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FRET ANALYSIS OF GREEN AND ORANGE FLUORESCENT PROTEINS FOR THEIR USE AS POTENTIAL FLUORESCENT PROTEIN PROBES

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Abstract

Fluorescent proteins are critical to the fields of biosensing, imaging, and molecular tracking for their use as fluorescent probes. Through Förster Resonance Energy Transfer (FRET), it is possible to study the chromophores of fluorescent proteins and the interactions of proteins attached to them. This study investigates various green fluorescent proteins and their ability to display intramolecular and intermolecular FRET with the Yukon orange fluorescent protein. The results shown in this study identify promising pairs that can be used as fluorescent probes in FRET sensing.

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Introduction

Fluorescent proteins (FPs) took center stage as a way to study protein-protein interactions after Chalfie, Shimomura, and Tsien were awarded the Nobel Prize in 2008 for their discovery and development of the green fluorescent protein (GFP).[1] After the initial discovery and application of GFP, modified fluorescent proteins now span the entire visible range.[2] The extended spectrum of fluorescent colors paved the way for previously invisible biological structures and processes to now be visible. As a result, new applications reliant on these colors are constantly being used in studies such as receptor-ligand complexes,[3-6] determination of three-dimensional structures of proteins,[7-10] and aiding in the modification of drugs and nucleic acids for medicinal purposes.[11-15] A main advantage of using fluorescence proteins as fluorescent probes for protein studies is the ability to undergo Förster Resonance Energy Transfer (FRET) between a donor protein and an acceptor protein.[16-17]

FRET is typically used to determine the interaction between biomolecules and plays a major role in biosensors.[18-20] FRET is well-suited to the study of protein interactions because the distance between proteins falls within the ideal range for fluorophores; less than 10 nm away from each other.[21] Studies using FRET usually involve two differently colored fluorescent protein tags which can be covalently bonded or infused to the protein and used either intermolecularly[22-25] (Figure 1a) or intramolecularly[22, 26] (Figure 1b). While intermolecular FRET is achieved by simply mixing the two proteins, intramolecular FRET involves chemically cross-linked proteins.

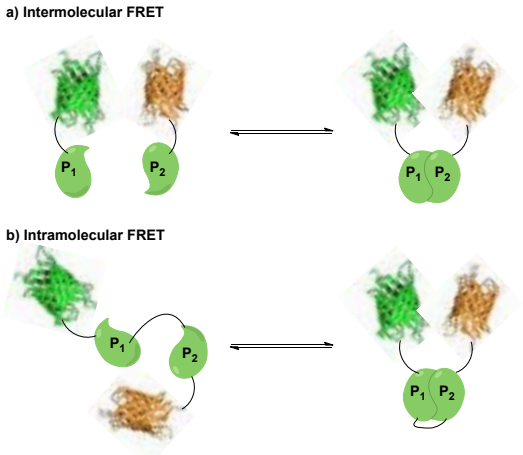

Figure 1: a) Intermolecular FRET between protein 1 (P1) and protein 2 (P2) where the donor and acceptor chromophores are on different molecules. b) Intramolecular FRET between P1 and P2 where the donor and acceptor chromophores are in the same molecule.

The process of chemical cross-linking involves chemically joining two or more molecules by a covalent bond via a cross-linking reagent possessing reactive ends for specific functional groups. Even though the process of cross-linking seems straightforward, identifying the products is challenging due to the complexity of the reaction mixture.[27] One way to overcome this challenge is to examine the products via mass spectrometry. In tandem with mass spectrometry, several strategies are currently being used to aid in the identification of the cross-linked products to include cleavable cross-linkers,[28-30] isotope-labeled cross-linkers/proteins,[31-33] and selective enrichment of the product via affinity purification.[34-36] These approaches allow access to the cross-linked product. However, because the cross-linked peptides are formed substoichiometrically, the large excess of non-cross-linked species leads to reduced yields of the product in the ion mass spectra[9, 37-39] and requires mass spectrometers with high sensitivity and high scan rates.[30] Fluorescence techniques provide an alternative approach to mass spectrometry.

When purified proteins are available, the ideal strategy for an unambiguous demonstration of protein-protein interactions in vitro is accomplished by chemical cross-linking.[40] Despite the complexity of protein structure, including composition with 20 different amino acids, only a small number of amino acids are used for chemical cross-linking. These reactive functional groups include primary amines via lysine (Lys, K), carboxyl via aspartic acid (Asp, D) and glutamic acid (Glu, E), and thiols via cysteine (Cys, C). Many cross-linkers have had great success in cross-linking proteins, and a variety of the cross-linking reagents are commercially available.[40] When considering cross-linking of FPs, the best FRET pairs require that the emission of the donor and excitation of the acceptor overlap well.[41] Recently we reported success with the cross-linking of green fluorescent proteins with an orange FP in the presence of glutaraldehyde.[42] This initial result prompted us to investigate green and orange FPs as likely pairs for intermolecular

FRET and the role cross-linkers play in the efficiency of FRET between the two FPs. Herein, we report intermolecular and intramolecular FRET of Yukon OFP with four green FPs. Additionally, we report on the success of the FRET pairs and discuss factors that impact cross-linking.

