Development of a CRISPR-cas12a Gene Editing System to Mutate GFP Gene to Produce BFP and CFP

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Introduction

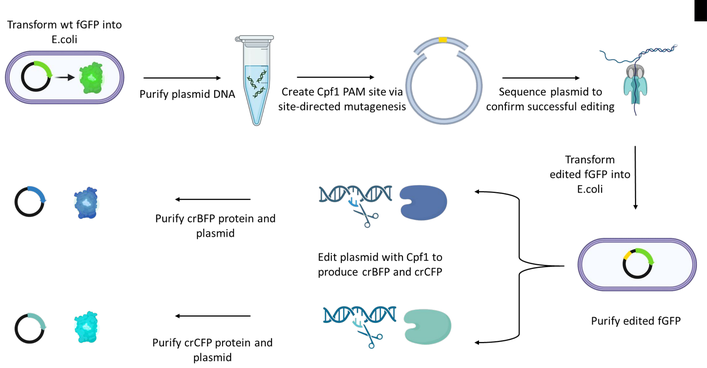
GFP is commonly used in molecular biology for reporter assays, cellular localization studies, fluorescence microscopy, FRET, and biosensors among numerous other applications. Directed mutations to the protein, particularly to the amino acids which form the central chromophore, produce proteins with different fluorescence characteristics. Two of these mutations, Y66H and Y66W, result in proteins which fluoresce blue and cyan respectively.

While educators have used a variety of techniques to mutate the GFP gene in an educational laboratory setting, the current project does so in a unique fashion. Specifically, modifying the GFP gene in vitro utilizing CRISPR/cas12a. CRISPR gene editing is a highly precise, direct, and straight forward method of introducing and modifying DNA sequence. While usually performed in living cells, in vitro gene editing of DNA outside of the cells (usually in the form of plasmids) is also highly effective. This method has been highly successful in editing the lacZ gene, which has been commercialized as the CRISPR in a Box educational kit. A similar approach of editing the GFP gene on a plasmid is described here.

With the high GC content of avGFP, we first performed the in vitro reaction using a CRISPR/cas9 system due to the presence of cas9 PAM sites located near the chromophore sequence. While the total number of successfully modified plasmids were low, the process yielded E. coli expressing BFP. Given the higher success rate of cas12a compared to cas9 in the lacZ system, we hypothesized a greater editing success rate of GFP with cas12a.

A different altered GFP gene (fGFP) was selected which has a higher AT content. A site-directed mutation of the fGFP gene introduced a cas12a PAM site as a silent mutation in the translated protein. sgRNA was designed to allow for the directed editing of fGFP using cas12a to produce a Y66H mutant (CRISPR produced BFP;crBFP). A protocol was developed to sequence the mutated plasmids using the Oxford Nanopore MinION. The newly developed crBFP, can be analyzed for fluorescent characteristics (excitation and emission spectra) by fluorescence spectroscopy. Together, this process touches upon genotype-phenotype associations, CRISPR gene editing, cloning, analytical analysis of proteins, among other key concepts of genetics, molecular biology, biochemistry, and analytical chemistry.

Methods



Results and Data

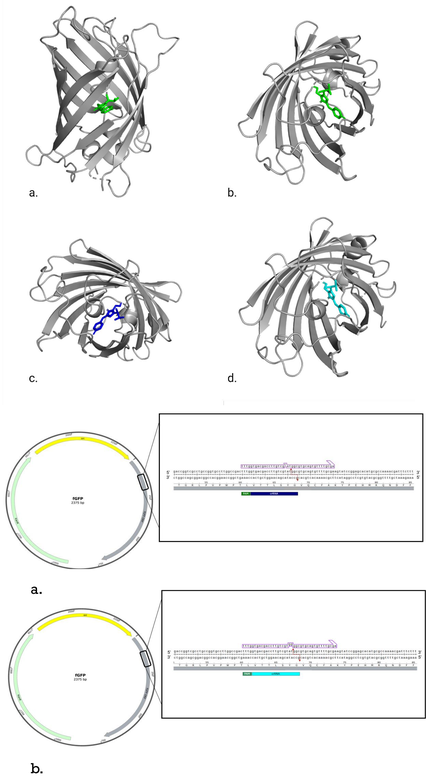


Figure 1. fGFP, BFP, and CFP Protein Structure. The single nucleotide changes made in this experiment change to fluorescence absorption and emission from green to blue and from green to cyan.

a. Depicts a barrel-up view of the protein structure and chromophore of wt GFP
b. Depicts a down-the-barrel view of the protein structure and chromophore of wt GFP
c. Depicts the protein structure and mutation phenotype of crBFP
d. Depicts the protein structure and mutation phenotype of crCFP

Figure 3. Cas12a gRNA, Cut Site and Corrective HDR Oligos Used to make T67 mutation(s) in fGFP Plasmid.

a. Depicts gRNA, cut site and corrective HDR oligos used to make Y66H mutation.
b. Depicts gRNA, cut site and corrective HDR oligos used to make Y66W mutation.

