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Geethika Malla April 09, 2018

Design of an experimental strategy to study the effect of rNTPs on transcriptional elongation by E. Coli RNA Polymerase

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Abstract

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Due to the high concentration of ribonucleotides (rNTPs) versus lower concentration of deoxy-ribonucleotides (dNTPs) in the cytoplasm, misincorporation of rNTPs into a DNA strand during replication is likely to occur. This strand of DNA may then participate in transcription by RNA Polymerase. The misincorporated rNTPs may serve as a transcriptional roadblock by affecting an RNA Polymerase as it transcribes the DNA strand. We hypothesize that rNTPs may act as an obstacle to transcription, slowing or even halting RNA Polymerase progress as it reads the misincorporated template. We used Protein Induced Fluorescent Enhancement (PIFE) to study the effects of rNTPs on RNA Polymerase by strategically placing a Cy3 fluorophore on the DNA strand. To analyze PIFE signals, an objective-type total internal reflection microscope with a 532 nm laser was used. Using a control strand—with no rNTPs—optimal conditions for the experiment were discovered. Due to non-specific interactions between RNA Polymerase and the DNA template, excessive protein concentration lead to spurious PIFE signals. Conversely, concentration that we too low prevented detection. We determined that an RNA Polymerase concentration of 0.05 U/uL was an optimal working condition. By comparing PIFE activity of different DNA constructs we found that RNA Polymerase enhances Cy3 fluorescence when placed within 67 bp of the dye; yet, when placed 245 bp away little fluorescence enhancement is observed. Together, these findings will help us to experimentally determine whether or not RNA Polymerase will behave as a roadblock during transcription and slow RNA Polymerase's progress down the strand. When observing PIFE in future experiments, we know that the RNA Polymerase less than 245 bp away and at least 67 bp away from the rNTPs and Cy3 fluorophore.

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I. Introduction

Biological mechanisms within prokaryotic and eukaryotic organisms have evolved over time to increase their probability of survival; however, there are still cellular processes where errors can occur. For example, due to the similarity of DNA and RNA molecules and the higher concentration of ribonucleotides (rNTPs) inside the cell compared to that of deoxy-ribonucleotides (dNTPs), it is possible for a DNA strand to contain multiple misincorporated rNTPs. While most organisms possess proof-reading machinery capable of correcting such mistakes, the high rate of misincorporation means that it is very unlikely all rNTPs are removed. The effects of rNTP misincorporation on down-stream processes such as transcription have been poorly studied. However, rNTPs may represent a transcriptional roadblock for RNA Polymerase during transcription.

Structural Differences between DNA and RNA Molecules

DNA, short for deoxyribonucleic acid is found as a double-stranded molecule where the two strands are held together by hydrogen bonds. The monomeric units are deoxy-ribonucleotides (dNTPs) consisting of a phosphate group, a deoxyribose and one of four nitrogenous bases: adenine, thymine, cytosine and guanine. RNA, short for ribonucleic acid, is also made up of four units, where uracil substitutes thymine and ribose replaces deoxyribose. The 2' carbon on the ribose ring of an rNTP binds a hydroxyl group, replaced by a hydrogen in deoxyribose (Lodish et al.). An image of the nucleotide of both DNA and RNA is shown in Figure 1. DNA and RNA contain genetic information that are used to synthesize proteins through the processes of transcription, and translation and is duplicated during replication. These three fundamental processes constitute the Central Dogma of biology (Thieffry and Sarkar).

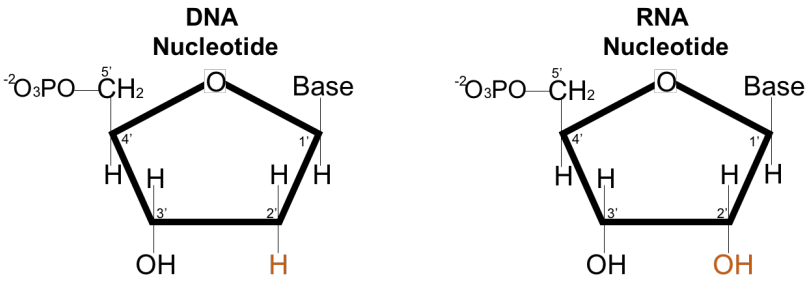


Figure 1 An RNA nucleotide (right) has an extra OH group on the 2' carbon compared to a DNA nucleotide (left).

A. Cellular Replication and Transcription

DNA replication is a biological process where a copy of an organism's genome is made. An enzyme known as DNA Polymerase breaks the hydrogen bonds that hold the two DNA strands together and moves along one of the parent strands assembling a complementary copy. The enzyme grabs deoxy-ribonucleotides (dNTPs) and matches them to a dNTP one after the other on the template strand, creating a daughter strand (Thömmes and Hübscher). This process is repeated for the other template strand resulting in the creation of a complete copy of the genome. Replication is followed by cell division and each daughter cell receives one of the two new DNA molecules.

The replicated DNA is then transcribed to make an RNA strand. RNA Polymerase is an enzyme that works similarly to DNA Polymerase, except for the fact that it grabs rNTPs. The enzyme moves down the DNA strand in the 3' to 5' strand, generating an RNA strand. The RNA strands are then used in translation to generate proteins ("Molecular Basis of Eukaryotic Transcription").

Cytoplasmic Nucleotides and Misincorporated Ribonucleotides

Both of these processes—replication and transcription—take place in the prokaryotic cytoplasm. Thus, DNA Polymerase and RNA Polymerase grab dNTPs and rNTPs, respectively, from this region of the cell where there are 10- to 2000-fold more rNTPs than dNTPs, depending on the organism (Gilmore). For example, in *S. cerevisiae*, there are about 500-3,000 µM rNTPs while dNTPs range from 12-30 µM. In particular, the ratio of rCTP:dCTP, where C stands for cytosine,

is 36:1, while rATP:dATP, where A stands for adenine, is 190:1 (Nick et al.). Due to the concentration difference, it is possible that during replication, rNTPs are incorporated into the genome rather than dNTPs (Gilmore). Misincorporated rNTPs can become sites for mutations, and create problems. These differences between DNA and RNA are significant enough that erroneous incorporation of several rNTPs in the DNA of murine embryos may be fatal (Yang et al.).

Due to these problems, there are mechanisms at the cellular level that are responsible for making sure that such kinds of incorporations do not occur. DNA Polymerase has an amino acid residue known for being a steric gate near the active site of the enzyme. This residue usually has a very large and bulky side chain, and it effectively blocks an incoming rNTP by hindering the 2' hydroxyl group on the ring of the sugar. However, some DNA Polymerases have gate residues with smaller side chains, and are therefore more prone to the incorporation of rNTPs (Yang et al.).

The DNA Polymerase of eukaryotic cells has an exonuclease proofreading activity that ensures that the base pairs of the two DNA strands are correctly matched (Gilmore). However, this repair mechanism does not work well to remove misincorporated rNTPs. Thus, in some cell types, there is another enzyme present called ribonuclease H2, known as RNase H2 that can go through a DNA strand and remove any misincorporated rNTPs. This removal is involved in a process called Ribonucleotide Excision Repair that helps fix the DNA strand that is "damaged" by the presence of rNTPs. However, there are also organisms that have RNase H2 mutations that lead to multiple misincorporated rNTPs not being removed. The case of the murine embryo mentioned previously, had a mutation with this enzyme that resulted in the death of the embryo (Yang et al.).

Transcription of Misincorporated Ribonucleotides by RNA Polymerase

DNA molecules with rNTPs that have not been removed will then go on to the next process in the Central Dogma: transcription. Prokaryotic RNA Polymerase has an active subunit called "clamp" that binds to the DNA molecule and recognizes dNTPs (Chakraborty et al.). However, there is little known about what happens when the RNA Polymerase comes across misincorporated rNTPs. It is likely that the clamp on the RNA Polymerase may not recognize an rNTP as easily as a dNTP. Thus, we postulate that RNA Polymerase will temporarily pause when it comes into contact with the misincorporated rNTPs before continuing elongation. The rNTPs may then represent transcriptional roadblocks. This work is centered on the design of an experimental strategy to test this hypothesis.

II. Experimental Design

The general scheme was to design the appropriate DNA strand that would serve as transcription template. This would have to contain a promoter to initiate transcription by RNAP, ribonucleotides, and a transcription terminator (Figure 2). At a minimum, all prokaryotic genes contain a promoter sequence and a terminator sequence. A promoter region is a sequence that an RNA Polymerase recognizes as a binding site, and a terminator region is a sequence that signals to an RNA Polymerase to detach from the strand and end transcription. For our experiment, we also inserted a region of 5 rCTPs between these two sequences. The RNA Polymerase used was taken from the bacterium *E. Coli* due to its accessibility and abundance. Second, we had to choose an experimental method that would allow the monitoring of RNAP movement along the DNA template strand. Protein Induced Fluorescence Enhancement (PIFE) and Total Internal Reflection Fluorescence (TIRF) are particularly suitable for this.

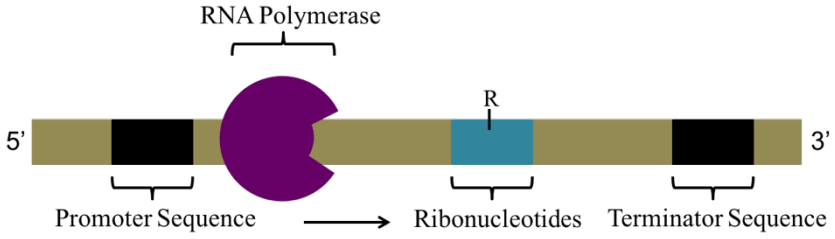


Figure 2 The basic features of the experimental strand are the promoter sequence, misincorporated rNTPs, and the terminator sequence.

Fluorescence

Excitation and emission from a molecule that exhibits fluorescence, a fluorophore, can be described using a Jablonski diagram, shown in Figure 3. The bottom level labelled "G" is the ground state of the molecule, the "E1" level is the first excited singlet state, and the "T" level represents the excited triplet state. When the molecule is excited by electromagnetic radiation, it can transition to the E1 level, and from there it can take two paths—either go straight back down to the G level (shown in green on the left) or transition over to the T level (shown in purple on the right). For both paths, after excitation, the molecule can relax to a band lower than the one it was excited to through non-radiative means (step 2). From there, the molecule can either follow the first path or second path. If it transitions directly back down to the G state, then a fluorescent

photon is emitted. If it instead transitions to the long-lived T state (which is a non-radiative transition) before going down to the G state, a phosphofluorescent photon is given off (Vicente et al.). The emitted photons give the fluorescence of the molecule.

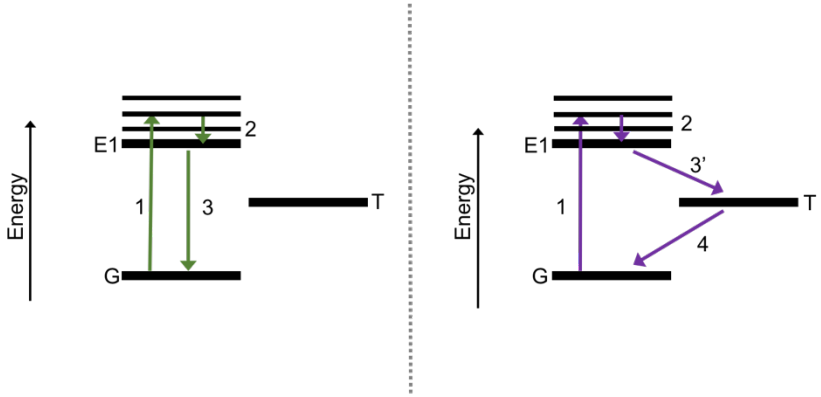


Figure 3 The Jablonski diagrams are used to understand fluorescence. G is the ground state, E1 is the excited singlet state, and T is the excited triplet state. Once a molecule is excited (step 1), it can drop down to a lower band non-radiatively (step 2), and then it can either directly go back down to the G state (emitting a fluorescent photon) shown in the left diagram, or it can non-radiatively transition to the T state and then back down to the G state (emitting a phosphofluorescent photon) shown in the right diagram.

A. Cy3 Fluorophore

The molecular structure of the fluorophore used in this experiment, Cy3, is shown in Figure 4 (Hwang et al.). It consists of similar aromatic groups joined by an unsaturated alkyl chain of three carbons with a single bond between C2 and C3. One of the two aromatic groups can rotate with respect to the other around this single bond, thus, Cy3 can either exist in either the cis or trans conformation.

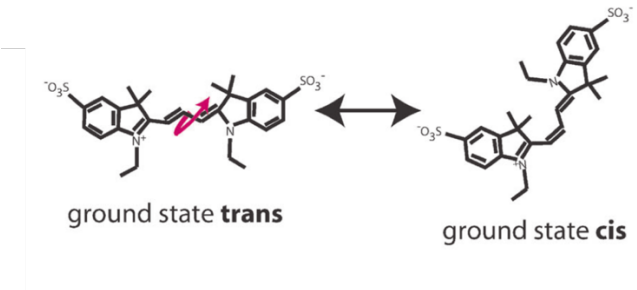


Figure 4 Cy3 is a fluorophore that can exist in two different conformations—cis and trans (Obtained rights from Hwang and Myong).

Excitation of the molecule causes it to move from the ground electronic state to the first excited electronic state, from which isomerization from the trans to the cis conformation, and vice versa, is easier and passes through a non-fluorescent intermediate. Cy3 can only fluoresce when in the trans conformation. A graph representing the excitation and de-excitation of the states of the molecule is shown in Figure 5 (Stennett et al.).

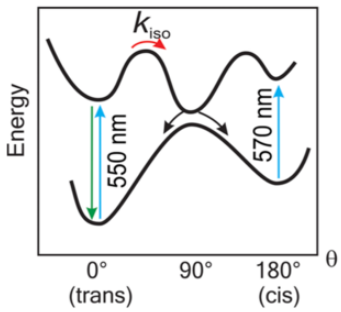


Figure 5 When excited, the Cy3 molecule goes through a cis-trans isomerization and also passes through a 90˚ intermediate during this transformation. The intermediate does not emit fluorescence, the cis state does not emit light when de-excited, and the trans conformation exhibits the most fluorescence (Obtained rights from Stennett et al.).

B. Protein-Induced Fluorescence Enhancement

The fluorescence of Cy3 when excited is an average of three the light emitted by all three confirmations and was labelled as the base state. When a large protein, such as an RNA Polymerase, comes near the fluorophore, the protein sterically prevents Cy3 from isomerizing and locks it into the trans state, thus increasing the fluorescence quantum yield of Cy3—we labelled this as the enhanced state. This fluorescence enhancement is called "protein-induced fluorescence enhancement" or PIFE. As the RNA Polymerase moves away from it, the Cy3 goes back to isomerizing between trans and cis and the fluorescence intensity decreases (Lerner et al.). Therefore, the Cy3 molecule was used as a reporter of the presence of RNAP in the vicinity of the rNTPs. For this purpose, it was placed on the opposite strand, directly across from the rNTPs, as shown in Figure 6. As RNA Polymerase reads the DNA strand in the 3' to 5' direction, Cy3 was placed on the sense strand—the 5' to 3' strand—to prevent it from interfering with elongating RNAP. Biotin was also covalently attached to the last base to allow the DNA to bind to the neutravidin coating the glass surface of the experimental chambers. In accordance with our hypothesis that the rNTPs will pause transcription, we expect the duration of the enhanced Cy3 fluorescence state to increase when RNAP reaches the rNTPs. This expected signal is represented in Figure 7.

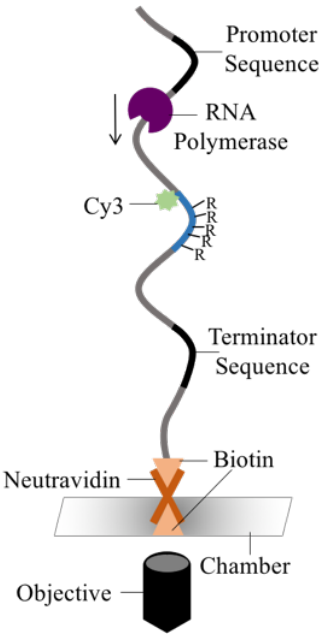


Figure 6 The experimental DNA contained a promoter sequence for the binding of RNA Polymerase, five rCTPs on the template strand, a Cy3 fluorophore on the opposite strand and a terminator sequence to cause the dissociation of RNAP. The end of the DNA was tagged with a biotin in order to anchor it to the bottom of the microscope chamber.

