MgSO₄) | 1 M in ddH₂O                                                | sterilize by autoclaving                                              |
| D-glucose            | 1 M in ddH₂O                                                | sterilize by filtration through a 0.45 μm pore size syringe filter   |
| sodium chloride (NaCl) | 5 M in ddH₂O                                             | sterilize by autoclaving                                              |
| calcium chloride (CaCl₂) | 1 g/L                                                    | sterilize by filtration through a 0.45 μm pore size syringe filter   |
| iron(II) chloride (FeCl₂) | 1 g/L                                                    | sterilize by filtration through a 0.45 μm pore size syringe filter   |
| thiamine             | 10 g/L                                                      | sterilize by filtration through a 0.45 μm pore size syringe filter   |
| biotin               | 10 g/L                                                      | sterilize by filtration through a 0.45 μm pore size syringe filter   |
| trace elements mix   | copper sulfate (CuSO₄), zinc chloride (ZnCl₂), manganese chloride (MnCl₂), ammonium molybdate ((NH₄)₂MoO₄); each 1 mg/L in ddH₂O | sterilize by filtration through a 0.45 μm pore size syringe filter |
| 19 amino acids mix   | 1.) Dissolve 0.5 g L-phenylalanine and 0.5 g L-tyrosine in 100 ml ddH₂O with dropwise addition of 1 M HCl under stirring until powder is dissolved. |                                                                      |
|                      | 2.) Weigh out 0.5 g of each of the remaining L-amino acids (except L-tryptophan). Mix with 22 mL fo 1 M KH₂PO₄ and 48 mL of 1 M K₂HPO₄. Add ddH₂O to about 800 mL. Stir until the solution becomes clear. |                                                                      |
|                      | 3.) Add the dissolved L-phenylalanine and L-tyrosine from step 1.) and adjust the volume to 1 L with ddH₂O. |                                                                      |
|                      | 4.) Sterilize the amino acid mixture by vacuum filtration with a bottle top filter unit. |                                                                      |
### Buffers and Media

| Buffer                          | Composition/Preparation |
|---------------------------------|-------------------------|
| SDS loading dye buffer, 5x      | 0.25 M Tris pH 6.8, 50 % w/v glycerol, 0.25 % w/v bromphenol blue, 0.5 M didithiothreitol (DTT; alternatively 5 % β-mercaptoethanol), 10 % w/v sodium-dodecylsulfate (SDS) |
| binding buffer                  | 50 mM sodium dihydrogenphosphate (NaH₂PO₄), 500 mM NaCl, 10 mM imidazole, pH 8 |
| elution buffer                  | 50 mM sodium dihydrogenphosphate (NaH₂PO₄), 500 mM NaCl, 250 mM imidazole, pH 8 |
| dialysis buffer                 | 50 mM sodium dihydrogenphosphate (NaH₂PO₄), 150 mM NaCl, 100 mL/L glycerol, pH 8 |
| MS buffer                       | 10 mM Tris-HCl, pH 8 |
| new minimal medium containing 19 L-amino acids except L-tryptophan (NMM19) | Mix all stock solutions to obtain the following final concentrations: 7.5 mM (NH₄)₂SO₄, 1.7 mM NaCl, 22 mM KH₂PO₄, 50 mM K₂HPO₄, 1 mM MgSO₄, 20 mM D-glucose, 50 mg/L of 19 amino acids mix, 1 µg/L CaCl₂, 1 µg/L FeCl₂, 10 µg/L thiamine, 10 mg/L biotin, 0.01 mg/L trace elements mix |
| LB medium                       | Composition: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.0 in ddH₂O |
|                                | Preparation: |
|                                | 1.) Weigh out 50 g tryptone, 25 g yeast extract, 5 g NaCl into a 1 L glass bottle. |
|                                | 2.) Add ddH₂O up to ~800 mL and dissolve components under stirring. |
|                                | 3.) Measure pH and adjust to pH 7 by dropwise addition of 1 M HCl or 1 M NaOH, if necessary. Add ddH₂O up to 1 L. |
|                                | 4.) Sterilize by autoclaving, check for volume loss afterwards and add sterile ddH₂O to compensate if necessary. Store at 4 °C until use. |
| LB agar plates                  | Composition: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar-agar, pH 7.0 in ddH₂O |
|                                | Preparation: |
|                                | 1.) Weigh out 50 g tryptone, 25 g yeast extract, 5 g NaCl, 7.5 g agar-agar into a 1 L glass bottle. |
|                                | 2.) Add ddH₂O up to 500 mL and dissolve components under stirring. |
|                                | 3.) Measure pH and adjust to pH 7 by dropwise addition of 1 M HCl or 1 M NaOH, if necessary. Add ddH₂O up to 1 L. |
|                                | 4.) Sterilize by autoclaving, check for volume loss afterwards and add sterile ddH₂O to compensate, if necessary. (Note: LB agar can be stored at 4 °C until use for preparation of LB agar plates. Carefully melt solidified agar using a microwave) |
|                                | 5.) When the solution is still warm (30-40 °C), add ampicillin to a final concentration of 100 µg/mL |
|                                | 6.) Pour about 15 mL of the liquid from step 5.) into a sterile 10 cm Petri dish under sterile conditions. When the agar is solidified, plates can be stored for 1 week at 4 °C until use. |
| phosphate-buffered saline (PBS) | Composition: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 1 mM CaCl₂, 0.5 mM MgCl₂, pH 7. Sterilize by autoclaving or filtration. |

