Stability of Super Folder Green Fluorescent Protein Under Varying Organic Solvent and Temperature Conditions

by
Lylan Ho

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science
(Honors Scholar)

Presented May 31, 2019
Commencement June 2019

AN ABSTRACT OF THE THESIS OF

Lylan Ho for the degree of Honors Baccalaureate of Science in Biochemistry and Molecular Biology presented on May 31, 2019. Title: Stability of Super Folder Green Fluorescent Protein Under Varying Organic Solvent and Temperature Conditions

Abstract approved:_____

Wei Kong

Obtaining high-resolution structures of biological macromolecules has become one of the biggest challenges in the scientific world. Dr. Wei Kong's lab is currently working to overcome the limitations of current structure resolution techniques, such as x-ray crystallography and nuclear magnetic resonance spectroscopy. Kong's group hopes to develop a new protein imaging method, called single-molecule serial electron diffraction imaging, that can be applied to determine protein structures without the need for crystallization and with no limitation on protein sizes. However, this protein imaging technique, like many other analytical techniques, requires exposing protein samples to unideal, non-native conditions. Understanding how these types of environments can alter protein conformations is important for determining the conditions that will optimize the reliability of the protein structure model that is obtained. Here, fluorescence measurements of super folder green fluorescent protein—an ultra-stable strain of green fluorescent protein engineered in 2006—were taken under extreme temperature and organic solvent conditions in order to assess its level of denaturation and aggregation within the conditions of single-molecule serial electron diffraction imaging. Our findings suggest that super folder green fluorescent protein tends to aggregate in the presence of acetonitrile and denature in the presence of methanol. The

protein is also capable of complete renaturation after exposure to temperatures up to 80ºC, and it retains 90% of its fluorescence in both organic solvent conditions at 80ºC for ≥4 s. This information is valuable for Kong's group as they continue to develop their protein imaging method and also provides insight into the structural equilibria of proteins when placed in non-native conditions.

Key Words: Super folder green fluorescent protein, protein structures, single-molecule serial electron diffraction imaging, non-native conditions, fluorescence measurements

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APPROVED:

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Lylan Ho, Author

1. Introduction

Developing new techniques to determine protein structures is a major research area in chemistry and biochemistry. Although the scientific world has made huge strides in understanding the chemical make-up of humans by sequencing the human genome, there is still much that is unknown about the three-dimensional structures of proteins. Understanding these structures and their dynamics has many applications and is crucial for elucidating the ways in which biological activity and protein targeting is regulated.[1] For example, by obtaining detailed protein structures, the structure-function relationship of proteins that contribute to human disease can be better understood, making it possible to tailor medications more specifically to target proteins.[2] Currently, the predominant techniques for protein structure determination are x-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy.[3,4]

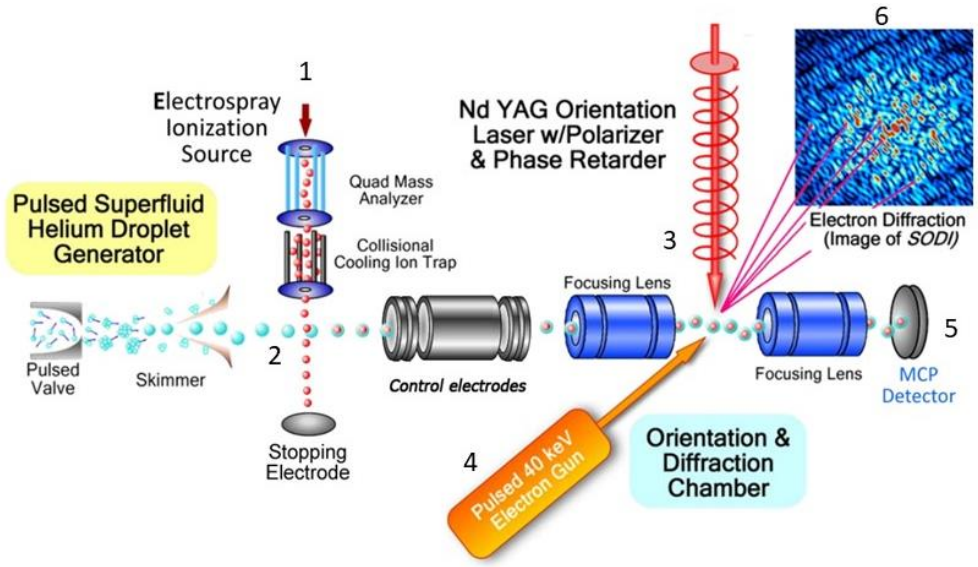
Although these techniques have expanded our ability to obtain high-resolution protein structures, significant limitations exist that prevent these methods from being applied to the vast majority of proteins. X-ray crystallography involves the collection of a series of diffraction images of a crystallized protein.[3] Obtaining a single crystal of a pure protein can be extremely challenging, requiring significant technical skill and some luck. Furthermore, many proteins are simply impossible to form crystal structures from. When a crystal is successfully obtained, x-ray crystallography only provides an image of a fixed protein conformation, which provides limited information about the protein's dynamics.[5] This snapshot of a protein in a fixed conformation may or may not reflect a conformation that the protein would take in its native state. In comparison, NMR is capable of investigating protein structures by applying a magnetic field to a protein sample in solution, thus providing a better representation of protein dynamics. However, a loss of

resolution occurs at larger protein sizes of approximately ≥35 kDa, and proteins larger than 50 kDa are not amenable to imaging by NMR at all.[4]

My research mentor, Dr. Wei Kong, and her research group are working to overcome the limitations of these structure resolution techniques by developing a method called single-molecule serial electron diffraction imaging (SS-EDI) that can be applied to develop protein structure models without the need for crystallization and with no limitation on protein sizes. The method requires that samples be prepared through electrospray ionization (ESI), then embedded in superfluid helium droplets. Electric-field induced alignment orients the molecules in a uniform direction, and a high-energy electron source can be used to obtain diffraction images from the millions of oriented sample molecules. Images of proteins oriented at different angles can be taken continuously by changing the polarization properties of the laser, and eventually sufficient information can be obtained to produce a 3-D image.

In order to be analyzed with this technique, the protein under study must survive the conditions of ESI, including high temperatures and low pH during the spray process, which lasts on the order of micro- to milliseconds. The protein sample will also be exposed to a significant concentration of organic solvent. These extreme conditions, although only applied for a short period of time, could potentially cause the protein to denature or aggregate before its image can be captured, thereby diminishing the representativeness or utility of the final image.[6]

a.



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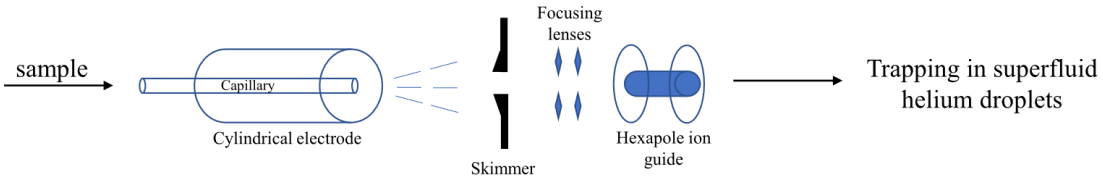


Figure 1: Experimental scheme of single molecule serial electron diffraction imaging and electrospray ionization. Dr. Wei Kong's experimental set-up for SS-EDI (a) includes (a1) the preparation of sample through ESI, then (a2) embedding the sample in superfluid helium droplets. (a3) Field induced alignment orients protein molecules via a laser, and (a4) a high energy electron source is used to obtain diffraction images. (a5) A diffraction image is developed from millions of sample molecules all oriented in the same direction. Electrospray ionization (b) places the protein samples under high temperatures, low pH levels, and moderate to high concentrations of organic solvents.

Similar to SS-EDI, many other analytical chemistry and biochemistry techniques involve exposing proteins to extreme environmental conditions. Understanding how factors such as organic solvent level, temperature, and pH can affect the equilibrium between stable, denatured, and aggregated protein conformations (Figure 2) is important for determining the relevant conditions that are optimal to the protein and the analytical technique being used. Examining this equilibrium under these conditions is limited in part by the fact that most enzymes act on chemical substrates that may not be accessible to enzyme active sites under solvent conditions that cause

denaturation or aggregation. Fortunately, green fluorescent protein (GFP) and its many unique chemical properties makes this type of analysis possible.

