The Effects of Oligonucleotide Concentration on Displacement Driven Triplex Formation Bioassays

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A thesis presented to

the faculty of

the College Arts and Sciences of Ohio University

In partial fulfillment

of the requirements for the degree

of chemistry with honors

Kelle D. Hart

April 2022

This thesis titled

The Effects of Oligonucleotide Concentration on Displacement Driven Triplex Formation Bioassays

by

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has been approved for

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Abstract

HART, KELLE D. Undergraduate, April 2022, Department of Chemistry and Biochemistry

The Effects of Oligonucleotide Concentration on Displacement Driven Triplex Formation Bioassays

Director of thesis: Jixin Chen

The purpose of this experiment is to investigate the phenomenon of triplex formation in a dye intercalated double stranded DNA and to quantify emission changes numerically throughout the triplex formation process. A secondary purpose is to investigate whether GA-Motif TFO follows Beer's Law of concentration proportionality with emission signal. Through experimentation it was found that ethidium bromide in aqueous solution had a weak emission signal, but when ds-DNA was added the signal increased drastically. It was also found that when ds-DNA and ethidium bromide are intercalated together and GA-Motif TFO is added, the emission signal will decrease. These are great conclusions to draw, however no numerical data could be obtained. Due to using very small concentrations of GA-Motif TFO, no quantifiable numerical conclusions were able to be drawn. The error associated with the measurements was too high and the error bars in each figure stated as much. It was also unable to be determined if GA-Motif TFO follows Beer's Law due to the error being too high during this stage of experimentation. This experiment gave mainly inconclusive results. Future experimentation should be conducted on this project to obtain quantifiable results.

Dedication

I would like to dedicate this document to Juvinch Vicente and Dr. Jixin Chen. Their help in this project is truly appreciated.

Acknowledgments

I would like to acknowledge Juvinch Vicente for his help in formulating the idea behind this experiment. The experiment conducted within this document was born from an idea that a graduate student, Juvinch Vicente, proposed during a group meeting within Dr. Jixin Chen's research group. Juvinch had an idea about studying the relationship between triplex forming oligonucleotide concentration and the displacement of ethidium bromide intercalator dye within double stranded DNA. This project was never officially started by Juvinch, but he had put the idea of experimentation into the research group. Juvinch then chose to complete his postdoctoral degree at the University of California, Irvine in industrial optics engineering and the idea of experimentation faltered. I ended up becoming interested in this idea and now I have written a whole thesis regarding something that Juvinch started. I am truly grateful that Juvinch put this idea into the research group and that he allowed me to continue with his idea and turn it into an experiment.