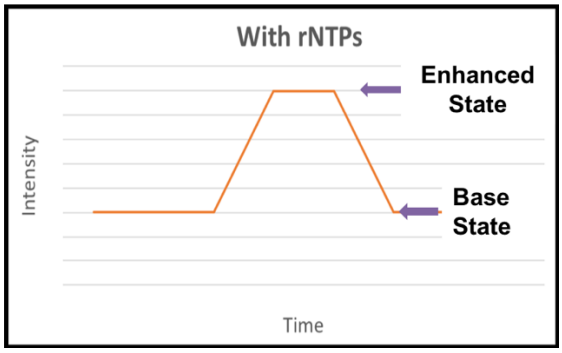


Figure 7 The expected PIFE signal when RNA Polymerase moves down the strand containing rNTPs. The x-axis is time in seconds, and the y-axis is intensity. We expect the signal to stay in the enhanced state for some time—representing the RNA Polymerase pausing when near the rNTPs—before the intensity drops back down as the enzyme moves past the ribonucleotides.

Total Internal Reflection Fluorescence Microscope

In order to observe both the fluorescence base and enhanced level of the Cy3 fluorophore as the RNA Polymerase moves down the experimental strand without seeing background molecules, total internal reflection fluorescence (TIRF) microscopy was needed. In objective-based

TIRF microscopy, a laser, whose path is shown in the left panel of Figure 8, hits an objective lens at the edge instead of in the middle; this ensures that the incident light hits the interface with the sample flow-chamber at a critical angle, θ_c, causing all of the light to be reflected away in accordance with Snell's law (Ross et al.). This law tells us that the critical angle occurs when the light moves from a medium with a high refractory index to one lower (Ockenga).

The right panel shows that if the incident light were to, instead, impinge the sample chamber at a right angle, the light would completely transmit through the interface. In order to reach the critical angle, the objective must have a high numerical aperture, which was achieved through the use of "immersion" oil. Most often, this oil with index of refraction matching that of the glass of the sample chamber is placed between the lens and this interface to ensure the light leaving the objective lens will hit the chamber at the critical angle.

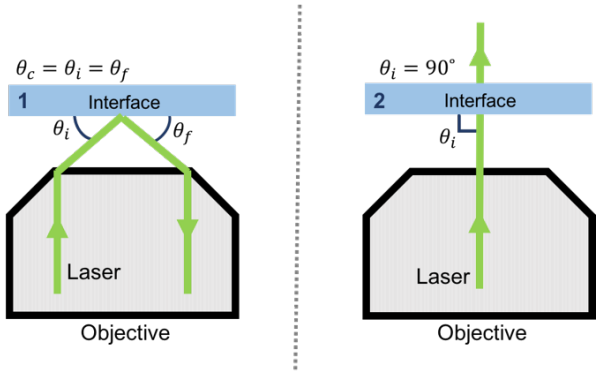


Figure 8 According to Snell's law, total internal reflection of light will happen at a certain critical angle (θ_c). At this angle the incident light (θ_i) and the reflected light (θ_f) are equal as shown on the left in side 1. In side 2, the incident ray is perpendicular to the interface so the incident angle (θ_i) is 90˚. Thus, all of the light is transmitted and none is reflected.

The total reflection of the light by the interface causes an electromagnetic field on the opposite side of the interface from the reflected light. This wave decays exponentially as it travels past the interface along the y-axis. Thus, the energy from the evanescent wave only excites molecules that are closer to the interface, and the molecules that are about 200 nm away from the interface are not excited (Ockenga). Therefore, TIRF is used to observe molecules that are closer to the surface.

A. TIRF with Cy3 Fluorophores

In order to observe the fluorescence intensity of a Cy3 molecule, an objective-type total internal reflection microscope with a 532-nm green light was used. This instrument belongs to Dr. Harold Kim from the Georgia Institute of Technology, and the project was conducted in collaboration with Jiyoun Jeong, a graduate student from the Kim Lab. Figure 9 shows the schematic diagram of the instrument used. The beam from a solid-state green laser was reflected by a mirror, travelled through the edge of the objective lens, bent when it came into contact with the immersion oil, was totally reflected by the coverslip and, then, was purposefully blocked so the camera would not detect it. An evanescent wave was given off from the total reflection of the green laser. This wave decayed as it moved across space and time, and, only the fluorophore molecules closest to the coverslip were excited and started to isomerize. The light emitted as fluorescence was then transmitted down from the coverslip into a CCD camera, which recorded the intensity levels of the light (Jeong et al.).

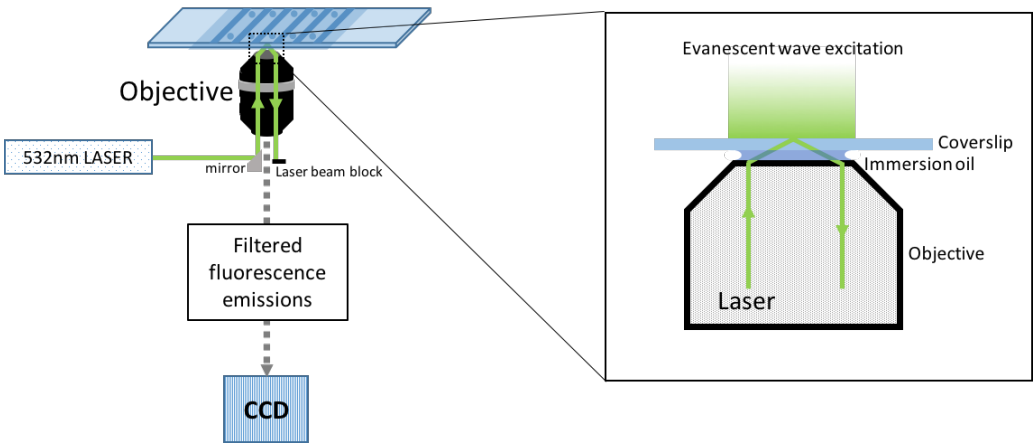


Figure 9 The optics behind the objective-type TIRF microscope used in the Kim Lab from the physics department of the Georgia Institute of Technology. The total reflection of the laser from the coverslip causes a decaying evanescent field to project upward. The light from the fluorophore generated by this wave travelled down into a CCD camera.

B. Preparation of the Experimental Chamber

In order to observe the DNA molecules and Cy3 fluorophores with the TIRF microscope, chambers were made using glass slides and coverslips. Holes were drilled at opposite ends of the slide to allow the exchange of solutions. Double-sided tape was put on the microscope slide, and parallel rectangular chambers were cut in the tape with each end stopping at the holes drilled. The

coverslip was then laid over the tape on top of the slide as a seal to the chambers and secured with epoxy glue. Figure 10 shows a schematic representation of the sample chambers that are so obtained.

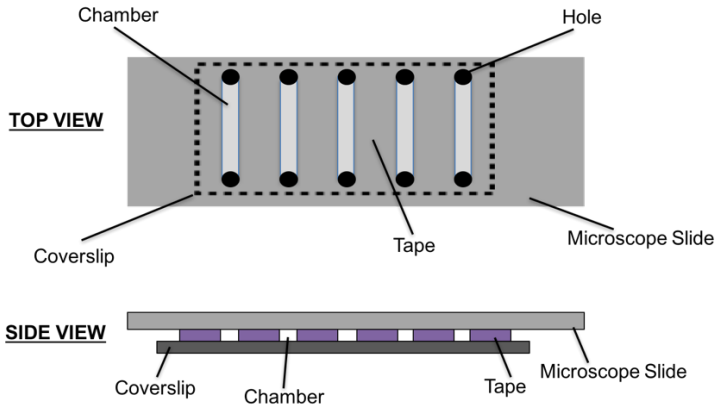


Figure 10 Assembly of the chamber used for the experiments using PIFE and TIRF. Holes were drilled on a microscope slide, double-sided tape was placed over it, small rectangular chambers were cut out of the tape, and then a coverslip was attached to the top of the tape.

Before putting the microscope slide and coverslip together with tape, they had to be prepared to ensure DNA would stick to the chamber. Thus, both were sonicated in water and then placed in a plasma cleaner. They were then immersed in a solution containing hexane and around 60µL of dimethyldichlorosilane (DDS). After about an hour, the slide and coverslip were placed in fresh hexane. At the end of this procedure, both the microscope slide and coverslip had a thin coating of DDS. The DDS coating ensured the surfaces were hydrophobic (Jeong et al.). The two pieces—the glass slide and coverslip—were then attached together.

C. Flowing in DNA into the Chamber

To stick to the bottom of the chamber, the DNA had to be labeled with biotin, which allowed the DNA to bind to the surface of the chamber. The biotin would bind to the neutravidin molecules already bound to the surface of the microchamber via biotinylated bovine serum albumin (BSA). A schematic representation of this process is depicted in Figure 11. Since neutravidin has four total binding sites for biotin, when biotin-labeled DNA was introduced in the flow-chamber, it bound to the surface (Jeong et al.).

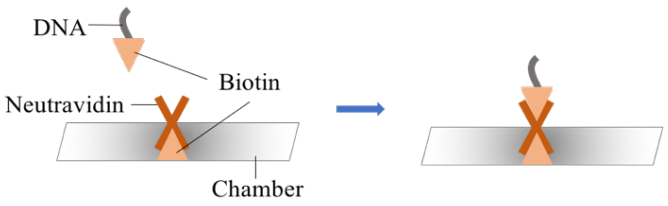


Figure 11 A biotinylated DNA molecule binding to a neutravidin protein stuck to the bottom of a chamber via a separate biotin-neutravidin .

To coat the chamber with biotin-neutravidin molecules, 50µL of biotin-BSA was flowed into the chamber and was incubated for five minutes. Then, 100µL of Tween-20 solution was added followed by a 10-minute incubation. This ensured that there was no non-specific binding of other molecules to the chamber and that only DNA molecules with a biotinylated anchor bonded to it. The chamber was then washed out with 100µL of T50 buffer (which contains 10mM Tris-Cl and 50mM NaCl). 20µL of Neutravidin (which contains 50% Glycerol and 1x PBS) was then added and incubated for two minutes. Then about 40pM of biotinylated DNA was introduced into the system (Jeong et al.).

D. Photobleaching

After a fluorophore is excited by a laser for an extended period of time, the emitted fluorescence disappears. This loss of fluorescence of a fluorophore is termed photobleaching, and it is an irreversible process (Diaspro et al.). Photobleaching involves the photo-induced chemical-destruction of a fluorophore. The molecules in the E1 and T state in the Jablonski diagram shown in Figure 3 can irreversibly lose the ability to fluoresce (Vicente et al.). While in the T state, the fluorophore can spend more time interacting with the surrounding environment before going back down to the G state. If the triplet fluorophore interacts with any oxygen molecules in the system, then a singlet oxygen may be formed, and the fluorophore may irreversibly lose its fluorescence (Diaspro et al.). Therefore, it was important to take precautions to minimize this effect with Cy3 inside the chamber. Introducing oxygen scavengers, which remove oxygen, to the chamber containing the fluorophores before exciting them with a laser may reduce photobleaching (Song et al.). In our experiments, an imaging buffer, which contained oxygen scavengers, was added.

The imaging buffer totaled 50µL, and it was made up of 2mM of Trolox buffer, 10mM protocatechuic acid (PCA), water, RNA Polymerase, RNA Polymerase buffer, and protocatechuate-3,4-dioxygenase (PCD). RNA Polymerase, RNA Polymerase buffer, and rNTPs were added to the imaging buffer depending on what was being tested. This solution was flowed through the

chamber, with excess coming out at the other end. After incubating this solution in the chamber for about five minutes, the laser was turned on and data was taken (Jeong et al.).

E. Data Readout from CCD Camera

Data was recorded from the CCD camera receiving signal from molecules excited by the evanescent field. With the help of MATLAB, specific molecules in the field of view of the objective were distinguished. The intensity of the entire field of view was averaged, and the areas with intensity greater than the average were distinguished as a Cy3 molecule. The area was labelled as background if the intensity was below the average. The intensity levels of the molecules on the coverslip were then measured for about 3 minutes and plotted. The idea was that in the absence of RNAP in the chamber, the signal should fluctuate due to instrumental noise, but its average should stay at a certain intensity value (at the base state). When RNA Polymerase and rNTPs are added into the chamber, PIFE should be observed as the enzyme transcribes the DNA molecules labeled with Cy3. In this case, the intensity level about doubles from the base state (Jeong et al.).

Trace Analysis

From the microscope, we received data that gave us the intensity of a molecule in the chamber over time. Plotting this data in MATLAB gives a graph similar to Figure 12. The graph represents one molecule in the chamber. The x-axis is time, and the y-axis is the intensity of the molecule. The fluctuation in the trace is due to background noise, which can result from fluctuations in laser intensity. The trace looks fairly constant between 400 A.U. and 500 A.U. at the base state. The intensity then doubles around 220 seconds and 330 seconds. The molecule was in the enhanced state at these spikes.

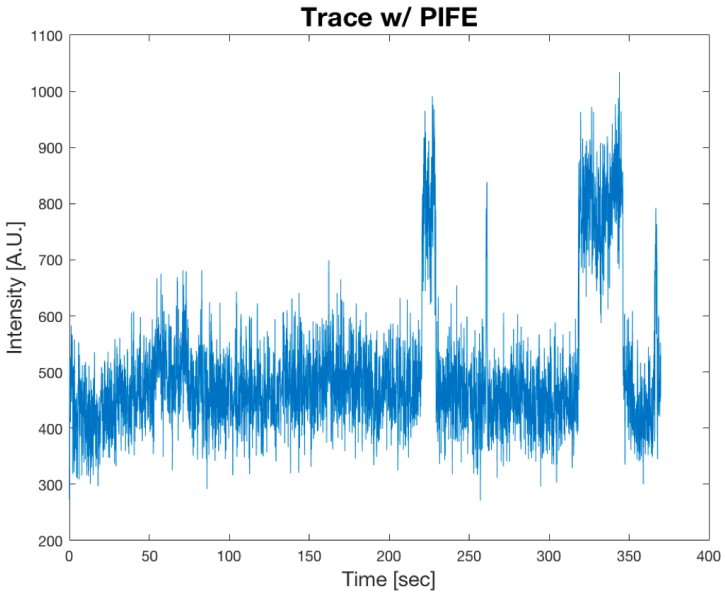


Figure 12 The data received from the microscope for one molecule in the chamber. The x-axis represents time in seconds, and the y-axis is the intensity of the molecule.

For our experiments, it was important to distinguish the base state and enhanced state within a trace. A graph helping to divide these two states is shown in Figure 13. A moving mean of the trace was taken (shown in red on the trace graph), and then a double-Gaussian was fitted to it. A cutline was established between the two Gaussian curves shown in the right graph (the dashed line in the graphs). The molecule was in the base state when the intensity was below this cut-line and was labelled to be in the enhanced state when the intensity was above the cut-line. Mu_1 is the midline of the bottom peak and Mu_2 corresponds to the top. Using this analysis, we were able to get a value for the fraction of time the molecule spent in the base state. After compiling these values for every molecule in the chamber, we generated a histogram and compared data from chamber to chamber.

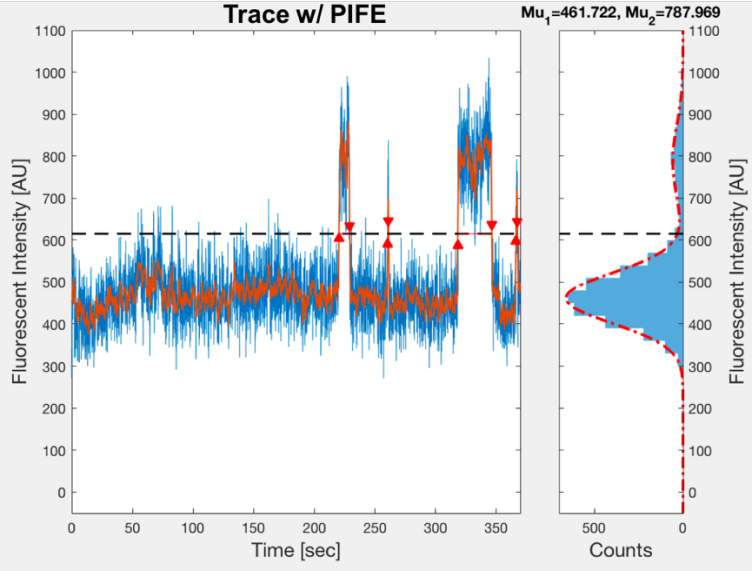


Figure 13 The left graph represents the raw trace (in blue) and a trace representing the moving mean (in red). The dashed line represents the cut-line, distinguishing between the base and enhanced state. The curve on the right represents a double-Gaussian used to fit the trace. Mu_1 and Mu_2 represent the midlines of the bottom and top peaks, respectively.