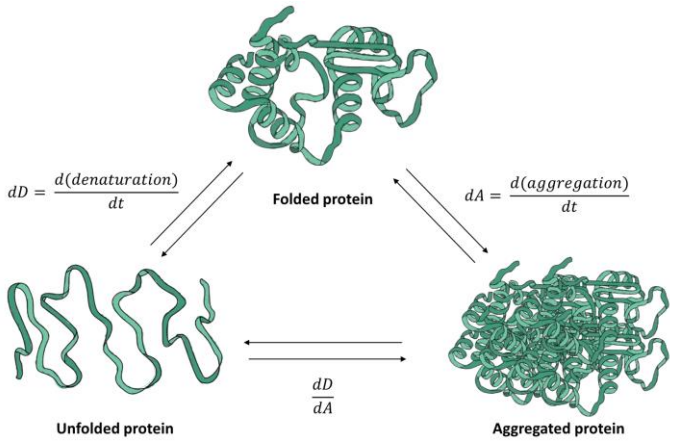


Figure 2: Chemical reaction scheme for folded, aggregated, and unfolded protein conformations. sfGFP, and any other protein, in solution is dynamic and constantly in changing in conformation. In native environments, the protein molecules are most often in a folded and functional state. However, this equilibrium can be shifted towards unfolding or aggregating when environmental factors are altered, such as increasing temperatures or the addition of organic solvents.

GFP was first discovered in *Aequorea victoria* jellyfish as a protein that interacts directly with aequorin, a chemiluminescent protein that emits blue light. GFP is 28 kD in size and can exist in a monomeric or dimeric form. Its structure consists of 11 β-strands that form a barrel structure through which an α-helix is threaded. This α-helix bears the protein's chromophore, which is composed of residues Ser, Tyr, and Gly. This chromophore is capable of absorbing blue light and converting it into fluorescent green light, which is reemitted by the protein.[7] This important ability to respond efficiently to external blue light stimuli has made GFP one of the most widely used proteins in biological and biochemical research, with applications in *in vivo* gene visualization,[8] fluorescence microscopy,[9] and fluorescence resonance energy transfer[10] (Figure 3). Beyond its highly efficient chromophore, the protein has low cytotoxicity, making it harmless to work with and heterologously express in new host organisms.[8] Most importantly, GFP is capable of autocatalyzing its own chromophore, requiring only oxygen to complete its synthesis.[11] Therefore, no extra substrates must be provided for GFP to exhibit activity. The activity of this chromophore

can thus easily be imaged and quantified through fluorescence measurements, without the need for a chemical substrate to physically access the active site. For our studies, we used a strain of GFP named superfolder green fluorescent protein (sfGFP), which was engineered by Pédelacq et. al[12] in 2006 to have enhanced folding capabilities and structural stability (Figure 3a).

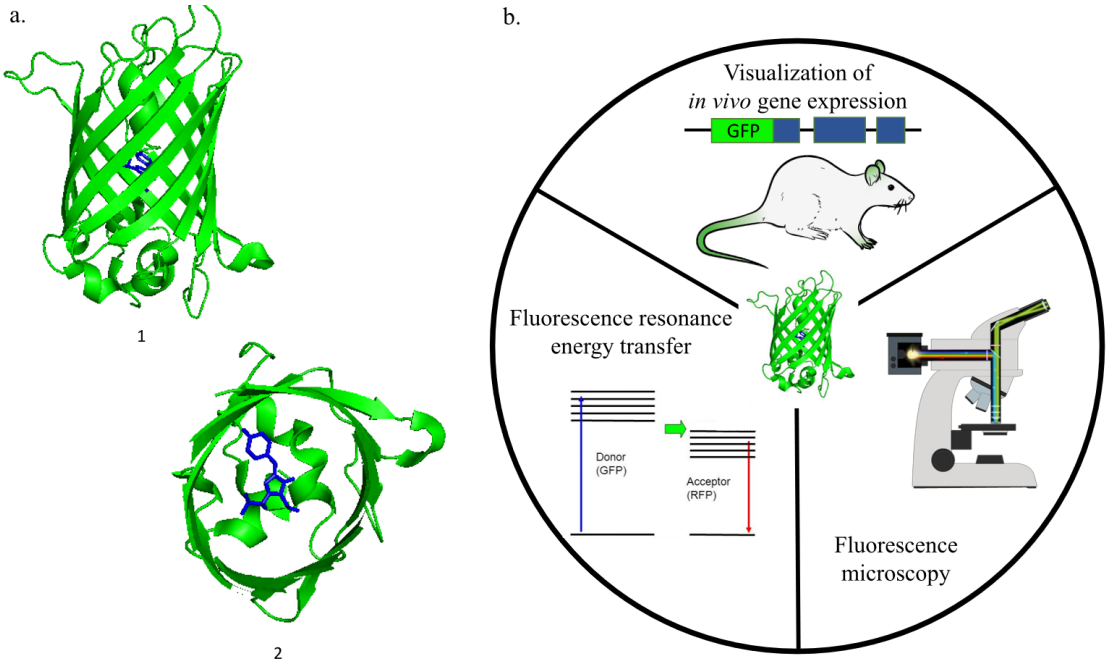


Figure 3: Structure and applications of green fluorescent protein. Superfolder green fluorescent protein (a) is composed of 11 β-strands which form a cylindric shape. An α-helix is threaded through the middle of the cylinder and contains the molecules chromophore (highlighted in blue) which is composed of Ser, Tyr, and Gly. (b) Some of GFP's major applications are shown. Fluorescence resonance energy transfer can be used to measure distances between fluorophores, allowing for the analysis of protein-protein interactions.[10] GFP can also be used as a gene marker to visualize gene expression *in vivo*, and this can be visualized on a microscopic level using fluorescence microscopy.[8,9]

Many previous studies have examined GFP's stability in varying conditions. It has been shown to be resistant to heat and alkaline pH levels.[13] It has also been found to remain stable to temperatures up to 100ºC in glucose-based solutions.[14] Here, we examine the stability of sfGFP under specific temperature and organic solvent conditions—particularly methanol and acetonitrile—that are expected to contribute to its structural equilibria under the conditions of SS-

EDI. This information will serve as a guideline for Kong's group as they continue to develop their protein imaging method and will also provide insight into the folded, denatured, and aggregated protein equilibrium reaction.

2. Materials and Methods

2.1 Materials. All reagents, purification columns, and desalting columns were supplied by Dr. Ryan Mehl's laboratory in the Biochemistry and Biophysics department at OSU. Fluorescence measurements were performed using a slightly customized fluorimeter built in the Colin Johnson lab with parts purchased from Photon Technology International. Fluorimeter filters were purchased from Thor Labs. A microcuvette and corresponding cuvette holder were purchased from Hellma Analytics.

2.2 Expression and Purification of sfGFP. pBad plasmid containing a gene encoding for sfGFP with a C-terminal 6xHis tag was transformed through $CaCl_2$ chemical transformation into a DH10B strain of *E. coli*. Starter cultures of these *E. coli* DH10B cells were grown in non-inducing media prepared based on protocols by Studier et. al.[15] Growth conditions included constant shaking at 250 rpm at 37ºC in 10-mL breathable capped culture tubes for a duration of 19 hours.

Starter culture was used to inoculate 50 mL of autoinduction media for expression.[15] Ampicillin (100 mg/mL) was added as a selection pressure, and the culture was grown in a 250-rpm shaker at 37°C for 25 hours until the optical density following a 10-fold dilution reached approximately 5.0. Cells were pelleted by centrifugation at 5525 rcf for 10 minutes at 4 ºC. Following centrifugation, the supernatant was carefully decanted, and the dry pellet was stored at -80°C until the following day.

In order to isolate sfGFP, the cell pellet was first resuspended by vortexing in 5 mL of lysis buffer (30 mM HEPES, 300 mM NaCl, pH 7.4) then lysed at 18,000 psi in a Microfluidics M-110P microfluidizer. The microfluidized sample was centrifuged at 5525 rcf for 20 minutes at 4°C to remove large cellular debris through pelleting. BD TALON cobalt affinity resin (500 µL total, 250 µL bed volume) was washed twice with lysis buffer then incubated with sample supernatant while gently rocking (<10 rpm) at 4°C for 30 minutes. Following incubation, resin-bound sample solution was poured into a Thermo Fisher Scientific elution column and washed three times with 10 mL of wash buffer (30 mM HEPES, 300 mM NaCl, 5 mM imidazole, pH 7.4). Sample that remained on the resin was eluted with 1 mL of elution buffer (30 mM HEPES, 300 mM NaCl, 250 mM imidazole, pH 7.4). A GE Life Sciences PD-10 desalting column was used to transfer eluted protein sample into imidazole-free lysis buffer to remove imidazole as a possible source of confusion during fluorescence imaging.

2.3 Protein quantification. Protein concentrations were estimated using the Bio-Rad Bradford protein assay method with bovine serum albumin as a standard. Samples were uniformly diluted to 50 µM in lysis buffer and kept at 4ºC for long-term storage.