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Theory

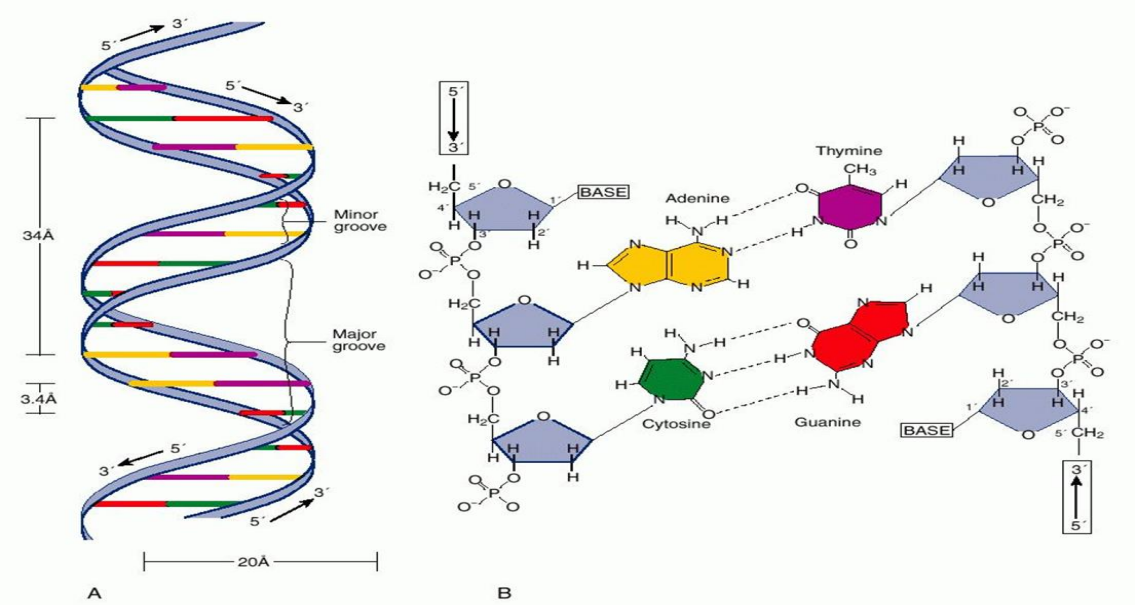
The experiment conducted within this document was born from an idea that a graduate student, Juvinch Vicente, proposed during a group meeting within Dr. Jixin Chen's research group. Through incredibly thorough literature evaluation and experimental planning, I came to a healthy hypothesis that would be testable within the limits of the laboratory equipment present at the Ohio University laboratories. The hypothesis is as follows: if ethidium bromide follows the intercalation mechanism proposed by current scientific literature, then a strong decrease in emission intensity signal proportional to the concentration of triplex forming oligonucleotide added to the ethidium bromide intercalated double stranded DNA should be seen.[23,20,17,15,14,11,8,6] A secondary hypothesis was to determine the quantifiable numerical increase and decrease in emission signal upon adding DNA to ethidium bromide and adding triplex forming oligonucleotide to a DNA and ethidium bromide solution respectively. To understand the meaning of this hypothesis, many aspects of the experiment must be discussed. These aspects include double stranded DNA properties and structure, triplex forming oligonucleotide properties and binding mechanisms, DNA triplex formation, ethidium bromide characteristics and applications, and the fluorescent properties of the ethidium bromide when in different chemical environments. The final paragraph of this section will sum up all the aspects previously discussed and relate them back to the primary hypothesis of this experiment. The first aspect to be discussed is double stranded DNA and its properties.

Double Stranded DNA

Double stranded DNA (ds-DNA) is the foundational molecule for this experiment. Ds-DNA can be visualized as if it were a ladder. Ds-DNA contains two separate strands that are linked together, much like a ladder has two sturdy edges linked together by rungs. Each separate strand present within ds-DNA has its own backbone, much like the sturdy edges of the ladder. These backbones are comprised of phosphate diester bonds that link together deoxyribose sugars. The backbones of ds-DNA run in an antiparallel fashion, meaning they are oriented in opposite directions.[18,35] The rungs of the ds-DNA ladder are analogous to the bases present within the ds-DNA structure. Ds-DNA contains four primary bases: adenine, guanine, cytosine, and thymine. These bases are also called nucleobases. The adenine and guanine bases are classified as purine bases and the thymine and cytosine bases are classified as pyrimidine bases. These bases within the ds-DNA hydrogen bond to both the pentose sugar portion of the backbone as well as to the complimentary base that is present across from it. This bonding completes the ladder comparison and there is now a fully attached ds-DNA molecule that is present in a structure very similar to a ladder. This method of ds-DNA base pairing is known as the Watson-Crick model and is widely accepted as the most probable way for ds-DNA to form.[18] A visual representation of this phenomenon is seen below.

Figure One

Watson-Crick Base Pairing Schematic [48]



-This figure shows the Watson-Crick base pairing theory. The importance of this figure is that adenine can be seen forming two hydrogen bonds with thymine and cytosine can be seen forming three hydrogen bonds with guanine.

This model of ds-DNA simplifies the base pairing, as it has been proven in this model that adenine binds with thymine and guanine binds with cytosine at a specific angular conformation. This is known as complimentary base pairing.[18,35] When two bases come together in DNA they are written in the notation of A:T and G:C. The letters refer to the corresponding nucleobases and the colon represents that the nucleobases are bonded through Watson-Crick base pairing. This has been proven to be the most stable form of ds-DNA due to the doubly hydrogen bound adenine and thymine to one another, along with the triply hydrogen bound cytosine and

