Establishing the Freeze-Dried Cell-Free Protein Synthesis System as a Paradigm for Studying Protein Folding on Earth and in Microgravity

Establishing the Freeze Dried-Cell Free Protein Synthesis System as a Paradigm for Studying Protein Folding Kinetics on Earth and in Microgravity

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Abstract

As virtually all fundamental processes for biological life are executed by proteins, the study of protein folding and folding kinetics is essential. In order to advance the study of protein folding in resource-limited settings, it is necessary to develop easy to use, inexpensive, and accessible assays to study protein folding, unfolding, stability, and kinetics. Through this research, I sought to develop a low-resource module that explores the parameters of protein folding in an open, cell-free system. The work proceeded in three stages: to establish an experimental set up, adapt said experimental set up for use in educational settings, and finally to lay the groundwork for space application. Here we show that the study of folding kinetics and the structural stability of fluorescent proteins can be achieved through the freeze-dried, cell-free platform called BioBits®. Combined, this research brings an enhanced study of protein synthesis and folding to the bench, the classroom, and to space.

Author's Biographical Sketch

Alia Qatarneh has had the pleasure to serve hundreds of high school science teachers and thousands of middle and high school students in the New England area as the Secondary School Implementation Coordinator for LabXchange and as the Program Coordinator for the Harvard Life Sciences Outreach Program and Amgen Biotech Experience Program. Consequently, she has developed skills at the lab bench and in the classroom that serve as the foundation for a hybrid approach to learning and teaching. Alia is one of the inaugural recipients of the New England Biolabs Passion in Science Award, with which she was recognized for her cross-disciplinary approach to better learning in the form of science raps. She believes that creativity in and out of the classroom can inspire young students to look at the field with an uncommon lens, resulting in an engagement and connection they never knew science could offer. She will begin her Ed.M. in the Learning and Teaching Program at the Harvard Graduate School of Education in the fall of 2020.

Dedication

I would like to dedicate this work to my family. To my loving parents, Francesca and Yahya Qatarneh, thank you both for always encouraging me to be my best self. Ma, your dedication to creating a loving home has propelled me forward since sixie year at Boston Latin School. Dad, you taught me to stay curious and be patient; two fundamental pillars of science. I would also like to dedicate this to my younger sister, Noel. "Happiness runs in a circular motion." To the one and only Nonna, *tu sei la ragione per cui sono qui*. And finally, to Roma, thank you for not only believing in me, but for pushing me to dream big, want more, and strive for greatness.

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I would also like to acknowledge my Program Director and biggest advocator, Tara Bristow, and the entire ABE Massachusetts community. You have collectively brought me to this very moment. Here's to changing science education together.

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Chapter I.

Introduction to Research

Cell-free protein synthesis (CFPS) technologies have facilitated inexpensive, high throughput protein expression for the production of therapeutics and biomolecules. A new wave of interest in the last decade has resulted in multiple CFPS platforms, commercial and otherwise, that support synthetic biology applications such as the synthesis of vaccines (Pardee *et al*., 2016). The power of CFPS to redefine recombinant protein expression technologies through synthetic biology is undeniable.

CFPS technologies have been pushed further by the development of a freeze-dried, cell-free protein synthesis system (FD-CF), which allows for a portable, shelf-stable, and more affordable platform for protein synthesis (Pardee *et al*., 2018). The FD-CF platform utilizes reaction pellets that contain lyophilized transcription and translation machinery, which are rehydrated with water and the DNA of the researcher's choice. With its proof-of-concept publication less than five years old, this FD-CF platform application has the potential to initiate profound changes in equity of and access to biotechnology benefits.

Protein yield efficiency, scalability, reproducibility, and high throughput are advantageous features of the FD-CF system. In addition, the FD-CF's open system characteristic is highly advantageous, as it is unencumbered by membranes or cellular compartments. The open system also allows for the straightforward regulation of input reagents. Holistically, these are desirable features in a system to study protein synthesis and folding, specifically for soluble proteins that do not require significant post-

translational modifications. Therefore, I sought to explore the suitability of the FD-CF system to study protein folding in resource-limited settings.

As virtually all fundamental processes for biological life are executed by proteins, the study of protein folding and folding kinetics is essential. A protein's shape determines its function, and in turn, its possible dysfunction. There is an extensive list of human diseases that stem from faulty protein folding, such as cystic fibrosis. Efforts to understand protein structure, folding, and kinetics are fundamental to drug discovery and protein design. The birth of protein folding experimentation started in 1961 with the Anfinsen experiment. Through denaturing protein RNaseA with urea and then slowly removing the urea through dialysis, Anfinsen demonstrated that 1) protein sequence determines structure, and 2) the native state of a protein is the minimum energy state. This experiment led to the thermodynamic hypothesis, which states that the native structure optimizes the thermodynamic properties of the polypeptide as a whole.

The field of protein folding has seen great progress since the Anfinsen experiment. This evolution in the field of protein folding, however, relies heavily on advanced experimental techniques. Nuclear magnetic resonance (NMR), mass spectrometry, and fluorescence resonance energy transfer (FRET) are modern yet expensive experimental techniques that have generated detailed protein-folding data. This presents challenges to studying protein folding in low-resource settings, for example, in classrooms. Arguably, the Anfinsen experiment is just as inaccessible as NMR, mass spectrometry, and FRET. In order to advance the study of protein folding in resource-limited settings, it is necessary to develop easy to use, inexpensive, and accessible assays to study protein folding, unfolding, stability, and kinetics.

The stability of the FD-CF system makes it suitable for unique environments where traditional techniques are not amenable, opening up the study of protein in environments where it was not previously possible. One such interesting environment is the realm of space and microgravity. The lack of equipment aboard the International Space Station (ISS), coupled with the numerous restrictions that surround research on the ISS, have hindered the area of protein folding studies in microgravity. NASA-supported projects regarding the behavior of proteins in microgravity have focused mainly on the observation of protein crystallization (McPherson *et al*., 2015). The stability, openness, and speed of the FD-CF system make it an attractive platform to explore the processes involved in protein synthesis and folding in microgravity.

Through this research, I sought to develop a module that explores the behavior of protein folding in an open, cell-free system. The work proceeded in three stages: 1. establish an experimental set up, 2. adapt for use in educational settings, and 3. lay the groundwork for space application. The first stage was to establish protocols focused on the use of the freeze-dried, cell-free platform called BioBits® to observe protein folding, denaturation, and fluorescence rescue. Given their diversity and visual outputs, I utilized fluorescent proteins as primary read-outs, allowing for easy detection without any specialized equipment. As this research expands on previous groundwork for educational applications of the FD-CF platform, the second stage was to adapt the assays for use in educational settings. The third and final stage of this research was to lay the groundwork for the application of this technology in the microgravity environment aboard the International Space Station. Combined, the stages of this research bring an enhanced study of protein synthesis and folding to the bench, the classroom, and to space.

Definition of Terms

ATP: adenosine triphosphate, utilized in protein synthesis

BioBits®: Freeze-dried, cell-free protein synthesis pellets developed by the Massachusetts Institute of Technology and Northwestern University, now currently owned by miniPCRbio and sold as educational synthetic biology kits.

Biomolecule: molecule produced by living cells, such as proteins, carbohydrates, lipids, and nucleic acids

Central Dogma of Biology: DNA to RNA to protein

CFPS: cell-free protein synthesis

Column chromatography: a method of separating substances, such as proteins in which the substances are dissolved in a liquid that is allowed to flow through a glass column filled with small resin beads

CTP: cytidine triphosphate, utilized in protein synthesis

DNA: deoxyribonucleic acid; double-stranded molecule made up of nucleotide subunits that encodes for all genetic information

Escherichia coli (E. coli): common, gram-negative, rod-shaped bacterium commonly used in biotechnology protocols

FD-CF: freeze-dried, cell-free (protein synthesis)

Fluorescence: production of light by a molecule (i.e. green fluorescent protein will release green light when exposed to ultraviolet light)

Genes in Space: educational competition for students to propose an experiment that utilizes molecular biology capabilities aboard the ISS, specifically polymerase chain reaction

Genetic engineering: a branch of biotechnology that uses specific procedures and techniques to change an organism's DNA

GFP: green fluorescent protein

GFPmut3b: a basic green fluorescent protein published in 1996, derived from *Aequorea victoria*; for the purposes of this thesis, GFPmut3b will be referred to as GFP

GTP: guanosine-5'-triphosphate, needed for the synthesis of RNA during transcription

HIC: hydrophobic interaction chromatography

iSAT: integrated synthesis, assembly, and translation; in-vitro protein synthesis

ISS: International Space Station; the ISS is a permanently crewed on-orbit laboratory that enables scientific research supporting innovation on Earth and future deep space exploration

Lyophilization: preservation method that freezes and vacuum seals reagents

Mg2+: magnesium cofactor, essential to protein synthesis

Microgravity: environmental condition where a reduction in gravity is observed, allowing an object to be in a state of free fall, in which the rate of falling is equal to the rate of gravitational acceleration

miniPCR™: miniaturized PCR machine

mRNA: messenger RNA; RNA molecule transcribed from the DNA of a gene and used as the template for protein synthesis

NASA: National Aeronautics and Space Administration

Ori: origin or replication; sequence of DNA at which replication of the DNA is initiated

P51™ visualizer: molecular fluorescence real-time viewer

PCR: polymerase chain reaction; a method to amplify specific regions of DNA

Plasmid: circular molecule of DNA

Protein: large biomolecule that is essential for cellular functions in cells

Promoter: specific DNA sequence that binds RNA polymerase and initiates transcription of the gene

Protein folding: physical process by which a polypeptide folds into its characteristic three-dimensional structure, which is essential to the protein's function

Recognition site: specific DNA sequence that is cut by a restriction enzyme

Recombinant DNA: DNA that contains sequences of genes from two or more sources

Resin: material used in a chromatography column to coat the beads

Restriction enzyme: a protein that can cut DNA at a specific sequence

RNA: ribonucleic acid; single-stranded biomolecule made up of a nitrogenous base, a ribose sugar, and a phosphate; RNA plays a critical role in protein synthesis, transmitting genetic information from DNA to the ribosome where proteins are then made

S150: ribosome-free *E. coli* crude cell extract

SDS: Sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

sfGFP: super folder green fluorescent protein

Synthetic biology: the interdisciplinary study of applying engineering principles to molecular and cellular biology to redesign natural biology systems

Transcription: process by which information encoded in DNA is transferred to messenger RNA, a single-stranded ribonucleic acid

Translation: process by which information encoded in messenger RNA is decoded and transformed into protein

tRNA: transfer RNA; RNA molecule that aids in decoding an mRNA sequence into a protein

UTP: Uridine-5'-triphosphate needed for the synthesis of RNA during transcription

VLPs: Virus-like particles; lack the viral genome but otherwise mimic the structure and organization of a native virus

Introduction to Synthetic Biology

Synthetic biology is a rapidly developing field that builds on engineering concepts to advance the field of biology. As an interdisciplinary area of study, synthetic biology connects various disciplines such as bioengineering, biotechnology, microbiology, biophysics, chemical biology, systems biology, and computer science to redesign existing and fundamental biological systems to approach problems in the life sciences and beyond. Through this novel redesign of natural biological processes, synthetic biologists have provided new methods for optimized biofuels, bioremediation, drug manufacturing, cell therapies, and clinical diagnostics.

The foundation of synthetic biology is the ability to control gene expression in a precise and predictable manner. This is achieved by the development of synthetic cellular networks that utilize regulatory circuitry. The first synthetic circuits appeared in early 2000 as toggle switches and repressilators, setting the characteristic experimental features of the field (Cameron *et al*., 2014). Through this understanding, applications of synthetic biology can be divided into two groups: the engineering of living cells to acquire new functionalities and the construction of *in vitro* biological systems made of altered, or rewired, biological components.

The field of synthetic biology has rapidly produced many novel technologies that utilize the circuit-like connectivity of biological entities. The redesign of natural biological systems for enhanced purposes directly impacts health, safety, and the environment. Furthermore, synthetic biology impacts science education by inviting young students into the interdisciplinary world of the field to tackle real-world problems.

Protein Structure and Folding

Proteins are organized into four levels of structure. The primary structure of a protein is the linear sequence of amino acids in its polypeptide chain. The second level of structure is the conformation taken on by contiguous amino acids. There are two common types of secondary structures in proteins: alpha helices (α) and beta sheets (β). This is the level where the protein first takes on a three-dimensional shape. The secondary structure is formed and stabilized by hydrogen bonding, electrostatic interactions, and Van der Waals forces. The third level of protein structure is the three-dimensional, folded composition that is the result of interactions between different units of secondary structures. Additionally, many proteins, but not all, exhibit quaternary structure where two or more polypeptide chains interact, forming a higher order protein complex.

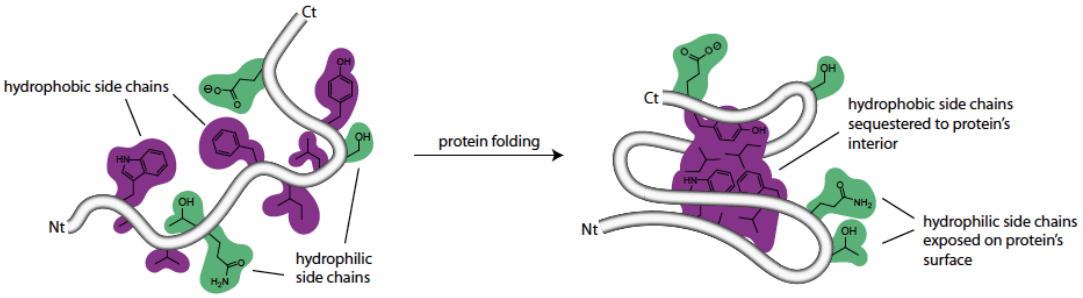


Figure 1. Protein Folding.

The arrangement of hydrophilic and hydrophobic side chains in a protein determines its folded structure.

More generally, in nature, protein folding provides a protein with structure, which in turn, determines its function, whether its function is to fluoresce a specific color or act as a recombinant therapeutic. Changes to the structure may alter normal function,

sometimes disrupting it. In some cases, we change the normal structure of a protein to obtain a new or enhanced function. During protein synthesis, a long, malleable chain of amino acids adopts a specific three-dimensional conformation. This is known as protein folding. Protein folding is achieved by the specific properties of the amino acids that make up said protein. Although protein folding optimizes both the burial of hydrophobic amino acids and the surface exposure of hydrophilic amino acids in an aqueous medium, it is the hydrophobic amino acids interactions that drive protein folding. The single, lowest energy shape a protein can adopt is called the native state or native conformation. This is determined by the protein's amino acid sequence.

Common Approaches to Studying Protein Folding

Over the last six decades, novel experimental techniques and computational methods have uncovered notable insights into folding mechanisms and kinetics. Protein folding is difficult to study for three reasons: 1) folding is very fast, 2) it involves very small changes in energy from the denatured to native state and 3) the intermediate states populated along the pathway are ensembles of structures and not discrete, single entities. For these reasons, complementary experimental techniques are typically required to study protein folding. Nuclear magnetic resonance (NMR), mass spectrometry, fluorescence resonance energy transfer (FRET), and X-Ray crystallography are experimental techniques that have generated detailed protein structure and folding data (Table 1).

Focus	Experimental Technique	Contribution to protein folding research	Reference
Mechanism of folding/unfolding	Equilibrium denaturation by guanidinium chloride	Suggestion of the two-state transition	S.E. Jackson, A.R. Fersht, Biochemistry 30 (1991) 10428–10435
Folding kinetics	Relaxation dispersion NMR	Identified and characterized low-population folding intermediates	D.M. Korzhnev *et al*. Nature 430 (2004) 586–590
Unfolded protein structure	NMS of unfolded proteins	Denatured proteins have a strong local conformational bias towards native state	D. Shortle, M.S. Ackerman, Science 293 (2001) 487–489
	Computational models of denatured proteins	Unfolded states feature local native-like structures	F. Ding, R.K. Jha, N.V. Dokholyan, Structure 13 (2005) 1047–1054

Table 1. Examples of experimental approaches to protein folding

Examples of experimental approaches to protein folding (adapted from Chen et al., 2007).

Importance of Developing a Low-Resource Protein Folding Assay

Although the use of advanced experimental techniques is common for studying protein folding, it should not be required. Development of an assay that requires little to no equipment or the use of living cells to observe protein folding will allow for democratization in and out of the laboratory. Two specific out-of-lab environments that motivated this research are the classroom and space. Both environments need the ability to study protein folding, however are limited in their access to advanced experimental equipment.

In the classroom, students could use a low-resource protein folding assay as an educational means to learn biochemical, molecular, and synthetic biology concepts. The topics of molecular and synthetic biology are best taught through interdisciplinary, inquiry-based, hands-on learning experiences. However, many secondary school science curricula do not support these experiences. There are a few initiatives that do currently exist, such as BioBuilder and iGEM (International Genetically Engineered Machines), which work to increase student engagement and interest in molecular biology, synthetic biology, and biotechnology. Even so, BioBuilder is an elective curriculum of the classroom teacher, and iGEM typically runs as an afterschool project. Furthermore, both BioBuilder and iGEM require educators to procure specialized equipment and reagents that need proper storage. These two requirements limit these specialized opportunities to educators who have the content knowledge, time, and funding.

The study of protein folding in space can benefit from a low-resource assay as well. Real estate aboard the International Space Station is restrictive. As such, experimentation generally uses portable pieces of equipment or requires parts of the experimentation to be done back on Earth. Since current protein folding and kinetics studies use highly specialized protocols, the exploration of protein folding in microgravity has been limited. Understanding how proteins fold and unfold in microgravity is necessary for the further development of a space-synthesized protein assay, which is essential to deep space exploration.

Cell-Free Protein Synthesis

Cell-free protein synthesis (CFPS) systems are not novel. In fact, the technology has been available for over 50 years (Perez *et al*., 2016). CFPS uses crude cellular extracts instead of intact biological cells to carry out transcription and translation. Although crude cell extracts contain the native biological transcription and translation machinery components from the cell, such as RNA polymerase and ribosomes, the cell-free system is an open system, allowing for precise control of additional reaction variables, such as amino acids, nucleotides, salts, and buffers (Figure 2). This control makes for easier optimization and scale-up opportunities of the application. A cell-free system for protein synthesis offers a quick and easy approach to studying biosynthetic pathways, as well as basic biomanufacturing, all without the hassles and limitations of large-scale cell growth.

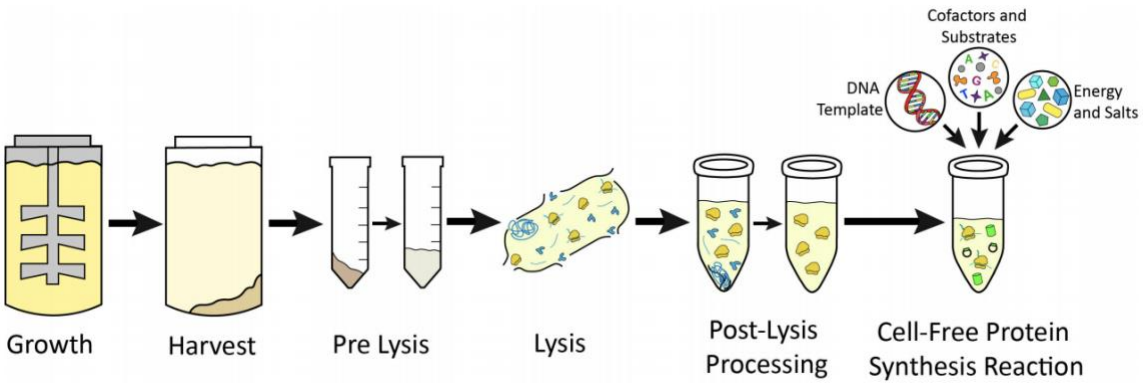


Figure 2. Cell-Free Process.