2.4 Fluorescence of sfGFP in organic solvents. The fluorescence spectra of sfGFP were examined after being diluted in either acetonitrile, methanol, a 1:1 mixture of acetonitrile and deionized water (AcN:H_2O), or a 1:1 mixture of methanol and deionized water (MeOH:H_2O). Samples were prepared by diluting 50 µM sfGFP in lysis buffer with the organic solvent of interest. Samples of sfGFP were prepared in increasing v/v percentages of acetonitrile or AcN:H_2O: 0%, 8.8%, 17.6%, 26.4%, 35.2%, 44%, 52.8%, 61.6%, 70.4%, and 79.2% as well as in increasing concentrations of methanol or MeOH:H_2O: 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%. These increasing concentrations of organic solvent corresponded with decreasing concentrations of

sfGFP: 50, 45, 40, 35, 30, 25, 20, 15, 10, and 5 µM. All samples were prepared to a final volume of 100 µL in a 1.7 mL Eppendorf microcentrifuge tube. Samples were transferred to a reusable quartz microcuvette with a cross-sectional area of 3x3 mm^2. The cuvette was held fixed in the fluorimeter by a cuvette holder. The fluorimeter chamber temperature was held at 25ºC during and between each reading. The excitation wavelength for all readings was set to 488 nm, which is the dominant excitation wavelength of sfGFP. The fluorimeter receiver channel was programmed to scan for emission peaks across the range 400 nm to 600 nm for all samples and solvent conditions. All samples were prepared immediately prior to fluorescence readings. After completion of fluorescence measurements for each sample, solution was vacuum aspirated out of the cuvette, and the cuvette was subsequently rinsed with deionized water. Samples were analyzed in biological replicate of $n_b = 2$, with two technical replicates performed for each biological replicate ($n_t = 2$). Diluted sfGFP in lysis buffer was used as a reference standard in all cases, and samples of acetonitrile and methanol without sfGFP were measured to control for intrinsic optical differences between media.

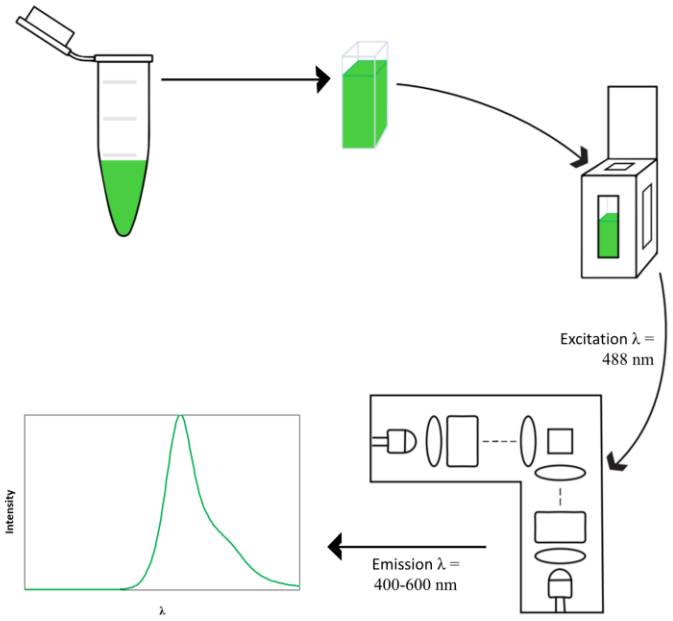


Figure 4: Experimental scheme for fluorescence data collection. Samples were prepared in a microcentrifuge tube immediately prior to fluorescence readings. These samples were transferred into a cuvette and the corresponding cuvette holder, then inserted into the fluorimeter. The excitation wavelength was set to 488 nm, and emission peaks were recorded from 400-600 nm.

2.5 Comparison of emission spectra of sfGFP in acetonitrile at excitation wavelengths of 488 nm and 600 nm. To explore the extent of scattering by large sfGFP particles in the various solvents, excitation was also performed at 600 nm. Samples of sfGFP were prepared in increasing v/v percentages of acetonitrile: 0%, 8.8%, 17.6%, 26.4%, 35.2%, 44%, 52.8%, 61.6%, 70.4%, and 79.2% as well as in increasing concentrations of methanol: 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%. These increasing concentrations of organic solvent correspond with decreasing concentrations of sfGFP: 50, 45, 40, 35, 30, 25, 20, 15, 10, 5, and 0 µM. The emission spectrum following excitation at 600 nm was taken as a baseline for scattering intensity, as this is outside of sfGFP's excitation range. Emission peaks were scanned for across the range of 450 nm to 800 nm. The difference in emission values after excitation at 600 and 488 nm can be attributed to excitation and fluorescence of the sfGFP chromophore. The scattering intensity measured after excitation at 600 nm can theoretically be used as a proxy, or at least a partial measurement, of aggregation effects. This experiment was also done with lysis buffer as a reference standard.

2.6 Denaturation and recovery of sfGFP at varying temperatures. Samples of sfGFP at 50 µM and 1 µM in lysis buffer were excited at 488 nm and their emission spectra were recorded at increasing temperatures of 25ºC, 40ºC, 60ºC, and 80ºC while keeping the same sample in the fluorimeter throughout. Samples were subsequently cooled to their starting temperature and their emission spectra were recorded again at the same temperature points in reverse order: 80ºC, 60ºC, 40ºC, and 25ºC. In total, each sample remained in the fluorimeter for approximately 30 minutes for a full set of measurements. Each concentration was analyzed in biological replicate of $n_b = 2$, and two technical replicates were performed for each biological replicate ($n_t = 2$).

2.7 Time-dependent effects of temperature on sfGFP. Samples of sfGFP at 50 µM in lysis buffer were placed in the fluorimeter at three set temperatures—25ºC, 50ºC, and 80ºC—for 200 s each.

Emission intensity at the 518-nm peak was recorded in one-second intervals, which was the time resolution limit of our fluorimeter. Samples were placed into the chamber 5 seconds before the first intensity reading started. This procedure was repeated for 30 μM and 1 μM sfGFP that had been diluted from 50 μM by methanol, acetonitrile, MeOH:H_2O, or AcN:H_2O. Each condition was analyzed in biological replicate of $n_b = 2$.

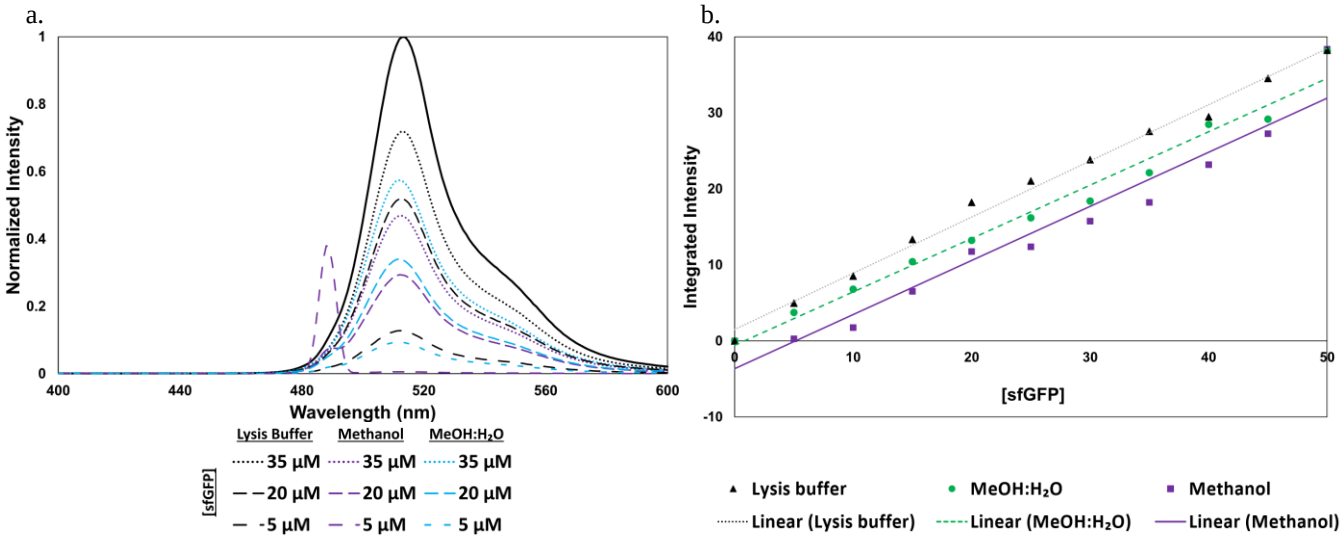
3. Results

3.1 Expression and Purification of sfGFP. A pBad expression plasmid containing a gene that encodes for sfGFP allowed us to express and purify a high yield of the protein for experimental use. The plasmid that encodes for sfGFP also contains a gene for β-lactamase, which confers ampicillin resistance. By growing our culture in the presence of ampicillin, we ensured that only cells containing the pBad plasmid expressing sfGFP were selected. sfGFP was successfully expressed and purified multiple times throughout the course of this project, with concentration yields ranging from 84 μM to 390 μM as growth and expression procedures were optimized.