### Table 1: Stock solution and buffer.

### Discussion

To achieve very high ncAA incorporation efficiencies, the auxotrophy-based SPI method relies on the use of metabolically engineered host cells, which are not able to synthesize the corresponding natural counterpart of the ncAA. For *E. coli*, such strains are readily available. Even the simultaneous incorporation of multiple ncAAs into the same protein is feasible using multiauxotrophic strains. The residual-specific mode of replacement and the chemical repertoire being restricted to similar chemical analogs can be seen as drawbacks. Nevertheless, a large number of protein variants can be produced as the natural bacterial translation apparatus tolerates numerous amino acid analogs. For example, more than 50 ncAAs could be incorporated into proteins using *in vitro* translation, accounting for about 73% of all codons of the genetic code to be available for reallocation[43]. Furthermore, SPI can also allow efficient multisite labelling of the target protein[41]. In principle, the SPI methodology is not restricted to *E. coli*, but can work in any other host and for each of the canonical 20 amino acids, provided that auxotrophic strains and defined cultivation media are available. For example, two methionine analogs, azidohomoalanine (Aha) and homopropargylglycine (Hpg), are commercially available and used for labeling proteins and proteomes in diverse organisms. In addition, Aha can be produced intracellularly and subsequently incorporated into proteins[44]. This ncAA is especially suitable for bioorthogonal conjugations such as click chemistry as developed by Tirrell and coworkers: For example, in plant tissue of Arabidopsis thaliana, in Bombyx mori larvae[43], Droso phila cells[44], larval zebrafish[45] as well as mammalian cells including neurons[46]; proteins can be labelled with Aha[47,48]. Similarly, Tpr analogs were successfully incorporated into antimicrobial peptides in Trp-auxotrophic Lactococcus lactis strains[49]. SPI is also useful for the field of Xenobiology[50,51], which explores alternatives to the basic chemical make-up of life. For example, based on previous works on *E. coli*[52] and *B. subtilis*[53], an *E. coli* strain was developed recently by an evolutionary strategy with selective pressure to utilize thienopyrrole instead of indole, resulting in proteome-wide substitution of tryptophan by thienopyrrole-alanine in the genetic code[54]. Generally, the canonical amino acid Trp, which is encoded by a single triplet (UGG), presents a promising target for protein engineering due to the rich facets of indole chemistry, which offers numerous chemical variations. Recently, and as an alternative to SPI-based incorporation, a novel SCS platform capable to incorporate Tpr analogs site-specifically
in both bacterial and eukaryotic hosts has been reported. This further broadens the toolbox of in vivo ncAA-based protein engineering, including the alteration of spectral properties.