Simplified workflow for preparation of cell-free protein synthesis reactions, highlighting the main steps of the process. (Gregorio et al., 2019).

Development of a CFPS system can be divided into three phases: host selection, catalyst synthesis (cell growth), and protein synthesis. During the first phase, one must select the organism from which the biological catalysts for transcription, translation, folding, and energy will be harvested. CFPS systems have used eukaryotic and prokaryotic organisms, and also archaea. Lysate extracts have been derived from *S. cerevisiae, E. coli,* wheat germ, rabbit reticulocytes, CHO cells, and HeLa cells (Zemella *et al*., 2016). Each source organism has its advantages and disadvantages, but also allows for diversification of the type of protein that can be made; for example, the *E. coli* cell lysate platform works best for antibodies, vaccines, and diagnostics, whereas the CHO and HeLa cell lysate platforms are best for glycoproteins, antibody-drug conjugates, and membrane proteins (Perez *et al*., 2016).

There has been a sudden surge in development of CFPS systems over the last 20 years (Perez *et al*., 2016). The advances made during the last two decades looked to find an inexpensive way to rapidly and efficiently synthesize recombinant proteins, resulting in multiple cell-free systems, enabling for the synthesis of a diverse group of proteins, shown in Table 2 (Perez *et al*., 2016). For example, the *E. coli* crude extract (ECE) platform has been used to synthesize vaccines such as a B-cell lymphoma vaccine (Ng. *et al*., 2012) and virus-like particles (VLPs) such as an anti-influenza VLPs (Lu *et al*., 2014). However, one of the most advantageous advances of the CFPS system isn't the synthesized output itself, but the development of the freeze-dried, cell-free system.

CFPS Platform	Advantages	Disadvantages	Applications
Prokaryotic			
E. coli (ECE)	● High protein yields ● Fast cell growth for lysate preparation ● Easy to genetically engineer ● Well-known system ● Commercially available	● Limited ability for post-translational modifications ● Eukaryotic proteins might not fold correctly	● High-throughput platform ● Vaccines ● Diagnostics ● Non-standard amino acids (nsAAs)
Archaeal	● Allows for extremophilic conditions, such as high temperature	● Low protein yield	● Synthesis of thermostable proteins
Eukaryotic			
Yeast (SCE)	● Ability to perform post-translational modifications ● Fast cell growth for lysate preparation ● Well-known system	● Low protein yield ● Does not allow for post-translational modifications	● Production of VLPs for anti-viral drug research ● Production of bioethanol in cell-free bioreactors
Wheat Germ (WGE)	● High yield of complex protein ● Ability to synthesize disulfide-bridged proteins ● High solubility of proteins ● Well-known system	● Lower protein yield compared to ECE and WGE platforms ● Relatively expensive lysate preparation ● Limited ability for post-translational modifications	● Production of malaria proteins to explore novel vaccines ● On-chip protein synthesis ● Used to synthesis 13,000 humans in a single study
Rabbit Reticulocytes	● Mammalian system ● Can be used in the presence supplemented, heterogenous microsomes	● Low protein yield ● Living animal treatment/ethical considerations	● Complex proteins ● Protein microarray technology ● Display technologies

CHO and HeLa Cells	● Well-known cell lines ● Mammalian post-translational modifications ● Allows for direct production of membrane proteins	● Lower protein yield compared to ECE platform ● Relatively expensive lysate preparation	● Complex proteins ● Antiviral drug applications

Table 2. Comparison of different cell-free protein synthesis systems, their advantages, disadvantages, and applications.

Overview of Freeze-Dried, Cell-Free (FD-CF) Systems

The freeze-dried, cell-free (FD-CF) protein synthesis platform was first introduced in 2016 by a cross-institutional group of scientists from Northwestern University and the Massachusetts Institute of Technology (Perez *et al*., 2016). Their research had shown that crude extracts from a typical cell-free system can be lyophilized, or freeze-dried, along with additional molecular components, into a reaction pellet. This reaction pellet only needs to be rehydrated with template DNA and water, and placed in optimal conditions for protein synthesis to occur (Figure 3). The stability of these FD-CF pellet reactions thus eliminates the need for reagents to be kept frozen, making this technology revolutionary to the fields of proteomics, synthetic biology, biotechnology, and education.

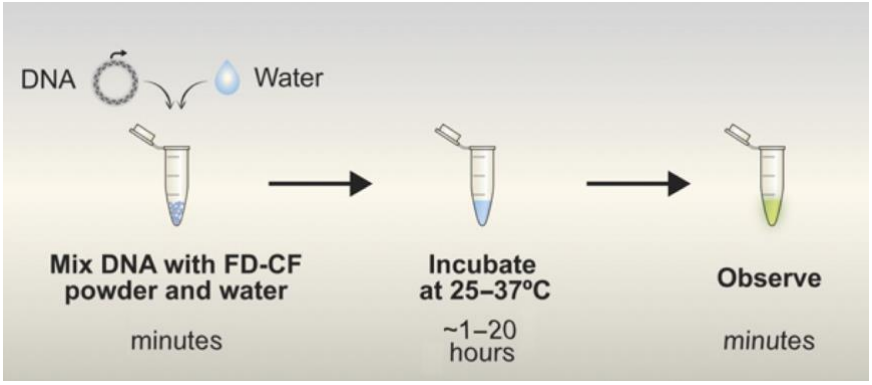


Figure 3. Simplified schematic of the FD-CF system, BioBits®.

This figure shows the addition of plasmid DNA to the freeze-dried pellet, resulting in a fluorescent output via protein synthesis. (Huang et al., 2018).

The FD-CF system has been used to produce on-demand antimicrobial peptides, vaccines, combinatorial antibody analogs, and combinatorial biosynthesis of small molecules (Figure 4) (Pardee *et al*., 2016). In one paper, the authors established the use of the FD-CF system to produce a diverse number of therapeutics and molecular diagnostic tools, with potential to influence the realm of global health. In fact, the same group of authors entered the global health field by establishing the use of the FD-CF platform to create a Zika virus diagnostic tool (Pardee *et al*., 2016).

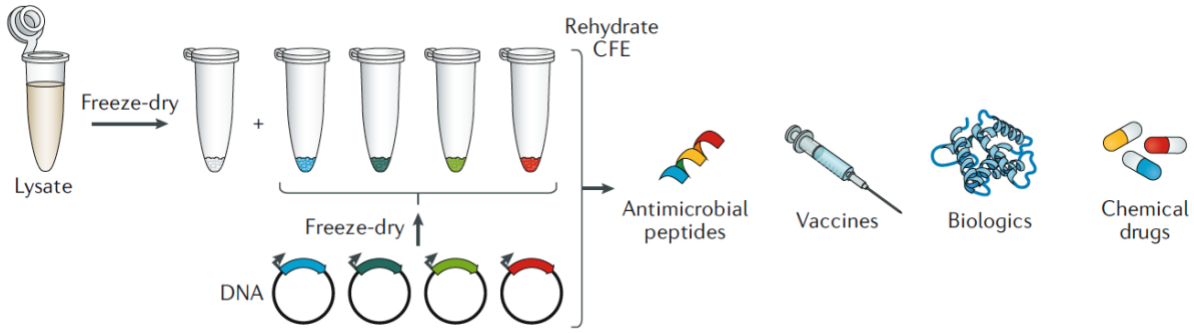


Figure 4. Diversity of FD-CF Proteins.

Examples of the wide variety of proteins and chemicals that can be synthesized using a freeze-dried cell free system. (Silverman et al., 2019).

Use of FD-CF System to Observe Protein Folding

It is clear from the literature that the FD-CF platform allows for a portable, low-cost, and rapid diagnostic and protein manufacturing tool due to its protein yield efficiency, scalability, reproducibility, and high throughput. The additional characteristic of being an open system is highly advantageous as well, allowing for the straightforward regulation of input reagents. Taken together, these features of the FD-CF system are ideal to study protein folding. Therefore, I sought to explore the suitability of the FD-CF system, specifically BioBits®, to study protein folding.

Selection of Fluorescent Proteins to Study Protein Folding

Green fluorescent protein (GFP) is among the most commonly used reporters for gene expression. GFP and other fluorescent proteins (cyan, cherry, tomato, etc.) have revolutionized biomedical and biotechnology research by making the invisible visible through genetic biomarker tagging. Derived from the jellyfish *Aequorea victoria*, GFP is most notable for its bright green glow when excited under specific light. Scientists have cloned the gene encoding GFP and have introduced multiple mutations to enhance folding and spectral characteristics (Liu *et al*., 2015). This has led to multiple versions of GFP, for example EGFP (Enhanced GFP, Cormack *et al*., 1996), GFPmut3b (Cormack *et al*., 1996), and sfGFP (superfolder GFP) (Pedelacq *et al*., 2006). I selected GFP variants GFPmut3b and sfGFP to study protein folding with the FD-CF system for three reasons.

First, these variants have been well studied, and data is freely available on open source databases, such as the Protein Data Bank and FPbase. Secondly, the output is visual. In its simplest form, protein folding can be studied by observations with the naked eye. Finally, the application of fluorescent protein folding in a FD-CF environment lends itself to an education platform to study protein folding in classrooms, without the need for sophisticated laboratory equipment.

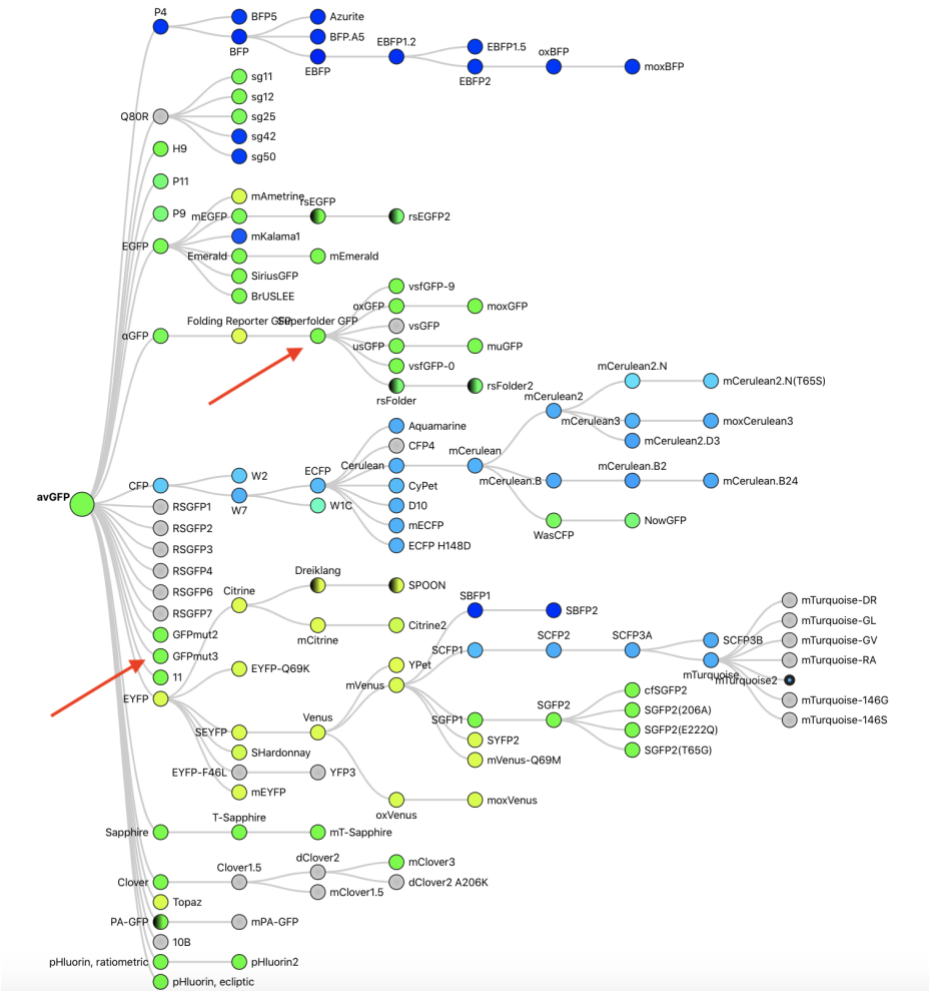


Figure 5. Green fluorescent protein tree.

This figure depicts the relationship between variants (schematic from FPbase).

GFPmut3b is a simple, green fluorescent protein first described by Cormack *et al.* in 1996. It was derived from wild-type GFP from *Aequorea victoria*. It is reported to be a rapidly maturing weak dimer. sfGFP is a green fluorescent protein published by Pedelacq *et al*. in 2006. It was derived from Folding Reporter GFP, with the mutations S30R/Y39N/N105T/Y145F/I171V/A206V. Pedelacq *et al*. observed that sfGFP exhibited enhanced folding performance compared to wild-type GFP and EGFP. Not only does sfGFP exhibit faster folding kinetics, but it also has a greater resistance to chemical denaturants.

Specifically, I selected GFPmut3b and sfGFP as ideal candidates for observing protein folding in a FD-CF system based on previous research where a panel of 12 different fluorescent proteins were visually analyzed for their brightness and reaction kinetics (Huang, 2019). Both GFPmut3b and sfGFP were shown to be highly expressed in the FD-CF system. Protein yield was confirmed by 14C-Leucine incorporation, as well as optimal excitation and emission spectra through relative fluorescent unit (RFU) data (Huang *et al*., 2018). Huang *et al*. demonstrated that fluorescence of these proteins with the FD-CF system was observable with the naked eye at room temperature for 24 hours, limiting the need for expensive equipment.

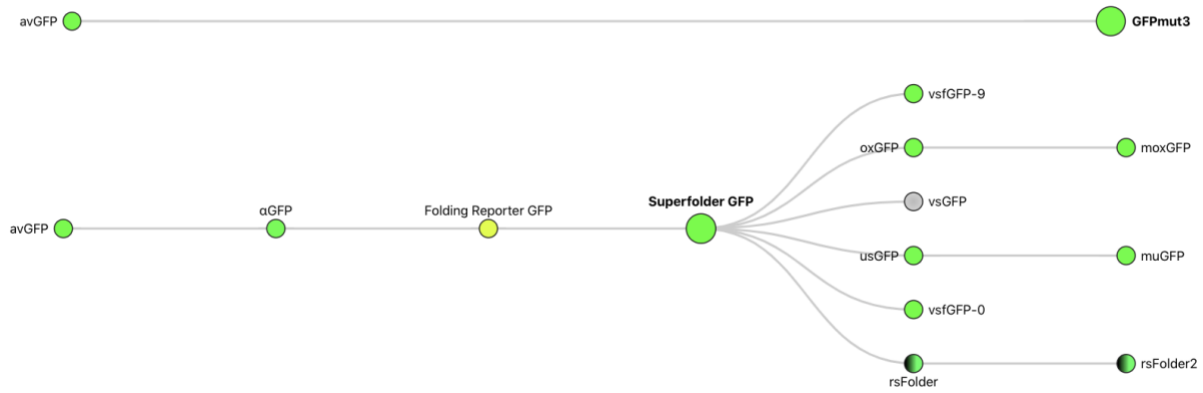


Figure 6. Relative Derivatives of Wild-Type GFP (schematic from FPbase).

GFP Variant	Molecular Weight	Excitation Maximum (Ex λ)	Emission Maximum (Ex λ)	Extinction Coefficient	Quantum Yield	Molecular Brightness
GFPmut3b	26.8 kDa	500	513	89,400	0.39	34.87
sfGFP	26.8 kDa	485	510	83,300	0.65	54.15

Table 3. Comparison between GFPmut3b and sfGFP

Figure 7. Sequence alignment of GFPmut3b and sfGFP proteins

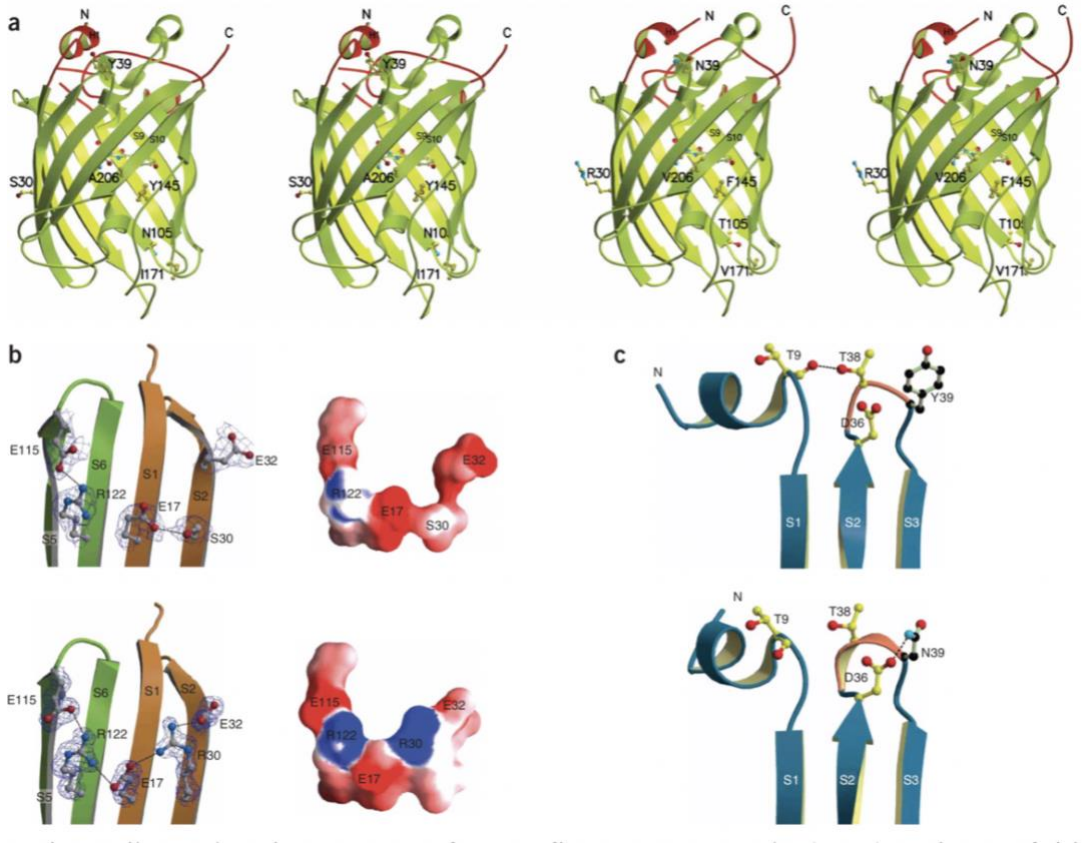


Figure 8. Three-Dimensional Structures of GFP and sfGFP.

Panel (a) shows the stereo views of both proteins, with GFP on the left and sfGFP on the right. Panel (b) shows the ribbon models and AA charge surface of GFP (top) and sfGFP (bottom). Panel (c) highlights one of the main AA changes between the two proteins that allows for the enhancing formation of sfGFP. Image from Pedelacq et al., 2006.

Chapter II.

Materials and Methods

The following section describes the materials and methods used throughout this research.

