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Generating Cyan Fluorescent Protein (CFP) From Green Fluorescent Protein (GFPUV) By Y66w Mutation

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Abstract: *This work seeks to generate CFP from GFPuv by Y66W mutation. Specific objectives include site directed mutagenesis on GFPuv and sequence alignment of generated CFP with wild type GFPuv and fluorimetry studies of CFP in relation to GFPuv. Several approaches that include plasmid digestion and GFPuv ligation, colony PCR,super-competent cell transformation, sequencing, sequence alignment, Ni-NTA chromatographic purification and fluorimetry were employed. Results show that GFPuv was successfully mutated to CFP as indicated on position 65 – 67 of aligned sequence thus, a clear confirmation that Y66W mutation in GFPuv generates CFP. Fluorimetry report 22.5nm difference in peak fluorescence between the proteins implying there was a stock shift.*
Keywords: *CFP, Generating, GFPuv and Y66W mutation.*

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I. Introduction

The discovery of green fluorescent protein (GFP) by Japanese scientist, Osamu Shimomura has unsealed secrets in biotechnology and molecular sciences [1]. The most important property of this protein is bioluminescence which has proved its fundamental use in several fields of science. GFP is marginally green in colour but appears yellowish under tungsten light [2]. Natively, the protein consists of 238 amino acid sequence and 27KDA molecular weight [3]. The cylindrical shape of GFP falls among her most famous attributes however, the exclusive property of the protein remains the ability to be genetically modified without loss of bioluminescence thus, the generation of several variants that include cyan fluorescent protein (CFP).

Serine, tyrosine and glycine that respectively occupy position 65 – 67 are important residues in the chromophore which is the critical structural component of GFP. Substitution of these residues is key in CFP development [4]. In generating CFP, the tyrosine (TAT) residue in GFPuv is substituted for tryptophan (TGG) thus, the major difference between the two proteins. CFP provides many advanced features such as increased pH balance, suitability for stable transfection protocols and photo stability among other variants [5,6]. These attributes explain the use of CFP in Fluorescence Energy Resonance Transfer (FRET) experiments as well as fusion tag in many molecular research [7].

Generating CFP for various experiments is challenging to many scientists. This work seeks to generate CFP from GFPuv by Y66W mutation. Specific objectives include site directed mutagenesis on GFPuv and sequence alignment of generated CFP with wild type GFPuv and fluorimetry studies of CFP in relation to GFPuv.

II. Materials and Methods

2.1. GFPuv extraction, site directed mutagenesis and super-competent cell transformation

GFPuv was purified from pET23-*gfpuv* plasmid digested with 2µl Nde I and 3µl Hind III via agarose gel electrophoresis. All concentration of reagents were used as provided in manufacturer kits. The extracted GFPuv band was sub-cloned into pET28c vector and ligated into *E.coli* cells. Colony PCR confirmed the transformation thereafter 1.2% agarose GFPuv DNA extraction. Forward and reverse primers (1µl each) designed to contain tryptophan codon that is required for Y66W mutation of the DNA was assayed with 1µl KOD hot start polymerase, 2µl 25mM $MgSO_4$,2µl of GFPuv- pET28c template DNA, 5µl of 2mM dNTP mix, 5µl 10x PCR buffer and 33µl sterile water that made up a total volume of 50µl. PCR cycling was done in accordance with Stratagene Quick Change site-directed mutagenesis protocol. The resulting solution (1µl) was incubated at 37^{o}C for one hour then mixed with pre-chilled 2x 50µl Dpn1 XL1 super-competent cells thereafter, heat pulsed for 90 seconds at 42^{o}C. Undigested pET28c DNA (1µl) was used as the transformation efficiency control. The obtained aliquots were incubated with 500µl NZY$^+$ broth after which LB/Kanamycin selection was done [8].

2.2. DNA purification, sequencing and alignment

The transformed cells (1500µl) were centrifuged at top speed for 2 minutes. P1 and P2 (250µl) buffers were used to avoid clumping of residue while N3 buffer (350µl) was used to obtain supernatant by top speed spinning for 10 minutes. PE (0.75µl) and elution buffer (50µl) were used to extract plasmid DNA that was concentrated using ethanol precipitation protocol. The obtained DNA (15µl) was sequenced and aligned with wild type GFPuv sequence using Clustal Omega database.