Experimental Methods

Reagents.

Chemicals were obtained from Sigma-Aldrich or Fisher Scientific unless specified. Synthesis of succinaldehyde was prepared as reported.[43] Plasmid DNA encodings were purchased from the following: modified teal fluorescent protein (mTFP0.7) (University of Alberta, Aquamarine fluorescent protein from CNRS, Orsay, France), enhanced consensus green protein (eCGP123) (Los Alamos), ultraviolet-exciting green fluorescent protein (GFPuv) (Clonetech), and Yukon orange fluorescent protein (OFP) (ATUM, Newark, CA). All proteins were isolated and purified as previously described.[42]

Fluorescence Measurements.

Fluorescence measurements were performed using a Greiner Bio-One 96-well microplate plate and a Synergy H4 Spectrofluorometer with a plate-reading accessory. Emission spectra were obtained by exciting at the appropriate wavelength to excite the green FP with minimal excitation of the OFP. Instrument parameters were optimized for each protein including adjustments of excitation and emission slit widths (9–20 nm), filters, and PMT tube voltage (50–150 V).

Preparation of Samples for Intermolecular FRET

The respective green FP (50 µg/mL) using protein concentrations calculated from a bovine serum albumin (BSA) assay were added to a 1.5 mL mini centrifuge tube. Potassium phosphate buffer (10 mM, pH = 7.0) was added to bring the final volume to 800 µL. Then Yukon OFP (50 µg/mL using protein concentrations calculated from a BSA assay) was added to the same tube of respective purified green FP. The solutions were shaken for 1 hour then 365 µL of samples of each solution were transferred to wells in a 96-well plate for analysis. Fluorescence measurements were taken at 370 nm for GFPuv and 420 nm for mTFP0.7, Aquamarine and eCGP123 and read for FRET at the OFP emission.

Cross-linking of Green FPs and Yukon OFP.

The same procedure was used as above except respective cross-linker solution (5% m/v) were added to facilitate cross-linking. Reactions were quenched with 50 µL of 1 M Tris then 365 µL of sample was transferred to the wells of the 96-well plates for analysis. Fluorescence measurements were taken at 370 nm for GFPuv and 420 nm for mTFP0.7, Aquamarine and eCGP123 and read for FRET at the OFP emission. Cross-linking was further verified by sodium dodecyl sulfate-polyacrylamide gen electrophoresis (SDS-PAGE) using a Bio-Rad Mini-Protean apparatus on 10% acrylamide Ready-Gels after staining with Coomassie Blue.

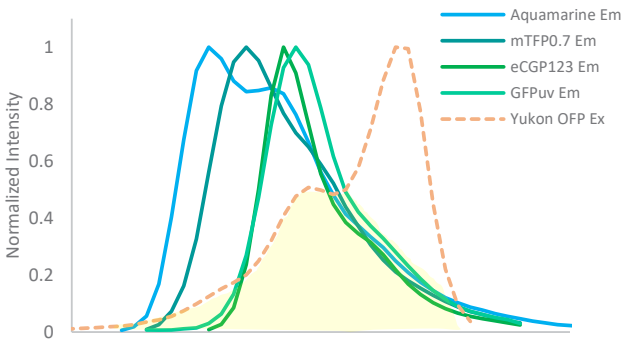

Figure 2: Emission spectrum of the green FPs (solid lines) and the excitation spectrum of the OFP (dashed line).

Results and Discussion

Spectroscopic Properties of FPs.

Efficient FRET pairs exhibit overlap between the emission of the donor and the excitation of the acceptor. Figure 2 compares the emission spectrum of the various green FPs and the excitation spectrum of Yukon OFP. All green FPs show extensive overlap with the OFP making them suitable candidates for this FRET study.

Synthesis of Cross-linked Proteins.

Equal concentrations of the respective green and orange FPs were combined in the presence of a cross-linking reagent. To determine the effect of the length of the cross-linkage chain on cross-linking efficiency, dialdehydes with increasing carbon backbone chain lengths were used. Figure 3 shows the cross-linkers investigated in this experiment. Difficulties of cross-linker 3 solubilty in the cross-linking reaction lead to the omission of this linker from this study.

Analysis of Intermolecular FRET.