III. Initial DNA Construct

Initially, the DNA to be used was designed as three different fragments to be eventually ligated. Fragment 1 was the part of the molecule that contained the promoter sequence and was 334 bp long. Fragment 2 contained the Cy3 fluorophore and the rNTP sequence, and it was 20 bp in length. The last fragment, Fragment 3, was 197 bp long, and it contained the terminator sequence and a biotin anchor at the end to allow the DNA molecule to stick to the bottom of the chamber.

Annealing Fragment 2

Each strand of Fragment 2 was designed and synthesized separately. Both the sense, with the Cy3 fluorophore, and antisense strand, with the five rNTPs, of this fragment were purchased from idtDNA (Coralville, IA). RNA Polymerase reads the antisense strand ("Molecular Basis of Eukaryotic Transcription"), so the rNTPs were strategically placed on this strand, and the Cy3 was placed on the other strand to prevent an additional variable, namely the presence of Cy3 in the antisense strand, from affecting transcriptional elongation by RNAP.

Just as a DNA molecule can be denatured at very high temperatures, two single strands of DNA can anneal together and associate to form a double-helix (Lodish et al.). Thus, the sense and

antisense strands of Fragment 2 were mixed together in solution, the temperature was raised to 95˚C and then lowered to 35˚C in 5-minute increments. As the temperature of the solution lowered, the two complementary strands bonded together, yielding a double-stranded DNA molecule.

Polymerase Chain Reactions of Fragment 1 and Fragment 3

Fragment 1 and Fragment 3 were synthesized in the lab via a Polymerase Chain Reaction (PCR), which consists in a repeated series of temperature-dependent reactions that produce, as an end result, multiple copies of a specific DNA molecule (Green et al.). The PCR mixture contains: i) the original DNA molecule with the desired sequence, ii) sense and antisense primers, which are the beginning of the 5' strand and the complementary 3' strand, respectively, of the DNA molecule to be amplified, iii) DNA Polymerase, and iv) the four dNTPs needed to synthesize new DNA. The first step of this reaction is to denature the original molecule of DNA by increasing the temperature to 95˚C for 50 seconds. Then, the solution is brought down to an annealing temperature—which is dependent on the sequence of DNA being amplified—for 30 seconds. This temperature is optimal for the sense and antisense primers to bind to the now separated original DNA strands. Then the temperature is raised to 68˚C for a time dependent on the length of the DNA to be amplified. This temperature is ideal for DNA Polymerase to elongate the primers, matching a dNTP to every base along the template DNA strand in a processive manner. These steps are repeated 45 times to increase by order of magnitude (amplify) the concentration of the desired DNA sequence (Joshi and Deshpande). Figure 14 illustrates the steps of a PCR reaction. After the cycles, the temperature was brought up to 72˚C for five minutes for a final elongation period.

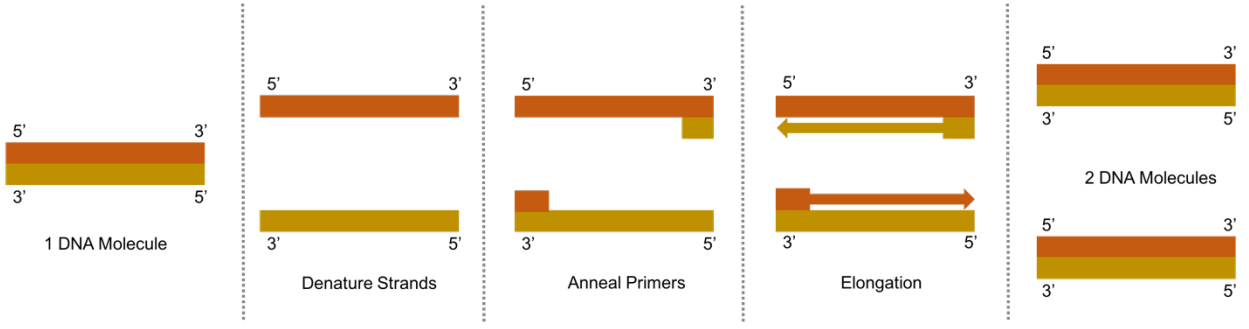


Figure 14 The steps of a Polymerase Chain Reaction (PCR). The first step is denaturation, the second annealing, and the third step elongation. These steps are then repeated to amplify the DNA product.

The annealing temperature used for Fragment 1 and Fragment 3 was 52˚C and 55˚C, respectively. The primers used to amplify both framgents were purchased from idtDNA (Coralville, IA) and are listed in Table 1. In our PCR experiments, we used Taq DNA Polymerase as it is known to withstand the high temperatures involved in the reactions (Joshi and Deshpande). This polymerase and the dNTPs used were purchased from New England Biolabs Inc. (Ipswitch, MA).

Fragment	Sense Primer	Antisense Primer
1	agcttgtctgtaagcggatg	cttatgcgggtgcgctac
3	ggattagcggtctcgataaaac-tatcccgac	gtctcgtctaacatgactctcacg-Biotin

Table 1 The sequence for the primers of Fragment 1 and Fragment 3.

Combining Fragment 1 with Fragment 2 and Fragment 3

A. Restriction Digestion

In order to combine the three fragments, Fragment 1 and Fragment 3 had to be digested by a restriction enzyme. These enzymes locate and bind to a recognition site, a sequence of nucleotides on a DNA sequence, and then break the molecule at that point. Different restriction enzymes have different recognition sites. After the enzyme cuts the sequence of DNA it recognizes, it leaves the molecule with a strand longer than the other, called an overhang or "sticky end." Another DNA molecule with a complementary sticky end can then take the place of the digested part and attach itself to the first molecule. ("Restriction Enzymes"). Figure 15 shows a schematic representation of the digestion of a DNA molecule. The reaction temperature depends on the restriction enzyme used.

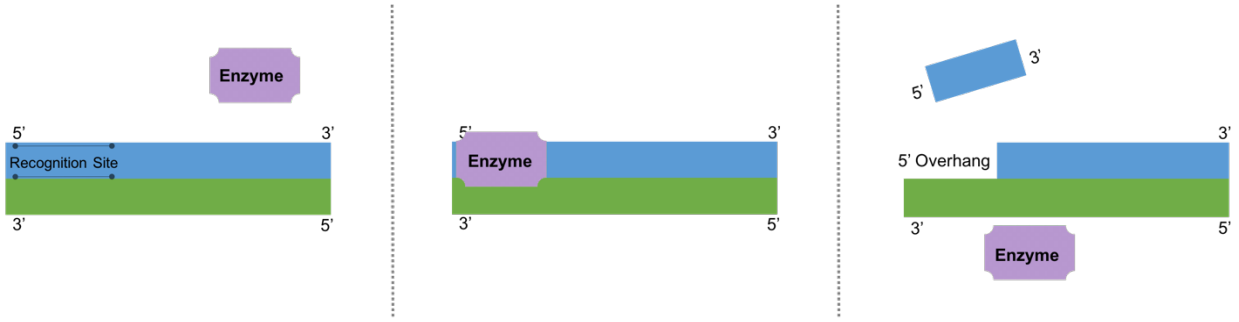


Figure 15 Schematic representation of the restriction process. DNA complementary strands are blue and green. The enzyme (purple) binds at the recognition site and cleaves off the sequence. This enzyme left a 5′ overhang in the DNA molecule ("Restriction Enzymes").

Before the three fragments of DNA could be combined, Fragment 1 and Fragment 3 had to be digested in order for them to contain the correct overhangs to insert Fragment 2 between them. Fragment 2 was designed and ordered with overhangs that matched the ones produced by digestion of Fragments 1 and 3. The restriction enzymes used for Fragments 1 and 3 were BsmBI, and BsaI, respectively. The reaction temperature for BsmBI was 55˚C and BsaI was 37˚C. Both enzymes were ordered from New England BioLabs Inc. (Ipswitch, MA). The sequences of Fragments 1 and 3 are shown in Figure 16 and Figure 17, respectively.

Figure 16 The sequence of Fragment 1. The primers are labelled with arrows, and the promoter sequence is in the middle labelled in green. The BsmBI binding site is also shown (pink) along with the overhang that is left after digestion (yellow).

Figure 17 The sequence of Fragment 3. The primers are labelled with arrows, and the terminator sequence is in the middle, labelled in red. The BsaI binding site is also shown (green on both strands) along with the overhang that is left after digestion (color?).

B. Ligation Reaction

Two DNA molecules that have complementary overhangs can be combined together via a ligation reaction. This reaction is catalyzed by a specific enzyme that forms phosphodiester linkages between the two strands of DNA and seals them together ("How to Ligate Plasmid DNA"). The process is illustrated in Figure 18. It is important that the DNA have overhangs in order to ligate the correct fragments together; thus, Fragment 1 and Fragment 3 were digested by their respective restriction enzymes, before they were combined with Fragment 2 in a ligase reaction. The reaction ran at 16˚C for 16 hours.

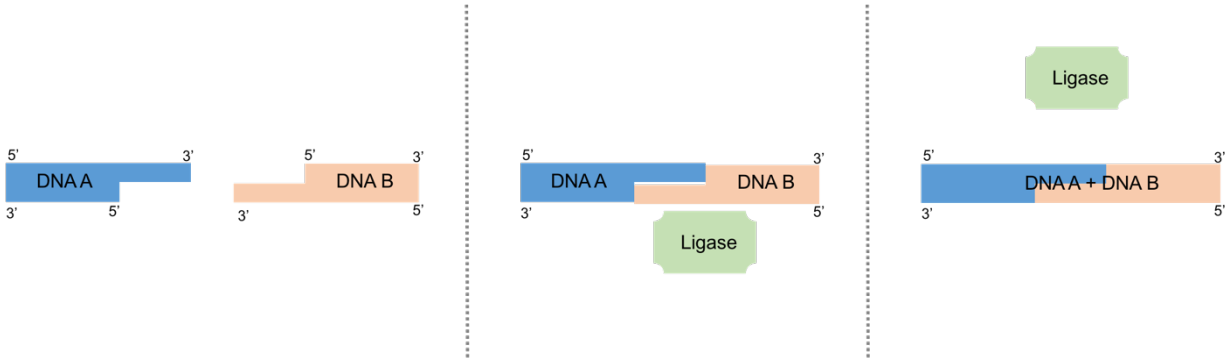


Figure 18 A ligase enzyme (green) working to combine DNA A and DNA B, which have complementary overhangs.

There are multiple types of DNA ligase enzymes. For our experiment, we first used T4 ligase; however, we soon learned that this enzyme facilitates the ligation of DNA without sticky ends (with blunt ends) (Packer). Thus, this type of ligase is not ideal as it will ligate DNA segments also by their non-sticky ends and will produce unwanted DNA molecules. For example, we could ligate multiple of either different or the same fragments together or ligate Fragment 1 and Fragment 3 together. Thus, we switched to T7 ligase. This enzyme does not partake in blunt-end ligation; therefore, the only fragments that should be combined are the ones that have complementary over-hangs.

We tried ligating the three fragments together following three different protocols. In one way, we mixed all three fragments together with ligase and run the reaction. When we did this, we tried different ratios of Fragment 1:Fragment 2:Fragment 3 in the mixtures. This allowed us to try and find the optimal ratio to ligate. We worked with 1:1:1, 1:2:1, and 1:3:1 ratios. We wanted to avoid the fragments sticking to each other so the concentrations of Fragment 1 and Fragment 3 were kept as low as possible. However, we wanted to optimize the concentration of Fragment 2 in order for there to be enough in the solution to make sure it ligated together with Fragment 1 and Fragment 3.

In another way, we ligated Fragment 1 to Fragment 2 and Fragment 2 to Fragment 3 in two separate reactions and tried to ligate the resulting fragments together. We also tried another procedure of ligating the fragments. We first ligated Fragment 1 to the antisense strand of Fragment 2, and then annealed the sense strand before finally ligating the two fragments together.

Gel Electrophoresis

A. Agarose Gel

After each of the above reactions—PCR, digestion, or ligation—we checked that the end product of DNA was what we wanted by using an agarose gel electrophoresis. This type of gel allows the DNA strands inside of a solution to separate depending on size. As DNA is negatively charged due to its phosphate backbone, when introduced to an electric field, the DNA will move from the negative end of the field to the positive end. The smaller the molecule, the faster it will travel to the positively charged anode, and the larger the molecule, the closer it will be to the negatively charged cathode (Lee et al.). A schematic representation of the gel electrophoresis method is shown in Figure 19.

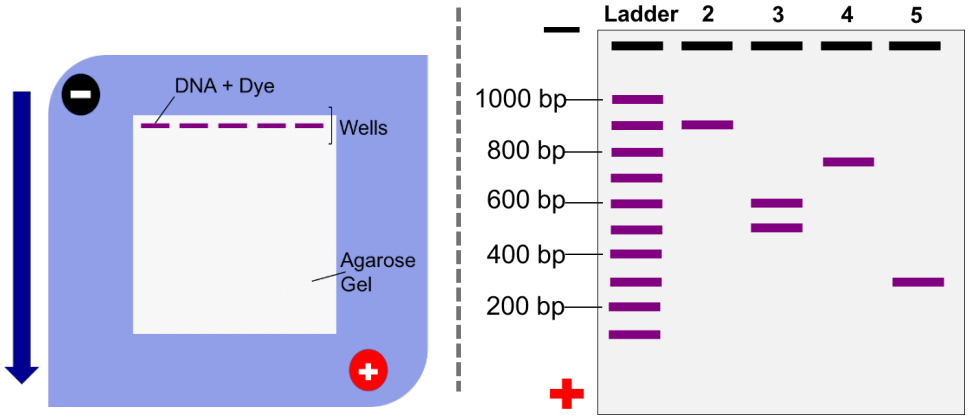


Figure 19 The image on the left is a gel box (blue) and an agarose gel (gray) that has been loaded with DNA (purple). DNA moves from the negative end to the positive end as it is negatively charged. The voltage of the system is around 100 V, and runs for 30 minutes to one hour depending on the length of the gel. The image on the right shows a gel after being placed in the electric field. The DNA has separated in relation to size—with the smallest molecule being closer to the bottom and the largest being near the top. A ladder, shows in lane one, is used as a standard to identify the size a band in lanes 2-5 corresponds to.

Thus, this method provided us with a way to test the success and yield of the DNA reactions. For example, when ligating two strands that are at two different lengths—100 bp and 200 bp—the end product should generate DNA molecules that are 300 bp long. When placing this solution in a gel and running it through an electric field, we should see a band around 300 bp long. The length is verified by comparing to a ladder on the gel. If we only saw two band at 100 bp and 200 bp, then this means that the ligation protocol did not work well. Many gels were taken throughout the experiment to ensure the accuracy of the reactions done.

An agarose gel was made by combining 1X Tris-Acetate- ethylenediaminetetraacetic acid (TAE) with agarose powder. A 1% gel consisted of 1 L:10 mg ratio of TAE to agarose powder. A 2% gel was used when we wanted to increase the distance between bands, and the ratio used was 1 L:20 mg. The mixture was heated up until all of the agarose was dissolved before it was cast inside of a gel box. After half an hour of cooling, the gel box was filled with 1X TAE to cover the gel. The DNA ladder and DNA, which was previously prepared with loading dye, were then loaded into the wells. The loading dye from New England Labs Inc. (Ipswitch, MA) was added into the DNA solution in order to sink the DNA and provide a way to visualize the bands (Lee et al.) as the DNA moved down the gel. A constant voltage of around 100 V was put across the box for about 30 minutes to one hour—until the bands reached near the bottom. The gel was then removed and placed into a 1X TAE solution containing EtBr in a 35 mL:1.7 mL ratio. EtBr is a reagent that stains the DNA within the gel. The gel was soaked in this solution for about 15 minutes before it was exposed to UV light, which excited the EtBr and gave off light, showing a distinct band at the location of the DNA. (Lee et al.)