Besides the use of auxotrophic expression hosts, the SPI protocol requires strict fermentation conditions, both in terms of target expression timing and the composition of the medium in order to reach high ncAA incorporation efficiency and target protein yield. Cultivation is conducted using chemically defined minimal media, which essentially contain besides major salts the sources for nitrogen (ammonium salt) and carbon (D-glucose), vitamins and trace elements. Although not strictly required in the absence of further auxotrophies, the remaining amino acids (20-n, if n amino acids are to be replaced) are commonly added to promote the bacterial growth. During an initial growth phase before induction of target protein expression, the n canonical amino acids to be replaced are added in limiting concentrations. Cellular growth proceeds until the targeted essential amino acids are depleted, as experimentally indicated by a stationary OD600. Subsequently, the culture medium is replaced by fresh medium that lacks the depleted amino acid and contains the ncAA in abundant concentrations. For the ribosomal incorporation of tryptophan analogs as shown in this protocol, an indole analog is fed, which becomes intracellularly converted to the corresponding tryptophan derivative by tryptophan synthase. Next, target protein expression is induced. At this stage, the cells are close to the end of logarithmic growth, as a balance between total cell number and fitness. As the presence and incorporation of the canonical amino would lead to wild-type protein production, it is critical to ensure that the essential amino acid is fully depleted prior to induction. Likewise, it is mandatory to examine the efficiency of ncAA incorporation into the target protein, commonly by mass spectrometry. In case of substantial presence of the canonical amino acid, the cultivation conditions need to be adjusted, e.g., by altering the concentration of the essential amino acid(s) for the initial growth phase or the duration of the latter. In case of low aaRS activity towards the ncAA, the overexpression of the endogenous enzyme or co-expression of a different aaRS, which is more active towards the ncAA, can be conducted.

The canonical amino acid Trp is endowed with three remarkable features: (i) its natural abundance in proteins is low; (ii) its biophysical and chemical properties are unique (e.g., it is usually the dominant origin of the intrinsic fluorescence of proteins and peptides), and (iii) it contributes to a variety of biochemical interactions and functions including π-stacking, H-bonding and cation-π interactions. All these features are radically changed upon Trp → 4-amino-Trp substitution in GdFP. Beyond doubt, the design of a "gold" class of avGFPs is a remarkable example for engineering tailor-made autofluorescent proteins. With distinct spectral properties, FPs can be tuned towards certain spectral windows via mutagenesis and ncAA incorporation. In case of GdFP, this is accomplished by a simple chemical exchange H → NH in the frame of the indole ring contained in the ECFP chromophore triad. Figure 5 displays the effects of ncAA incorporation within the chromophore. The introduction of the electron-donating group originating from 4-amino-indole (intracellularly converted to 4-amino-Trp) enables a variety of mesomeric structures that can explain a stabilized excited state. Spectroscopically, its enlarged Stokes shift and red-shifted fluorescence emission result from these distinct properties of the extended conjugated system. As reported earlier, the enhanced intramolecular charge transfer within the GdFP chromophore is inherently sensitive to pH (Figure 4B) and accompanied by a larger change in dipole moment between the S0 ground and S1 excited state relative to ECFP. As alternative electron-donating groups, tryptophan analogs bearing an indole ring substituted with hydroxy groups could be used, as reported in a comparative study with the model protein barstar. The absorption and fluorescence spectra of GdFP are broadened compared to ECFP and EGFP (Figure 3C and D). Homogeneous broadening of the absorption and fluorescence spectra is generally caused by vibrational modes in the chromophore and, additionally, by coupling of the chromophore to further vibrational modes present in the protein. The coupling to the local protein environment is supported by specific groups localized on the chromophore. As the structural inhomogeneity of the protein leads to local variations of the vibronic spectrum, such coupling between the vibronic spectra of the chromophore and the rest of the protein are supported by charge delocalization and mesosmeric states as indicated in Figure 5. This coupling also supports the large Stokes shift and necessarily reduces the fluorescence quantum yield. In comparison to other red-shifted FPs, GdFP even exhibits improved protein stability and a low tendency for aggregation. It not only differs in color from other FP variants but also exhibits a substantially increased thermostability and enhanced cooperative folding. Its fluorescence intensity is at least 90% preserved upon heating to 60 °C, while ECFP fluorescence is reduced to about 30%. In proteins, aromatic amino acids often contribute to networks of interacting side chains, which commonly have a stabilizing effect on the protein's tertiary structure. avGFPs harbors such a side chain network, which consists of the chromophore itself, as well as Phe-165, His-148, and Tyr-145. These side chains are not only quite rigid in the GdFP structure, but importantly, they form hydrophobic contacts with the chromophore. The most prominent novel feature identified in GdFP is that the amino-chromophore is more proximal to Phe-165. This interaction is a feature not observed in other known avGFPs. As the two residues are 3.2-4.5 Å apart, amino-aromatic interactions might be also present. Together with the amination-induced resonance stabilization of the chromophore, these most likely stabilize this hydrophobic network of amino acids in a cooperative fashion. A more effective intramolecular charge transfer might be supported by these interactions in the excited state in comparison to the ground state of the chromophore, and it at least partly accounts for the 108 nm Stokes shift.