3.2 *Effects of methanol on sfGFP fluorescence.* Emission spectra for sfGFP diluted by methanol and MeOH:H_2O are plotted in Figure 4a. Two technical replicates per biological replicate were collected, equaling a total of four spectra collected for each condition. These spectra were averaged, then normalized to the intensity peak of 50 μM sfGFP in lysis buffer taken at the time of each experiment. Integrated intensities of the normalized spectra are shown in Figure 5b in comparison to dilution with lysis buffer. The reference standard of 50 μM sfGFP in lysis buffer was chosen because it is concentrated enough to be easily measured by the fluorimeter but without oversaturation in signal or significant background aggregation of the protein in aqueous solution. Using this standard concentration, we were able to clearly observe the decrease in fluorescence

intensity due to interaction with organic solvent as methanol or MeOH:H_2O was added to the protein solution.

As methanol concentration increases, the intensity of sfGFP fluorescence decreases linearly (Figure 5b). Intensity values at each concentration of sfGFP are also significantly lower than the corresponding concentrations of sfGFP diluted by lysis buffer. This effect is partially reduced when methanol is diluted by water (Figure 5b). At the highest concentrations of methanol, when sfGFP concentrations range from 10 µM to 1 µM, the excitation peak at 488 nm becomes approximately 4x more prominent (Figure S1). However, this trend is not present for sfGFP in MeOH:H_2O.



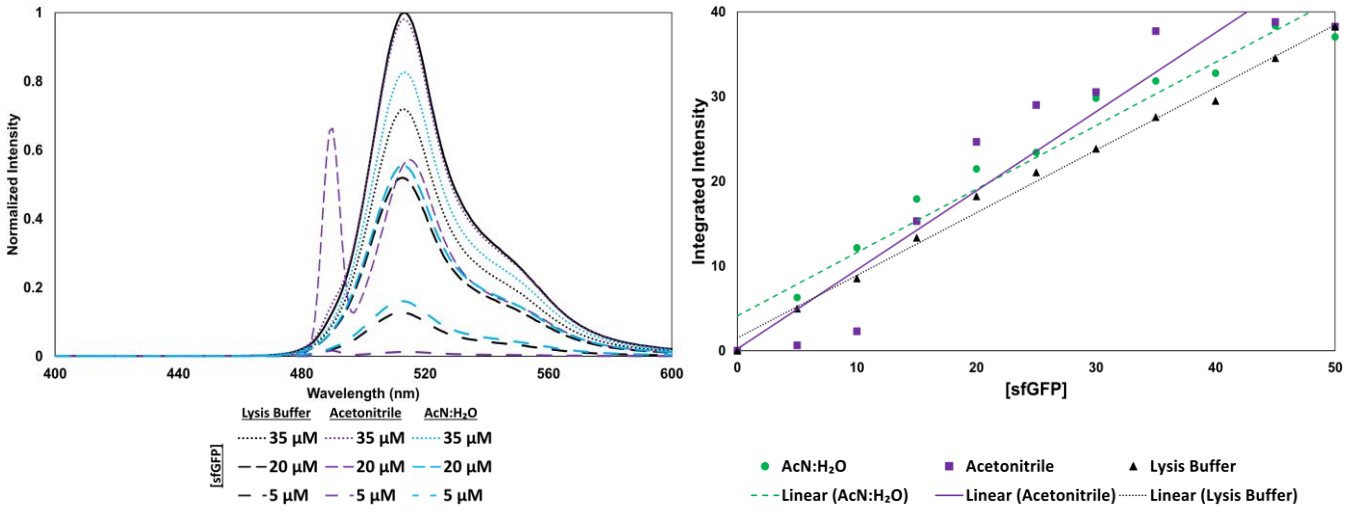
** All spectra were normalized to 50 µM sfGFP in lysis buffer, which is included as a reference standard*

Figure 5: Effects of Methanol on sfGFP Fluorescence. (a) Emission spectra of sfGFP diluted to 35 µM, 20 µM, and 5 µM by methanol and MeOH:H_2O are shown relative to sfGFP diluted by lysis buffer. A standard emission spectrum of 50 µM sfGFP in lysis buffer, to which all displayed spectra are normalized, is also included. (b) Integrated emission peak trends for sfGFP diluted with methanol and MeOH:H_2O are shown in comparison to those obtained by dilution with lysis buffer. Decreasing concentrations of sfGFP correspond to increasing concentrations of the diluent used.

3.3 *Effects of acetonitrile on sfGFP fluorescence.* Emission spectra for sfGFP diluted by acetonitrile and AcN:H_2O are plotted in Figure 6a. Integrated intensities of the normalized spectra

are shown in Figure 6b in comparison to dilution with lysis buffer. The decrease in fluorescence as acetonitrile concentration increases was observed as a linear trend, with a coefficient greater than 1. At 8.6%-61.6% v/v acetonitrile, the observed intensity was higher than the intensities at the corresponding concentrations of sfGFP in lysis buffer. At higher concentrations of acetonitrile (≥70.4% v/v), the intensity values fell below those of the corresponding sfGFP samples that were diluted by lysis buffer.

a. b.



** All spectra were normalized to 50 µM sfGFP in lysis buffer, which is included as a reference standard*

Figure 6: Effects of Acetonitrile on sfGFP fluorescence. (a) Emission spectra of sfGFP diluted to 35 µM, 20 µM, and 5 µM by acetonitrile and AcN:H_2O are shown relative to sfGFP diluted with lysis buffer. A standard emission spectrum of 50 µM sfGFP in lysis buffer is also included. (b) Integrated emission peak trends for sfGFP diluted with acetonitrile and AcN:H_2O are shown in comparison to sfGFP diluted with lysis buffer. Decreasing concentrations of sfGFP correspond to increasing concentrations of the diluent used.

3.4 *Aggregation and denaturation effects of acetonitrile and methanol.* While preparing samples for testing, aggregation of sfGFP could be observed by eye at concentrations of ≥61.6% v/v acetonitrile (Figure 7c). At these high concentrations, there was an immediate separation of the two solutions upon mixing. In high concentrations of methanol, aggregates of sfGFP also

appeared in solution (Figure 7c). However, in comparison to the aggregates in acetonitrile, aggregates formed in methanol were much smaller and appeared to be a lighter, more muted shade of green. They also appeared to be flaky in shape rather than globular (Figure 7c). Secondly, aggregate formation in methanol did not occur immediately upon mixing. Only after letting the methanol-containing mixture sit overnight at 4ºC were we able to observe aggregation. Scattering intensity measured after excitation at 600 nm can theoretically indicate aggregation for sfGFP in acetonitrile and methanol, since the intensity of scattered light correlates with particle size (Equation S6). As a control, this experiment was also repeated for sfGFP diluted by lysis buffer.

For each solvent condition, there was no apparent emission peak at any of the concentrations examined. However, there was clear variation in the size of the excitation peak. For sfGFP in lysis buffer, the intensity of the excitation peak remained relatively constant, with a slight decrease in size at lower concentrations of sfGFP ranging from 5 µM to 15µM. In the presence of organic solvent, however, there was no clear trend in emission peak size. At concentrations of sfGFP at 20 µM or below, there were some significant spikes in excitation peak size (Figure 7b). The highest excitation peak observed out of all three sets of data was for 20 µM sfGFP diluted by acetonitrile which was approximately 7x larger than the excitation peak observed for our standard 50 µM sfGFP sample in lysis buffer. All excitation peaks were normalized to this highest peak value. For methanol, the highest excitation peak observed was at 10 µM sfGFP.