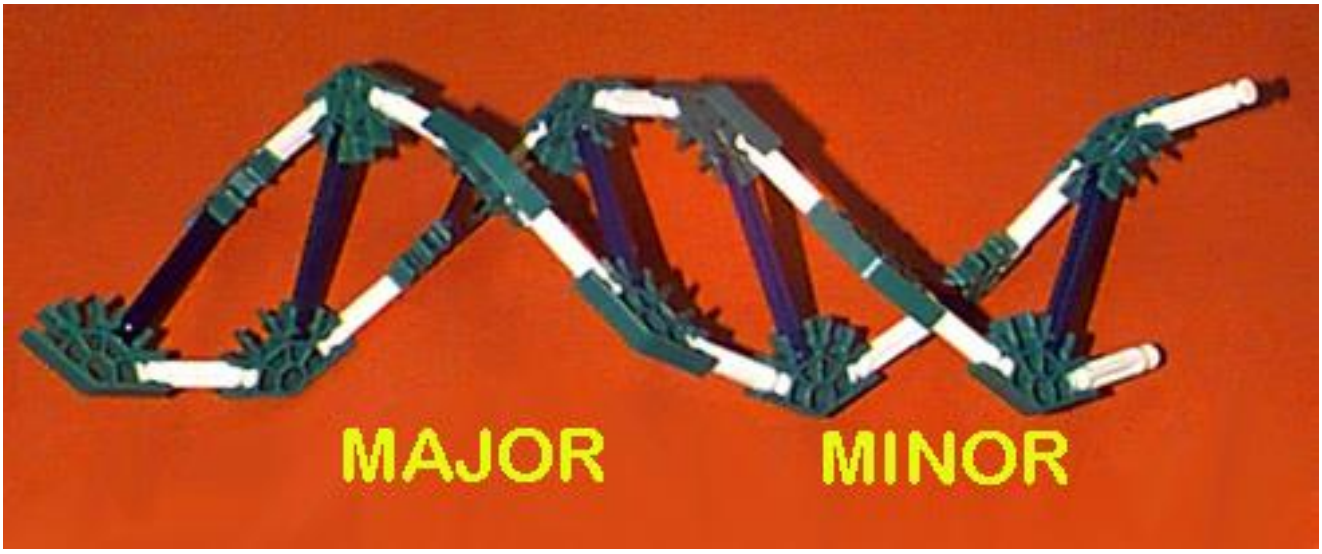
guanine to one another.[35] It is important to note that ds-DNA is not conformed to rigidity like that of a ladder. Ds-DNA is in the shape of a double helix as shown in figure one. The double helix is possible due to pi-pi stacking interactions between the base pairs of ds-DNA. The double helix is also possible due to hydrophobic interactions between the bases present within the ds-DNA. These interactions occur due to the planar nature of the base pairs.[18] DsDNA that is in the shape of a double helix and has not been modified or altered is known as B-form, right-handed ds-DNA. The specificity of this distinction is that B-form ds-DNA has ten and a half base pairs per turn and the backbones conform in a right-handed manner. This is the most common form of ds-DNA at the physiological pH of 7.35-7.45 and is the conformation present within this experiment.[4,37]

The double helix shape of ds-DNA is critical within this experiment, as a ds-DNA double helix contains sites classified as major and minor grooves. These grooves occur due to the helical nature of the ds-DNA and the angles in which the purine and pyrimidine bases hydrogen bond with each other. If ds-DNA were to be in the shape of a ladder the bases and backbone would bond at an angle of one hundred and eighty degrees, whereas a double helix bonds the bases and backbone together at one hundred and twenty degrees. This form of double-helical bonding allows for ds-DNA to have two asymmetrical grooves present within its structure. These grooves expose the bases within the ds-DNA to the outer environment.[1] A healthy way to visualize ds-DNA grooves is to think of the grooves as a window to peer inside of the ds-DNA. Most of the ds-DNA would appear to be a backbone, while the window would allow for an observer to view the individual base pairs attached to the backbone. The larger groove present

on ds-DNA is classified as the major groove and the smaller groove present on ds-DNA is the minor groove.[1] A visual representation of ds-DNA grooves can be seen below.

Figure Two:

Ds-DNA Major and Minor Grooves[49]



-This figure shows the major and minor grooves present within ds-DNA. The major groove is the groove of study in this experiment and can be seen exhibiting a larger surface area for binding.