B. Polyacrylamide Gel

Agarose gels are only useful for observing DNA that has a length greater than 100 bp; therefore, to analyze DNA below this length, polyacrylamide (PAGE) gels are used. The pore size of polyacrylamide allows a greater separation of smaller fragments than agarose (Chory and Pollard). The gel apparatus used for this type of electrophoresis is different from the one for an agarose gel as in that the gel runs vertically rather than horizontally. 0.5 µL of 10x Tris-borate-ethylenediaminetetraacetic acid (TBE, which contained 21.8 g Tris, 11 g Boric Acid, and 1.488 EDTA), 40% of acrylamide stock (which contained 30% acrylamide and 1% methylene bisacrylamide), 100µL of 10% ammonium persulphate, 10µL of tetramethyl-1,2-diaminoethane (TEMED), and water were casted in the gel box to create the gel. After it solidified, it was covered with 10x TBE. The DNA was then loaded and run at about 100-200 V. After the bands reached the end, the gel was submerged in buffer and Sybr Safe (a stain similar to EtBr) for about 10 minutes and was then excited by UV light to see the bands (Harwood).

IV. Results and Analysis

Data with the Initial DNA Construct

When observing the DNA inside a chamber, we should only see PIFE signals when there are both RNA Polymerase and rNTPs in the chamber. If there is neither molecule and only DNA, then all of the molecules should be in the base state. After adding just RNA Polymerase into a chamber with DNA, there should still be a constant base state signal as the enzyme should not be moving down the strand to sterically hinder the fluorophore. A PIFE signal should only be seen after the rNTPs are added.

Using a previously made stock of DNA, in which Fragment 1, no rNTP Fragment 2, and Fragment 3 were all ligated together in one reaction, we ran experiments using the microscope in Dr. Kim's lab at the Georgia Institute of Technology. The DNA molecule with no rNTPs was used as the control molecule. After flowing in the DNA molecules without the addition of any other molecules, such as RNA Polymerase or rNTPs, we graphed the data collected (Figure 20). The left graph shows the intensity trace of a singular molecule on the chamber—the x-axis representing time and the y-axis fluorescence intensity. Combining all 147 molecules in the chamber, a histogram was generated that showed the fraction of time the molecule spent in the base state. The histogram in Figure 20 shows that molecules in the chamber spent most of their time in the base state. As there was no RNA Polymerase added into the chamber, this is an expected result.

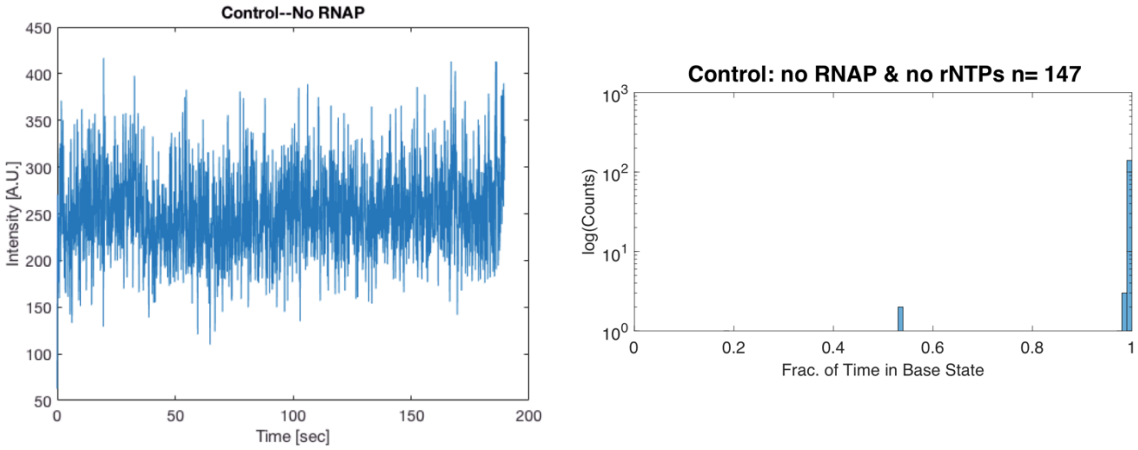


Figure 20 The graph on the left represents the level of fluorescence intensity given off by a single DNA molecule in the chamber, which only contained DNA molecules. The x-axis represents the time measured in seconds, and the y-axis is the intensity. The graph on the right represents a histogram of the fraction of time all 147 molecules in the chamber were in the base state. The x-axis represents the fraction of time the molecule spent in the base state, and the y-axis represents the log of the counts of molecules.

We then added 1 U/µL of RNA Polymerase into the chamber, shown in Figure 21, and we see more fluctuation in the data.

We then added 1 Units/µL of RNA Polymerase into the chamber and obtained a graph shown in Figure 21. The graph is representative of all 73 molecules in the chamber and shows how long the molecules spent in the base state. From the graph, we see there were less molecules in the base state. However, since there were no rNTPs in the chamber, this is the opposite of the expected effect. We predicted this histogram would look similar to that of Figure 20.

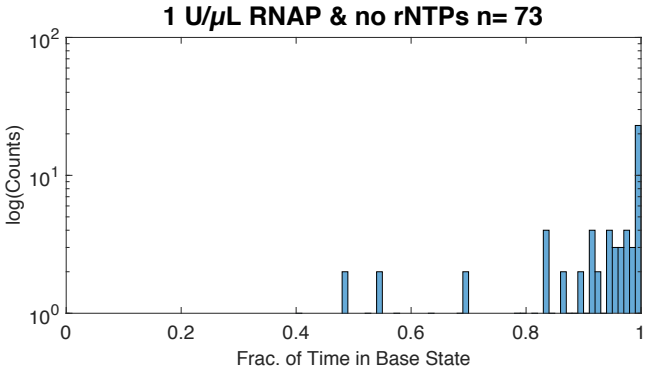


Figure 21 A histogram of the fraction of time 73 DNA molecules in a chamber with 1 U/µL RNA Polymerase spent in the base state.

Due to these unexpected results, we came to the conclusion that if the RNA Polymerase concentration inside of the chamber was too high, then the enzymes were more likely to hit the DNA molecule while floating around. If an RNA Polymerase hits the sequence near the Cy3 during its free diffusion, then the RNA Polymerase may induce a spurious PIFE signal. This hypothesis is shown in Figure 22.

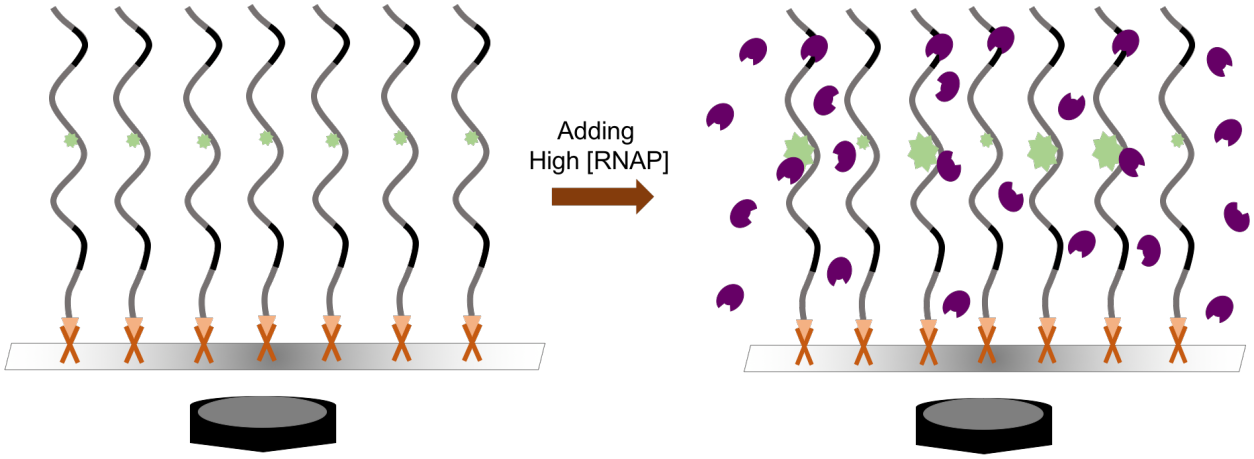


Figure 22 The left image represents a chamber only containing the DNA molecules, and the right chamber shows what might happen when concentration of RNA Polymerase added with DNA molecules is too high.

Therefore, the RNA Polymerase concentration added into the chamber had to be optimized to ensure the Cy3 was only in the enhanced state when RNA Polymerase was in the process of transcribing the DNA strand. We then tried to see how the intensities would change with decreasing RNA Polymerase concentration. We made 5 chambers containing the following RNA Polymerase concentrations: 0.05 U/µL, 0.01 U/µL, 0.005 U/µL, 0.001 U/µL, 0.0001 U/µL. The histograms representing the fraction of time the molecules are in the base state is shown in Figure 23. The molecules seem to have spent more time in the base state as the enzyme concentration decreased. This means that there were less PIFE signals present as the concentration went down.

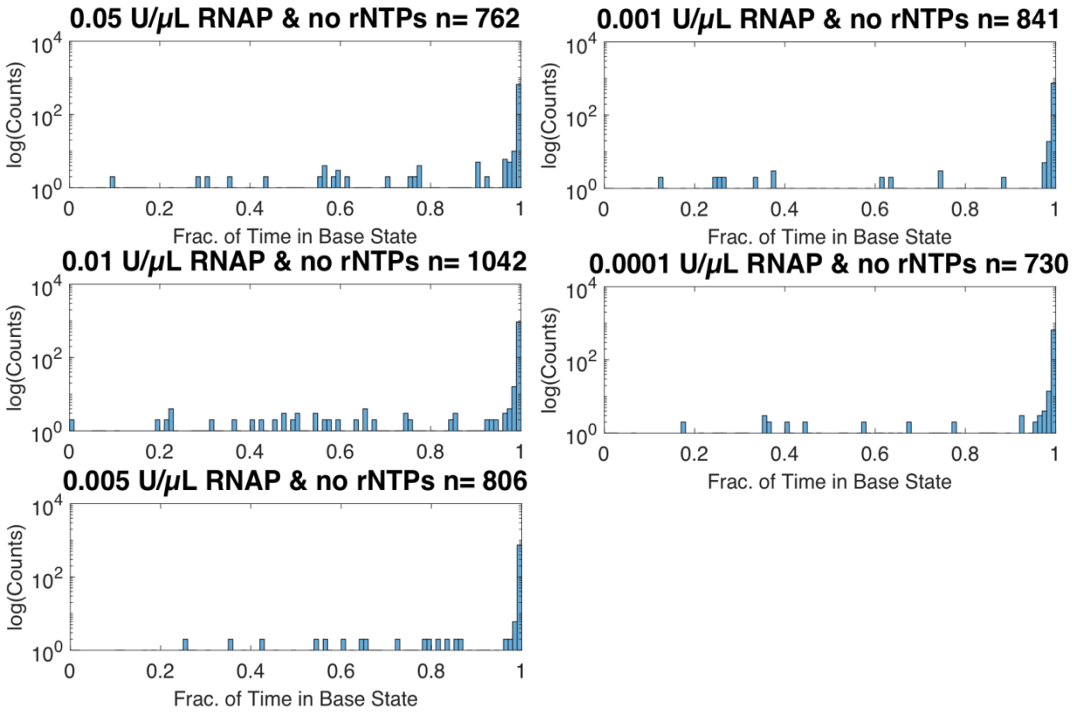


Figure 23 Histograms of the fraction of time molecules in a chamber spent in the base state. The five chambers contained differing concentrations of RNA Polymerase. These include 0.05 U/µL, 0.01 U/µL, 0.005 U/µL, 0.001 U/µL, and 0.0001 U/µL. As the concentration of RNA Polymerase decreased, PIFE signal decreased.

We then focused on 0.01 U/µL concentration of RNA Polymerase and added 1 µL of 25 mM of rNTPs to the chamber. One chamber only contained RNA Polymerase and another had both the enzyme and rNTPs. The histograms representing the data acquired is shown in Figure 24. The addition of rNTPs into a chamber containing RNA Polymerase should show us an increase in PIFE signal, which is what we see from the histograms. The histogram with rNTPs shows more molecules spending time in the enhanced state. Comparing the chamber with 0.01 U/µL RNA Polymerase and no rNTPs in Figure 24 to the similar chamber in Figure 23, we see that there were more molecules in the enhanced state in Figure 23. This difference may be due to the fact that there were 1042 molecules in the chamber shown in Figure 23 but 133 molecules in the chamber shown in Figure 24. This difference may have resulted in a greater fluctuation of results.

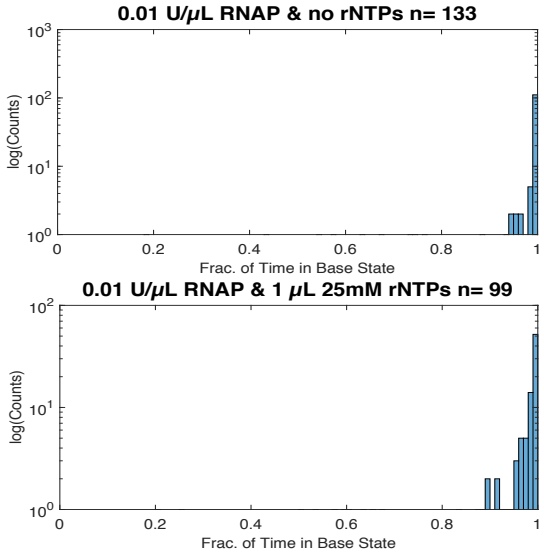


Figure 24 The top graph represents a histogram of the fraction of time 133 DNA molecules in a chamber with 0.01 U/µL RNA Polymerase spent in the base state. The bottom represents a chamber with 99 DNA molecules, 0.01 U/µL RNA Polymerase, and 1 µL of 25 mM rNTPs. Addition of rNTPs increased PIFE signal.

We then monitored the effect of the addition of 0.1 mM of rNTPs to different concentrations of RNA Polymerase (Figure 25). This rNTP concentration was added to chambers with different RNA Polymerase concentrations—0.005 U/µL, 0.001 U/µL, and 0.0001 U/µL. The results indicate that the addition of rNTPs did not increase the number of molecules in the enhanced state. The concentration of RNA Polymerase inside of the chamber may have been so low that there were not enough enzymes in the chamber to induce PIFE.

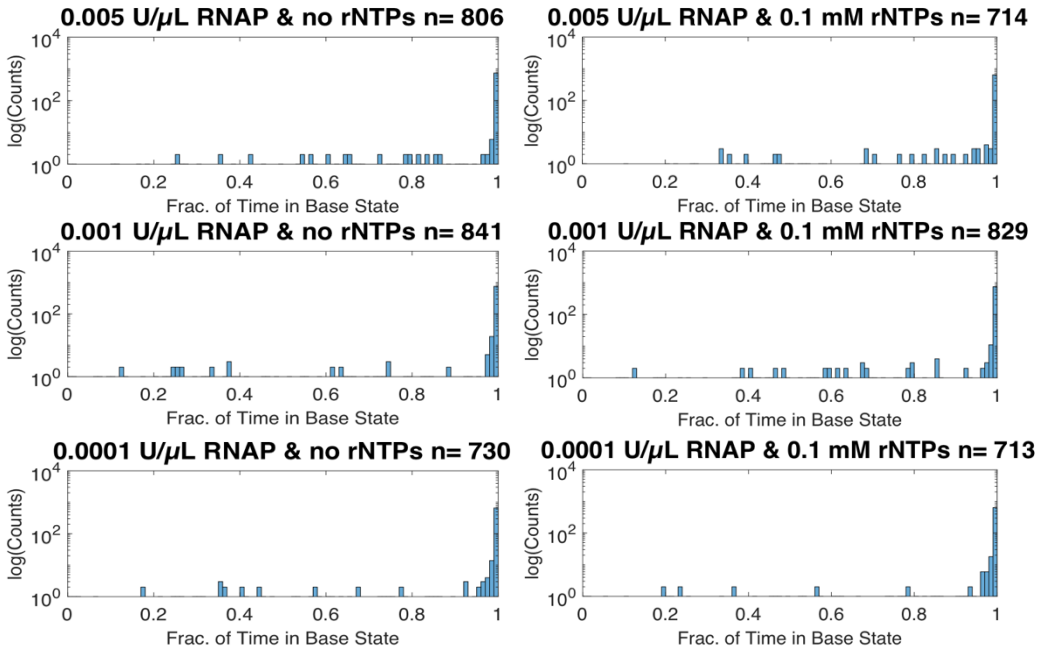


Figure 25 Histograms of the fraction of time molecules in a chamber spent in the base state. Each concentration of RNA Polymerase—0.005 U/μL, 0.001 U/μL, and 0.0001 U/μL—was added into chambers with 0 mM of rNTPs and 0.1 mM of rNTPs. Addition of rNTPs did not increase PIFE.