Design of the FD-CF Reaction Pellets: BioBits®

The FD-CF reaction pellets, available under the trade name BioBits® (Figure 9), were created through a collaboration between Northwestern University and the Massachusetts Institute of Technology. The cell lysate used to create the BioBits® pellets for this research came directly from the Jewett Lab at Northeastern University. The lyophilization process occurred in-house at miniPCR bio™ laboratory. The protocol used was adapted from the published literature (Soye *et al.*, 2018 and Stark *et al.*, 2018). NEB® DH5-alpha competent *E. coli* cells were used for the CFPS extractions. The cells were grown in 2X YTPG media at 37°C, until an OD_{600} of 3.0 was reached. Once optimal growth was achieved, the cells were pelleted by centrifugation. In preparation for lysis, the cells pellets were first resuspended and then washed in S30 buffer (10 mM Tris-acetate (pH 8.2), 14 mM magnesium acetate, and 60 mM potassium acetate) three times. The cells were spun to create a cell pellet once again. A final addition of 1 mL of S30 buffer was used to resuspend the pellet for lysis. The cells were lysed using a sonicator, using parameters already established for minimal cell damage. The cells were spun down for the last time, allowing for the cell extract to be aliquoted out of the tube and stored in a separate tube, which was then flash-frozen in liquid nitrogen and stored at -80°C until

shipment. Previous research has outlined a standard reaction mixture to be added to the cell lysate (Table 4). An appropriate amount of 5 µL of 27 % (v/v) cell lysate was used in each reaction pellet. Lyophilization of the cell-free reaction pellets was achieved by using a VirTis BenchTop Pro Lyophilizer overnight, until completely freeze-dried.

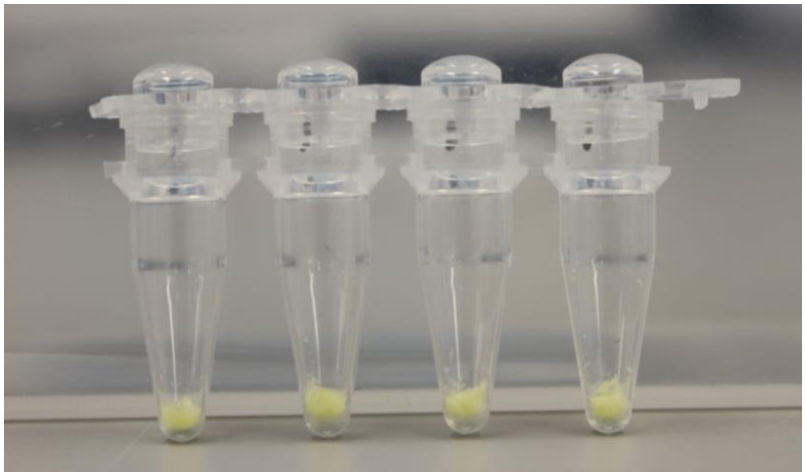


Figure 9. BioBits® Reaction Pellets.

Reagent	Concentration
Adenosine 5′-triphosphate	1.2 mM
Guanosine-5′-triphosphate	0.85 mM
Uridine 5′-triphosphate	0.85 mM
Cytidine 5′-triphosphate	0.85 mM
l-5-formyl-5,6,7,8-tetrahydrofolic acid	34.0 mg ml−1
E. coli transfer RNA mixture	170.0 mg ml−1
Potassium glutamate	130 mM
Ammonium glutamate	10 mM
Magnesium glutamate	12 mM
All 20 amino acids	2 mM each
Nicotinamide adenine dinucleotide	0.33 mM
CoA	0.27 mM
Spermidine	1.5 mM
Putrescine	1 mM
Sodium oxalate	4 mM
Phosphoenolpyruvate	33 mM
T7 RNA polymerase	100 mg ml−1

Table 4. BioBits® Reaction Mixture.

List of reagents and concentration added to the cell lysate to prepare final FD-CF protein synthesis pellet.

Plasmids Construction: GFP and sfGFP

For the purposes of this thesis, GFPmut3b will be referred to as GFP. This research compares folding between a standard, green fluorescent protein, called GFP (GFPmut3b), and the superfolder GFP (sfGFP) protein. As fluorescence of these proteins is directly linked to their properly folded protein structure, I will be using the fluorescence of GFP and sfGFP to observe protein folding and stability.

A recent study has cloned GFP and sfGFP into the pJL1 cell-free expression vector, which has been made available through Addgene (Stark *et al*., 2018). Through our collaboration with the Jewett Lab at Northeastern University, I was able to obtain both plasmids, named pJL1-GFP and pJL1-sfGFP. Both plasmids include a T7 promoter (Figures 10 and 11). They both include a strep tag (C terminal on insert), which can be utilized for an affinity chromatography protocol. Creation of these plasmid constructs utilized the same vector backbone, pJL1, through Gibson assembly cloning. This allowed for the same features to be consistent between the two constructs, including the T7 promoter and the strep tagging. The Gibson assembly procedure included designing the plasmid and associated primers, generating DNA segments by polymerase chain reaction (PCR), verifying product size using gel electrophoresis, combining the segments with the Gibson Assembly master mix, transforming NEB® DH5-alpha competent *E. coli,* screening for correct plasmid product, and sequencing for final verification of desired construct.

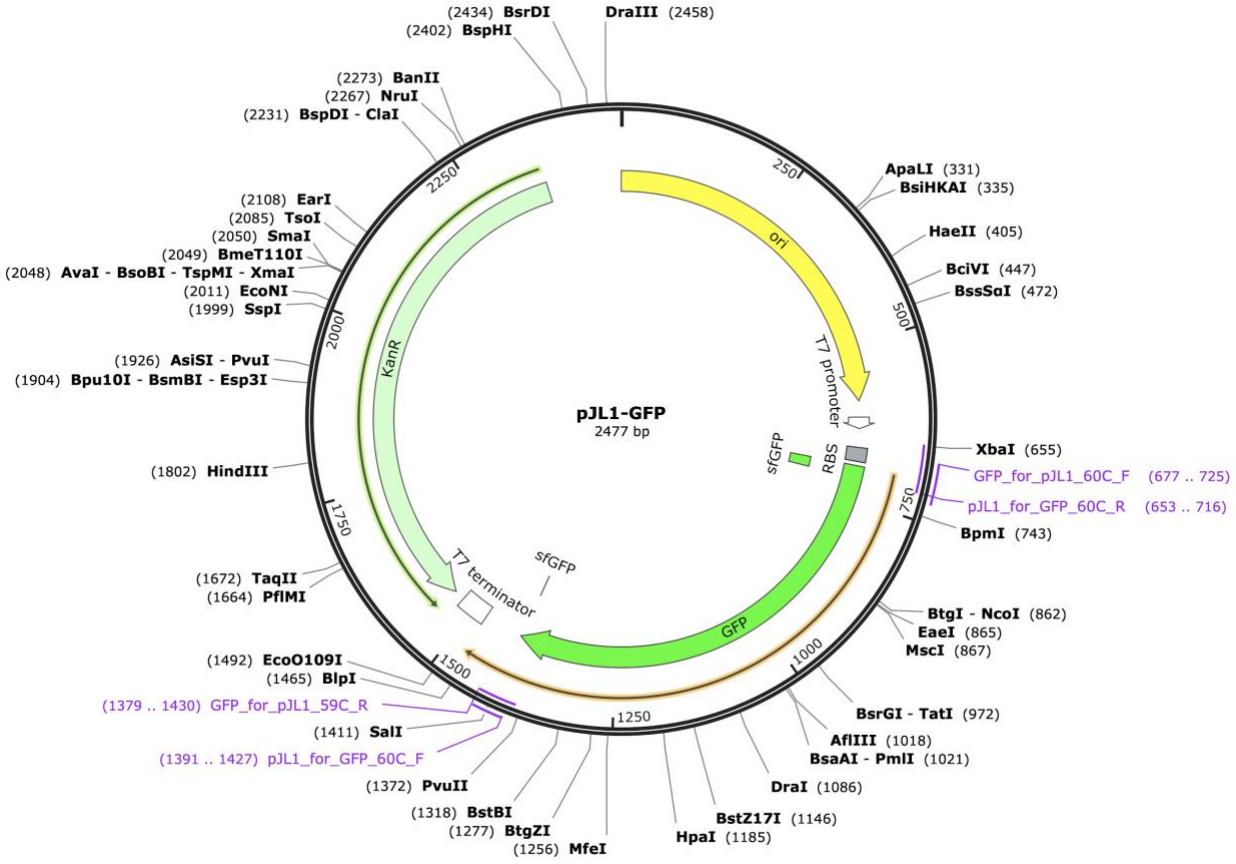


Figure 10. Plasmid map of pJL1-GFP.

This is the plasmid map of pJL1-GFP, with the GFPmmut3b insert, which was created with the pJL1 expression vector backbone (supplied by Jewett Lab, Northwestern University).

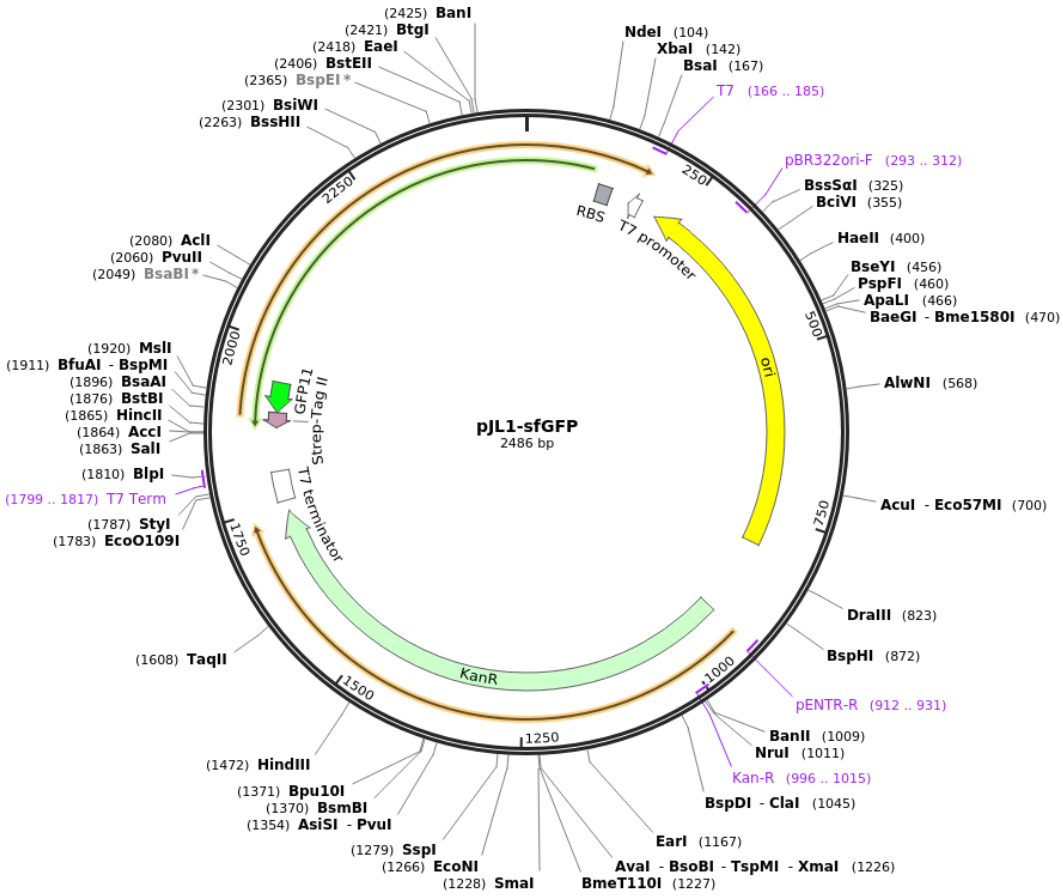


Figure 11. Plasmid map of pJL1-sfGFP plasmid.

This is the plasmid map of pJL1-sfGFP, which is created with the pJL1 expression vector backbone and the addition of the sfGFP gene (Addgene plasmid #102634; RRID:Addgene_102634)

Protein Synthesis

Incubation using miniPCR™

BioBits® pellet reactions were reconstituted with 5 µL either GFP or sfGFP plasmid DNA and incubated at room temperature, 30 °C, or 37 °C. The concentration of DNA, incubation temperature, and incubation time were all experiment-dependent (detailed below). For incubation temperatures greater than room temperature, a miniPCR™ thermocycler was used (Figure 12).

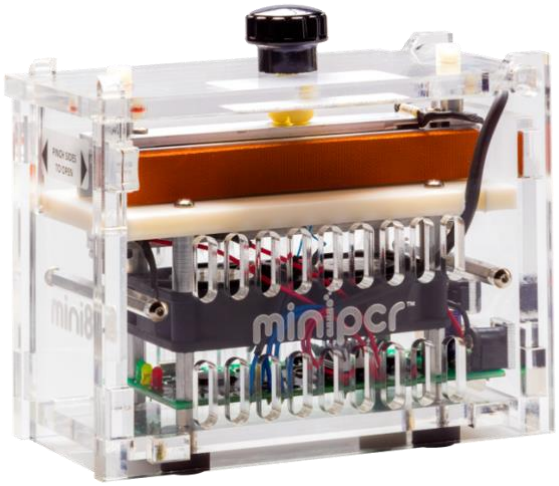


Figure 12. miniPCR™ Machine. The miniPCR™ machine is a PCR machine, but was used for controlled incubation protocols.

Quantifying Fluorescence of the FD-CF Reactions

It is common practice to use a plate reader to quantify fluorescence, measuring excitation and emission wavelengths of fluorescent protein constructs. However, it is possible to quantify fluorescence without a spectrophotometer. By using a portable, low-cost visualizer called the P51™, digital image capture, and subsequent fluorescence analysis in ImageJ, a free image-processing program supported by the NIH, I collected semiquantitative data of the FD-CF reactions for this research.

P51™ Visualizer

After the incubation period, the freeze-dried, cell-free synthesized fluorescent proteins were visualized using a P51™ visualizer (Figure 13). The P51™ visualizer is a molecular fluorescence viewer from miniPCR bio™. It is an all-in-one molecular visualization system with an integrated power source that utilizes blue light to excite fluorescence. The front display window allows for real time visualization of samples. The P51™ visualizer's cardboard design also allows this powerful tool to be relatively inexpensive. An orange filter was used for the selective wavelength of emission of green fluorescence and blockage of the blue excitation light.

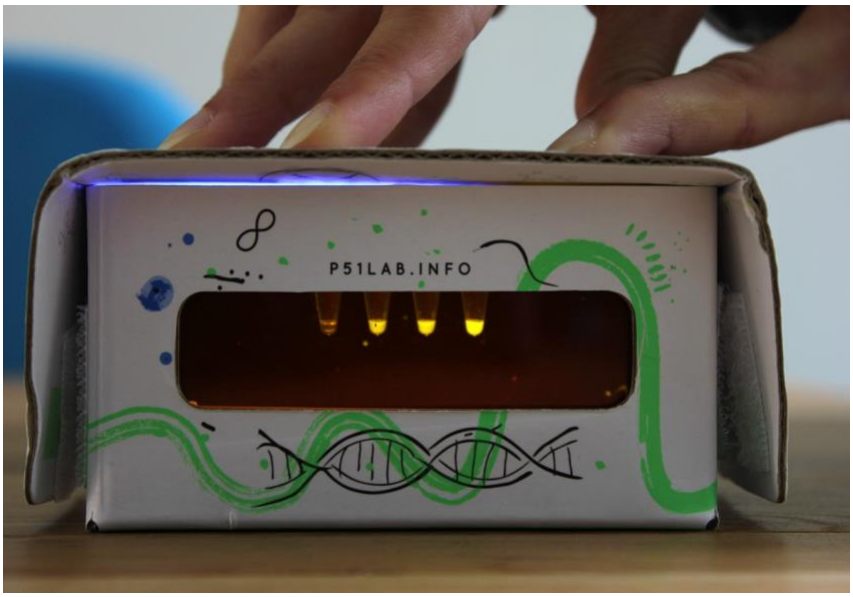


Figure 13. Photo of P51™ visualizer with BioBits® reactions inside.

DSLR and ImageJ photo analysis

The quantification of fluorescence of each FD-CF pellet was achieved by using ImageJ, a free software program developed by the National Institute of Health. ImageJ was used to measure relative pixel intensity (or brightness) values. Images taken with a DSLR camera were first converted to grayscale. For each reaction tube, a region of interest (ROI) was carefully selected using the oval shape icon to make a perfect circle that completely fit inside the FD-CF reaction part of each tube. Since the software acquired the average of every pixel within that selected area, it was important for the selection area to not include space outside of the reaction area. After all tubes had appropriate regions selected, they were then added to the ROI Manager window, where the Mean Gray Value box was selected. This then calculated the average pixel brightness value for each ROI selected.

Experiments

The following section details the experimental design used in this research, which can be divided into three tiers: protein synthesis, denaturation, and fluorescence rescue. First, various concentrations of DNA were used to explore the optimal visual difference between the two fluorescent proteins. Secondly, different denaturation techniques were applied to the synthesized fluorescent proteins for observation of unfolding. Finally, the denatured proteins were evaluated for potential fluorescence rescue, or refolding.

Experimental Focus	**Goal**
Protein Synthesis	Establish optimal visual difference between GFP and sfGFP (folding)
Denaturation	Establish parameters for denaturation of both GFP and sfGFP (unfolding)
Fluorescence Rescue	Establish feasibility of fluorescence rescue post protein denaturation (refolding)

Table 5. Overview of experimental focus, divided into three tiers

Protein Synthesis

The first goal of the experimental process was to establish at what concentration the greatest visual difference (brightness) occurred between GFP and sfGFP. Establishing this optimal concentration of plasmid where differences would be revealed in folding kinetics between two very similar proteins known to fold at different rates would help

determine the feasibility of using BioBits® as an assay to study folding. This was done by observing fluorescence of GFP and sfGFP samples with different DNA input concentrations over time. Four different concentrations of DNA were observed: 0.5 ng/µL, 1 ng/µL, 5 ng/µL, and 10 ng/µL. For both GFP and sfGFP, 5 µL of DNA were added per BioBits® reaction pellet. The reaction tubes were left at room temperature and only moved to a P51™ visualizer for photographing at specific timepoints over the course of 24 hours. After 24 hours, all reaction pellets were stored in the freezer at -20°C.

Protein Synthesis Experimental Design	
Part I	Comparison between 1 ng/µL, 5 ng/µL, and 10 ng/µL DNA concentrations for observation of optimal visual difference between GFP and sfGFP
Part II	Comparison between 0.5 ng/µL and 1 ng/µL DNA concentration for observations of optimal visual difference between GFP and sfGFP

Table 6. Overview of Protein Synthesis Experimental Design

Protein Denaturation

The second goal of the experimental process was to establish the parameters for GFP and sfGFP denaturation. Observing the denaturation of the two similar proteins by thermal and chemical disruptions would give insight into each protein's structural stability. The denaturation experimental design was divided into the following four parts.

Denaturation Experimental Design	
Part I	Progressive heat denaturation of stored proteins synthesized using 1 ng/µL concentration of starting plasmid
Part II	0.5% SDS denaturation at room temperature of proteins synthesized using a 0.5 ng/µL concentration of plasmid
Part III	0.5% SDS + progressive heat denaturation of proteins synthesized using a 0.5 ng/µL concentration of plasmid
Part IV	Three concentrations of SDS + progressive heat denaturation of stored proteins from previous experiment, using 1 ng/µL concentration of starting plasmid
Part V	Non-progressive heat denaturation of stored proteins synthesized using 1 ng/µL concentration of starting plasmid

Table 7. Overview of Denaturation Experimental Design

Part I: Progressive heat denaturation of stored proteins synthesized using 1 ng/µL concentration of starting plasmid

Protein Synthesis

GFP and sfGFP (both 1 ng/µL concentration) utilized for this denaturation experiment came from a previous experiment where four BioBits® pellets (two reactions per each condition) were placed in either a miniPCR machine and incubated at 37°C or in a waistband (body heat, roughly 37 °C) for 30 minutes and then left at room temperature overnight.