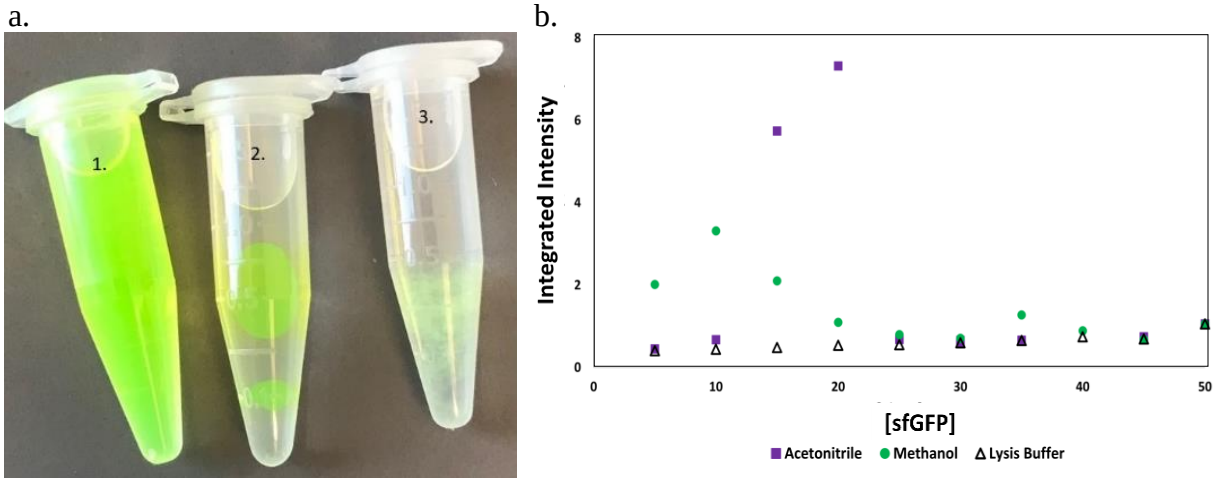
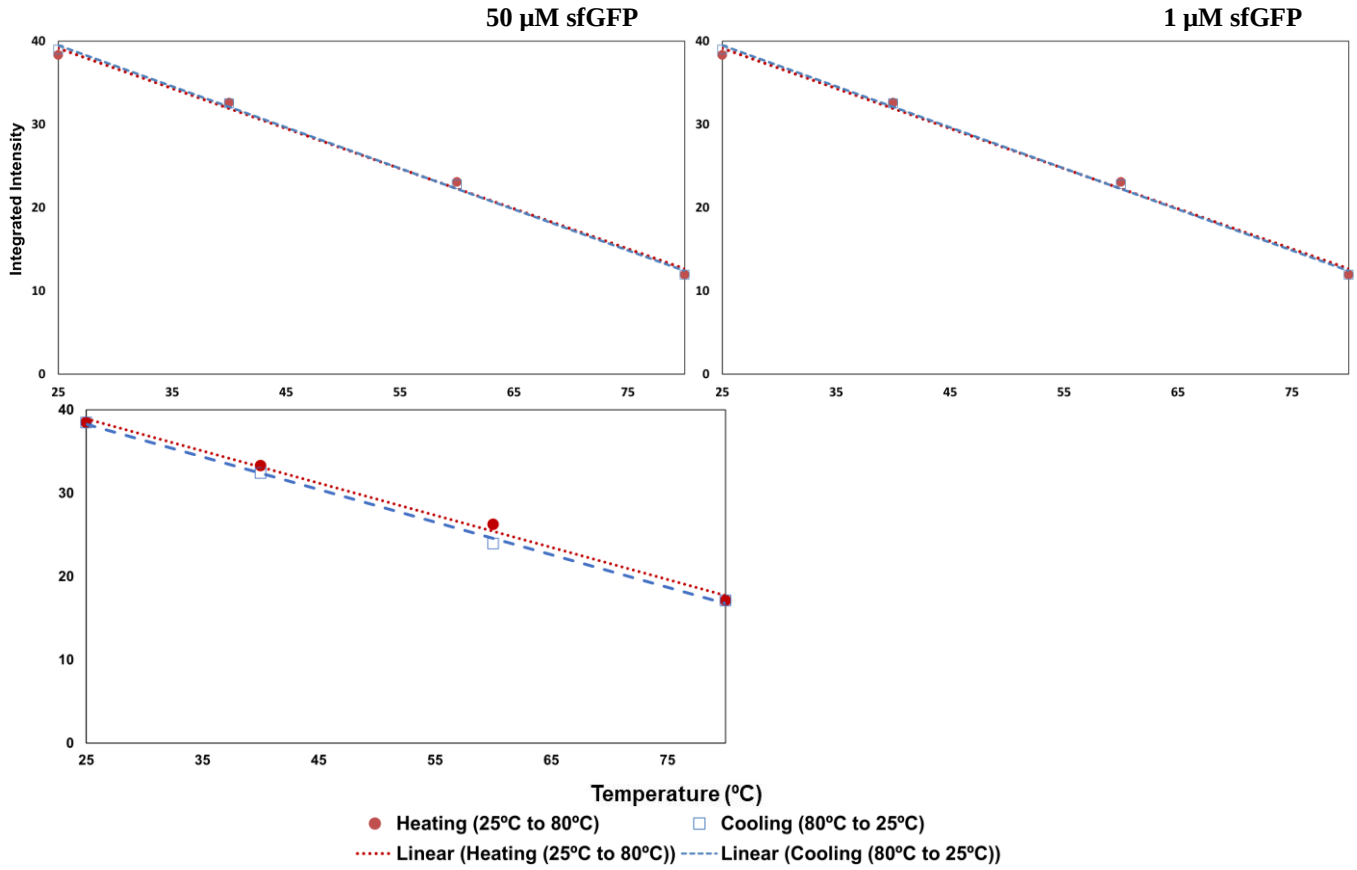


Figure 7: Effects of aggregation on excitation peak of sfGFP after exciting at 600 nm. (a) A photo comparison of (a1) 50 µM sfGFP in lysis buffer, (a2) 10 µM sfGFP in acetonitrile, and (a3) 10 µM sfGFP in methanol displays the aggregate characteristics of sfGFP in each solvent. (b) A plot of the integrated intensity values of normalized excitation peaks after exciting at 600 nm reveals an increase in intensity at lower concentrations of sfGFP in both acetonitrile and methanol.

3.5 Effects of temperature on sfGFP fluorescence. At both 1µM and 50µM, sfGFP displayed complete renaturation after being heated to 80ºC then cooled back down to 25ºC (Figure 7a). Time-dependent experiments of sfGFP in organic solvents at various fixed temperatures were also conducted. When diluted from 50 to 30 µM by either organic solvent, sfGFP lost 11-22% of its fluorescence after being held at 50ºC for 200 s. Diluting sfGFP with pure acetonitrile caused the protein to denature at 80ºC approximately 200% faster than protein diluted with 1:1 AcN:H_2O. In pure methanol, sfGFP completely lost fluorescence at ~104 s, whereas sfGFP in MeOH:H_2O lost fluorescence at a slower rate and still retained ~17% of its original fluorescence at the end of the 200 s measurement period. At 1 µM, a similar pattern was observed; pure acetonitrile and methanol caused a faster denaturation than the 1:1 mixtures containing water. At this low concentration, sfGFP was able to retain 90% of its original fluorescence for >4 s in all solvent and temperature conditions.

a.



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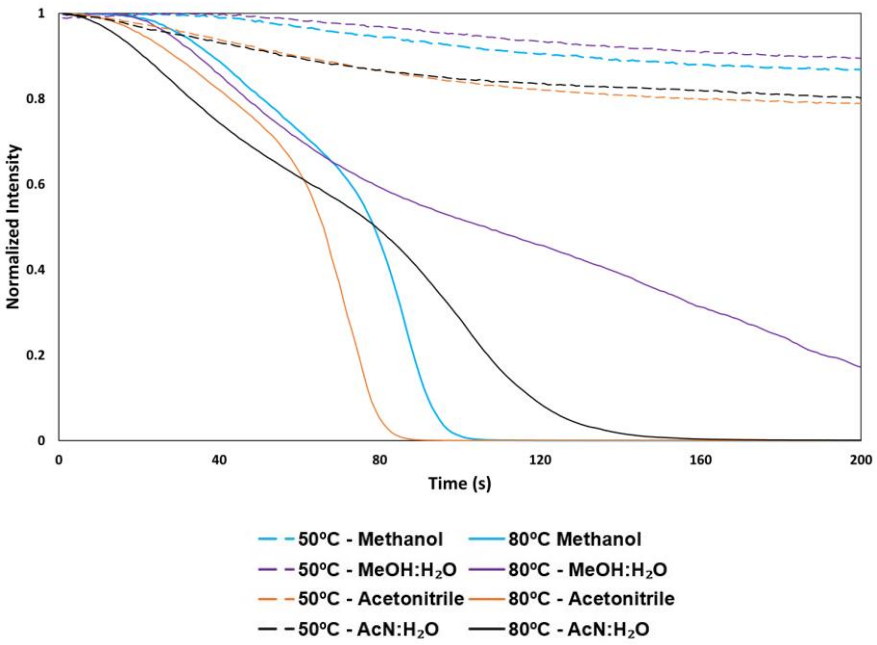

Figure 8: Effects of temperature on green fluorescent protein. sfGFP showed complete renaturation after (a) being heated to 80ºC then immediately cooled back down to 25º C in lysis buffer at concentrations of 1 and 50 µM. (b) Samples of sfGFP at 30 µM in methanol, MeOH:H_2O, acetonitrile and AcN:H_2O were placed in the fluorimeter at two set temperatures—50ºC, and 80ºC—for 200 s each. Emission intensity at the 518-nm peak was recorded in one-second intervals, which was the time resolution limit of our fluorimeter.