We went back to optimizing the concentration of enzyme in the chamber with repeated washes. We hypothesized that the excess RNA Polymerase could be removed by flowing in 50 μL of imaging buffer, which did not contain any RNA Polymerase or rNTPs. This idea is illustrated in Figure 26.

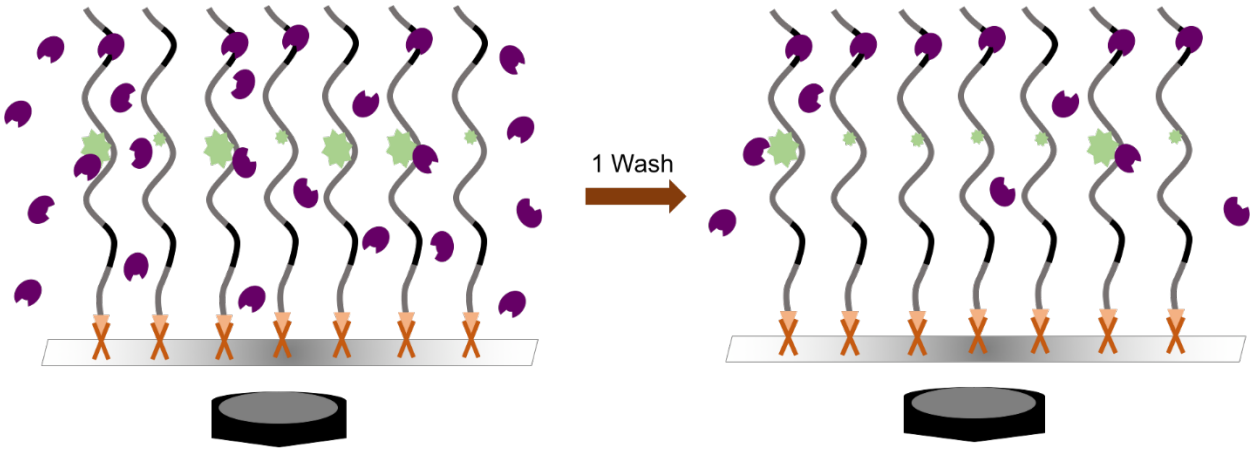


Figure 26 The left image represents a chamber containing DNA with a very high concentration of RNA Polymerase, and the right chamber shows what results after washing the chamber once with 50 µL of imaging buffer. This buffer did not contain any RNA Polymerase or rNTPs. Washing the chamber removed excess enzyme inside of the chamber.

We tested this method by flowing in 1 U/µL of RNA Polymerase and washed the chamber four times with 50 µL of imaging buffer (which did not contain any RNA Polymerase or rNTPs). Four more washes followed, and on the fifth wash, 0.5 mM rNTPs were included in the imaging buffer. Looking at the histograms in Figure 27, we see that the PIFE signal decreased as the number of washes increased before adding the rNTPs. Less and less molecules were in the enhanced state. However, after adding in 0.5 mM rNTPs in with the imaging buffer on the fifth wash, the number of molecules in the base state seemed to increase rather than decrease. This may have happened because the level of RNA Polymerase molecules left inside of the chamber was too low.

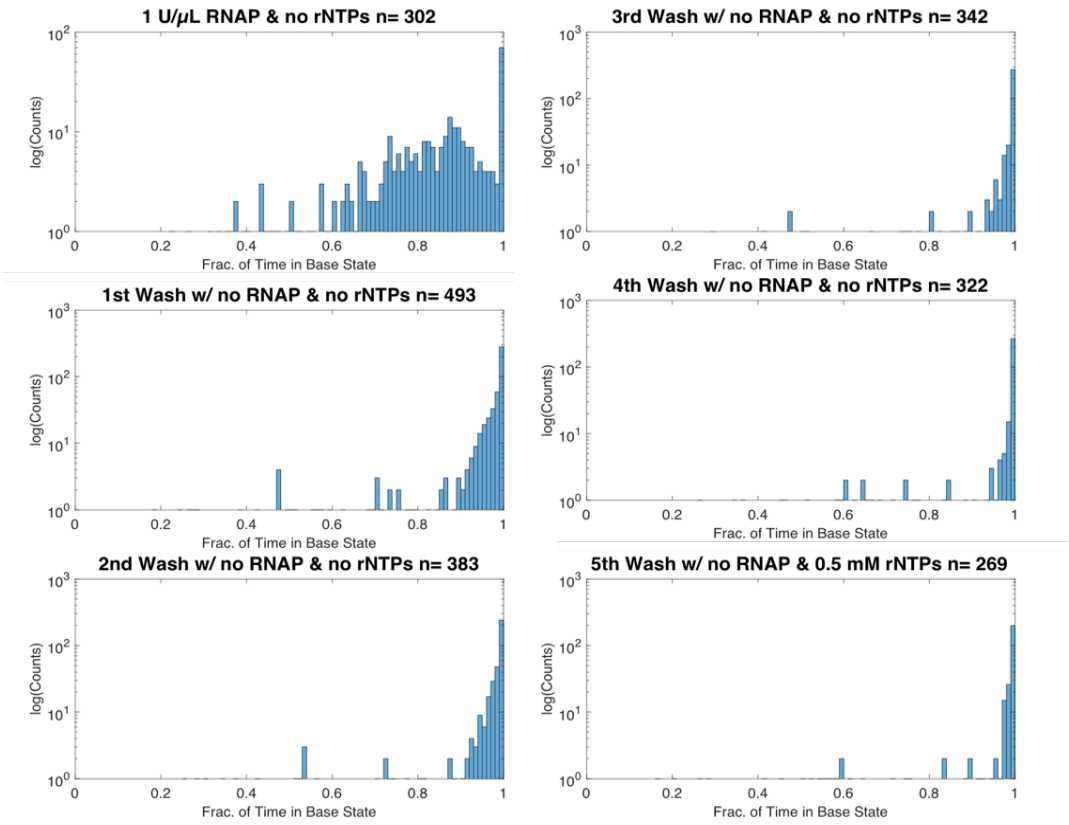


Figure 27 Histograms of the fraction of time molecules in a chamber spent in the base state. 1 U/µL of RNA Polymerase was added into a chamber and was then washed out 4 times with 50 µL of imaging buffer without any RNA Polymerase or rNTPs. On the 5th wash, 0.5 mM of rNTPs was added with the imaging buffer. Addition of rNTPs did not increase PIFE.

Thus, we repeated the experiments; however, this time, we added 0.1 mM rNTPs into the chamber on the third wash instead of the fifth to make sure there was more RNA Polymerase. We still started out with 1 U/µL RNA Polymerase in the original chamber; however, this time we decreased the number of washes. The histograms of this data are shown in Figure 28. We see that the level of enzyme left inside of the chamber was again too low as there were less molecules exhibiting PIFE.

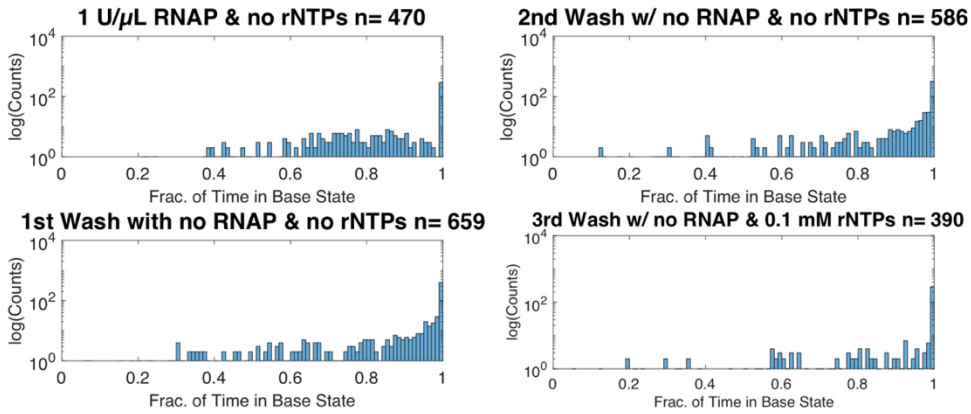


Figure 28 The graphs represent histograms of the fraction of time molecules in a chamber spent in the base state. 1 U/μL of RNA Polymerase was added into a chamber and was then washed out 2 times with 50 μL of imaging buffer without any RNA Polymerase or rNTPs. On the 3rd wash, 0.1 mM of rNTPs was added with the imaging buffer. Addition of rNTPs did not increase PIFE.

As these results were not what we were expecting, the aliquots of DNA used in these experiments were run through a PAGE gel to verify their length. A PAGE gel was used as less amount of DNA was needed to run the gel than with an agarose gel. There were two original stock solutions that were used, and both of these gels are shown in Figure 29. In both cases, the first and last lane contained a 1 kb plus ladder, the second lane contained DNA with rNTPs, and the third lane contained DNA without rNTPs. The ribonucleotide lane on the first batch (the second lane) showed bands around 500 bp, 450 bp, 400 bp, 300 bp, and 200 bp. The third lane on the first batch without ribonucleotides showed bands around 450 bp, 400 bp, 300 bp, and 200 bp. Both the second and third lanes in the second batch showed bands in similar places—1000 bp, 850 bp, 650 bp, 500 bp, 450 bp, 400 bp, 370 bp, and 200 bp. The DNA that was used in the experiments above was in the lanes without rNTPs. As restricted Fragment 1 was 297 bp, Fragment 2 was 20 bp, and restricted Fragment 3 was 188 bp, the complete ligated strand should appear around 505 bp. Thus, a band around this length should be seen on the gel. However, we saw that there were a lot of other molecules with different lengths inside of the stock solutions. Though we saw very faint bands around 500 bp for the lane with rNTPs on the first batch and in both lanes of the second batch, we concluded that there were too many other unwanted molecules to conduct accurate experiments. Therefore, our next step was to synthesize the DNA molecule again.

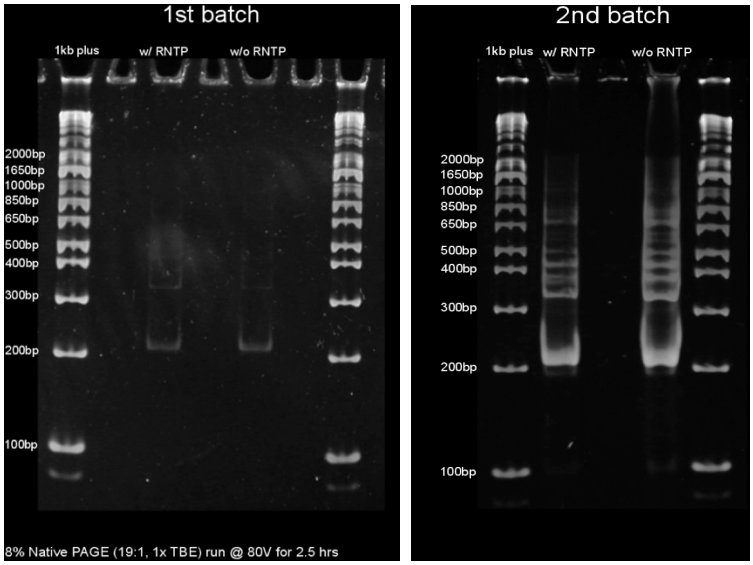


Figure 29 PAGE Gels of two stocks of DNA solutions. For both batches, the lanes from left to right are 1 kb plus ladder, DNA with rNTPs, DNA without rNTPs, and 1 kb plus ladder. The lane with rNTPs in the first batch shows bands at 500, 450, 400, 300, and 200 bp. The lane without rNTPs in the first batch shows bands around 450, 400, 300, and 300 bp. Both lanes of the second batch show bands around 1000, 850, 650, 500, 450, 400, 370, and 200 bp. The DNA stock we had been using was not pure.

Synthesizing the DNA Molecule using Initial DNA Design

The result of the PAGE gels in Figure 29 induced us to work on a way to combine the three fragments more efficiently using the initial DNA construct design. PCR was used to generate Fragment 1 using the primers shown in Table 1. The DNA was then run through an agarose gel, shown in Figure 30. The first lane had a 2-log DNA ladder (which shows bands from 0.1 kb to 10.0 kb), and the second lane had Fragment 1. A band around 350 bp could be seen. This was an expected result as unrestricted Fragment 1 was 334 bp in length.

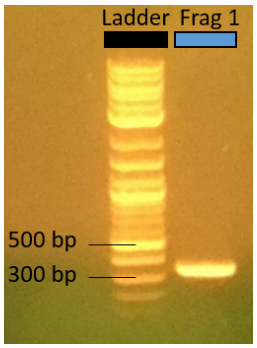


Figure 30 1% agarose gel of Fragment 1. Lane one has a 2-log DNA ladder, and lane two has Fragment 1. A band around 350 bp can be seen. The fragment was correctly synthesized.

Fragment 3 was then synthesized by PCR, using primers shown in Table 1, and the agarose gel is shown in Figure 31. The first lane contained a ladder, and the second lane had Fragment 3 with a band around 200 bp, which was an expected result as unrestricted Fragment 3 was 197 bp in length.

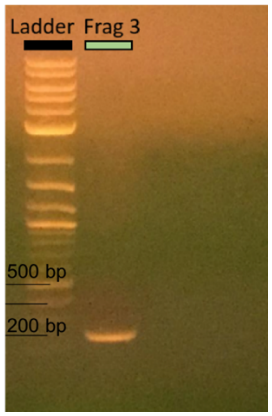


Figure 31 A 1% agarose gel of Fragment 3. Lane one has a 2-log DNA ladder, and lane two has Fragment 3. A band around 200 bp can be seen. The fragment was correctly synthesized.

After synthesis, Fragment 1 was restricted using BsmBI. After multiple trials, optimal reaction conditions were determined, with the reaction time running for 6 hours. A gel of an optimal restriction reaction of Fragment 1 is shown in Figure 32. The first lane had the 2-log DNA ladder, the second lane had Fragment 1, and the third lane had restricted Fragment 1. Restricted Fragment 1 was 297 bp in length, which is what we observed in the gel. The second lane showed a band around 350 bp, and the third lane showed a band less than 350 bp around 300 bp.

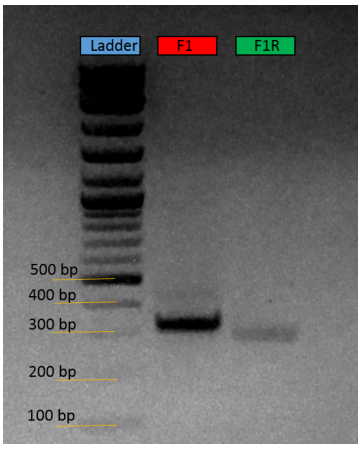


Figure 32 A 2% agarose gel of Fragment 1 and restricted Fragment 1. Lane one has a 2-log DNA ladder, and lane two has Fragment 1—showing a band around 350 bp—and lane three has restricted Fragment 1 with a band around 300 bp. The fragment was correctly digested.