Protein Denaturation

Starting with 37 °C, the samples, which included 5 µL of water, were heated for 30 minutes. The samples were imaged every 5 minutes (0, 5, 10, 15, 20, 25, and 30 minute timepoints). After the 30 minute mark, the samples then progressed to the next temperature (37 °C, 50 °C, 70 °C, 80 °C, and finally 90 °C). The samples were placed in a P51™ visualizer with an orange filter. Images were taken with a DSLR camera with manual settings.

Part II: 0.5% SDS denaturation at room temperature of proteins synthesized using a 0.5 ng/µL concentration of plasmid

Protein Synthesis

Eight BioBits® pellets (two reactions per each condition) were used to produce GFP and sfGFP samples. 5 µL of 0.5 ng/µL concentration GFP or sfGFP were added to four tubes each. The tubes were incubated at 37°C in a miniPCR™ machine for 60 minutes and then left overnight for the denaturation experiment next day.

Protein Denaturation

5 µL of water were added to two GFP samples and two sfGFP samples. 5 µL of 1% SDS were added to the remaining four samples (two GFP and two sfGFP). The samples were incubated at room temperature for 30 minutes and observations were made every 5 minutes (0, 5, 10, 15, 20, 25, and 30 minute increments).

Part III: 0.5% SDS + progressive heat denaturation of proteins synthesized using a 0.5 ng/µL concentration of plasmid

Protein Synthesis

Eight BioBits® pellets (two reactions per each condition) were used to produce GFP and sfGFP samples. 5 µL of 0.5 ng/µL concentration GFP or sfGFP were added to four tubes each. The tubes were incubated at 37°C in a miniPCR™ machine for 60 minutes and then left overnight for the denaturation experiment next day.

Protein Denaturation

5 µL of water were added to two GFP samples and two sfGFP samples. 5 µL of 1% SDS were added to the remaining four samples (two GFP and two sfGFP). The following day, the samples were treated with either 0.5% SDS or water and incubated in a miniPCR™ machine at 70°C for observation for 30 minutes. After 30 minutes, the temperature increased to 80°C (followed by 90 °C). Samples were placed in a P51™ visualizer with an orange filter. Photos were taken with a DSLR camera with manual settings.

Part IV: Various concentrations of SDS + progressive heat denaturation of stored proteins from previous experiment, using 1 ng/μL concentration of starting plasmid

Protein Synthesis

GFP and sfGFP (both 1 ng/μL concentration) used for this denaturation experiment came from a previous experiment where four BioBits® pellets (two reactions per condition) were placed in a waistband (body heat, roughly 37 °C) for 60 minutes and then left at room temperature overnight to simulate typical classroom implementation.

Protein Denaturation

2 μL of either water, 1.7% SDS (final concentration 0.5%), 0.875% SDS (final concentration 0.25%), or 0.5% SDS (final concentration 0.143%) were added to the appropriate BioBits® tube. Starting with 70°C, the samples were heated for 30 minutes in a miniPCR™ machine. The samples were imaged every 5 minutes (0, 5, 10, 15, 20, 25, and 30-minute timepoints). After the 30-minute mark, the samples then progressed to the next temperature (80 °C). Unlike previous denaturation experiments where the full 30 minutes were observed for each temperature, samples progressed to the next temperature once a visual change in fluorescence was observed. Samples were placed in a P51™ visualizer with an orange filter. Photos were taken with a DSLR camera with manual settings.

Part V: Non-progressive heat denaturation of stored proteins synthesized using 1 ng/µL concentration of starting plasmid

Protein Synthesis

24 BioBits® pellets (three strips of eight) were used to produce GFP and sfGFP samples. 5 µL of 1 ng/µL concentration GFP and sfGFP were added to 12 tubes each. The tubes were incubated at room temperature overnight for the denaturation experiment next day.

Protein Denaturation

5 µL of either water or 1% SDS (final concentration 0.5%), were added to the appropriate BioBits® tube. Previous temperature denature experiments used a progressive temperature protocol. Here, each strip of eight samples was placed in a miniPCR™ machine and denatured at one temperature only. Strip 1 was denatured at 70 °C, strip 2 was denatured at 80°C, and strip 3 was denatured at 90 °C. The samples were imaged every 5 minutes (0, 5, 10, 15, 20, 25, and 30 minute timepoints). Samples were placed in a P51™ visualizer with an orange filter. Photos were taken with a DSLR camera with manual settings.

Fluorescence Rescue

The third goal of the experimental process was to determine parameters for GFP and sfGFP fluorescence rescue after previous denaturation protocols. Observing fluorescence rescue would reveal refolding capabilities and differences between the two similar proteins.

Fluorescence Rescue Experimental Design	
Part I	Room temperature overnight rescue
Part II	Temperature ramp down rescue
Part III	Dilute out SDS with the addition of water

Table 8. Overview of Fluorescence Rescue Experimental Design

Part I: Room temperature overnight rescue

Samples from previous denaturation experiments were left on the benchtop at room temperature overnight and observed the next day for fluorescence rescue.

Part II: Temperature ramp down rescue

A ramp down protocol was programmed onto the miniPCR™ machine, where the starting temperature was 90°C (this was the last temperature of the denaturation experiment). The cooling rate was 1°C per minute, until the samples reached a final temperature of 25°C.

Part III: Dilute out SDS with the addition of water

In an attempt to dilute out the SDS in the pellet reaction, 2 µL of sterile water were added to samples that had been previously denatured at 90°C and then ramped down at 1°C per minute to 25°C. After this initial observation, an additional 2 µL of sterile water were added twice more (for a total volume of 4 µL and 6 µL of sterile water). After each addition of water, the samples were left on the benchtop for 15 minutes before being placed in a P51™ visualizer and photographed for comparison.

Chapter III.

Results

The focus of this research was to explore the suitability of the FD-CF system to study protein folding in resource-limited settings. To do this, laboratory work focused on three areas: protein synthesis, denaturation, and fluorescence rescue. The data collected from the experiments outlined in the previous section are described in further detail in this section.

Protein Synthesis

The goal of Part I of the Protein Synthesis experiment was to determine the optimal visual difference between GFP and sfGFP; that is, at what concentration was the greatest difference in fluorescence between the two proteins observed. Establishing this optimal concentration of plasmid where differences would be revealed in folding kinetics between two very similar proteins known to fold at different rates would help determine the feasibility of using BioBits® as an assay to study folding. It was also necessary to establish this plasmid DNA concentration for all further experiments, as it is important for all experimental procedures to be carried out with only one DNA concentration for classroom feasibility.

I first explored fluorescence of the two proteins at 1 ng/µL, 5 ng/µL, and 10 ng/µL plasmid concentrations (not protein concentration) over a 24-hour incubation period at room temperature. Previous research using BioBits® explored plasmid DNA

concentrations between 2.5 ng/µL and 25 ng/µL (Huang *et al*., 2018), thus I expected to see a greater difference in fluorescence at the higher concentrations. Surprisingly, the difference in fluorescence between GFP and sfGFP was greatest at 1 ng/µL, compared to 5 ng/µL and 10 ng/µL (Figure 14). After 24 hours, the 1 ng/µL GFP reaction exhibited an average pixel intensity (brightness) of 146.299 compared to 190.41 of the 1 ng/µL sfGFP reaction. At both 5 ng/µL and 10 ng/µL concentrations, the difference in brightness between the two constructs was minimal (Table 9). The point of saturation was observed at 20 hours for both constructs.

Concentration	**GFP Pixel Intensity**	**sfGFP Pixel Intensity**	**Difference**
1 ng/µL	146.299	190.41	44.111
5 ng/µL	171.489	190.067	18.578
10 ng/µL	169.358	190.751	21.393

Table 9. Calculated pixel intensity.

Brightness after 24 hours of both protein construct reactions at 1 ng/µL, 5 ng/µL, and 10 ng/µL concentrations.

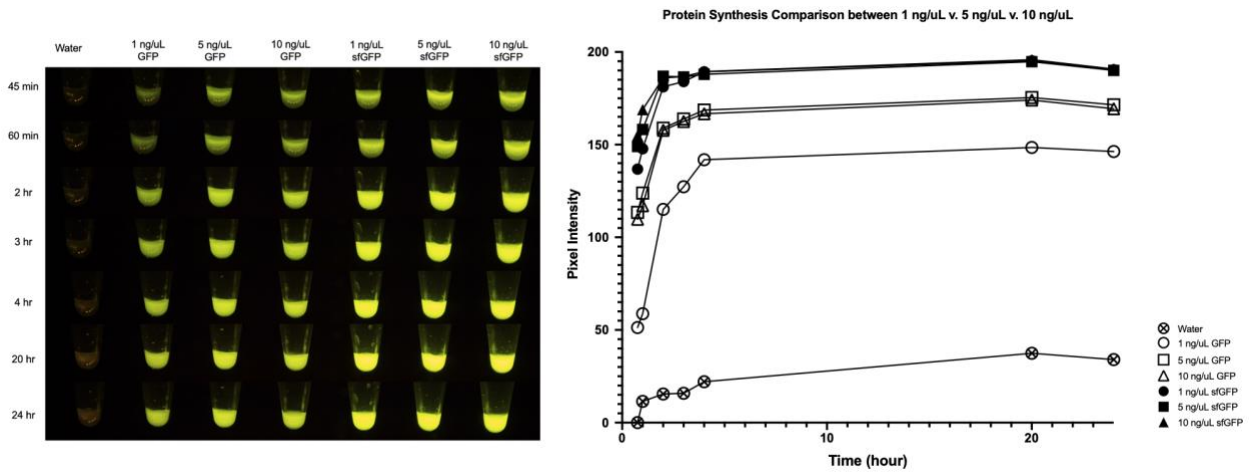


Figure 14. Optimal visual difference between GFP and sfGFP at 1 ng/µL, 5 ng/µL, and 10 ng/µL.

The left panel shows observed fluorescence over time from 45 minutes to 24 hours at room temperature. The first column serves as the control, as 5 µL of water were added to the BioBits® pellet. The preceding columns use 1, 5, and 10 ng/µL of GFP then sfGFP respectively. At the appropriate time, the tubes were placed in a p51™ visualizer and photographed.

With the greatest optimal difference at the lowest DNA concentration tested in Part I, it was only natural to explore concentrations even lower. In Part II, an optimal visual difference in fluorescence was observed at 0.5 ng/µL, compared to 1.0 ng/µL (Figure 15). After 60 minutes, the 0.5 ng/µL GFP reaction exhibited a pixel intensity (brightness) of 65.634 compared to 156.06 of the 0.5 ng/µL sfGFP reaction (difference of 90.426). The 1 ng/µL GFP reaction exhibited 70.263 pixel intensity (brightness) compared to 172.238 of the 1 ng/µL sfGFP reaction (difference of 101.975). The data suggest that using half the initial concentration of plasmid DNA does not drastically change the measured fluorescence after 60 minutes, thus using 0.5 ng/µL is the minimum amount of plasmid DNA added to the FD-CF pellet for both robust expression and optimal visual difference. Taken together, these data show that the system saturates at

high plasmid concentrations, but at lower concentrations I could observe expected differences, suggesting this is a helpful assay to explore differences in folding rates. Controlling for the differences in the amount of protein being made in order to know whether the difference in brightness reflects only a protein folding difference or a synthesis difference could not be done in house. This is further considered in the discussion section.

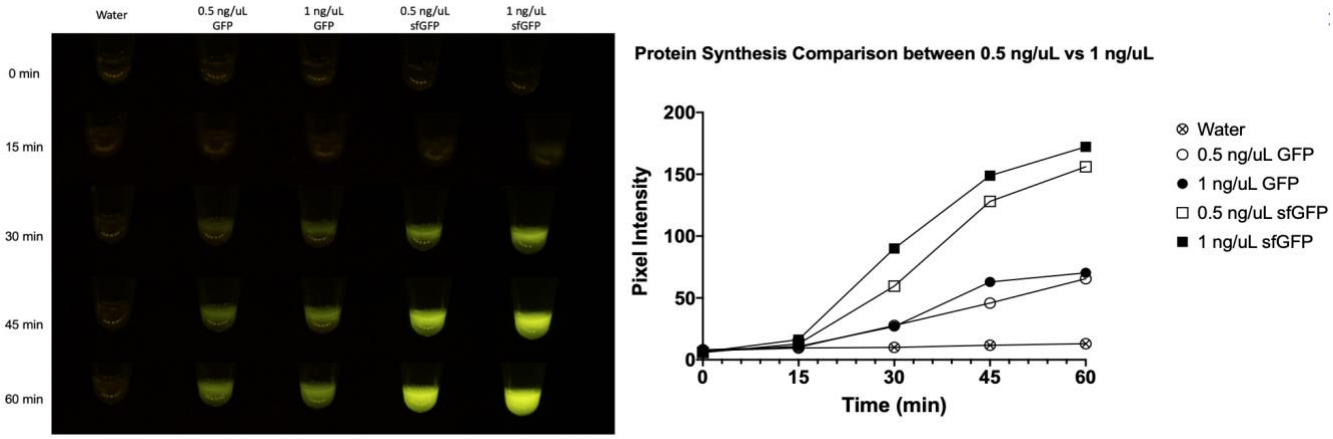


Figure 15. Optimal visual difference between GFP and sfGFP at 0.5 ng/µL and 1 ng/µL.

The left panel shows observed fluorescence over time from 0 to 60 minutes at room temperature. The first column serves as the control, as 5 µL of water were added to the BioBits® pellet. The preceding columns use 0.5 and 1 ng/µL of GFP then sfGFP respectively. At the appropriate time, the tubes were placed in a p51™ visualizer and photographed.

Concentration	GFP Pixel Intensity	sfGFP Pixel Intensity	Difference
0.5 ng/µL	65.634	156.06	90.426
1 ng/µL	70.263	172.238	101.975

Table 10: Calculated pixel intensity (brightness) after 60 minutes of both protein construct reactions at 0.5 ng/µL and 1 ng/µL concentrations.

Denaturation

The second goal of the experimental process was to establish the parameters for GFP and sfGFP denaturation. As limited data was available regarding the denaturation of GFP and sfGFP synthesized with the FD-CF system, it was necessary to establish the impact of multiple denaturants on fluorescence. The denaturation experimental design was divided into the five parts using heat, SDS, and a combination of both.

Part I: Heat denaturation of stored proteins from previous experiment, using 1 ng/µL concentration of starting plasmid

In this experiment, I wanted to test the effect of heat on already synthesized GFP and sfGFP reactions. I started the incubation temperature at 37°C and increased every 30 minutes to the next temperature condition. The fluorescence of both GFP and sfGFP reactions did not decrease until the higher incubation temperatures (Figure 16). GFP fluorescence did not decrease significant amounts at 37°C, 50°C, and 70°C. However, a noticeable decrease in fluorescence was observed visually with the GFP reactions after 5 minutes at 80°C (Figure 17.D). sfGFP fluorescence did not decrease significant amounts at 37°C, 50°C, 70°C, or even 80°C. sfGFP fluorescence decreased after 5 minutes at 90°C (Figure 17.E). The data suggests that sfGFP is able to stay in its folded and stable state at higher temperatures compared to GFP, shedding light on the structural stability of sfGFP at different temperatures.

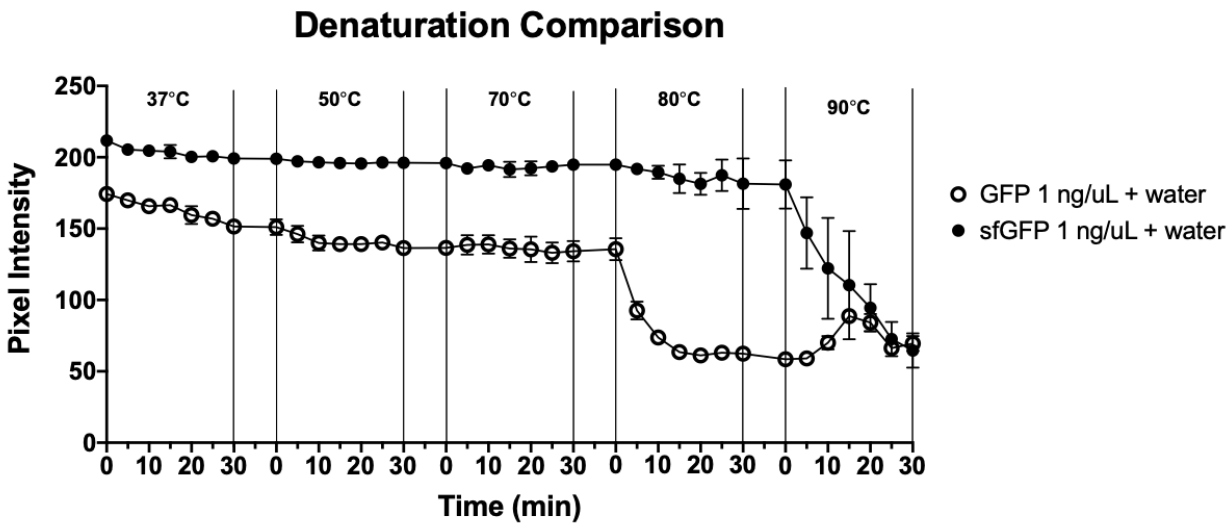


Figure 16. Heat denaturation comparison between GFP and sfGFP.

GFP and sfGFP at 1 ng/µL over time and across five temperatures: 37, 50, 70, 80, and 90°C. Pixel intensity data was generated by analyzing the previous images with ImageJ.

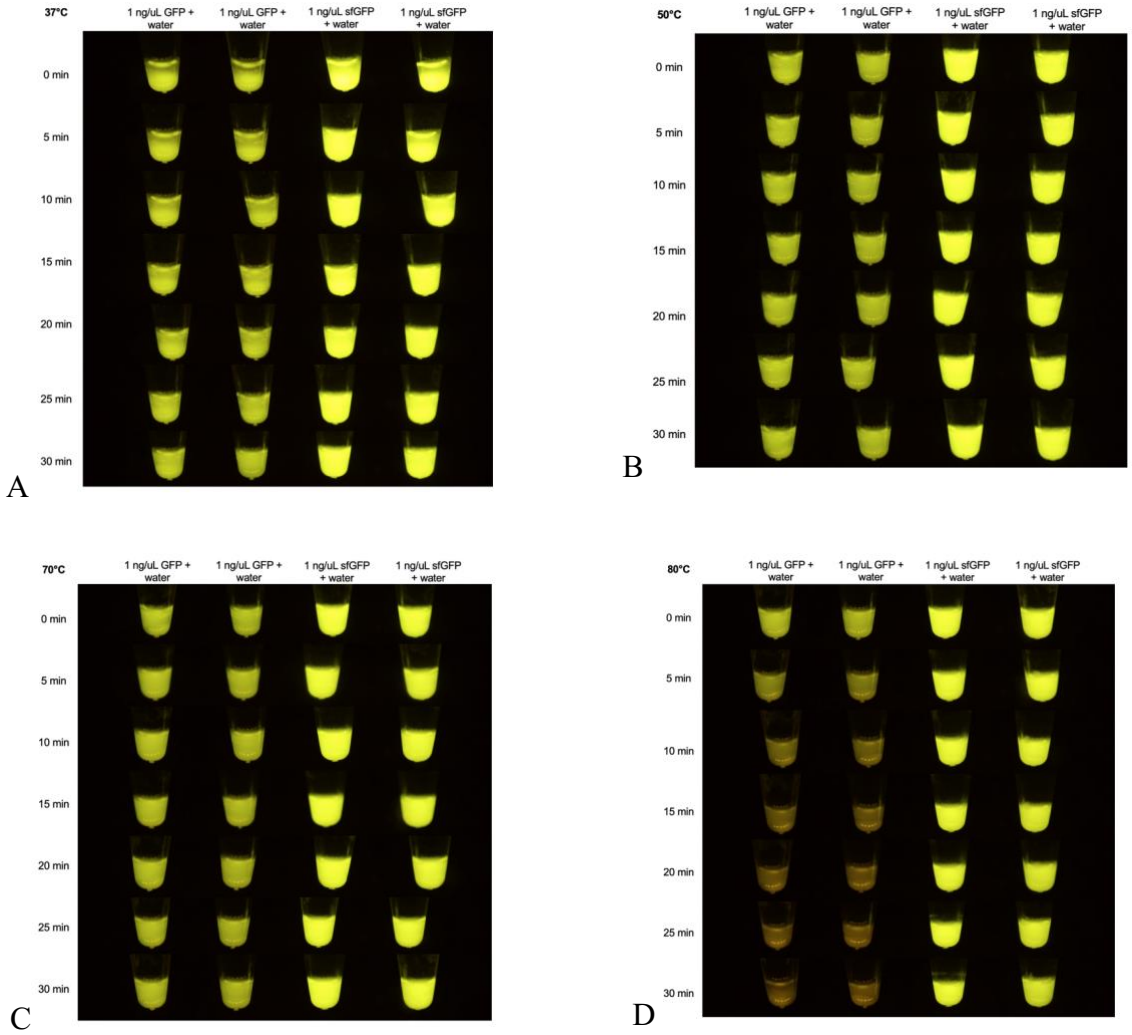


Figure 17. Heat denaturation of stored proteins.