4. Discussion

4.1 Effects of methanol on sfGFP fluorescence. The presence of methanol consistently caused a decrease in the detected intensity of sfGFP, which suggests that sfGFP may be less stable in methanol. We hypothesize that this observed decrease in emission intensity of sfGFP in methanol is due to denaturation of the protein. Since methanol's absorption wavelength is at 210 nm, far below the excitation wavelength of 488 nm, we can assume that methanol itself is not absorbing any of the incident light. Secondly, methanol's refractive index of 1.32 would not affect the detected intensity of light emitted. When considering the protein's structure, sfGFP has a multitude of locations where hydrogen bonding can occur,[16] and it is possible that methanol is forming hydrogen bonds with the protein along its outer surface. As methanol is significantly less polar than water, oversaturation of methanol on sfGFP's surface could destabilize the protein. Methanol also has an affinity for hydrophobic side chains, which increases the likelihood that methanol is disrupting hydrophobic interactions that are crucial for chromophore activity in sfGFP.[17] Lastly, methanol has 3 hydrogen binding sites—two donors and one acceptor—and its mass is approximately 0.1% that of sfGFP's. Methanol's small size and hydrogen bonding abilities also increase its affinity for binding to sfGFP. In the case of sfGFP in MeOH:H_2O, the intensity loss was approximately half that of sfGFP in methanol, which exactly tracks with the ratio of methanol present between the two cases.

At high concentrations of methanol (≥80%), some aggregation of sfGFP was observed, as a light precipitate that was light green in color. There was also a prominent excitation peak at these low concentrations of sfGFP, which we suspected may have been due to scattering. By exciting the samples of sfGFP in methanol at 600 nm, we were able to more clearly observe changes in the excitation peak without the presence of a fluorescence signal. The same trend in excitation peak

increase at lower concentrations of sfGFP was observed, and no other peaks appeared along the spectrum. This led us to hypothesize that the aggregated sfGFP sample in methanol may be causing scattering of light at the same wavelength as the incident light. This phenomenon, called Rayleigh's scattering,[18] could explain the random increases in excitation peak size at high concentrations of methanol, as light detected via scattering could appear to increase the intensity of absorbance.

4.2 Effects of acetonitrile on sfGFP fluorescence. When comparing lysis buffer, methanol, and acetonitrile, sfGFP should theoretically exist in its most stable form in lysis buffer. Therefore, it is expected that sfGFP fluorescence should be greater in buffer than in acetonitrile. Because of this, the observed increase in fluorescence intensity of sfGFP in acetonitrile relative to lysis buffer was surprising.

Initially, we suspected that light scattering of sfGFP aggregates in acetonitrile may be contributing to the apparent increase in fluorescence intensity. However, after exciting the samples at 600 nm to evaluate light scattering patterns in the absence of fluorescence, there was no change in intensity at 518 nm, the location of sfGFP's emission peak. When the sample was in high concentrations of acetonitrile (≥60%), however, random spikes in the excitation peak sizes were observed, indicating the presence of light scattering of the protein aggregates. Since there were no other intensity peaks observed along the spectrum, we can infer that this scattering does not contribute to the increase in intensity of the emission peak that is observed after exciting sfGFP at its dominant excitation wavelength in acetonitrile.

Although scattering does not appear to be the mechanism behind the increased fluorescence signal for sfGFP in acetonitrile, it is possible that aggregation still plays a role in the observed phenomenon. Acetonitrile is a hydrophobic molecule and only contains one hydrogen bonding

donor site. Because of these properties, sfGFP in acetonitrile is likely to preferentially dimerize with other sfGFP molecules or interact with water molecules that are present in solution.[19] This effect can "push" the sfGFP molecules together, which may simulate the effects of molecular crowding that occurs within living cells.[20] Within any cell, there is an abundance of biological macromolecules that limit the amount of free water present. In this environment, proteins are stabilized because they are less likely to take on unfavorable conformational states.[21] Estimated protein concentrations in a cell of *E. coli* are varied, ranging widely from 0.19 mg/mL to 300 mg/ml.[22] The observed aggregates of sfGFP in acetonitrile, in comparison, are estimated to be roughly 0.81 mg/ml (Equation S2). If the true concentration of proteins within a cell is closer to the lower end of the range of values that are found in literature, it is possible that acetonitrile may be acting on sfGFP in a similar way as macromolecular crowding by modulating its conformational states and thereby reducing the likelihood of protein misfolding. With less protein in an unstable state, the sample would be expected to have higher fluorescence.

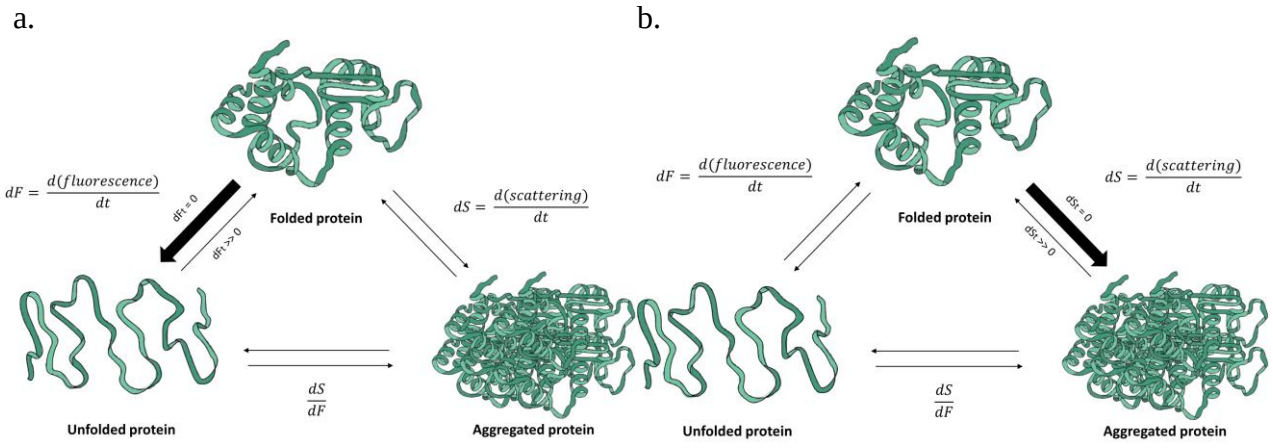

Figure 9: Theoretical model of the conformational equilibria of sfGFP in methanol and acetonitrile. In the presence of (a) methanol, we hypothesize that sfGFP is pushed towards a denatured state. Whereas in (b) acetonitrile, sfGFP tends to aggregate.

Effect of temperature on sfGFP fluorescence. sfGFP displays remarkable stability and renaturation abilities under high temperatures. These findings suggest that the protein is capable of withstanding the temperatures of SS-EDI and any other analytical technique that exposes the protein to temperatures up to 80ºC for 4 s or less.

5. Conclusion

By exposing sfGFP to varying temperatures and organic solvents, we were able to gain insight into the conformational stability of sfGFP in extreme conditions. After undergoing sustained exposure to high temperatures of 80ºC, sfGFP ss able to renature completely after being cooled back down to room temperatures, indicating that the protein is able to withstand the temperature conditions of SS-EDI. In the presence of methanol, sfGFP seems to be pushed towards a denatured state, losing complete fluorescence in high concentrations of methanol. In acetonitrile, on the other hand, sfGFP appears to be pushed towards an aggregated state, which may be contributing to the higher intensity observed for sfGFP in this organic solvent. These findings suggest that acetonitrile may be a more suitable organic solvent for SS-EDI measurements, since the protein is more likely to remain in a folded state when diluted with acetonitrile and AcN:H_2O in comparison to methanol.

Although we now have a better idea of the behavior of sfGFP in acetonitrile and methanol, the actual mechanisms behind the increased emission signal of sfGFP in acetonitrile, as well as the growth in excitation peak in both organic solvents, remains unknown. Further analytical tests would have to be conducted in order to elucidate the causes of these observed phenomena. Dynamic light scattering (DLS), for example, is a reliable method for measuring the size and movements of molecules in suspension, and our group has started preliminary testing to further characterize sfGFP aggregates through this method. To gain more insight into the rate at which

denaturation and aggregation is occurring, time-resolved DLS measurements could be conducted in tandem with time-resolved fluorescence measurements. Further testing also involves the evaluation of sfGFP in varying pH conditions.