The major and minor grooves of ds-DNA are instrumental within this experiment, as they allow for a convenient opening into the base pairs of the ds-DNA. Having a pathway into the base pairs of the ds-DNA allows for investigation of the chemical environment present within ds-DNA. The phrase "within ds-DNA" referring to within the base pairs of the ds-DNA. The chemical environment within ds-DNA has been thoroughly studied and is a foundational pillar

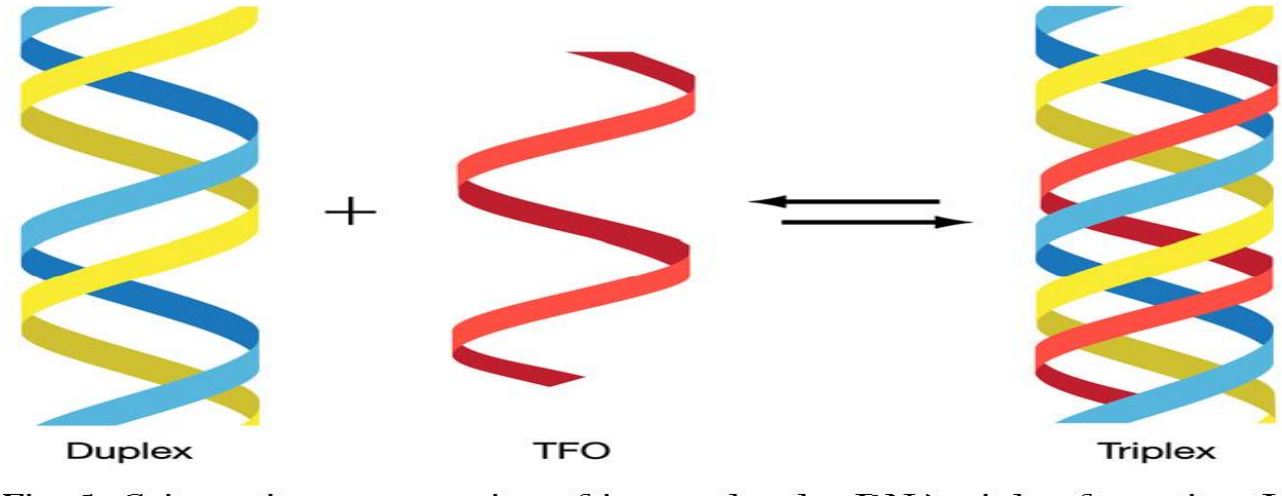
for this experiment. The base pairs within ds-DNA cause the chemical environment inside of the double helix to be highly hydrophobic. This hydrophobicity is due to the nucleobases of adenine, guanine, thymine, and cytosine containing large, planar, aromatic rings which contribute to hydrophobic affinity[37,17]. This hydrophobicity within ds-DNA contributes to the stability and overall shape of the double helix as mentioned previously. When ds-DNA is thrown into aqueous solution, the only part of that entire solution that is hydrophobic is located within the nucleobases of ds-DNA. Some intercalator dyes, such as the ethidium bromide used in this experiment, require a hydrophobic environment to be able to show fluorescent properties.[8] This requirement of hydrophobic environments is crucial to the application of this experiment and will be discussed further when deliberating upon the ethidium bromide theoretical information of this experiment. Before ethidium bromide can be discussed, triplex formation and triplex forming oligonucleotides must be elaborated upon.

Triplex DNA

As discussed previously, ds-DNA is in the shape of a double helix. Two backbone strands wrap around an axis that consists of nucleobases within the ds-DNA. The double helical shape of ds-DNA also allows for the formation of the major and minor grooves present within the ds-DNA. A major aspect of this experiment is the manipulation of the major groove to form a structure known as a triple helix DNA (triplex DNA). Triplex DNA is a form of DNA in which there are three strands in the form of a helix rather than the normal two stranded ds-DNA in a double helix. An image of a triplex DNA can be seen below.

Figure Three

Triple Helix Formation and Retention of Helical Shape[39]



-This figure shows how a third strand of TFO can bind to a double helix of ds-DNA. This figure is important in visualizing how the third strand attaches without prompting a conformational change.