Starting with 37°C, the samples, which include 5µL of water, were heated for 30 minutes. The samples were imaged every 5 minutes. At the 30 minute mark, the samples then progressed to the next temperature (Panels A: 37°C, B: 50°C, C: 70°C, D: 80°C, and E: 90°C). Samples were placed in a p51™ visualizer with an orange filter and photographed. GFP and sfGFP (both 1 ng/µL concentration) utilized for this denaturation experiment came from a previous experiment.

Part II: 0.5% SDS denaturation at room temperature of proteins synthesized using a 0.5 ng/μL concentration of plasmid

In Part II of the denaturation phase, I sought to test the effect of SDS on both GFP and sfGFP proteins. This was first done at room temperature. Minimum loss of fluorescence was observed in both the GFP of sfGFP samples with the addition of 0.5% SDS at room temperature over the course of one hour, however this change was difficult to confirm visually (Figures 18 and 19). The data suggest that both GFP and sfGFP were stable in the presence of SDS at a final concentration at 0.5% at room temperature. Both GFP and sfGFP retained their relative fluorescence, suggesting no structural shift in this condition. This was unexpected as SDS is a known denaturant. After further research, I discovered that a previous study using 0.5% SDS to denature GFP without heat was dependent on the pH of the reaction buffer (Alkaabi *et al.*). I went on to test the general pH of a BioBits® reaction, which was shown to be 7.0 (data not shown). Thus, the data was no longer surprising, as Alkaabi *et al.* showed GFP retains its fluorescence in the presence of 0.5% SDS, but only when the protein was in buffers that ranged from pH 7 to 8. Alkaabi *et al.* went on to show that as soon as the pH dropped to 6.5 or below, GFP lost all of its fluorescence. Considering that it would be difficult to decrease the pH of a BioBits® reaction specifically in a classroom, I then interrogated the coupled effect of SDS and heat.

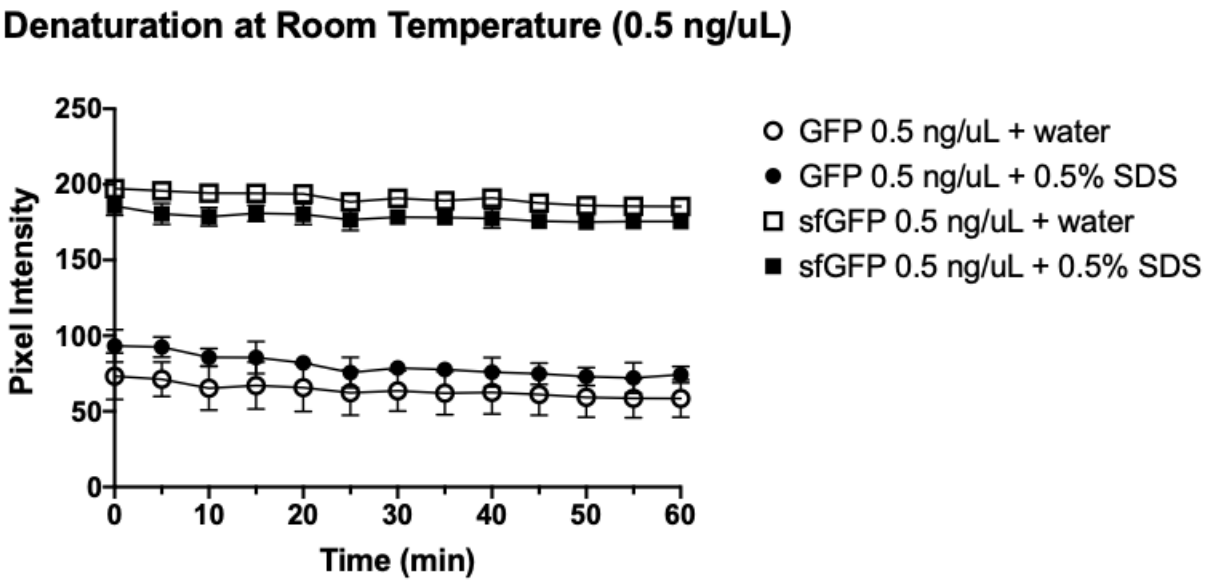


Figure 18. Denaturation at Room Temperature with SDS.

GFP and sfGFP (both 0.5 ng/μL concentration) samples were treated with water or 0.5% SDS and left at room temperature for observation for one hour. No heat was added.

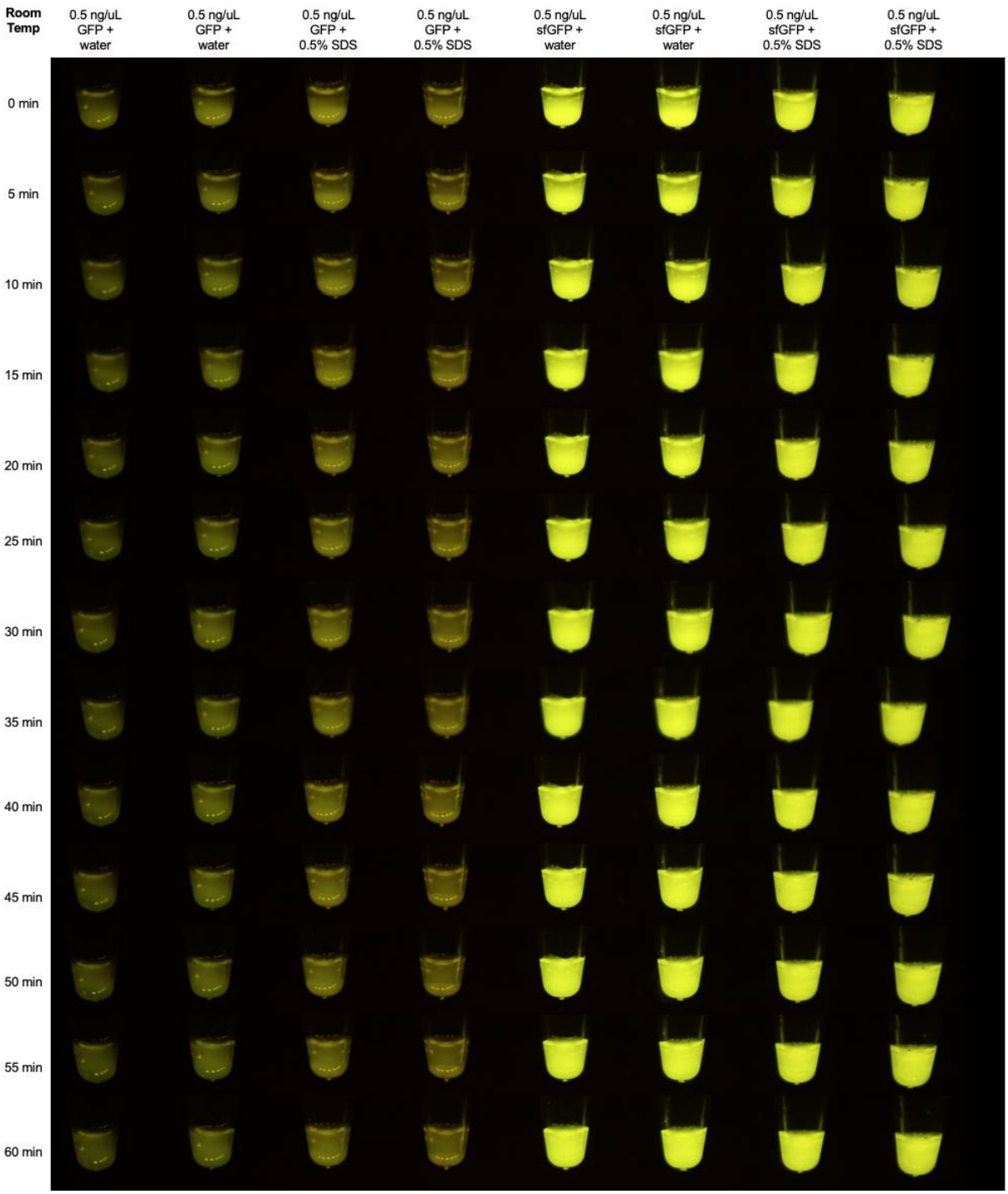


Figure 19. Visualization of Denaturation at Room Temperature with SDS.

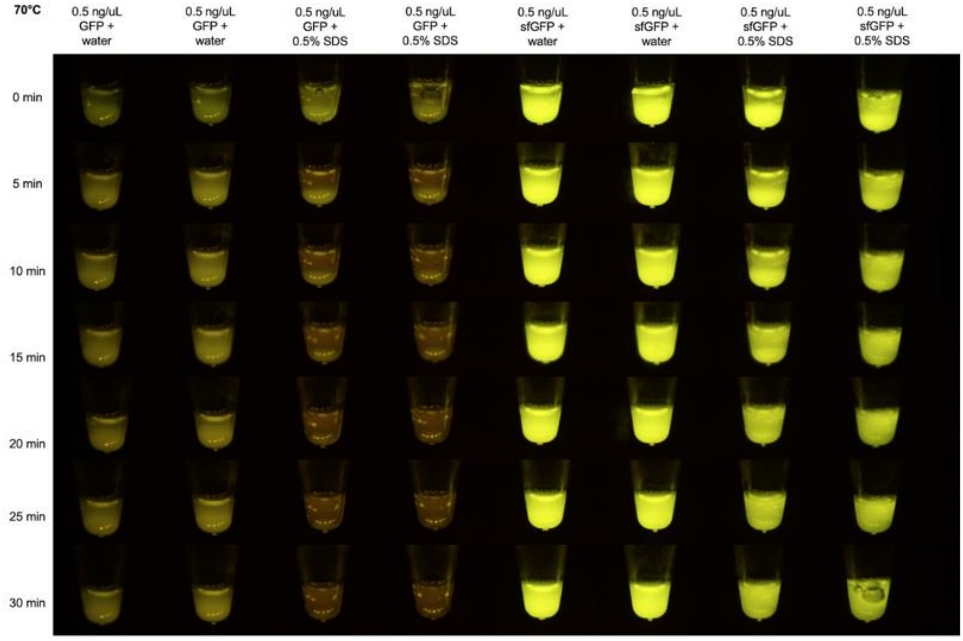
GFP and sfGFP (both 0.5 ng/µL concentration) samples were treated with water or 0.5% SDS and left at room temperature for observation for one hour. No heat was added. Visually, no loss of fluorescence was observed. Samples were placed in a P51™ visualizer with an orange. Photos were taken with a DSLR camera with manual settings.

Part III: 0.5% SDS + heat denaturation of proteins synthesized using a 0.5 ng/µL concentration of plasmid

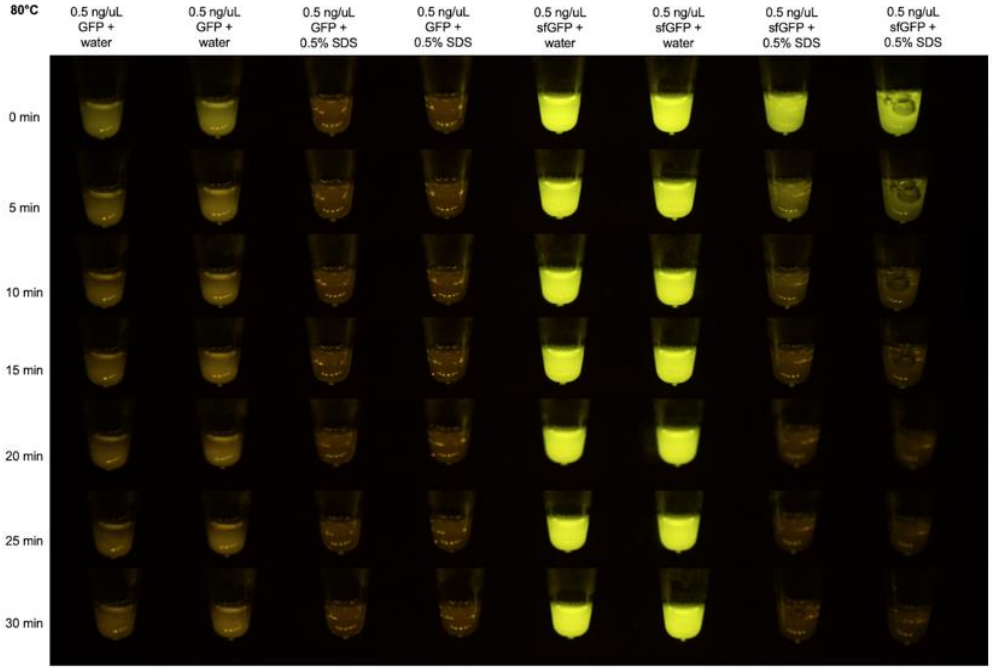
Using the data from Part I which showed that heat denatures both GFP and sfGFP, and previous work that SDS is a common denaturant, I sought to observe the coupled effect of heat and SDS on GFP and sfGFP fluorescence. At 0.5 ng/µL, sfGFP has a significant decrease in fluorescence after 5 minutes at 80°C with the addition of 0.5% SDS. With only the addition of water, sfGFP fluorescence decreased after 5 minutes at 90°C. This is consistent with previous data, where 1 ng/µL was used. This suggests that the structural shift is independent of input concentration of the DNA.

However, decreasing the GFP plasmid concentration from 1 ng/µL to 0.5 ng/µL does influence the visual impact of denaturation. This may be a result of the GFP's initial weaker fluorescence at 0.5 ng/µL compared to 1 ng/µL or this could suggest that the structural shift in stability of GFP is dependent on input DNA concentration. As a result of this observation, it was suggested to move forward with using 1 ng/µL concentration of input DNA for further experiments for feasibility of classroom applications.

A


70°C
0.5 ng/uL GFP + water
0.5 ng/uL GFP + water
0.5 ng/uL GFP + 0.5% SDS
0.5 ng/uL GFP + 0.5% SDS
0.5 ng/uL sfGFP + water
0.5 ng/uL sfGFP + water
0.5 ng/uL sfGFP + 0.5% SDS
0.5 ng/uL sfGFP + 0.5% SDS
0 min
5 min
10 min
15 min
20 min
25 min
30 min

B


80°C
0.5 ng/uL GFP + water
0.5 ng/uL GFP + water
0.5 ng/uL GFP + 0.5% SDS
0.5 ng/uL GFP + 0.5% SDS
0.5 ng/uL sfGFP + water
0.5 ng/uL sfGFP + water
0.5 ng/uL sfGFP + 0.5% SDS
0.5 ng/uL sfGFP + 0.5% SDS
0 min
5 min
10 min
15 min
20 min
25 min
30 min

C

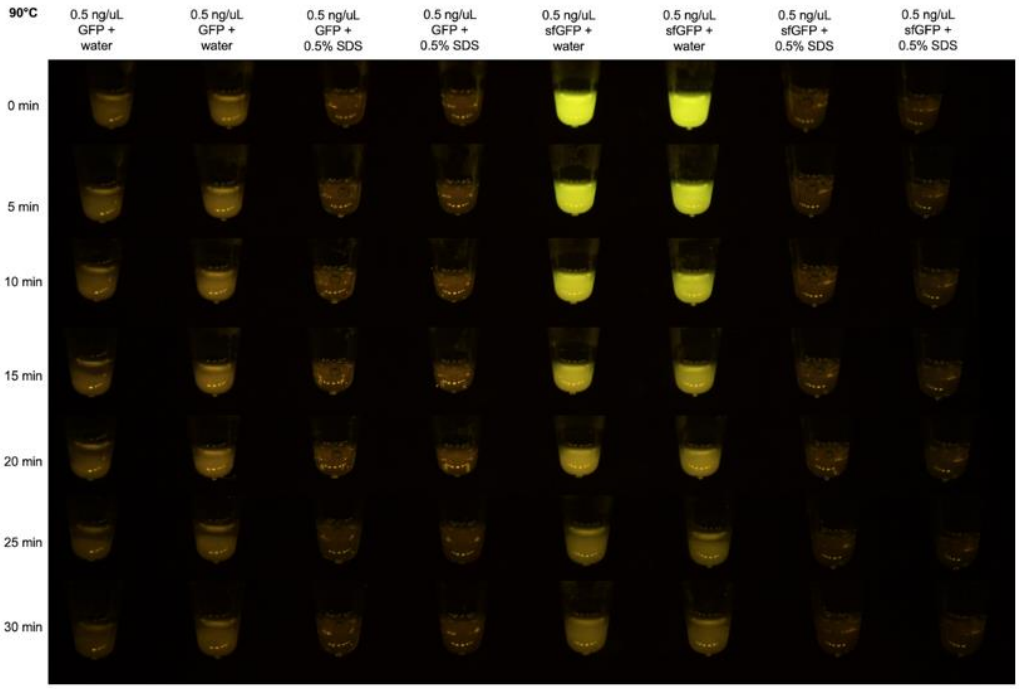


Figure 20. Denaturation with Heat and 0.5% SDS

GFP and sfGFP (both 0.5 ng/µL concentration) were expressed using eight BioBits® pellets. These tubes were incubated at 37°C in a miniPCR machine for 60 minutes and then left overnight for the denaturation experiment next day. 5 µL of water were added to two GFP samples and two sfGFP samples as controls. 5 µL of 1% SDS were added to the remaining four samples. The samples were treated with either 0.5% SDS or water and incubated in a miniPCR machine at 70°C for observation for 30 minutes. After 30 minutes, the temperature was increased to 80°C (followed by 90°C). Samples were placed in a p51™ visualizer with an orange filter. Photos were taken with a DSLR camera with manual settings.