Fragment 3 was restricted using BsaI, and the product was run through a PAGE gel to distinguish between small difference of length in the bands of unrestricted and restricted Fragment 3. The gel is shown in Figure 33. The first lane contained the 1 kb DNA ladder, the second lane had unrestricted Fragment 3, and the third lane had restricted Fragment 3. The second lane showed a band around 200 bp, and the third lane showed bands around 200 and 290 bp. There are also some smeared bands from 200 bp to 300 bp. Restricted Fragment 3 was around 188 bp in length, which is a band in the gel. However, not all of the DNA restricted as there was still a faint band around 200 bp. The reaction time was already 6 hours, and we were having trouble optimizing the conditions for this reaction; therefore, the bottom band was cut out of the gel and purified using a QIAGEN® (Netherlands) gel extraction kit. Due to the fact that the bottom band was not completely separated from the unrestricted band, there may have been some unrestricted molecules in the purified sample. We believed that the presence of the unrestricted molecule would not affect ligation of the restricted one with other molecules, thus we continued with the experiments.

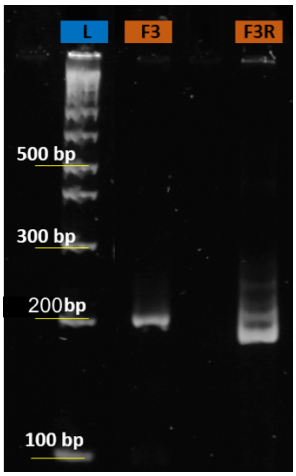


Figure 33 The PAGE gel of Fragment 3 and restricted Fragment 3. Lane one has a 1 kb DNA ladder, and lane two has Fragment 3—showing a band around 200 bp. Lane three has restricted Fragment 3 with a band around 200 bp and 190 bp—with smearing between 200 bp and 300 bp. The restricted fragment had to be purified using gel purification kit.

After restricting Fragment 1 and Fragment 3, we added them to a ligase reaction with Fragment 2 using T4 ligase. We tried different ratios of Fragment 1:Fragment 2:Fragment 3 in the mixture. After the ligase reaction, we ran them through an agarose gel, which is shown in Figure 34. The first lane had a 2-log DNA ladder, the second lane contained a 1:1:1 ratio of ligated DNA, the third contained 1:2:1, and the fourth lane had a 1:3:1 ratio. As the lanes did not contain a band around 500 bp, which is what was expected, we concluded that the ligation was not successful. The last three lanes show faint bands around 300 bp (which is the length of Fragment 1) and 200 bp (length of Fragment 3) and brighter bands below 100 bp (which corresponds to the length of Fragment 2). Thus, the three fragments did not ligate together and instead stayed as individual molecules during the reaction.

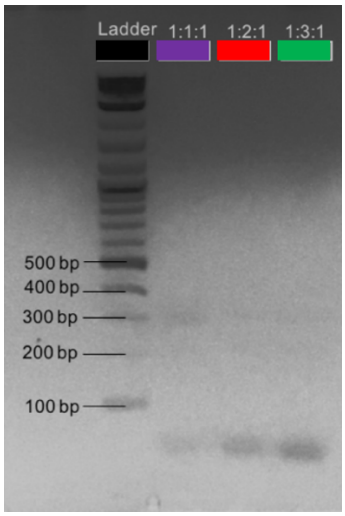


Figure 34 A 1% agarose gel of Fragment 1, Fragment 2, and Fragment 3 ligated together using T4 ligase. Different ratios of Fragment 1:Fragment 2:Fragment 3 were mixed together. Lane one has a 2-log DNA ladder, and lane two has a 1:1:1 ratio, lane three has a 1:2:1 ratio, and lane four has a 1:3:1 ratio. The last three lanes showed bright bands below 100 bp, and faint bands around 300 bp and 200 bp. The ligation was not successful for any of the ratios.

We then tried ligating the fragments together in different ways. We first tried ligating Fragment 1 and Fragment 2 together, and then, in a separate reaction, Fragment 2 and Fragment 3. A ligated molecule containing ligated Fragment 1 and Fragment 2 was 317 bp in length, and ligated Fragment 2 and Fragment 3 was 208 bp. The PAGE gel of these results is shown in Figure 35. The first lane had the 1 kb DNA ladder, the second lane contained Fragment 1 that had been restricted, the third lane had Fragment 1 and Fragment 3 that was ligated together using T4 ligase, lane four had restricted Fragment 3, and lane five had ligated Fragment 2 and Fragment 3 using T4 ligase. The second lane showed a band around 290 bp. The third lane showed distinct bands around 300 and 330 bp but also showed a smear of bands from 500 bp to 1000 bp. The fourth lane showed bands around 190 bp and 200 bp—with some smearing to about 350 bp. The last lane showed a band around 200 bp. Looking at the ligation of Fragment 1 and Fragment 2, there seemed to be unwanted molecules floating around in the solution as there were bands appearing at more than just around 300 bp. The ligation of Fragment 2 and Fragment 3 showed a band around 208 bp; thus, the ligation may have been a success.

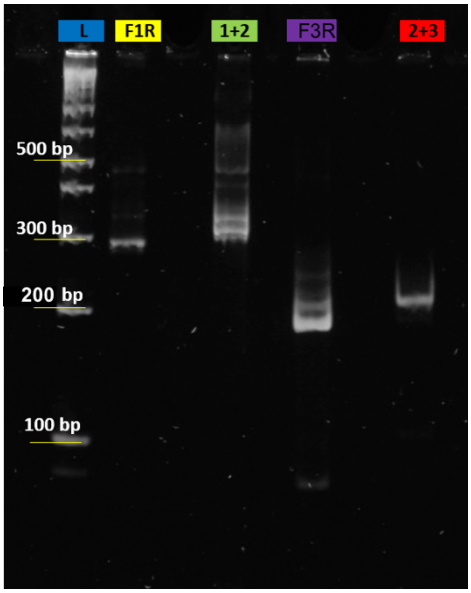


Figure 35 PAGE gel with lane one is the 1kb DNA ladder, lane two is restricted Fragment 1, lane three is ligated Fragment 1 and Fragment 2, lane four is restricted Fragment 3, and lane five is ligated Fragment 2 and Fragment 3 using T4 ligase. Lane two shows bands around 290 bp, lane three has bands that are smeared from 300 bp to 1000 bp, lane four shows a smear of bands from 190 bp to 350 bp, lane five shows a band around 200 bp. The individual ligation method was not successful.

We then worked on optimizing the ligase reaction of Fragment 1 and Fragment 2. The control antisense strand of Fragment 2 was first ligated with Fragment 1, and then the sense strand was annealed and ligated—completing the ligation of Fragment 1 and Fragment 2. T4 ligase was used for the reaction. The PAGE gel representing this ligase reaction is shown in Figure 36. There was a smear of bands from 300 bp to 850 bp, with distinct bands at 300, 325, 350, 640, 700, and 850 bp. As we should only see a band around 300 bp for the ligation of Fragment 1 and Fragment 2, we concluded that there was error in the reaction since we received unwanted product.

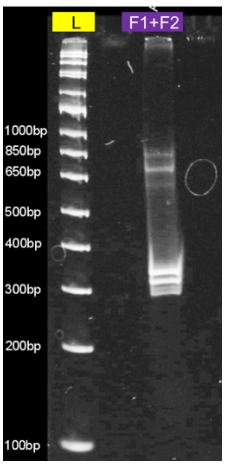


Figure 36 PAGE gel of ligated Fragment 1 and Fragment 2. Lane one is the 1kb DNA ladder, lane two is ligated Fragment 1 and Fragment 2. In this ligation, Fragment 1 was first ligated with the single strand of Fragment 2 that did not contain rNTPs or Cy3 (the control antisense strand). Then the sense strand was then annealed and ligated to the mixture. T4 ligase was used in the reaction. The second lane shows a smearing of bands from 300 bp to 850 bp. Distinct bands are around 850 bp, 700 bp, 640 bp, 350 bp, 325 bp, and 300 bp.

We then learned that T4 ligase performs blunt-end ligation, which may be the cause of unwanted bands appearing in our ligation. We discovered that T7 ligase performs sticky-end ligations (unlike T4 ligase) and so we tried to ligate Fragment 1, Fragment 2, and Fragment 3 together using T7. The gel of this ligation is shown in Figure 37. The first and last lane were 2-log DNA ladders, lane two was the ligation, lane three was Fragment 1, lane four was restricted Fragment 1, lane five is Fragment 3, and lane six is restricted Fragment 3. Two faint bands around 300 bp and 200 bp could be seen in the second lane. The third lane showed a band at 300 bp, the fourth lane showed a band lower than 300 bp, the fifth lane showed a band at 200 bp, and the sixth lane showed a faint band at 200 bp and a brighter band lower than 200 bp. As the second lane does not show a band around 500 bp as expected, we concluded that the ligation reaction did not work. However, due to time constraints, we could not focus on working to optimize the synthesis of the DNA molecule used to test whether or not RNA Polymerase would pause when it came across rNTPs.

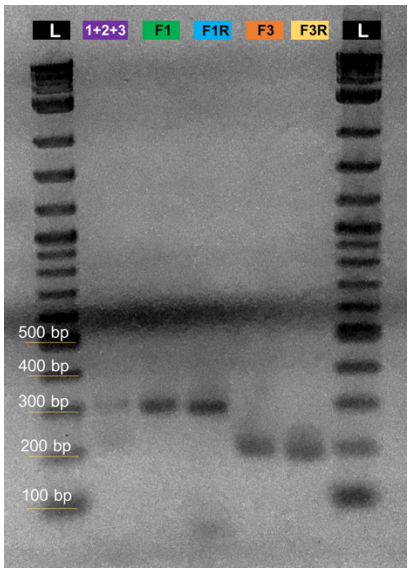


Figure 37 2% agarose gel—where lane one and lane seven is a 2-log DNA ladder, lane two is ligated Fragment 1, Fragment 2, and Fragment 3 using T7 ligase, lane three is Fragment 1, lane four is restricted Fragment 1, lane five is Fragment 3, lane six is restricted Fragment 3. Lane two shows faint bands around 300 bp and 200 bp. Lane three shows a band at 300 bp, and lane four has a band a little lower than 300 bp. Lane five shows a band at 200 bp, and lane six shows a faint band at 200 bp and a brighter band below 200 bp. Ligation with T7 ligase was not successful.

V. Reliability of RNA Polymerase Affecting Cy3 Fluorophore

In our last set of experiments, we further analyzed the interaction of RNA Polymerase and Cy3. As the enzyme moves down the strand of DNA and gets closer to the Cy3, it sterically hinders the fluorophore. However, we wanted to test the distance from which the RNA Polymerase may sterically hinder the Cy3 molecule and cause it to enter the enhanced fluorescence state. Thus, two new strands of DNA molecules were generated to observe this relation.

Design of DNA Constructs

Both of these molecules had a Cy3 molecule attached to the top, a promoter sequence, and a biotinylated anchor at the bottom. The difference between the two strands, which is shown in Figure 38, was the distance between the fluorophore and the promoter. The distance in the case of DNA A was 245 bp while in DNA B it was 67 bp. A third type of DNA was used, DNA C, which was similar to the structure of the other two molecules except it did not contain a promoter region.

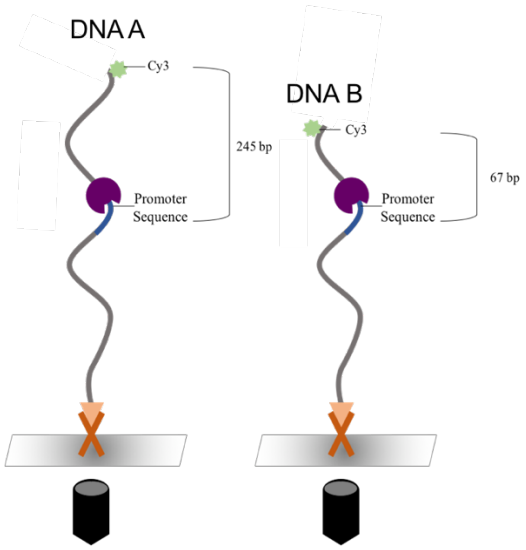


Figure 38 The left image represents DNA A, where the distance between the Cy3 molecule and the promoter sequence is 245 bp. The right image shows DNA B, where the same distance is 67 bp.

As the RNA Polymerase would only be 67 bp away from the Cy3 in DNA B, we hypothesized that this distance would be close enough for the enzyme force the molecule into the enhanced state. We believed that the distance within DNA A would be too long for the enzyme to have any sort of effect on the Cy3; thus, we hypothesized that the molecule would stay in the base state. DNA C was thought to be the control molecule as the RNA Polymerase would have no place to bind on this molecule, meaning the fluorophore would stay in the base state.

The total length of Construct A DNA was 517 bp and Construct B DNA was 335 bp. A gel containing these DNA products is shown in Figure 39. Lane one contained a 2-log DNA ladder, lane two was Construct A DNA, and lane three was Construct B DNA. Lane two showed a bright band around 500 bp, and lane three showed one around 300 bp. Both of these results were expected. As both Construct A and Construct B also had faint bands around 1000 bp and 800 bp, respectively, the bright bands for both Sample A and Sample B were gel purified using a QIAGEN® (Netherlands) gel extraction kit. This step was taken to ensure that the only DNA we used experimentally was of the proper length.

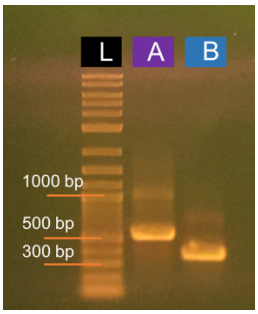


Figure 39 1% agarose gel of Construct A and B. The left lane contains a 2-log DNA ladder, lane two contains Construct A DNA, and lane three contains Construct B DNA. Lane two shows two bands around 500 bp and 1000 bp. Lane three shows a band around 800 bp and 300 bp. Both constructs were correctly synthesized.

Experimental Results and Analysis

The first test we did was to see whether or not different fields of view on the same chamber would give us similar results. To run this check, we used Construct B DNA. A histogram of the time averaged intensity of the molecules in the chamber was generated, and the results are shown in Figure 40. Different concentrations of RNA Polymerase were added to the chamber—0, 0.005, and 0.05 U/µL. Three fields of view were tested for each concentration and the data from each field is plotted on the same histogram. We then ran ANOVA test for each concentration. For 0 U/µL, the p-value obtained was 0.3362, p equaled 0.3773 for 0.005 U/µL, and p equaled 0.0567 for 0.05 U/µL. All three of these p-values are above 0.05; thus, the data had been found to be consistent across all fields of the chamber. In the experiments to follow, we took data across three different fields of view on the same chamber for each concentration.

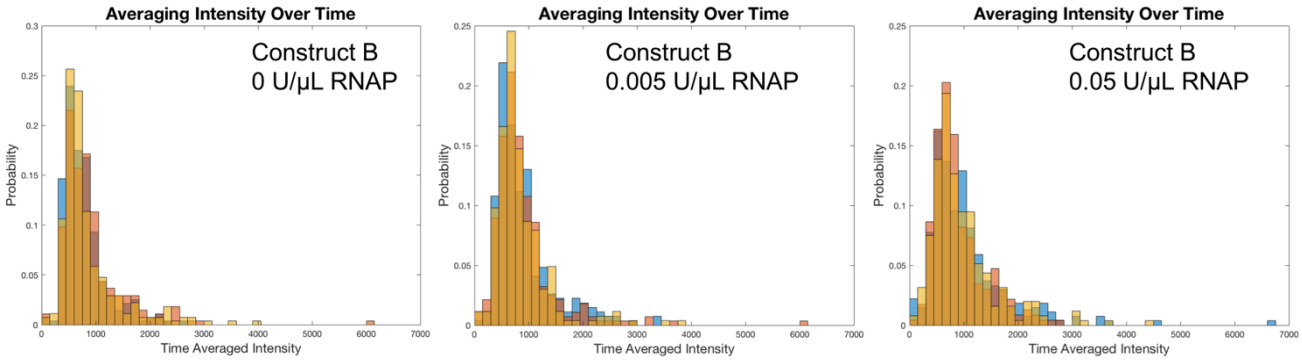


Figure 40 The histograms represent the data received from Construct B DNA. Three fields of view were tested for each concentration and laid on top of each other (shown in the different colors). The y-axis is the intensity of the molecules averaged over time, and the x-axis is the probability of finding the molecules at the certain intensity. The left most histogram shows a chamber with 0 U/µL RNA Polymerase, the middle represents a chamber with 0.005 U/µL RNA Polymerase, and the last graph represents the chamber with 0.05 U/µL. Three fields of view on the same chamber gave results that were comparable.