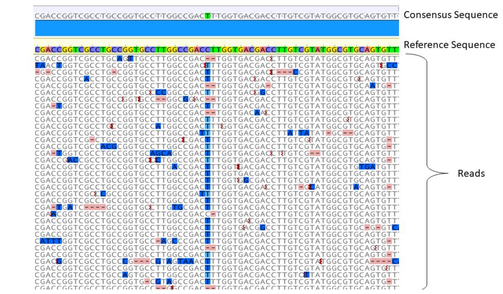
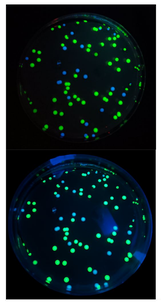


Figure 2. Sequence Verification. Verification of correct PAM site editing using Oxford Nanopore Technologies MinION.



	Total Colonies	Green Colonies	Correct Colonies	Grey Colonies
BFP+	75	60	12	3
BFP-	70	40	26	4
CFP+	9	4	5	0
CFP-	75	71	4	0

Figure 4. Growth of crBFP and CFP on Nutrient Agar

a. The top left picture depicts crBFP on nutrient agar.
b. The bottom left picture depicts crCFP on nutrient agar.
c. Table consists of numerical data on how many colonies contained successfully and unsuccessfully edited fGFP.

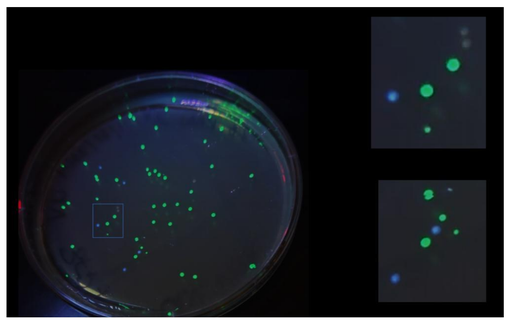


Figure 5. Genotypic and Phenotypic Outcomes: Phenotype. Editing of fGFP with CRISPR-cas12a results in three distinct genotypic and phenotypic outcomes.

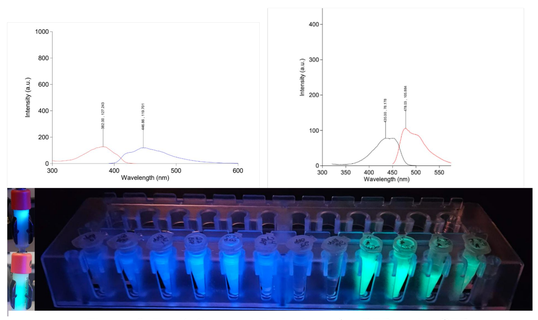


Figure 6. Excitation and Emission of crBFP and crCFP

a. Depicts excitation and emission spectra for crBFP (top left)
b. Depicts excitation and emission spectra for crCFP (top right)
c. Depicts purified crBFP and crCFP in varying concentrations.

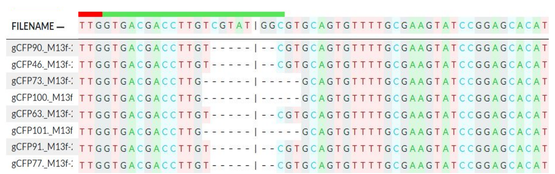


Figure 7. Genotype of Gray CFP colonies. Genotypic outcome of non fluorescent colonies after editing with CRISPR-cas12a to produce CFP. Image depicts INDELS in the region where the corrective HDR was designed to go. Sequence was analyzed using Decodr.

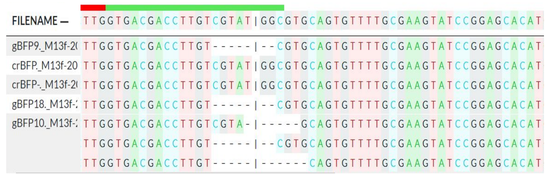


Figure 7. Genotype of Gray BFP colonies. Genotypic outcome of non fluorescent colonies after editing with CRISPR-cas12a to produce BFP. Image depicts INDELS in the region where the corrective HDR was designed to go. Sequence was analyzed using Decodr.

Conclusion

An *in vitro* CRISPR gene editing procedure was developed that allows for editing mEGFP to produce Blue Fluorescent Protein [BFP] and Cyan Fluorescent Protein [CFP] using a CRISPR cas9 system. However, the success rate was relatively low and the fluorescence of the BFP and CFP was difficult to observe using standard methodology.

To overcome the issues, the procedure was updated for editing fGFP using a CRISPR cas12a system. Although both forms of GFP produce fluorescent protein, there are some key differences between mEGFP and fGFP. fGFP expression is constitutive to a high level producing a greater amount of highly fluorescent protein which should also allow for production of higher levels of BFP and CFP fluorescence following editing.

Secondly, the fGFP gene sequence is more highly AT rich allowing for more readily located cas12a PAM sites. The mEGFP plasmid has a cas9 PAM site near Y66 but the specific Cas12a PAM site for fGFP had to be constructed via site directed mutagenesis [SDM]. As a result of the SDM, a plasmid sequence verification protocol was also developed based on with Oxford Nanopore sequencing technologies.

With the tools in place, next steps will be to complete the in vitro reaction to assess CRISPR-cas12a mediated gene editing fGFP to BFP as an enhancement to the previous CRISPR-cas9 editing of meGFP. This experimental procedure would facilitate teaching of CRISPR gene editing using a readily viewable and engaging fluorescent protein system.

Acknowledgements

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2. The "Methods" figure, along with Figures 4 and 6 were created with biorender.com

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