To observe FRET spectroscopically it is necessary to excite the green FP in a range where the OFP is not directly excited, therefore, any emission of the OFP would have to come from resonance energy transfer from the excited green FPs. To determine the best wavelength to excite for the respective green FPs, the excitation spectra of the green FPs was compared with the excitation spectrum of the OFP (Figure 4). GFPuv was excited at 370 nm; Aquamarine, mTFP0.7 and eCGP123 were excited at 420 nm. We began our studies by investigating the extent of intermolecular FRET between the respective green FPs and OFP.

Emission spectra of the isolated GFP and equal concentrations of the GFPs and OFP in the absence of cross-linkers following excitation of the GFP are shown in Figure 5. All the protein mixtures

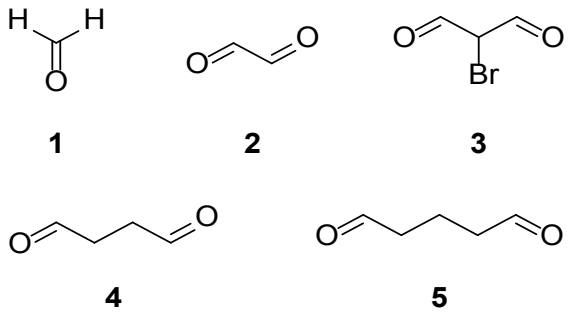

Figure 3: Aldehyde cross-linkers used in this study: formaldehyde (1), oxalaldehyde (2), 2-bromomalonaldehyde (3), succinaldehyde (4) and glutaraldehyde (5).

show a slight band at 570 nm, which corresponds to emission from the OFP. The weak bands in mTFP0.7 and Aquamarine mixtures likely arise from direct excitation of the OFP due to an overlap in the excitation wavelength with the tail of the OFP absorption. The eCGP123 and GFPuv solutions with OFP show higher emission from the OFP, indicating that FRET is occuring between the two proteins in solution. GFPuv shows a substantial amount suggesting it is a suitable canidate for doing intermolecular FRET.

Analysis of Intramolecular FRET.

To investigate the success of cross-linking the proteins to achieve intramolecular FRET, the efficiency of the cross-linked proteins were determined by fluorescence spectroscopy. If the cross-linking was successful, FRET would occur between the green and orange FPs as evident by an emission band at 570 nm. The emission spectra of the different green FPs with OFP in the presence of the different cross-linkers are shown in the Figure 6.

When examining cross-linked proteins, ideally the best FRET pair would have a decrease in the green protein emission and an increase in the OFP emission. From the results shown in Figure 6, mTFP0.7, Aquamarine and eCGP123 are the best FRET pair when cross-linked with 5. This result is further supported by kinetic analysis of the cross-linking process in which the orange emission increases and by SDS-PAGE where a faint new band can be seen around 50 kDA, the expected molecular weight for the

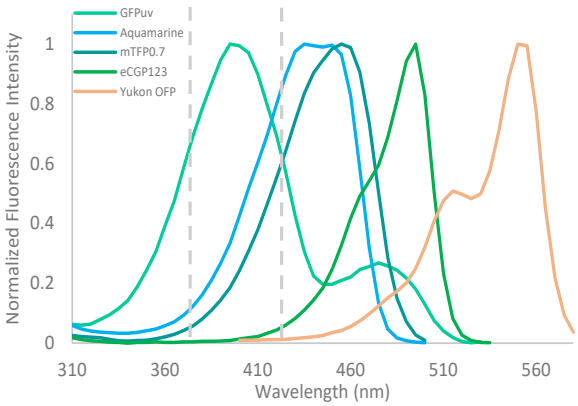

Figure 4: Excitation spectrum of green FPs and Yukon OFP. Dotted lined indicate excitation wavelengths for the respective green FPs to avoid direct excitation of OFP.

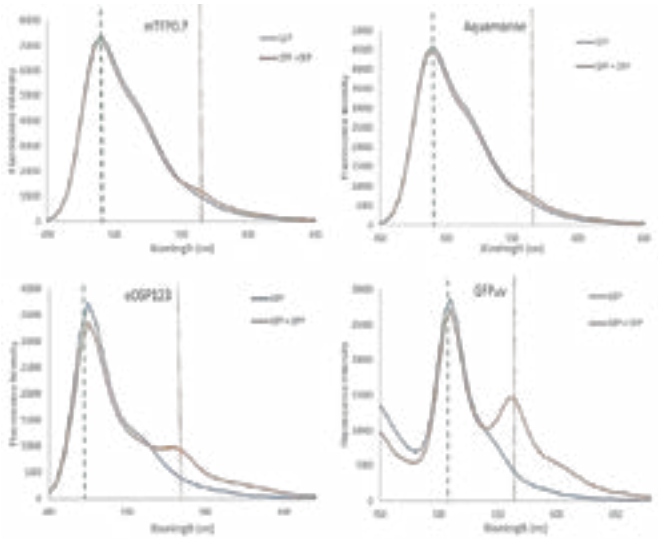

Figure 5: Emission spectra of equal concentration mixtures of green and orange FP in the absence of a cross-linker. Green dashed lines represent emission max of the green FP while orange dotted lines represent emission maximum of the OFP.