We then ran full-length experiments using Construct B DNA to see the fraction of time the molecules in the chamber spent in the base state. The histograms of this data are shown in Figure 41. The different concentrations of RNA Polymerase used were 0, 0.005, 0.05, 0.5, and 1.0 U/µL. From analyzing the graphs, we see the molecules did spend some time in the enhanced state, suggesting that placing the RNA Polymerase 67 bp away from the fluorophore still sterically hindered the molecule. The number of molecules spending time in the base state decreased as the RNA Polymerase concentration increased, which is what we saw when we ran experiments using the impure stock of DNA.

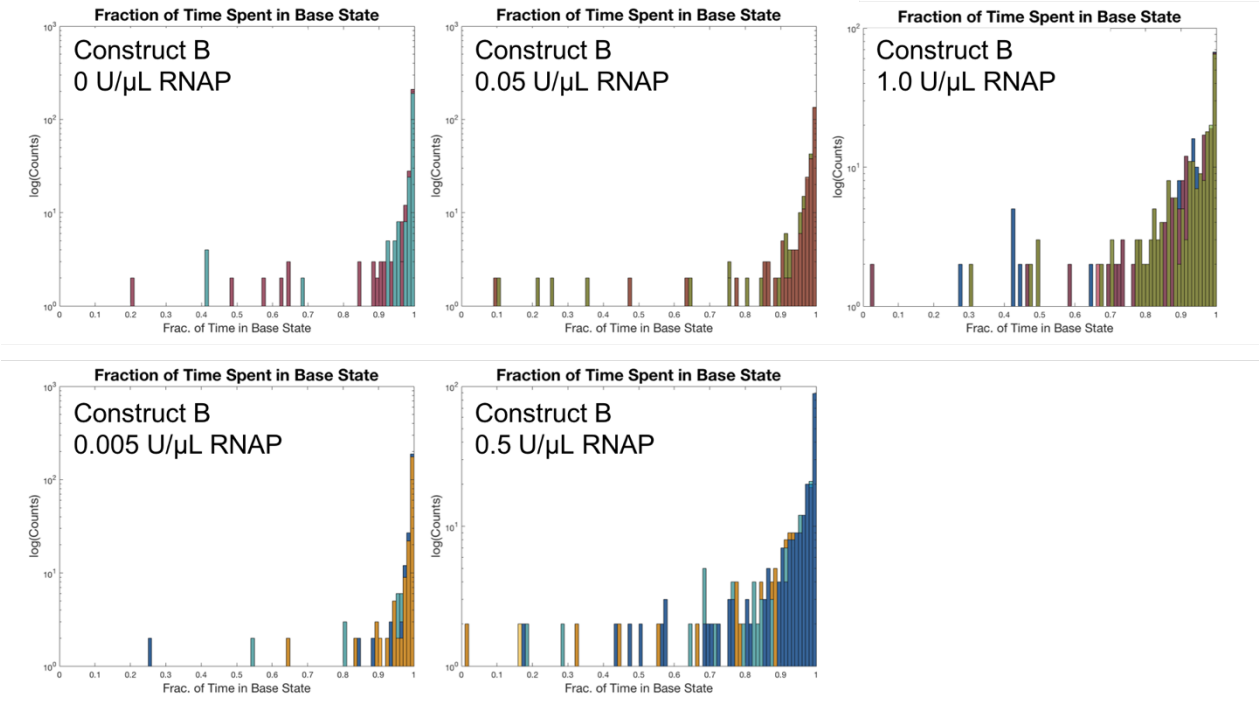


Figure 41 The histograms represent the data received from Construct B DNA. The x-axis is the fraction of time the molecules spent in the base state, and the y-axis is the counts. The different RNA Polymerase concentrations used were 0, 0.05, 1.0, 0.005, and 0.05 U/μL. As the RNA Polymerase concentration increased, PIFE increased.

We also generated histograms of the time averaged intensity of each molecule in the chamber at different RNA Polymerase concentrations. These graphs are shown in Figure 42. As the RNA Polymerase concentration increased, there were more molecules with higher intensity values. Thus, as RNA Polymerase concentration increased, PIFE increased. Comparing the method of analysis where we analyzed the fraction of time the molecules spent in the base state to the method where we averaged the intensity over time of each molecule we noticed that both gave similar results. Therefore, both forms of analysis could be used to look at the data. We also generated a boxplot, shown in Figure 43, on the averaging intensity over time of the molecules of Construct B with each RNA Polymerase concentration. We then ran an ANOVA test on the consecutive RNA Polymerase concentrations to see if they were significantly different or not, and the table containing the p-values is also in Figure 43. The mean of the averaged intensity over time of the molecules seems to increase as the RNA Polymerase concentration increases—thus, confirming that the PIFE signal is increasing.

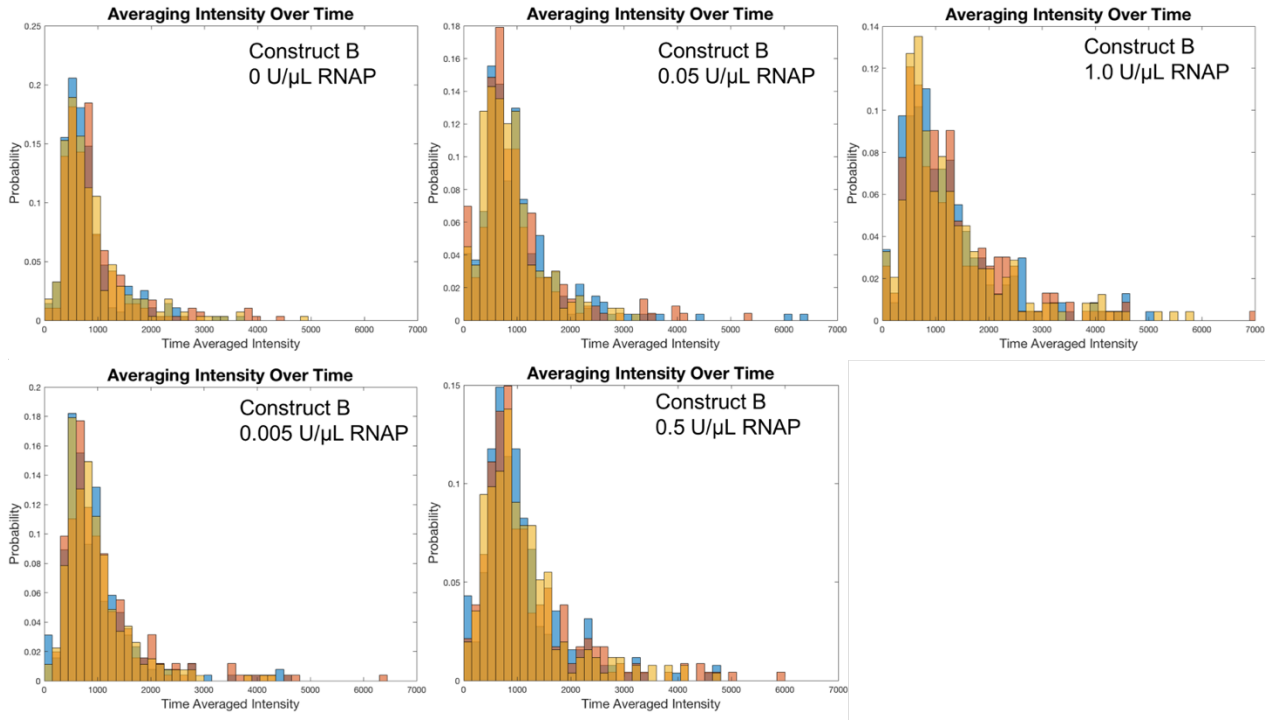
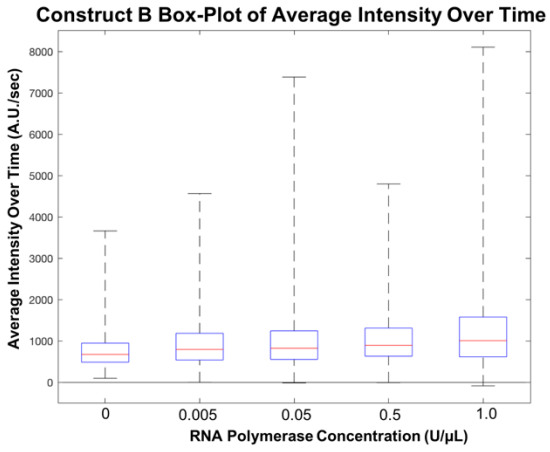


Figure 42 The histograms represent the data received from Construct B DNA using the new analysis showing the time averaged intensity of the molecules in the chamber. The different RNA Polymerase concentrations used were 0, 0.05, 1.0, 0.005, and 0.05 U/μL. As RNA Polymerase concentration increased, PIFE increased.



Change in [RNAP]	P-Value
0 to 0.005	0.0100*
0.005 to 0.05	0.3066
0.05 to 0.5	0.4667
0.5 to 1.0	0.0468*

Figure 43 Box-plot of averaging the molecules from Construct B over time. The table on right represents p-values from ANOVA tests ran between consecutive RNA Polymerase concentrations. * signifies a significant p-value. The mean of the intensity seems to steadily increase as the RNA Polymerase concentration increases.

After testing Construct B, we moved onto Construct A DNA. We tested the same RNA Polymerase concentrations as Construct B's experiments. The histograms of this data are shown

in Figure 44. As the RNA Polymerase concentration increases, the number of molecules in the enhanced state increase. Though we do not expect any PIFE with Construct A, the high concentration of RNA Polymerase must be inducing spurious PIFE. When running the data through the new analysis method, we generated histograms of the time averaged intensity of the molecules, shown in Figure 45. From these graphs we see that increasing RNA Polymerase concentration increased the number of molecules with higher intensities—corresponding with PIFE. A box-plot of Construct A's data of averaged intensity over time is shown in Figure 46 along with the p-values received after running ANOVA tests on consecutive RNA Polymerase concentrations. Again, the mean intensity seems to increase as the RNA Polymerase concentration increases—thus, PIFE seems to be increasing.

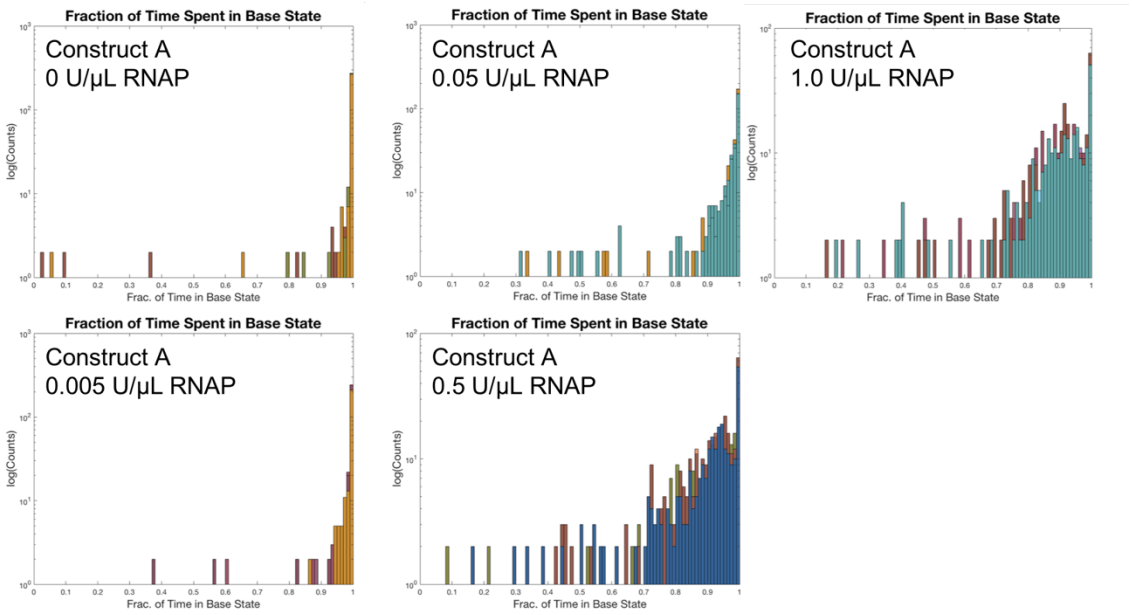


Figure 44 The histograms represent the data received from Construct A DNA. The x-axis is the fraction of time the molecules spent in the base state, and the y-axis is the counts. The different RNA Polymerase concentrations used were 0, 0.05, 1.0, 0.005, and 0.05 U/μL. As the RNA Polymerase concentration increased, PIFE increased.

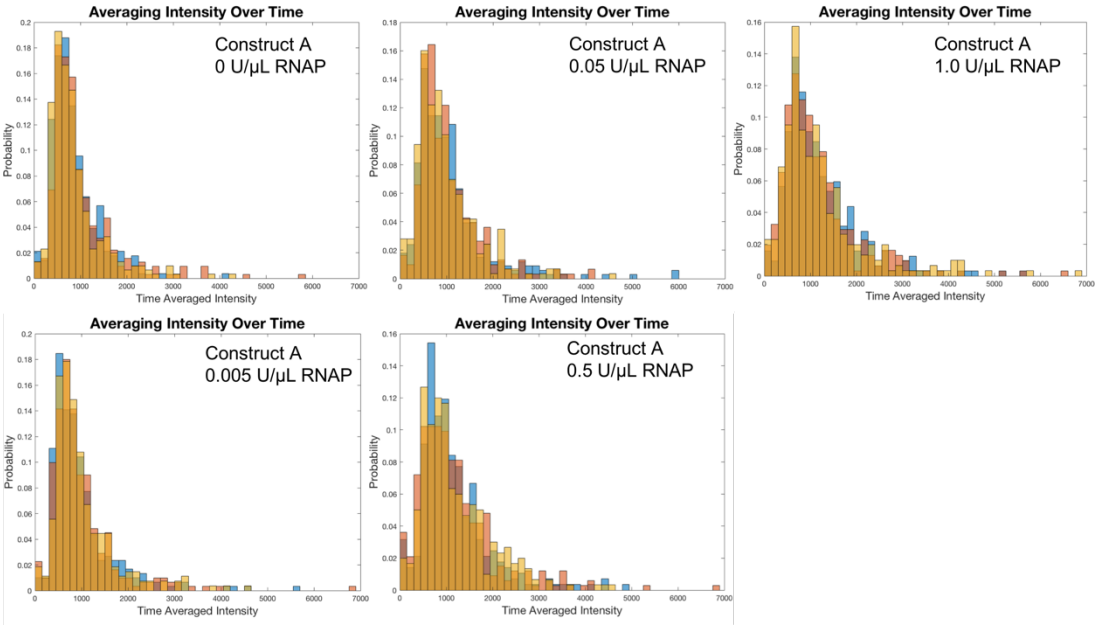
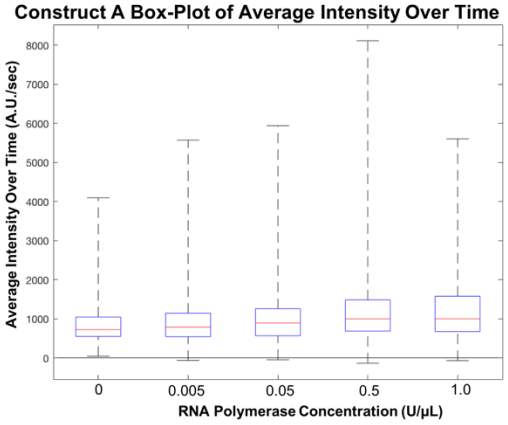


Figure 45 The histograms represent the data received from Construct A DNA using the new analysis showing the time averaged intensity of the molecules in the chamber. The different RNA Polymerase concentrations used were 0, 0.05, 1.0, 0.005, and 0.05 U/μL. Increasing RNA Polymerase concentration, increased PIFE.