2.3. BL21 (DE3) cells transformation, auto-induction, western blotting and fluorimetry

BL21 (DE3) cells of 0.297 *OD* were respectively suspended in ice chilled RF1 (RbCl 100mM, $MnCl_2.4H_20$ 50mM, K Acetate 30mM, Glycerol 15%, pH 5.8) and RF2 (MOPS 10mM, RbCl 10mM, $CaCl_2.6H_20$ 75mM, Glycerol 15%, pH 6.8). The resulting cells were transformed with 1µl pET28c-CFP plasmid DNA as described earlier ensuring 50µl of pure cells as the mock sample. Cell recovery was performed using 250µl SOC at 37°C for 30 minutes thereafter, LB/Kanamycin selection [8]. SB-5052 (900µl) with 100µl pre-cultured cells of 1.4 *OD* at 600nm made up the induced sample while 100µl pre-culture of 0.487 *OD* was the non-induced sample. The induced sample was spun to obtain pellets of which 0.651g was mixed with 2000µl bugbuster and re-suspended with 1.5µl DNAse1. The resulting solution was spun at 13000rpm to obtain the supernatant which was the soluble sample thereafter, the pellets were mixed with 2000µl binding buffer that was the insoluble sample. Samples (5µl each) were analysed on 12% polyacrylamide gel.The presence of CFP was also verified in a western blot experiment [9] thereafter, confirmed by Ni-NTA chromatographic purification of histagged CFP [10]. Purified sample was sent to the fluorimetry unit of the University of Leeds for analysis.

III. Results

Fig. 1showssequence alignment of CFP andGFPuv. Y66W mutation is indicated in yellow at position 65-67 of alignment report. Presence of tryptophan codon in CFP that contradicts tyrosine in GFPuv proves that the muation was succesful.

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CFP       ----------AATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACTT
GFPuv     ----------AATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACTT
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CFP       ACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACT
GFPuv     ACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACT
          ************************************************************

CFP       ACTTTCTCTTGGGGTGTTCAATGCTTTTCCCGTTATCCGGATCATATGAAA---------
GFPuv     ACTTTCTCTTATGGTGTTCAATGCTTTTCCCGTTATCCGGATCATATGAAA---------
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Fig. 1: Sequence alignment of CFP and GFPuv. Confirms Y66W mutation indicated in yellow.

Fig. 2 reportsemission spectra of CFPandGFPuv. Results show both CFP and GFPuv have an emission intensity of 1 but have peak fluorescence of 481.5nm and 504nm respectively.

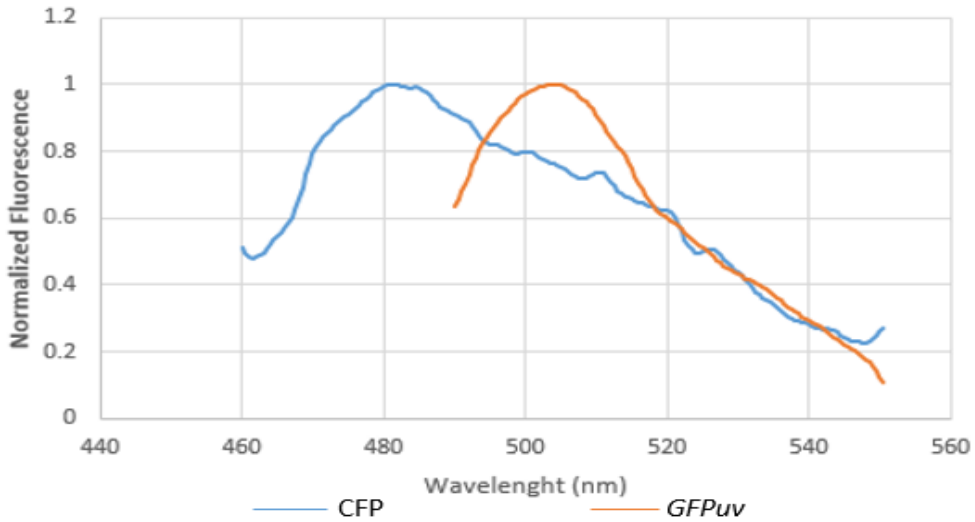

Fig. 2: Emission spectra of CFP and *GFPuv.* Shows peak fluorescence of 481.5nm and 504nm respectively.

IV. Discussion

Alignedsequence (Fig. 1) confirmed Y66W change as indicated by the double base pair mismatch in yellow within the open reading frame. This proves that residues 65 – 67 are critical in CFP development[4]. This result agrees with the findings of Shaneret al.(2005) who succesfully performed T65S single point mutation to arrive at a different variety of CFP [6]. The obtained result further agrees with Ai et al.(2006) that reported tryptophan (W) to be a major codon in CFP mutagenesis [5]. Tryptophan accounts for the blue shift in the absorption spectra and indole formation that improves luminosity thus, the edge of CFP in several biotechnological experiments [11,12].