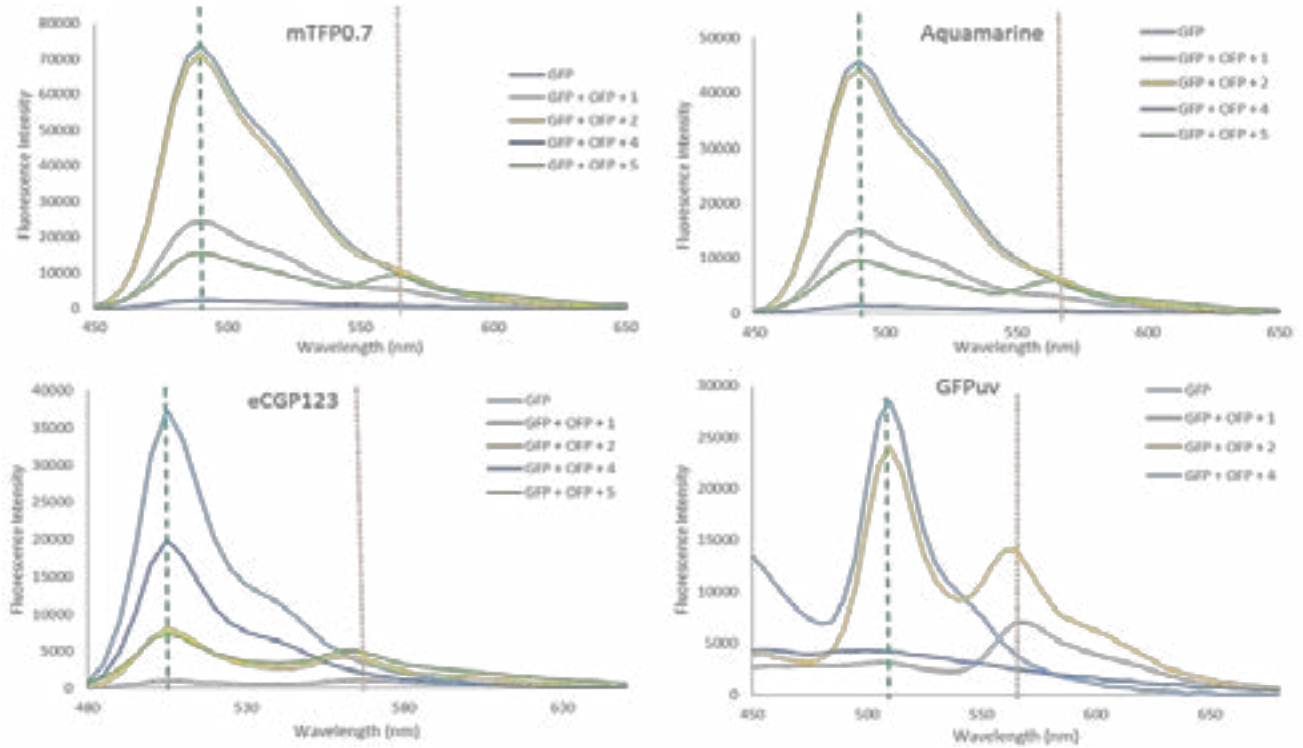

Figure 6: Emission spectra of cross-linked proteins after 1 hour of mixing. Green dashed lines represent emission max of the green FP while orange dotted lines represent emission maximum of the OFP.

respective cross-linked proteins.

GFPuv is an ideal candidate for cross-linking with OFP due to the low excitation wavelength (370 nm) used for FRET to OFP. For GFPuv, Figure 6 shows the best FRET pair with 1 as it appears that when GFPuv is cross-linked with 1 the greeen emission of GFPuv is completely gone, making it an idea canidate for this process. However, kinetic studies and SDS-PAGE do not support the notion of signifcant cross-linking between GFPuv and 1. Upon further evaluation, 1 is quenching the fluorescence of GFPuv and the other three green FPs as well. 4 is a quencher as well since all green emission is substantially or completely quenched for all four proteins. The results seen with GFPuv and 1 is more likely due to the orange emission coming from intermolecular FRET of unquenched GFPuv.

Conclusion

In summary, four different green FPs were investigated for intermolecular and intramolecular FRET with OFP. For intermolecular FRET, GFPuv shows the most promise. For intramolecular FRET, under this procedure, the best cross-linker was glutaraldehyde for mTFP0.7, Aquamarine and eCGP123 as indicated by fluorescence studies, kinetic data and SDS-PAGE.

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