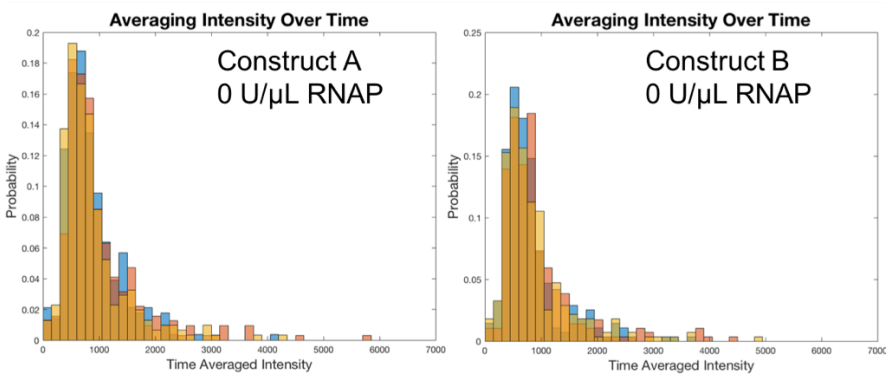


Change in [RNAP]	P-Value
0 to 0.005	0.0137*
0.005 to 0.05	0.0807*
0.05 to 0.5	0.0751*
0.5 to 1.0	0.6741

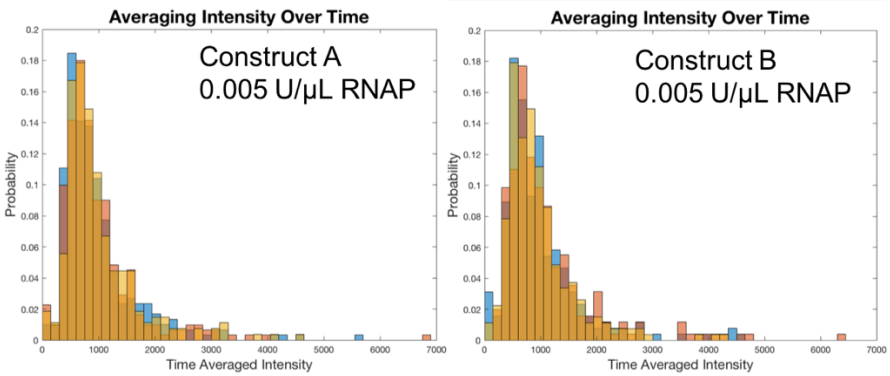
Figure 46 Box-plot of averaging the molecules from Construct A over time. The table on right represents p-values from ANOVA tests ran between consecutive RNA Polymerase concentrations. * signifies a significant p-value. The mean of the intensity seems to steadily increase as the RNA Polymerase concentration increases.

We next looked at the average intensity values over time for each RNA Polymerase concentration in both Construct A and Construct B. A comparison of the histograms between both samples are shown in Figure 47. We ran an ANOVA test between the two samples for each RNA Polymerase concentration, and the results are shown in Table 2. As there is no RNA Polymerase

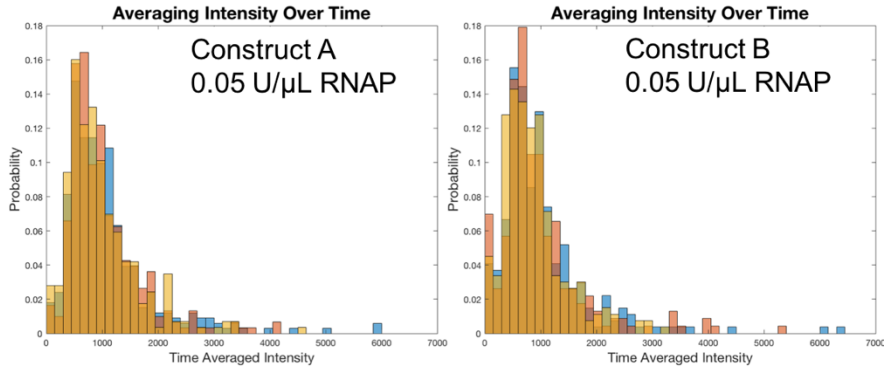
inside the chamber at the 0 U/μL concentration level, the Cy3 molecules should always stay in the base state for both chambers; therefore, the p-value should not be significant when comparing the two constructs. This result is what we see as the p-value for 0 U/μL is 0.0528, which is higher than 0.05. At 0.005 U/μL, the p-value is 0.1248, meaning that the two chambers again gave similar results. This means that the RNA Polymerase concentration was too low inside of the chambers to induce any PIFE. At 0.05 U/μL the p-value was 0.0076, which means that the two chambers were different from each other. When going higher than this concentration to 0.5 and 1.0 U/μL, we get the p-values 0.4673 and 0.6446, respectively. Thus, both of the constructs of DNA gave similar results at both of these concentrations. The concentration may have been too high at these levels, inducing spurious PIFE in all of the chambers regardless of the construct. Therefore, it seemed as though 0.05 U/μL was the optimal concentration of RNA Polymerase to use, and the RNA Polymerase was close enough to sterically hinder the Cy3 fluorophore when 67 bp away but not 245 bp away.


Averaging Intensity Over Time
Construct A
0 U/μL RNAP
Probability
Time Averaged Intensity

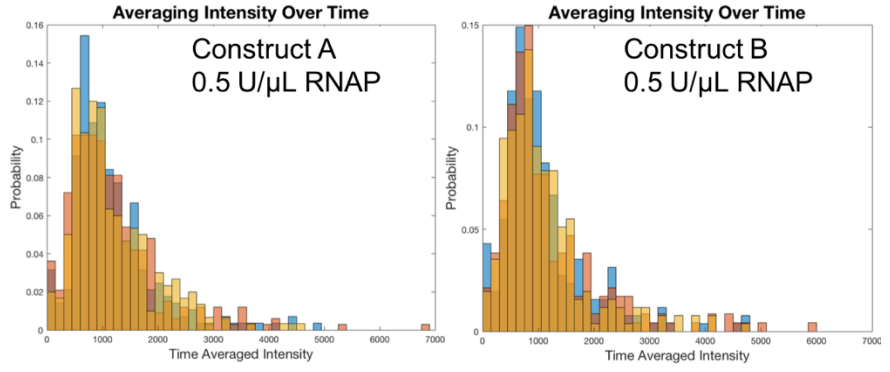
Averaging Intensity Over Time
Construct B
0 U/μL RNAP
Probability
Time Averaged Intensity


Averaging Intensity Over Time
Construct A
0.005 U/μL RNAP
Probability
Time Averaged Intensity

Averaging Intensity Over Time
Construct B
0.005 U/μL RNAP
Probability
Time Averaged Intensity


Averaging Intensity Over Time
Construct A
0.05 U/μL RNAP
Probability
Time Averaged Intensity

Averaging Intensity Over Time
Construct B
0.05 U/μL RNAP
Probability
Time Averaged Intensity


Averaging Intensity Over Time
Construct A
0.5 U/μL RNAP
Probability
Time Averaged Intensity

Averaging Intensity Over Time
Construct B
0.5 U/μL RNAP
Probability
Time Averaged Intensity

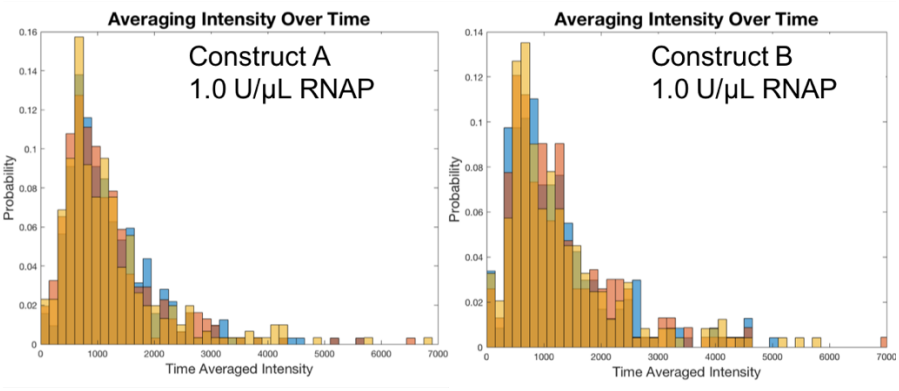


Figure 47 The histograms represent the data received from Construct A DNA and Construct B DNA. The x-axis is the intensity of the molecules averaged over time, and the y-axis is the probability of finding the molecules at the certain intensity. The plots show a comparison between the two samples of DNA. At low and high concentrations, the graphs seem to show similar results.

Construct A vs. Construct B

RNAP Concentration	P-Value
0 U/μL	0.0528
0.005 U/μL	0.1248
0.05 U/μL	0.0076*
0.5 U/μL	0.4673
1.0 U/μL	0.6446

Table 2 The table above represents the p-values received after running an ANOVA test between the two constructs of DNA, A and B. The symbol * represents a significant p-value. The optimal RNA Polymerase concentration seems to be 0.05 U/μL.

To confirm our results, we also compared Construct B DNA to Construct C DNA. We tested different RNA Polymerase concentrations—0, 0.05, 0.5, and 1.0 U/μL. As Construct C does not have a promoter region, we expect that there will be no PIFE signals present. The histograms of both samples with the average intensity over time for the different concentrations are shown in Figure 48. To test the significance of our results, we ran an ANOVA test on the data for the two different constructs and received the p-values shown in Table 3. Again, when the concentration of RNA Polymerase is 0, we saw that the two chambers were not different as we received a p-value of 0.0969, which is not significant. At 0.05 U/μL, we saw a significant difference between the two chambers with a p-value of 0.0037. This was expected as 0.05 U/μL seems to be a working concentration inside of the chamber. At 0.5 U/μL RNA Polymerase concentration, the p-value was significant at 0.0818, meaning the two chambers of DNA did not react differently to the addition

of RNA Polymerase. Again, this may have been because the concentration was too high and induced spurious PIFE. However, for 1.0 U/μL, the p-value received was 4.95e^-05, meaning that there was a significant difference between the two chambers. When looking at the data for this concentration with Construct C DNA, it seemed as though there was an error inside of the chamber. This may have due to a contamination inside of the chamber during experiments.

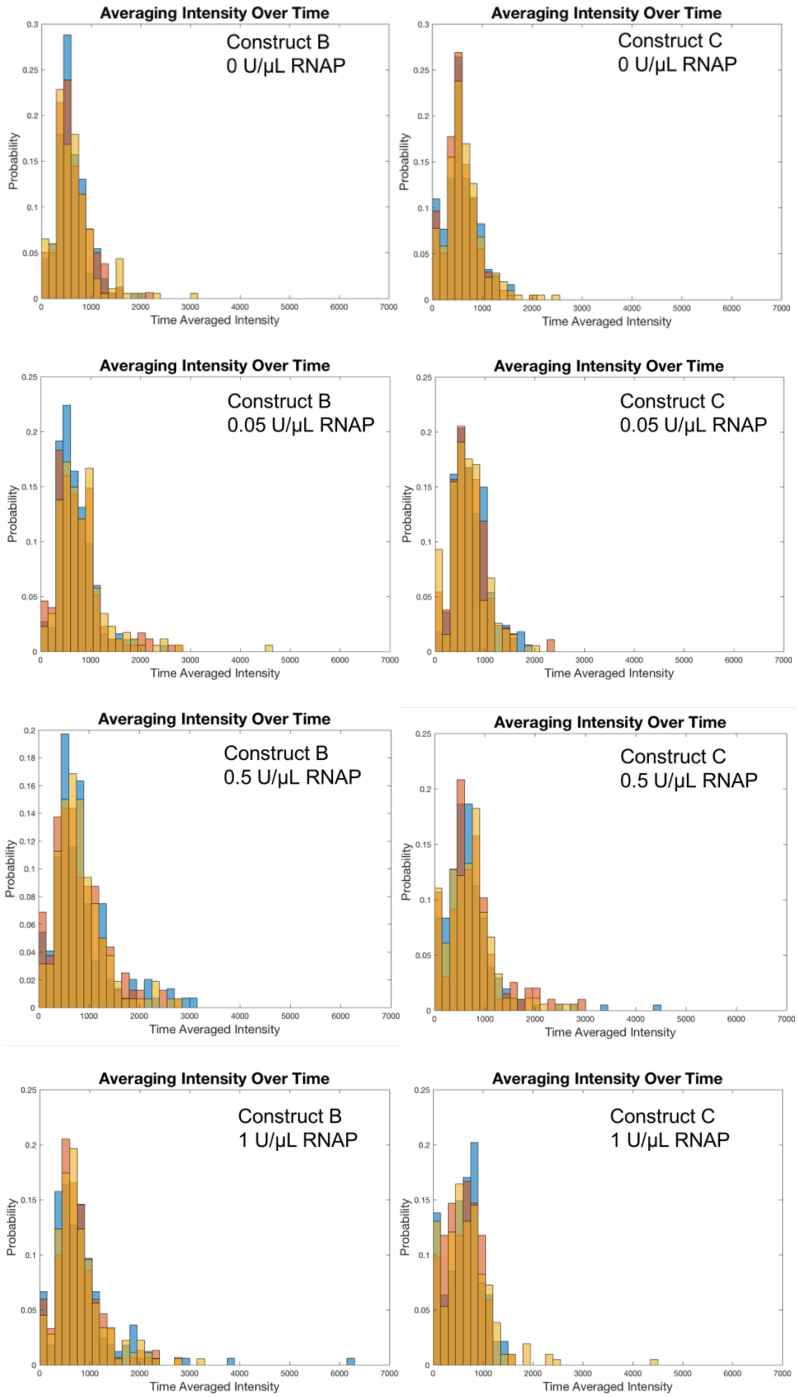

Averaging Intensity Over Time
Construct B
0 U/μL RNAP
Probability
Time Averaged Intensity
Averaging Intensity Over Time
Construct C
0 U/μL RNAP
Probability
Time Averaged Intensity
Averaging Intensity Over Time
Construct B
0.05 U/μL RNAP
Probability
Time Averaged Intensity
Averaging Intensity Over Time
Construct C
0.05 U/μL RNAP
Probability
Time Averaged Intensity
Averaging Intensity Over Time
Construct B
0.5 U/μL RNAP
Probability
Time Averaged Intensity
Averaging Intensity Over Time
Construct C
0.5 U/μL RNAP
Probability
Time Averaged Intensity
Averaging Intensity Over Time
Construct B
1 U/μL RNAP
Probability
Time Averaged Intensity
Averaging Intensity Over Time
Construct C
1 U/μL RNAP
Probability
Time Averaged Intensity

Figure 48 The histograms represent the data received from Construct B DNA and Construct C DNA. The x-axis is the intensity of the molecules averaged over time, and the y-axis is the probability of finding the molecules at the certain intensity. The plots show a comparison between the two samples of DNA. At low and high concentrations, the graphs seem to show similar results.

Construct B vs. Construct C

RNAP Concentration	P-Value
0 U/µL	0.0969
0.05 U/µL	0.0037*
0.5 U/µL	0.0818
1.0 U/µL	4.95×10^{-5} *

Table 3 The table above represents the p-values received after running an ANOVA test between the two constructs of DNA, B and C. The symbol * represents a significant p-value. The optimal RNA Polymerase concentration seems to be 0.05 U/µL.

VI. Conclusion

The goal of this experiment was to observe how RNA Polymerase reacted to rNTPs on a DNA strand during transcription. Though the old stock of DNA used was incorrectly synthesized (as shown in Figure 29), we managed to learn information when running experiments using it. We learned that RNA Polymerase concentration had to be optimized before running PIFE experiments on the DNA as too high of a concentration leads to spurious PIFE signals. When trying to synthesize a new DNA molecule, we ran into trouble putting the entire molecule together. Through this process we discovered that T7 ligase might be better than T4 ligase as it did not conduct blunt-end ligation. After multiple attempts at piecing together the DNA, we only had time left to run a few more experiments. Thus, we decided to create new DNA molecules to test the reliability of RNA Polymerase sterically hindering Cy3 and inducing a PIFE signal. We discovered an optimal concentration of RNA Polymerase to be around 0.05 U/µL. We also found that when 245 bp away from Cy3, RNA Polymerase is too far away to sterically hinder it. However, when placed 67 bp away, the large enzyme induces PIFE. These results can be used in future experiments when working with our original DNA construct as we will know more about the path of the RNA Polymerase

down the strand. When we see PIFE in a molecule, we will know that the RNA Polymerase is less than 245 bp away from the Cy3 fluorophore and is at least 67 bp away in distance.

For future experiments, we suggest conducting more tests to further analyze RNA Polymerase and its steric hindrance of Cy3 by conducting the same experiments to confirm the validity of the results received. Though we did not get the chance to answer whether or not RNA Polymerase will pause when near rNTPs, we developed and learned many ideas that will help with future experiments.

VII. References

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