Arbitrary allocation of maximum emission was used to normalize fluorescence in CFP and GFPuv (Fig. 2). An intensity of 1 was noted for both proteins however, at 504nm, GFPuv had its peak fluorescence as with 481.5nm in CFP. Peak emission observed in CFP is in accordance with the findings of Heim et al.(1994) who reported 480nm emission peak after performing similar experiment[13]. Hussain (2009) alsorevealeda fluorescence peak of about 480nm inhis Fluorescence Resonance Energy Transfer analysis which is in consonance with our result [14]. The 22.5nm difference in peak fluorescence between the proteins imply there was a stock shift which agrees with Heim et al.(1994) that observed changes in the emission peak of wildtype GFP having substitutedhistone for tyrosine[13].

V. Conclusion

This work is designed at passing the necessary knowledge for generating CFP from GFPuv. Although mass spectrometry analysis of both proteins is absent due to some challenges, this study reveals that substituting residues 65 – 67 of GFPuv with tryptophan will generate CFP and there is variation in peak fluorescence of both proteins which suggests a shift in the emission spectra.

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References

[1]. M. Zimmer, Green fluorescent protein (GFP): Applications, structure, and related photophysical behavior, *Chemical Reviews, 102(3)*, 2002, 759 -781.
[2]. R.Y. Tsien, The green fluorescent protein. *The Biochemical journal , 67*, 1998, 509 - 544.
[3]. M. Zimmer, GFP: from jellyfish to the Nobel prize and beyond, *Chemical Society reviews, 38(10)*, 2009, 2823 -2832.
[4]. S.G. Olenych, N.S. Claxton, G.K. Ottenberg, and M.W. Davidson, The fluorescent protein color palette, *Current protocols incell biology, 21*, 2007, 21.5.
[5]. H. Ai, J.N. Henderson, S.J. Remington, and R.E. Campbell, Directed evolution of a monomeric, bright and photostable version of Clavularia cyan fluorescent protein: structural characterization and applications in fluorescence imaging, *The Biochemical journal, 400(3)*, 2006, 531 - 540.
[6]. N.C. Shaner, P.A. Steinbach, and R.Y. Tsien, A guide to choosing fluorescent proteins,*Nature methods, 2(12)*, 2005, 905 - 909.
[7]. N.C. Shaner, Fluorescent proteins for quantitative microscopy: Important properties and practical evaluation, C. W. Jennifer and W. Torsten (Ed),*Methods in Cell Biology*, 123, (Cambridge: Academic press 2014) 95 – 111.
[8]. A.T. Tu, Transformation of Escherichia coli Made Competent by Calcium Chloride Protocol. *American Society for Microbiology*, 2013, 3 – 7.
[9]. S. Radulovic, J.M. Troyer, M.S. Beier, A. O. Lau, and A .F. Azad, Identification and molecular analysis of the gene encoding Rickettsia typhi Hemolysin, *Infection and immunity, 67(11)*, 1999, 6104 - 6108.
[10]. Babec. (2012). Purification of Green Fluorescent Protein (GFP) by Nickel Affinity Chromatography Student Guide. [Accessed 21 February, 2016]. Available from: www.babec.org.
[11]. J.Goedhart, D.von Stetten, M. Noirclerc-Savoye, M. Lelimousin, L. Joosen, M.A. Hink, L. van Weeren, T.W. Gadella Jr, and A. Royant, Structure-guided evolution of cyan fluorescent proteins towards a quantum yield of 93%. *Nat Commun, 3*, 2012, 751.
[12]. M.Lelimousin, M. Noirclerc-Savoye, C. Lazareno-Saez, B. Paetzold, S. Le volt R. Chazal, P. Macheboeuf, M. J. Field, D. Bourgeois, A. Royant, Intrinsic dynamics in ECFP and Cerulean control fluorescence quantum yield, *TheBiochemistry Journal, 48(42)*, 2009, 10038 - 46.
[13]. R. Heim, D.C. Prasher, and R.Y. Tsien, Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc. Natl. Acad. Sci*, USA, 1994, 12501–12504.
[14]. S.A. Hussain, An Introduction to Fluorescence Resonance Energy Transfer (FRET), *Energy, 132(6)*, 2009, 4.

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