Triplex DNA can be formed in two ways, both intermolecularly and intramolecularly. The intermolecularly formed triplexes are established through the introduction of triplex forming oligonucleotides (TFOs). Intermolecularly formed triplexes are established in the major groove through Watson-Crick base pairing, Hoogsteen base pairing, and reverse Hoogsteen base pairing[13,15]. These terms only reference the way in which the purine nucleobase pairs with the pyrimidine nucleobase and is dependent upon the type of TFO chosen for experimentation. In Watson-Crick base pairing the nucleobases are formed in the most energetically favorable way,

hence why Watson-Crick base pairing is the most common in nature. In Hoogsteen base pairing, the purine that is base pairing with the pyrimidine is rotated 180 degrees so that the hydrogen bonds formed are in a different configuration than in Watson-Crick base pairing. The same scenario occurs for reverse Hoogsteen base pairing, however this method of base pairing sees the adenine further rotated to make yet another configuration of the base pairs.[2,19,20] These methods of base pairing are crucial in the notation of triplet base pairs. Triplet base pairs refer to the three bases that will be bonded together once a triplex DNA is formed. Triplet base pairs function as the ladder rungs for triplex DNA, they are just longer rungs due to another base pair being added. An example of the notation for triplet base pairs is T*A:T. The asterisk is present to distinguish that one thymine is bonded in a Hoogsteen or reverse Hoogsteen manner. The distinction between the ways in which the nucleobases bond is important because the ways a nucleobase bonds will determine the stability of the triplex. This will be discussed further when determining the TFO probe that gives the best triplex stability.[2,17,21] It is also important to note that intramolecularly formed triplexes are established when the ds-DNA supercoils onto itself forming H-DNA.[3,20,15] For this experiment only the intermolecular triplex formation will be investigated.

For triplexes to be formed intermolecularly there are certain criteria that must be met for this phenomenon to be viable. First, the ds-DNA must have sequences of purines or pyrimidines available in the major groove for bonding.[3] Recent studies show that TFOs can also bind to other sequences of DNA base pairs as well, but this experiment assumes that purine or pyrimidine sequences are present within the target ds-DNA used for experimentation.[12,13] Another criterion that must be met relates to the guanine content of the ds-DNA target. For stable triplex

formation, at least 40-50% of the ds-DNA target sequence should be comprised of guanine nucleobases. Below this threshold, secondary structures begin to form within the ds-DNA and triplex formation is not feasible. There also cannot be more than four guanine nucleobases per stretch and no multiple repetitions of triple guanine nucleobase sequences. This parameter is set to avoid secondary structures from forming within the dsDNA. Another criterion to be met for stable triplex formation is the implementation of ionic concentrations. A thorough literature evaluation showed that one hundred millimolar sodium chloride and ten millimolar magnesium chloride have been seen assisting rapid triplex formation. It is important to note that triplex formation has been completed without magnesium ions, but magnesium ions are very useful in triplex formation and stability. [21] The criteria based upon triplex formation that has been discussed thus far relates to the target ds-DNA double helix composition, as well as the chemical environment present within the solution containing the target. There is one final aspect that influences triplex formation, that being the topic of choosing the correct TFO to form a triplex.

Choosing the Correct TFO Probe

Choosing the correct TFO probe for experimentation boils down to choosing what the experimenter needs to meet experimental parameters. There are three distinct motifs of TFO probes that are popular amongst DNA studies. It is worth mentioning that a motif is a distinctive pattern or sequence of nucleotides present within a strand of DNA or oligonucleotides.[18] The first motif popular amongst DNA studies is the TC motif, motif one. The TC motif contains strings of the thymine and cytosine oligopyrimidines. This motif creates C+*G:C and T*A:T