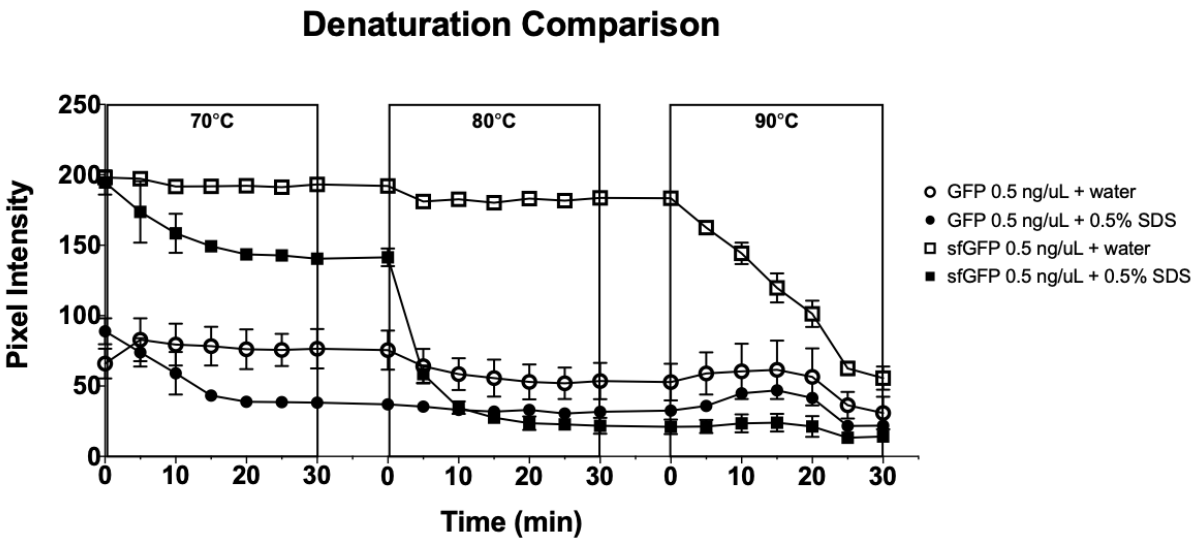


Figure 21. Comparison of Denaturation with Heat and 0.5% SDS GFP and sfGFP.

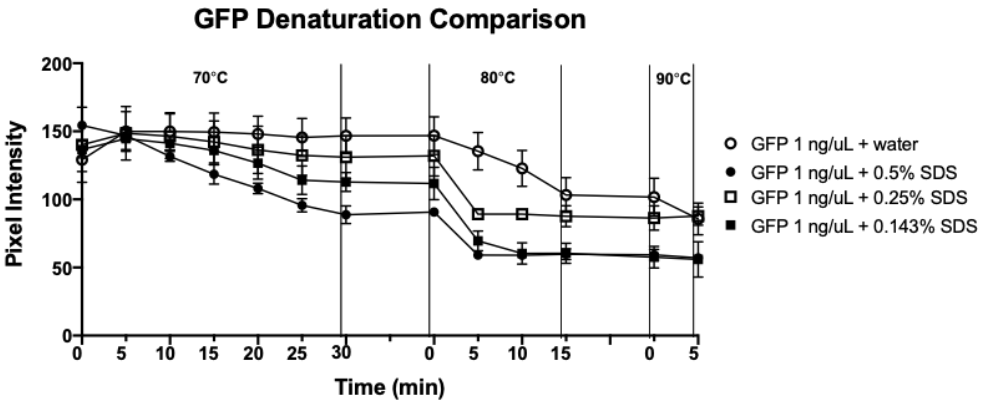
At 0.5 ng/µL, sfGFP has a significant decrease in fluorescence after 5 minutes at 80°C with the addition of 0.5% SDS. With only the addition of water, sfGFP fluorescence decreased after 5 minutes at 90°C. This is consistent with the data from a previous experiment, where 1 ng/µL of DNA was used for protein synthesis. In this respect, cutting the DNA concentration in half from 1 ng/µL to 0.5 ng/µL does not affect the time at which fluorescence starts to decrease for sfGFP with the addition of water. However, going from 1 ng/µL to 0.5 ng/µL for the GFP does influence the visual impact of denaturation.

Part IV: Three Concentrations of SDS + Heat Denaturation of Proteins Synthesized using 1 ng/µL DNA

With data from Part III showing that a combination of heat and 0.5% SDS results in a decrease in fluorescence of both GFP and sfGFP, I wanted to then explore the effect of lower SDS concentrations on fluorescence. Not surprisingly, GFP with the highest concentration of SDS exhibited the greatest loss of fluorescence (Figure 22). Even during the initial stages of the experiment at 70°C, GFP fluorescence decreased more with the 0.5% SDS condition, compared to the 0.25% and 0.143% conditions. The most interesting observation was observed at the 5-minute time point at 80°C. All three SDS concentration conditions exhibited a steep drop in fluorescence at this time point. However, fluorescence then plateaued at this point. Even when the temperature increased to 90°C, the fluorescence for all three SDS concentrations were relatively similar.

The effect of the three SDS concentrations on sfGFP fluorescence exhibited a different trend (Figure 23). sfGFP reactions with the highest concentration of SDS added exhibited a slight drop at the 15-minute time point at 80°C. However, all three SDS conditions exhibited steep decreases in fluorescence after 5 minutes at 90°C. The data show sfGFP is sensitive to denaturation by heat and SDS at higher temperatures.

A


GFP Denaturation Comparison
70°C
80°C
90°C
Pixel Intensity
Time (min)
GFP 1 ng/uL + water
GFP 1 ng/uL + 0.5% SDS
GFP 1 ng/uL + 0.25% SDS
GFP 1 ng/uL + 0.143% SDS

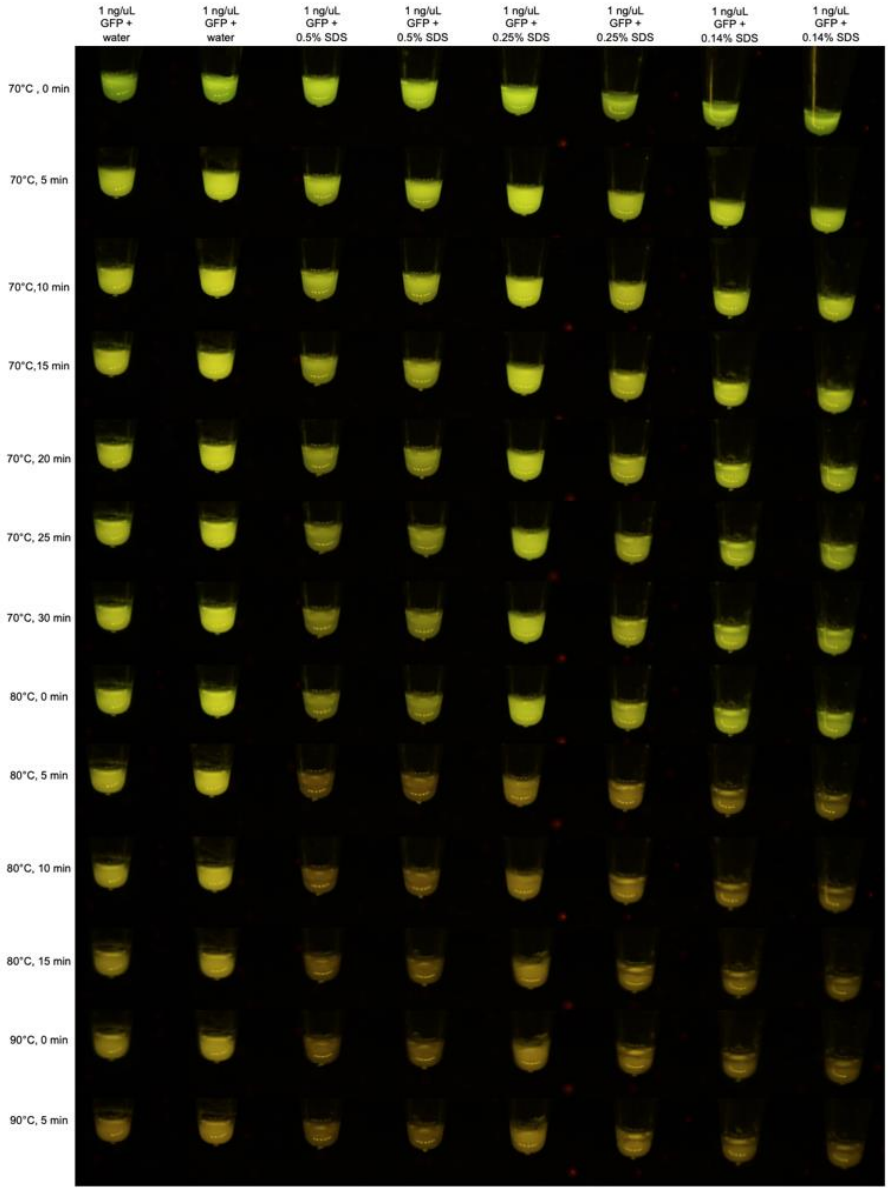

1 ng/uL GFP + water
1 ng/uL GFP + water
1 ng/uL GFP + 0.5% SDS
1 ng/uL GFP + 0.5% SDS
1 ng/uL GFP + 0.25% SDS
1 ng/uL GFP + 0.25% SDS
1 ng/uL GFP + 0.14% SDS
1 ng/uL GFP + 0.14% SDS
70°C , 0 min
70°C, 5 min
70°C,10 min
70°C,15 min
70°C, 20 min
70°C, 25 min
70°C, 30 min
80°C, 0 min
80°C, 5 min
80°C, 10 min
80°C, 15 min
90°C, 0 min
90°C, 5 min

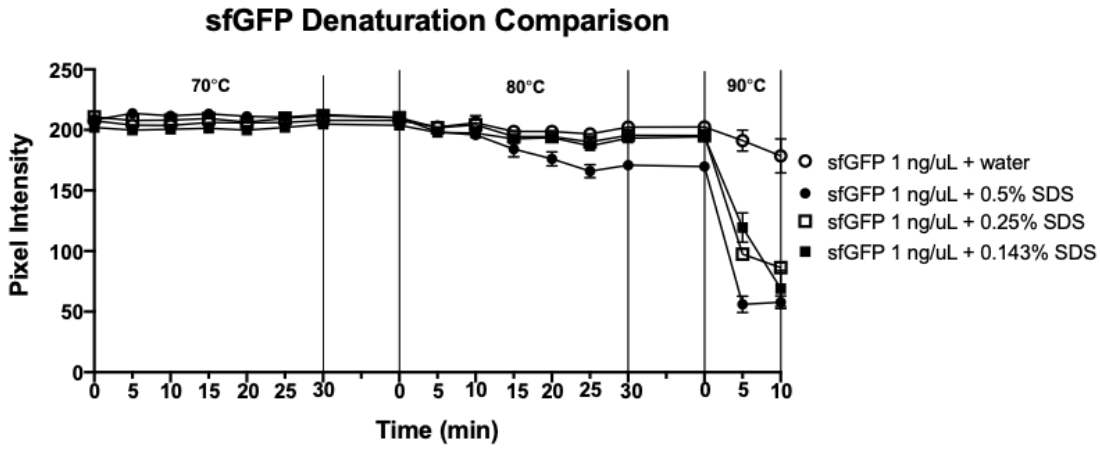
Figure 22. GFP Denaturation with Varied Concentration of SDS

GFP samples (1 ng/µL concentration) utilized for this denaturation experiment came from a previous experiment where four BioBits® pellets (two reactions per each condition) were placed in a waistband (body heat, roughly 37°C) for 60 minutes and then left at room temperature overnight to simulate typical classroom implementation. 2 µL of either water, 1.7% SDS (final concentration 0.5%), 0.875% SDS (final concentration 0.25%), or 0.5% SDS (final concentration 0.143%) were added to the appropriate BioBits® tube. Starting with 70°C, the samples were heated for 30 minutes in a miniPCR machine. The samples were imaged every 5 minutes (0, 5, 10, 15, 20, 25, and 30-minute timepoints). After the 30-minute mark, the samples then progressed to the next temperature (80° C.) Unlike previous denaturation experiments where the full 30 minutes were observed for each temperature, samples progressed to the next temperature once a visual change in fluorescence was observed. Samples were placed in a p51™ visualizer with an orange filter. Photos were taken with a DSLR camera with manual settings.

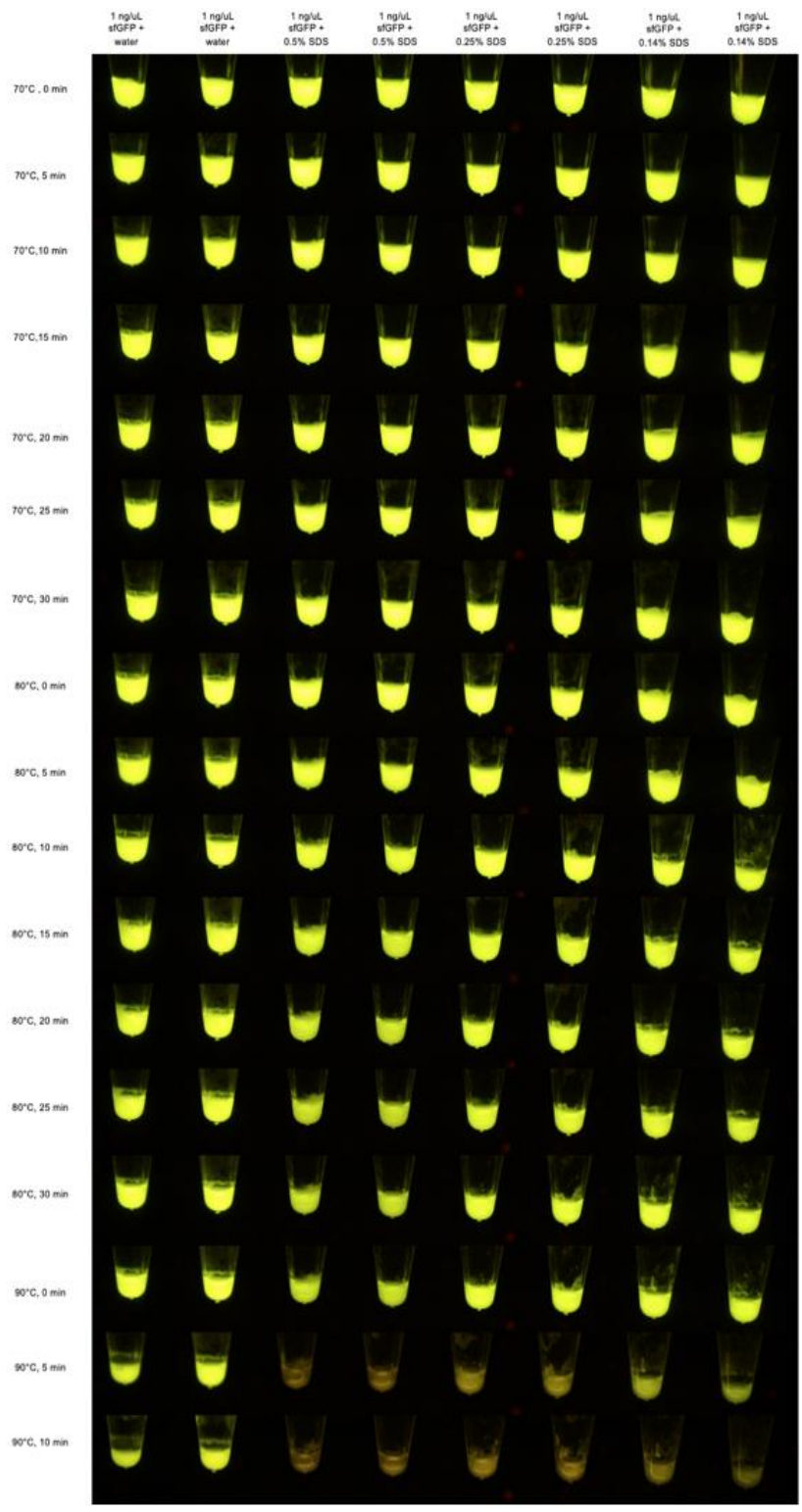
Figure 23. sfGFP Denaturation with Varied Concentration of SDS

sfGFP samples (1 ng/µL concentration) utilized for this denaturation experiment came from a previous experiment where four BioBits® pellets (two reactions per each condition) were placed in a waistband (body heat, roughly 37°C) for 60 minutes and then left at room temperature overnight to simulate typical classroom implementation. 2 µL of either water, 1.7% SDS (final concentration 0.5%), 0.875% SDS (final concentration 0.25%), or 0.5% SDS (final concentration 0.143%) were added to the appropriate BioBits® tube. Starting with 70°C, the samples were heated for 30 minutes in a miniPCR machine. The samples were imaged every 5 minutes (0, 5, 10, 15, 20, 25, and 30-minute timepoints). After the 30-minute mark, the samples then progressed to the next temperature (80°C.) Unlike previous denaturation experiments where the full 30 minutes were observed for each temperature, samples progressed to the next temperature once a visual change in fluorescence was observed. Samples were placed in a p51™ visualizer with an orange filter. Photos were taken with a DSLR camera with manual settings.

A



B


1 ng/uL sfGFP + water
1 ng/uL sfGFP + water
1 ng/uL sfGFP + 0.5% SDS
1 ng/uL sfGFP + 0.5% SDS
1 ng/uL sfGFP + 0.25% SDS
1 ng/uL sfGFP + 0.25% SDS
1 ng/uL sfGFP + 0.14% SDS
1 ng/uL sfGFP + 0.14% SDS
70°C , 0 min
70°C, 5 min
70°C,10 min
70°C,15 min
70°C, 20 min
70°C, 25 min
70°C, 30 min
80°C, 0 min
80°C, 5 min
80°C, 10 min
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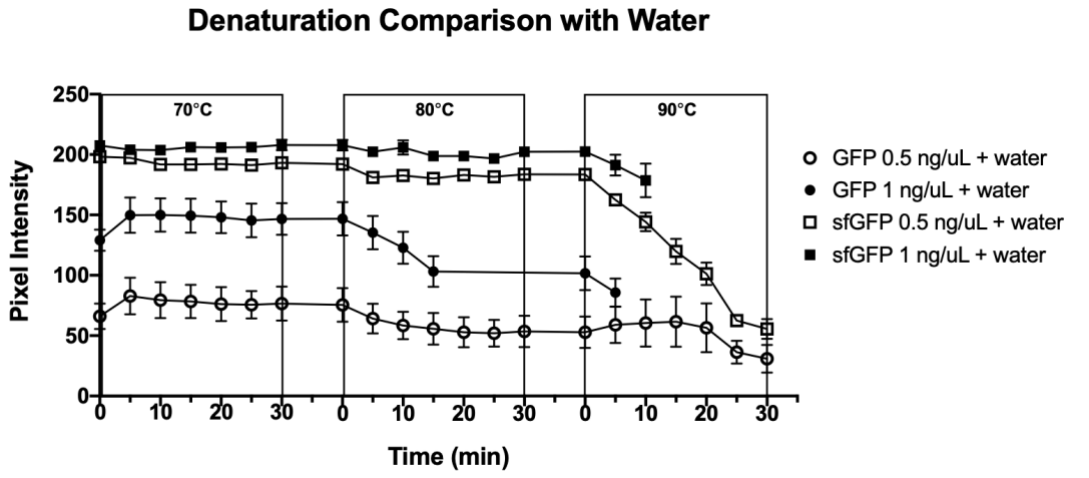
80°C, 30 min
90°C, 0 min
90°C, 5 min
90°C, 10 min



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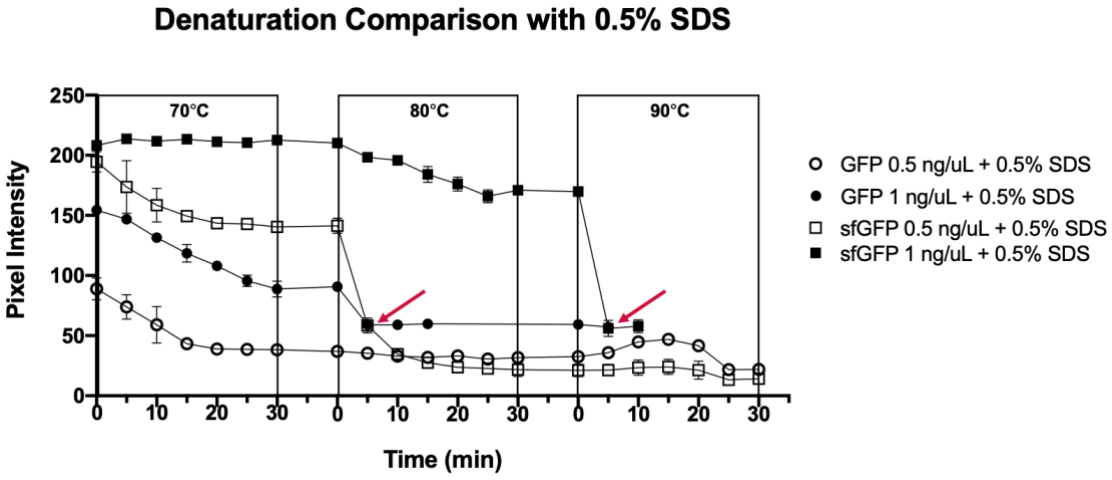


B

Figure 24. Protein Denaturation Overview.