In rational design of fluorophore properties, an increase in the size of the delocalized π-system is predicted to result in a red-shifted excitation wavelength. This rule of thumb is obeyed by the series of amino acids in position 66 leading to neutral chromophores: Phe (λmax = 395 nm) < His (λmax = 386 nm) < Tyr (λmax = 395 nm) < Trp (λmax = 436 nm). In nature, this extension of the chromophore's conjugated system of π-bonds has been achieved by different strategies. For DsRed from Discosoma striata, it is extended by the integration of an additional amino acid, thus shifting λmax to 573 nm. The chromophore of asFP955 (λmax = 595 nm) from Anemonia sulcata was extended by an imino group, enlarging its π-system. Since the chromophore of GdFP and other avFPs is of the same size, a different principle must entail an emission wavelength in the range of the expanded DsRed and asFP955 chromophores. The profound Stokes shift of 108 nm is attributed to the distinct structure of the GdFP chromophore, which reveals a new photophysical principle in the design of autofluorescent proteins. Preliminary calculations (as reported in ) have shown that the dipole moment of the excited-state chromophore of GdFP is substantially larger than in the ground state, in contrast to the respective values of ECFP. Whereas the dipole moment of GdFP increases from ~3 D (Debye) in the S0 state to ~15 D in S1, the change for the ECFP chromophore was rather moderate (from ~4 D to ~6 D). Thus, the unique golden fluorescence of GdFP is caused by substantial intramolecular charge transfer within the chromophore, which increases the variety of possible mesomeric structures (see Figure 5) that can alter resonance stabilization. This reduces the energy level from which emission occurs. As a consequence of the profound change in the dipole moment upon excitation, the intramolecular charge separation is the principal reason for the changes in the electrostatic potential of the chromophore environment. The surrounding protein matrix, in turn, adjusts to the changes in the charge distribution after chromophore excitation. The subsequent structural relaxation lowers the energy level of the excited chromophore, which shifts the fluorescence.
spectrum to the red due to its charge transfer character. For the same reason, as a consequence of the large Stokes shift and enhanced rates of radiationless processes, the fluorescence quantum yield of GdFP is reduced compared to ECFP.

The high quantum yield and small Stokes shift of ECFP and EGFP are usually ascribed to a rigid protein environment of the chromophore, which reduces the degrees of freedom and, consequently, internal conversion to favor the radiative relaxation of the excited state. Consequently, the molecular design of more rigidly embedded chromophores with reduced coupling to the remaining protein matrix might serve as a guide to produce farther red-shifted GFP derivatives with high fluorescence quantum yield. Therefore, for further engineering approaches to produce red-shifted autofluorescent proteins, enlargement of the π-electron system and a rigid chromophore structure with weak coupling to the protein environment is highly desirable. Such modifications could also be introduced either directly into GFP-based chromophores or by placement of desired ncAAs in the chromophore vicinity.

Disclosures

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