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7. Supplemental Figures

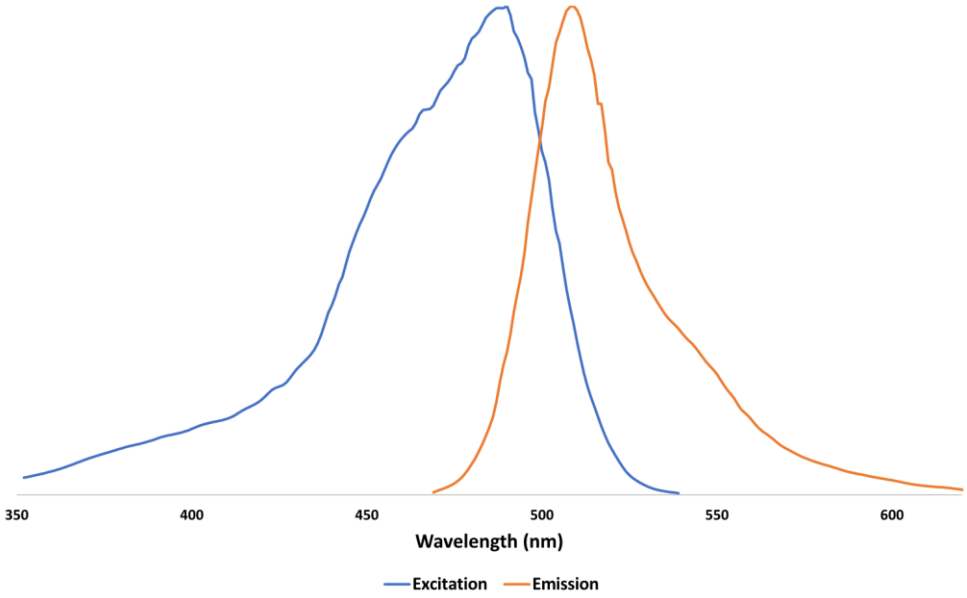

Figure S1: Excitation and Emission spectra of sfGFP. sfGFP has a wide excitation range, from ~350 to 540 nm, with a peak excitation wavelength of 488 nm. sfGFP's peak emission is at ~412-418 nm.

Solvent	**Refractive Index**	**Absorbance cutoff λ**
Acetonitrile	1.3404	190 nm
Methanol	1.3270	210 nm
HEPES	1.37	280 nm
Water	1.33	190 nm

Figure S2: Refractive indices and absorbance cutoff wavelengths for acetonitrile, methanol, and HEPES. The refractive index value represents the ratio between the speed of light in a vacuum to the speed of light in a specified medium, or organic solvent. An absorbance cutoff wavelength is the wavelength below which the organic solvent absorbs all the light. Excitation of sfGFP was at 488 nm, far above any of the solvent's cutoffs.

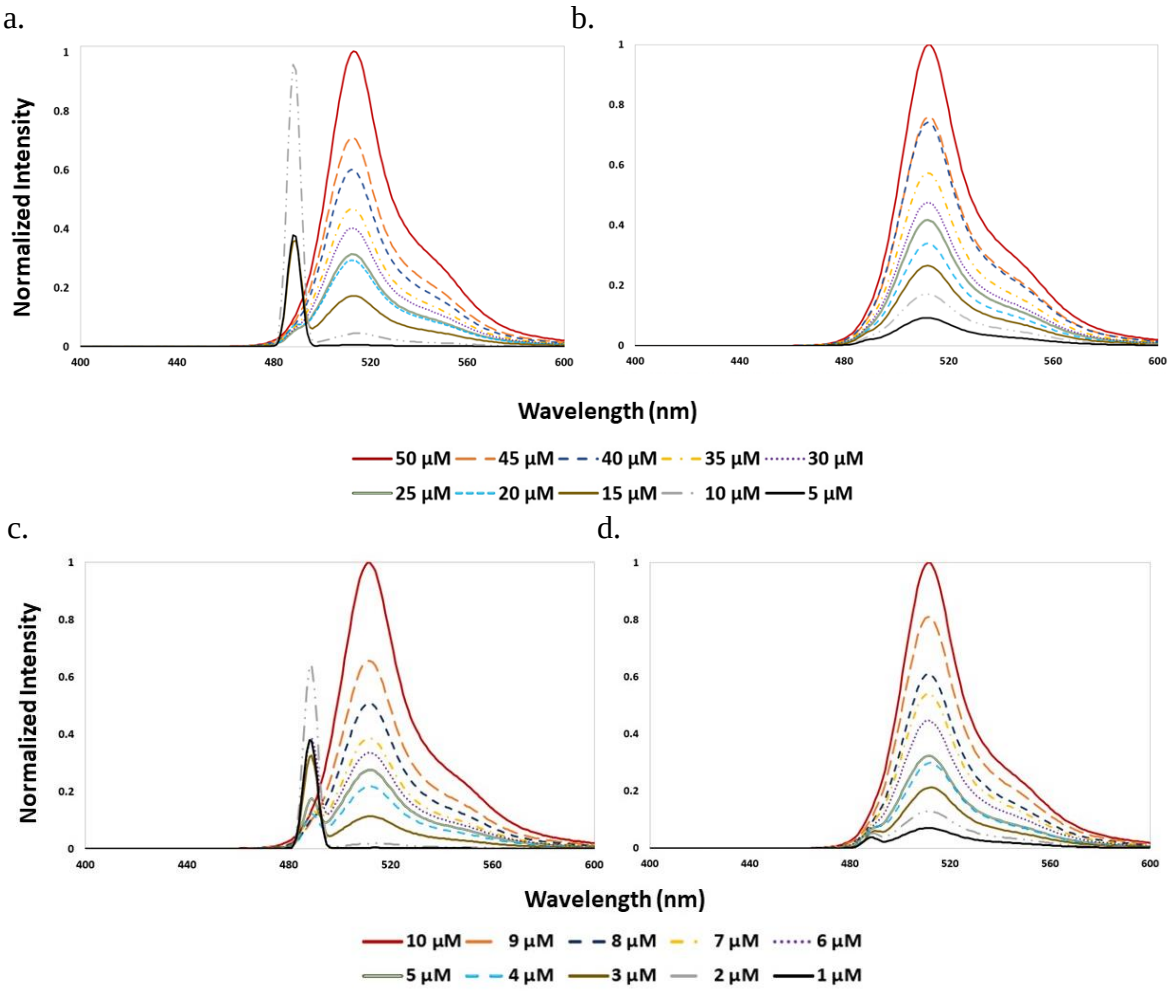

Figure S3: Emission spectra of sfGFP diluted by methanol. 50 µM sfGFP was diluted in increments of 5 µM by (a) methanol and (b) MeOH:H_2O. This was also done for 10 µM sfGFP in decreasing increments of 1 µM by (c) methanol and (d) MeOH:H_2O.