Panel A: Denaturation comparison of GFP and sfGFP at 0.5 and 1 ng/µL with water. Regardless of the concentration, the point at which a decrease in fluorescence is observed is comparable for both GFP and sfGFP. Panel B: Denaturation comparison of GFP and sfGFP at 0.5 and 1 ng/µL with 0.5% SDS. With sfGFP, there is a difference in when denaturation occurs. At 0.5 ng/µL, sfGFP starts to lose fluorescence within 5 minutes at 80°C. At 1 ng/µL, sfGFP starts to lose fluorescence within 5 minutes of 90°C

	0.5 ng/µL Concentration	1 ng/µL Concentration	Comparison
GFP	With water, GFP fluorescence ↓ within 5 minutes at 80°C	With water, GFP fluorescence ↓ within 5 minutes at 80°C	✔
	With 0.5% SDS, GFP fluorescence ↓ within 10 minutes at 70°C	With 0.5% SDS, GFP fluorescence ↓ within 10 minutes at 70°C	✔
sfGFP	With water, sfGFP fluorescence ↓ within 5 minutes at 90°C	With water, sfGFP fluorescence ↓ within 5 minutes at 90°C	✔
	With 0.5% SDS, sfGFP fluorescence ↓ within 5 minutes at 80°C (steep ↓)	With 0.5% SDS, sfGFP fluorescence ↓ within 5 minutes at 90°C (steep ↓)	**X**

Table 11. Ideal input DNA concentration for expression and denaturing.

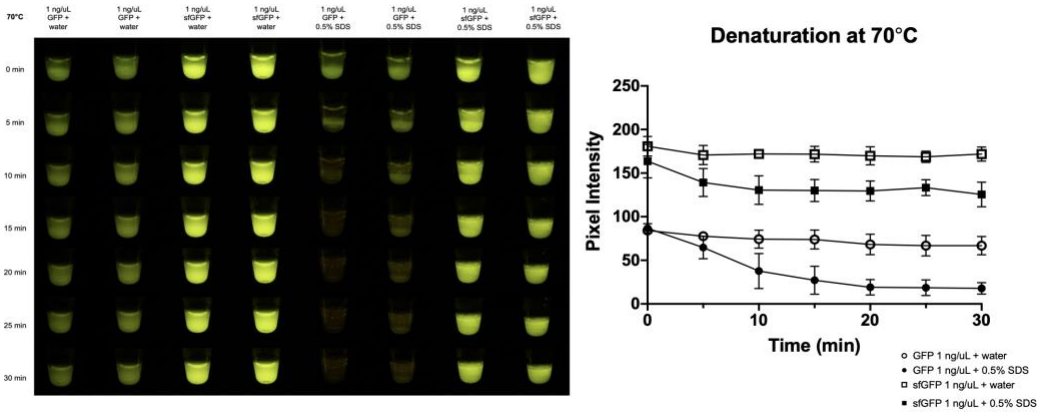
Part V: Non-progressive heat denaturation of stored proteins synthesized using 1 ng/µL concentration of starting plasmid

Previous temperature denature experiments used a progressive temperature protocol, where samples were heated continuously through specific temperature points. Here, I wanted to test non-progressive temperature denaturation through exploration of three temperatures independent of each other. The goal with this experiment was to observe the true effect of each temperature condition as individual treatment to eliminate the compounding effect of temperature on fluorescence (Figure 25).

This data was not initially surprising until I plotted it against the data from the previous denaturation experiment that used a progressive temperature protocol (Figures 26, 27, and 28). Although the starting brightness between the progression and non-progressive treatments were not identical, all conditions that included 0.5% SDS and heat showed a greater decrease in fluorescence with the non-progressive heat denaturation compared to the continuous heat denaturation (Figures 26, 27, 28, panels B and D for each). Both GFP and sfGFP systems exhibited sharp decreases in fluorescence within 5 minutes of incubation at 80°C and 90°C with 0.5% SDS. This was surprising as I assumed continuous exposure to temperatures above 70°C would cause GFP and sfGFP to experience a structural shift that would decrease fluorescence faster. This could be a result of intermediate structures forming during the progressive temperature protocol, allowing fluorescence to persist longer.

These findings suggest that, holistically, protein denaturation can be studied using the FD-CF BioBits® system, a miniPCR™ machine, and p51™ visualizer.

A



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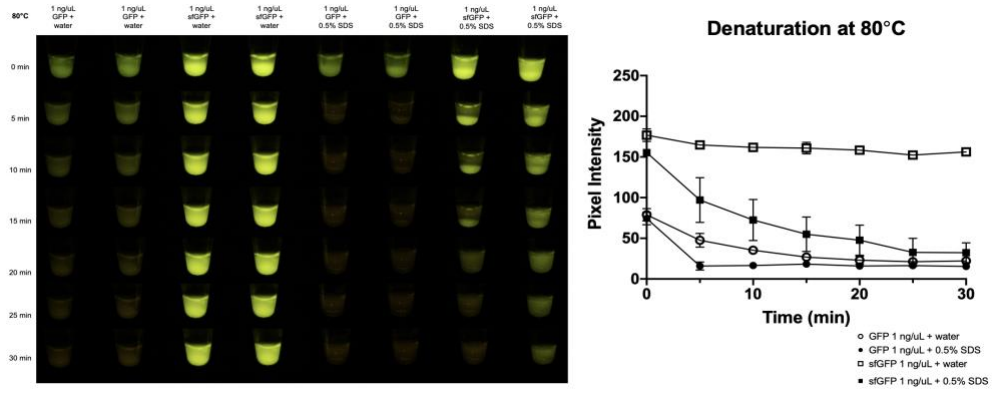

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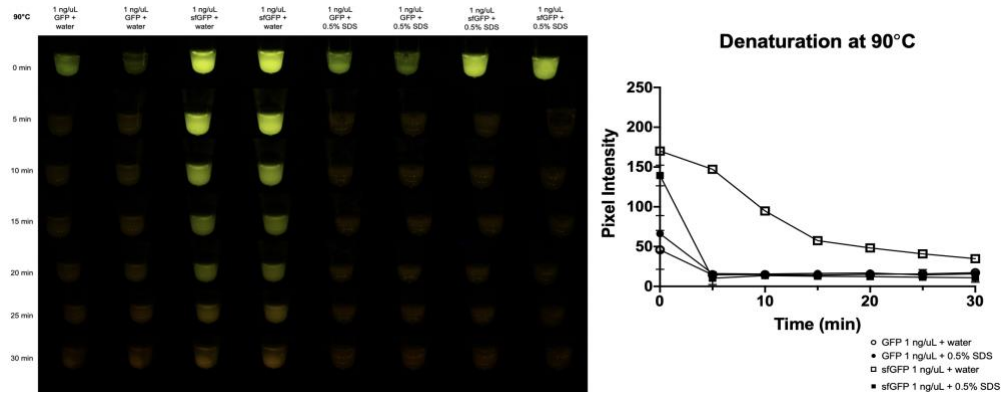


Figure 25. Exploration 70°C, 80°C, and 90°C independent denaturation.

This figure shows the non-progressive heat denaturation of stored proteins synthesized using 1 ng/µL concentration of starting plasmid.

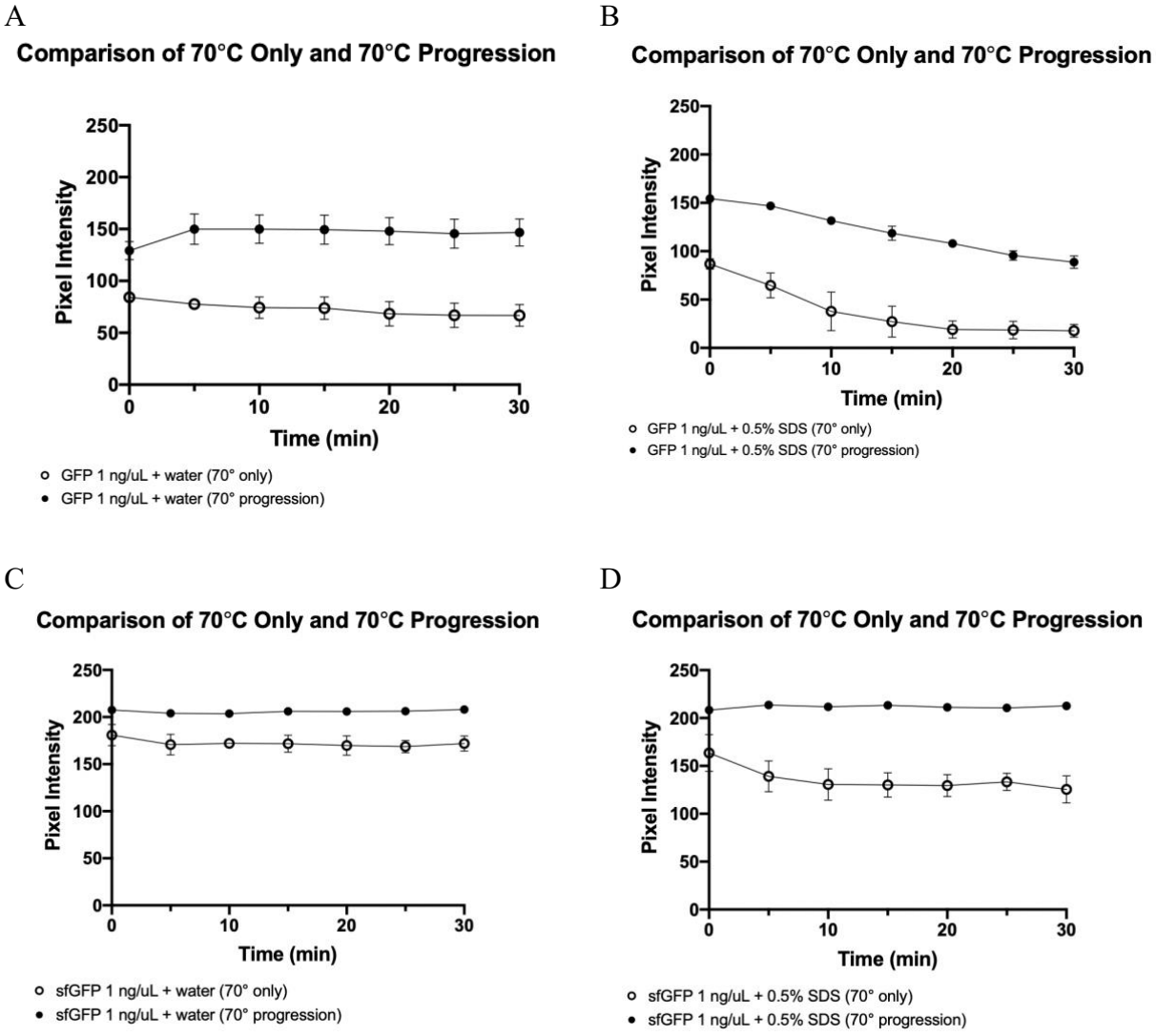
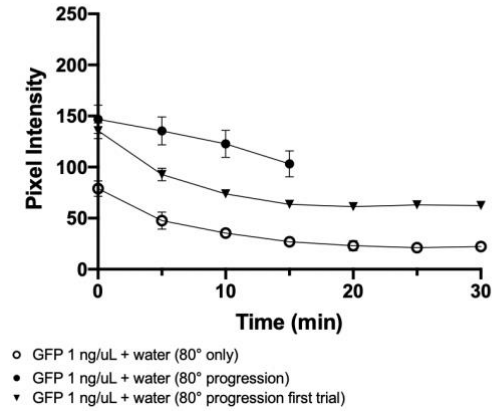


Figure 26. Comparison of 70°C Only and 70°C Progression

A



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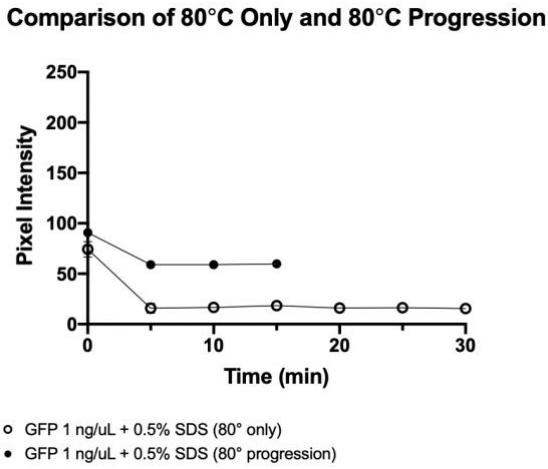
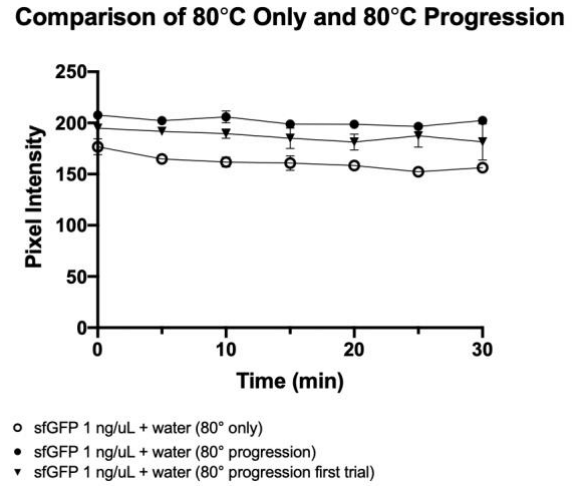


C



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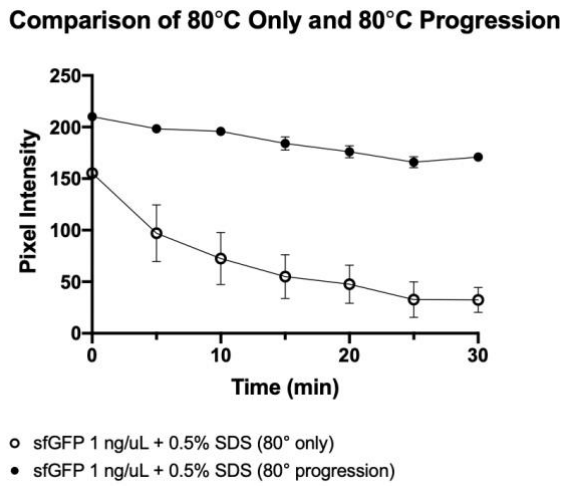


Figure 27. Comparison of 80°C Only and 80°C Progression

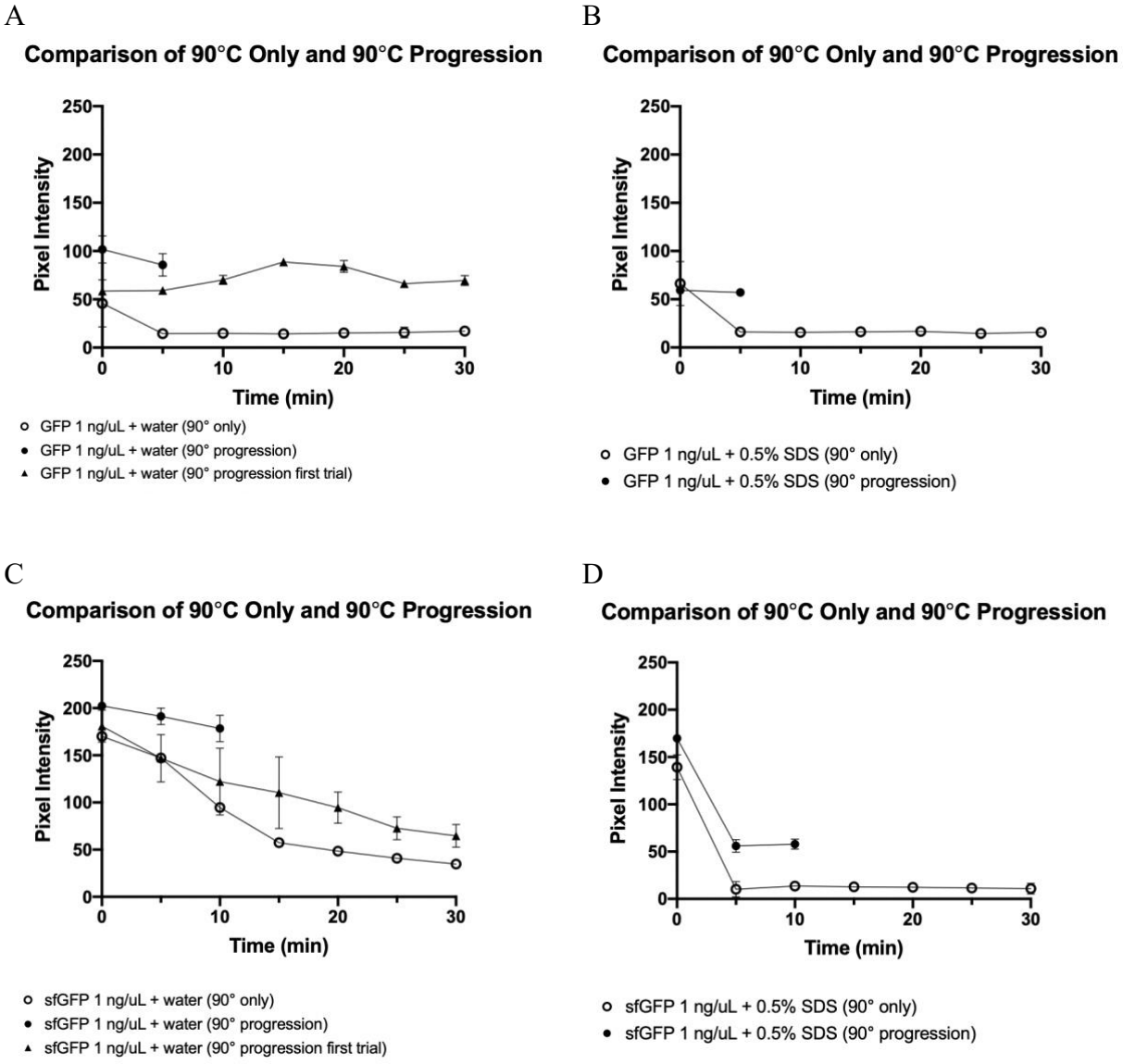


Figure 28. Comparison of 90°C Only and 90°C Progression

Fluorescence Rescue

The third goal of the experimental process was to establish the parameters for GFP and sfGFP rescue, as an observation of fluorescence would indicate renaturation or refolding. In Part I, I observed the effect of room temperature on already-denatured GFP and sfGFP samples from two separate denaturation experiments (Figure 29). The samples in Panel A were from a previous room temperature denaturation experiment. The samples in Panel B were from a previous heat denaturation experiment. In both instances, there seemed to be no observational change in fluorescence after 20 hours at room temperature. For the samples treated with water and 0.5% SDS and incubated at room temperature (A), no loss of fluorescence was observed, thus the 20-hour recovery observation looks identical. For the samples treated with water and 0.5% SDS and incubated at 70°C, 80°C, and 90°C (B), no fluorescence recovery was observed. This failure of either GFP or sfGFP renaturing effectively post denaturation using heat or heat and SDS suggests that both proteins undergo some structural shift that inhibits their fluorescent properties. Both proteins are sensitive to denaturation at increased concentrations of SDS and increased temperatures.