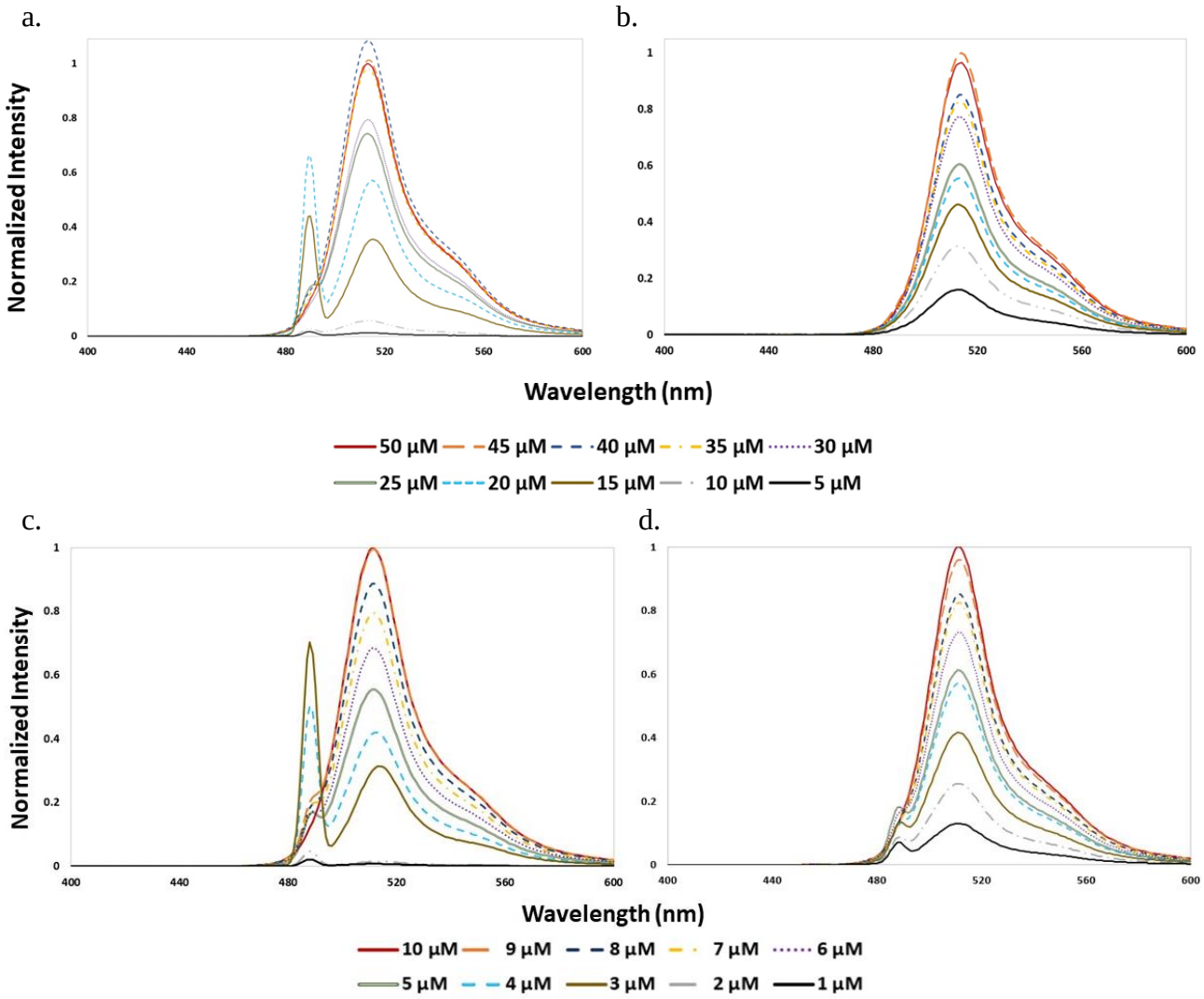

Figure S4: Emission spectra of sfGFP diluted by acetonitrile.. 50 µM sfGFP was diluted in increments of 5 µM by (a) acetonitrile and (b) AcN:H_2O. This was also done for 10 µM sfGFP in decreasing increments of 1 µM by (c) acetonitrile and (d) AcN:H_2O.

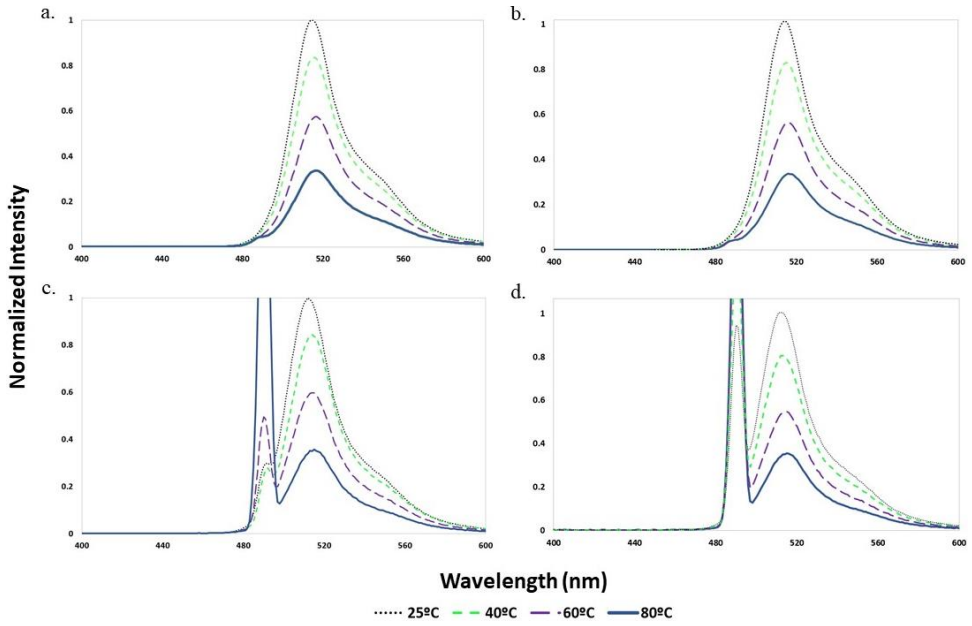

Figure S5: Emission spectra of sfGFP in increasing and decreasing temperatures. 50 µM sfGFP was (a) incrementally heated up to 80ºC, then (b) immediately cooled back down incrementally to 25ºC. This procedure was repeated for 1 µM sfGFP (c and d). At this low concentration, the excitation peaks appear significantly larger relative to the emission peak.

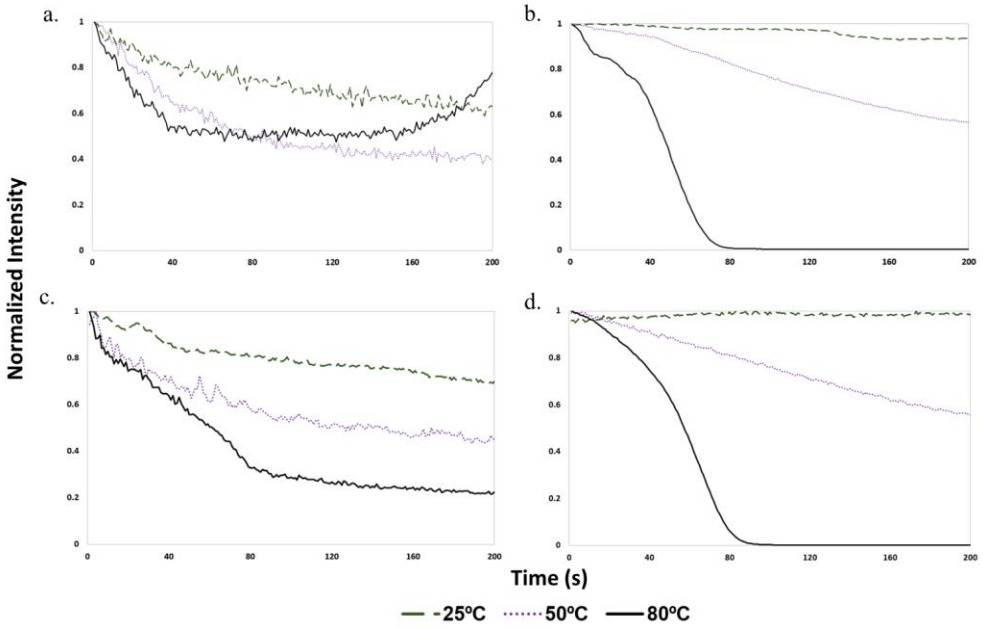

Figure S6: Samples of sfGFP at 1 µM in (a) methanol, (b) MeOH:H_2O, (c) acetonitrile and (d) AcN:H_2O were placed in the fluorimeter at three set temperatures—25ºC, 50ºC, and 80ºC—for 200 s each. Emission intensity at the 518-nm peak was recorded in one-second intervals, which was the time resolution limit of our fluorimeter.

a. 30 µM sfGFP:

Organic Solvent	Time (s)	
	50ºC	80ºC
MeOH	126	38
MeOH:H_2O	184	34
AcN	59	28
AcN:H_2O	57	21

b. 1 µM sfGFP:

Organic Solvent	Time (s)	
	50ºC	80ºC
MeOH	12	4
MeOH:H_2O	54	9
AcN	6	4
AcN:H_2O	41	20

Table S1: Time for 30 µM and 1 µM sfGFP diluted by varying organic solvents to lose 10% fluorescence at 50ºC and 80ºC. Each temperature and solvent condition was able to withstand temperatures of 50ºC and 80ºC for >20 s for (b) 30 µM sfGFP. At (b) 1µM, samples were able to retain 90 % of fluorescence for >4 s.

$$I \propto d^6$$

Equation S1: Rayleigh's approximation. The diameter of a particle in solution, d, correlates with the intensity, I, of light scattered.

$$10\ \mu M\ sfGFP = 0.00001\ mols/L$$

$$GFP\ molecular\ weight: 27kDa = 27{,}000\ g/mol$$

$$\frac{0.00001\ mols}{L}\left(\frac{27000\ g}{mol}\right) = \frac{0.27\ g}{L} = \frac{0.27\ mg}{ml}$$

$$Estimated\ volume\ of\ sfGFP\ aggregate = 0.5\ mL\ x\ \frac{2}{3} = \frac{1}{3}$$

$$Estimated\ concentration\ of\ sfGFP\ aggregate = \frac{0.27\ mg/ml}{\frac{1}{3}}$$

$$= 0.81\ mg/ml$$

Equation S2: Calculations for sfGFP concentration estimation in acetonitrile aggregates. Photo shown on the left is a sample of 10 µM sfGFP in acetonitrile.

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I would like to thank Dr. Ryan Mehl's lab for their assistance and for giving me access to the necessary reagents for this project. I would also like to thank Dr. Colin Johnson for being a part of my committee as well as his lab for allowing me to use their fluorimeter. Both labs have been welcoming towards me throughout this process, and this project would not have been possible without them.

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