Part I: Room Temperature Overnight

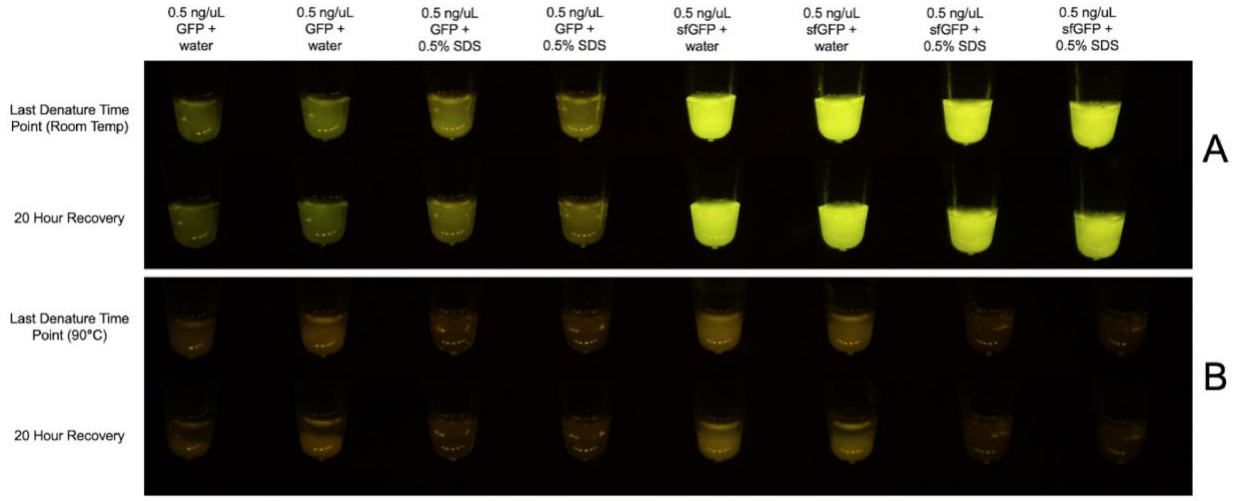


Figure 29. Fluorescence Rescue Overnight at Room Temperature.

Comparison between the last time point of each experiment. Samples in Panel A from the room temperature denaturation experiment. Samples in Panel B are from the heat denaturation experiment. All samples were left at room temperature and observed for a rescue of fluorescence.

Part II: Temperature Ramp Down

In Part II, I attempted to use a temperature ramp down in order to rescue fluorescence. The GFP samples in Figure 30 Panel A had previously been denatured at 90°C for 5 minutes, whereas the sfGFP samples in Panel B had previously been denatured at 90°C for 10 minutes. As soon as the denaturation protocol was complete, the ramp down protocol commenced using a miniPCR™ machine. The samples decreased by 1°C per minute until they reached a final temperature of 25°C. Visually, no rescue of fluorescence was observed in either set of samples.

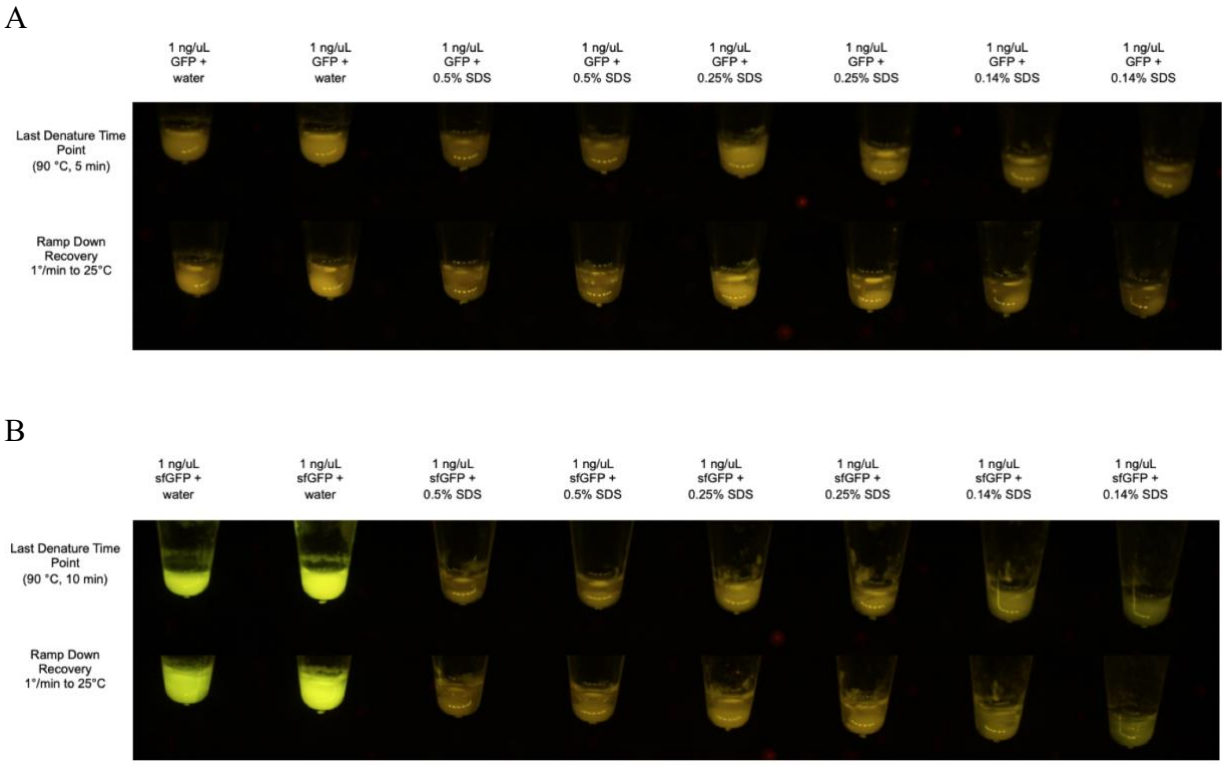


Figure 30. Temperature Ramp Down.

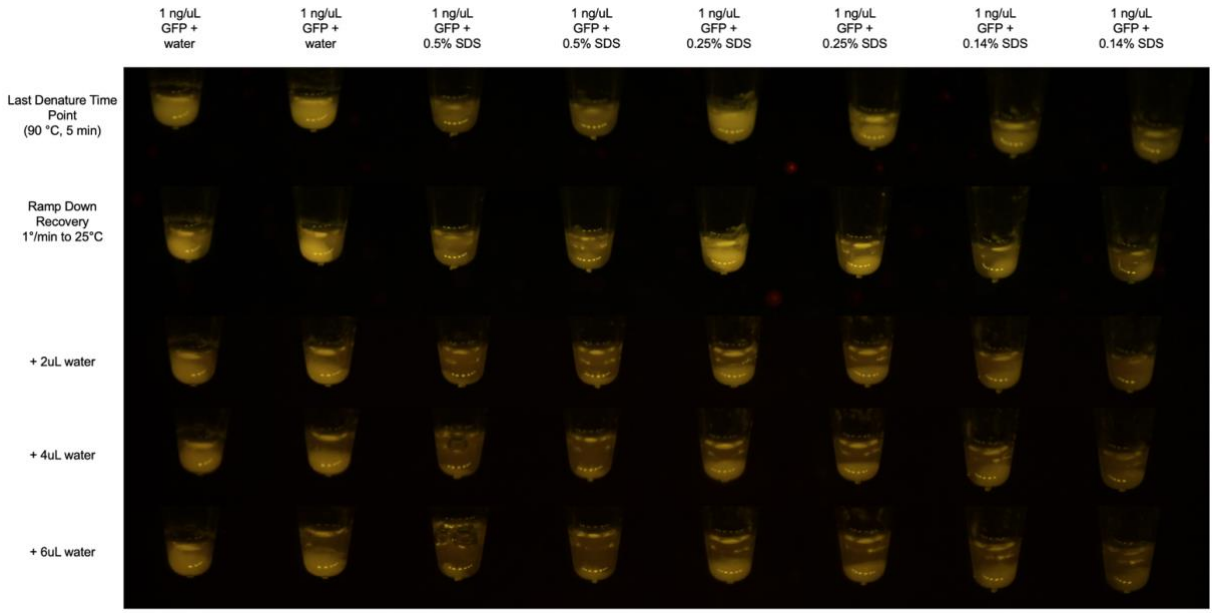
A ramp down protocol was programmed on a miniPCR machine, where the starting temperature was 90°C (the last temperature of the denaturation experiment). The cooling rate was 1°C per minute, until reaching a final temperature of 25°C.

Part III: Dilute Out SDS

In Part III, I attempted to dilute out the SDS from the samples used in Part II. After no fluorescence rescue was observed post the temperature ramp down protocol, I added increments of 2 µL of water to each sample and incubated at room temperature for 15 minutes before imaging. No rescue of fluorescence was observed after a total of 2 µL, 4 µL, and 6 µL of water were added to the previously denatured protein samples. It is important to note that the addition of water also diluted out the protein. This was controlled for by normalizing the data to the pixel intensity of the samples imaged after the ramp down.

Taken together, the parameters explored in the fluorescence rescue experiments do not stimulate refolding of FD-CF synthesized proteins.

A



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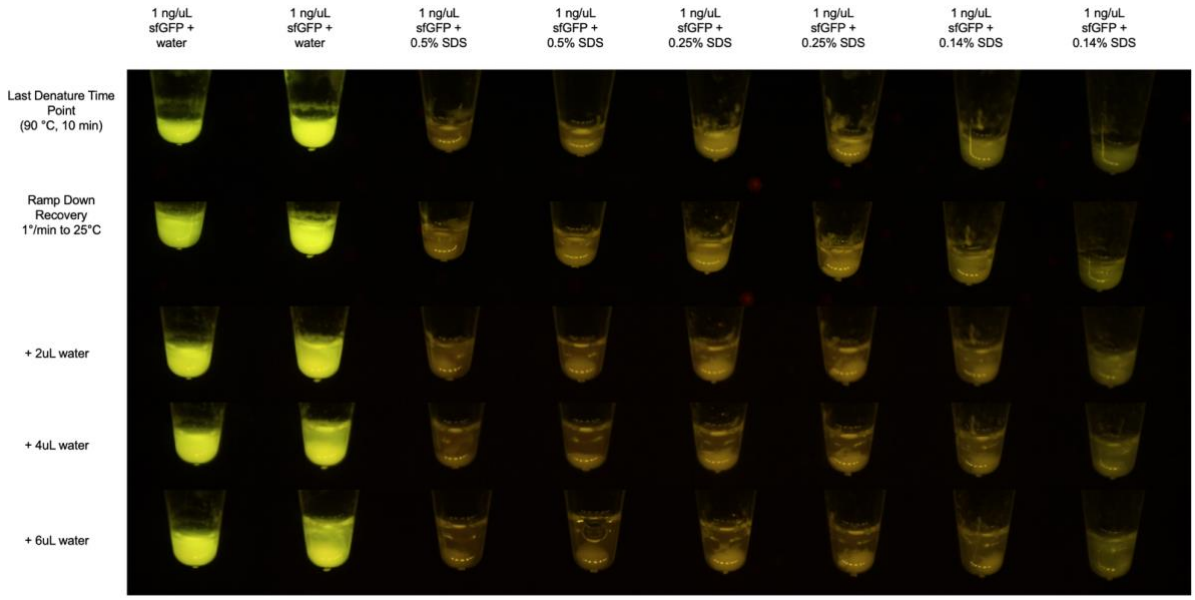

Figure 31. Addition of water in Effort to Dilute Out SDS.

Water was added in increments of 2 µL to GFP (A) and sfGFP (B) reaction pellets and left at room temperature for 15 minutes before being placed in the P51™ visualizer and photographed for analysis.

Chapter IV.

Discussion

Through this research, I sought to develop a low-resource module that explores the parameters of protein folding in an open, cell-free system. The work proceeded in three stages: to establish an experimental set up, adapt said experimental set up for use in educational settings, and finally to lay the groundwork for space application.

Taken together, the data presented in this research show that the study of folding kinetics and the structural stability of fluorescent proteins can be achieved through the freeze-dried, cell-free platform called BioBits®. The majority of the research presented in this thesis is focused on the use of the low-resource system of BioBits® to observe protein folding, denaturation, and attempted fluorescence rescue. The research presented is divided into three main experimental sections, each demonstrating the ability to use non-advanced techniques to study protein folding. In the first section, I demonstrated the ability to use BioBits® to observe real-time protein synthesis of GFP and sfGFP at low plasmid DNA concentrations (0.5 ng/µL and 1 ng/µL), as previous research only explored as low as 2.5 ng/µL DNA concentration (Stark *et al.,* 2018). These data show that the system saturates at high plasmid concentrations, but at lower concentrations I could observe expected differences, suggesting this is a helpful assay to explore differences in folding rates. Since controlling for the differences in the amount of protein being made in order to know whether the difference in brightness reflects only a protein folding difference or a synthesis difference could not be done in house, it is, of course, important to consider whether this is a true difference in folding kinetics, a difference in

brightness, or a difference in absolute amount of protein synthesized, as all of these factors might contribute to the observed difference.

In the second section, I demonstrated the ability to use BioBits® to observe fluorescent protein denaturation through exploration of thermal and chemical means. In the third and final section, I explored the ability of fluorescence rescue, or renaturation, of proteins synthesized with BioBits® reaction pellets. Although I was unable to observe fluorescence rescue, which would have demonstrated protein refolding, with the techniques presented, valuable data was gained.

This research has the ability to bring an understanding of protein folding beyond the bench. Both secondary school classrooms and the environment of space can utilize the BioBits® system to study protein folding. For example, students can use BioBits® to explore the difference in protein folding rates between fluorescent proteins such as GFP and sfGFP. Students can collect visual data that could be quantified to investigate protein kinetics. Students not only can synthesize their own proteins, but can introduce denaturants in a visual, inquiry-based learning way. Furthermore, students can use computational programs to explore bioinformatics in this lens. The realm of microgravity is no different. As real-time protein synthesis studies have yet to be executed aboard the International Space Station because of resource and equipment restraints, this research lays the groundwork for the potential use of BioBits® in microgravity.

The next steps of this research should explore other fluorescent protein constructs, namely ones that have fewer mutations between each other but exhibit profound folding differences. This will allow for a more direct interpretation of protein synthesis results. Additionally, fluorescence rescue, or protein refolding, should be explored deeper.

Previous research has shown that GFP fluorescence can be rescued post thermal and chemical denaturation, however I was unable to reproduce these results using FD-CF synthesized proteins (Alkaabi *et al.,* 2005).

The research presented in this thesis has the ability to create a novel and out-of-the-textbook learning opportunity, not only for students, but for educators globally, changing how science happens through our global community of practice. Our educators want more competency-based inquiry learning opportunities for their students, but not at a price. There is certainly work to be done in science education so that all students have the ability to explore through hands-on learning, however it is my hope that students in classrooms and astronauts in space will explore protein folding alongside each other using the BioBits® system.

Impact

Application to the Classroom

The research presented in this thesis is in the process of being used to create a shelf-stable, affordable, inquiry-based, and hands-on educational kit for secondary-school students to explore molecular biology, synthetic biology, and biotechnology. The curriculum will mirror two of the three tiers of my experimental design: protein synthesis and denaturation. This is because I was unable to observe proper protein refolding during the fluorescence rescue experiments. Additionally, the curriculum will explore bioinformatics, in which students will use BLAST to gather data to make their own hypothesis on protein synthesis and denaturation of GFP and sfGFP.

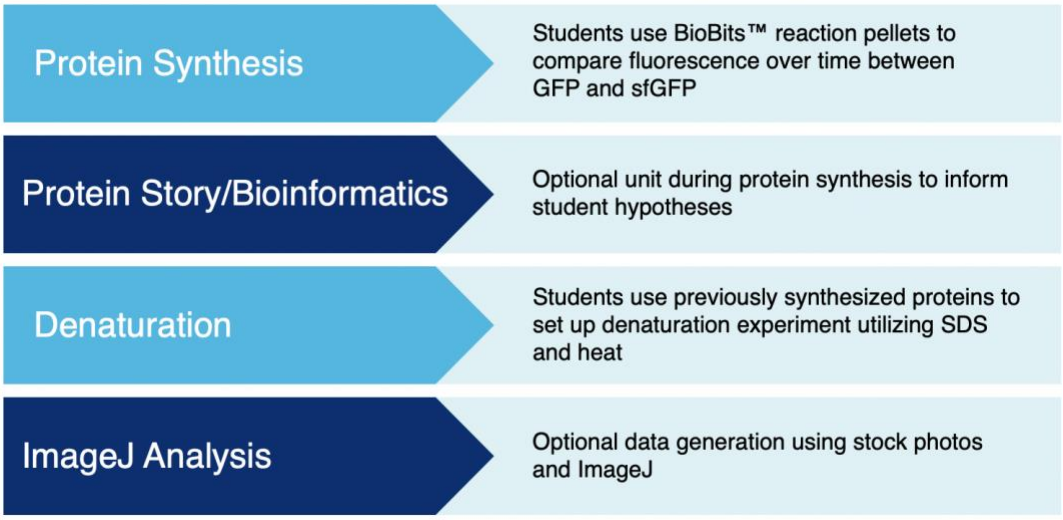

Figure 32. Application to Classroom Overview

Application to Microgravity

The study of proteins, specifically protein synthesis, in space is foundational to the goal of deep space exploration. However, the technology over the past 20 years aboard the International Space Station (ISS) has only allowed for the study of protein crystallization (McPherson *et al*., 2015). NASA-funded studies have shown that protein crystals grown aboard the ISS in a microgravity environment are more perfect; that is, their structural morphologies are more precise than the crystals grown on Earth. This niche of research is important, as understanding the effects of microgravity on protein crystals growth can lead to optimized methods of protein crystallization.

Protein synthesis in a simulated microgravity environment has, in fact, been studied to a degree. Based on a paper published within the last three years, researchers showed that protein synthesis in a simulated microgravity environment induced protein changes in cardiomyocytes, which are cardiac muscle cells (Feger *et al*., 2016). By using a rotating wall bioreactor engineered by NASA, the researchers were able to simulate microgravity, allowing for the continuous suspension of cells in zero gravity. However, this paper focused on maintaining mitochondrial homeostasis in response to the stressful stimulus of microgravity. The goal of this project was to show that in stressful environments, actively dividing cells tend to reallocate energy to cellular division over protein synthesis, increasing the chance of overall survival. Even though this study was performed on Earth, the authors were still unable to truly explore to what degree of misfolding was present in microgravity, let alone why misfolding occurred.

Protein studies in space can take leaps forward with the FD-CF protein synthesis system. Real-time protein synthesis studies have not been achieved aboard the ISS due to the necessary equipment and reagents. With the FD-CF system, there are no reagents that need to be stored at a specific temperature, as the FD-CF pellet, and constituent reagents, are freeze-dried and stable at room temperature. Furthermore, the use of fluorescent proteins, specifically GFP and sfGFP, will allow for easily detectable, visual outputs that an ISS crew member can observe. The FD-CF pellets can also be returned to Earth for yield studies, using affinity chromatography and SDS-PAGE gel electrophoresis (currently, these technologies are not available on the ISS). With micropipettes, a miniPCR™ machine, and a p51™ visualizer in the hands of an ISS crew member, basic protein folding can be observed and understood in microgravity.

Figure 33. miniPCR™ Machine aboard the ISS.

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