triplet base pairs, with the cytosine in the first triplet base pair requiring protonation hence the "+" sign. This motif is chosen if the experiment is to be taking place in acidic pH (to protonate the cytosine) and if the target ds-DNA contains stretches of mostly adenine and guanine oligopurines. This motif also allows for the third strand to sit parallel to the oligopurine sequence of the ds-DNA. The next popular motif in DNA studies is the GT motif, motif two. The GT motif contains strings of the guanine and thymine nucleobases. This motif creates the C:G*G and T:A*T triplet base pairs and the third strand sets either parallel or antiparallel to the oligopurine strand. This motif is chosen if the target strand of ds-DNA is difficult to characterize or if the ds-DNA target has a major groove that is difficult to determine compositionally. This motif is rarely chosen however due to the instability of the C:G*G triplet base pair. The final TFO motif used in DNA experimentation is the GA motif, motif three. This motif contains strings of guanine and adenine oligopurine sequences. This motif creates A*A:T, T*A:T, and G*G:C triplet base pairs. It is worth noting that the G*G:C base pair is very unstable and is unlikely to form, though it is possible to be formed. This motif also positions the third strand antiparallel to the target ds-DNA oligopurine sequence. This motif is chosen when the target ds-DNA contains oligopyrimidine sequences. This motif also does not contain a pH requirement, making this motif easier to utilize.[3,12,13,19,20] With all this information put into place, the choice of TFO motif depends heavily upon the sequence of the target ds-DNA. The target sequence of ds-DNA used in this experiment contains both oligopurine and oligopyrimidine sequences, so motif one or motif three could theoretically be chosen. Due to the experiment taking place at physiological pH, motif one is disqualified. This leaves motif three, the GA motif, to be the motif chosen for experimentation. The specific sequences for the target ds-DNA and the GA motif can be viewed in the methods

portion of this document. The final discussion to be had relating to the introduction information is the topic of ethidium bromide intercalation.

Ethidium Bromide Intercalator Dye

Ethidium bromide is a nonradioactive intercalator dye used widely for ds-DNA studies due to its fluorescent properties and planar configuration.[7] The planar configuration of ethidium bromide allows for ds-DNA intercalation of ethidium bromide upon introduction of ds-DNA. Ds-DNA intercalation can be defined as the ability for a small, planar, molecule to insert itself between the ds-DNA base pairs within the double helix. It must be noted that intercalation can cause structural changes within the ds-DNA and the backbone.[40] There is a plethora of intercalator dyes on the market, such as YOYO-1, SYBR Gold, and SYTOX Green.[41] Ethidium bromide was chosen for this experiment due the structural changes it imparts on ds-DNA being minimal, as well as its affinity to fluorescence when placed in a hydrophobic environment. The intercalation of ethidium bromide into ds-DNA has the consequences of the ds-DNA being no longer able to synthesize new nucleic acids in vivo, as well as the ethidium bromide inhibiting polymerase activity due to binding to the ds-DNA template molecule.[6,44] Neither of these parameters are studied in this experiment, furthering the reason for choosing ethidium bromide as the intercalator dye. Ethidium bromide is also classified as a nonspecific intercalator dye at physiological pH, which means the ethidium bromide could intercalate the ds-DNA in either the major or minor groove, there is not distinct path it will follow.[42] It has also been proven that at ethidium bromide could bind to the backbone of ds-DNA through electrostatic interactions if the

solution is held at low pH.[44] Again, this strengthens the idea of using ethidium bromide as the intercalating agent, as the major groove is the topic of study for the experiment. Since intercalation has been discussed for ethidium bromide, the fluorescent properties of ethidium bromide need to be addressed.

Ethidium bromide is a fluorescent intercalator dye, meaning it has excitation and emission properties when placed in the right environment. Ethidium bromide is quenched in aqueous solution, meaning there is a minimal fluorescent signal present. When ethidium bromide is placed in a hydrophobic environment however, the dye begins to fluoresce and give off a quantifiable emission signal. The hypothesized reasoning behind why ethidium bromide is quenched in aqueous solution is due to the proton transfer from the ethidium bromide to the proton acceptors found in aqueous solutions. The hypothesized reasoning behind why ethidium bromide fluoresces when in a hydrophobic environment is attributed to a reduction in the rate of excited state proton transfer to solvent molecules when in a hydrophobic environment, as solvent molecules are not present in this environment.[43] Ethidium bromide can be excited by light of a wavelength between 460-480nm, with the optimum excitation wavelength being 469.5nm. This wavelength can be found doing an excitation scan of ethidium bromide in aqueous solution. Ethidium bromide has a measurable emission wavelength of 605nm, as proven by the literature.[43] With these parameters in mind, along with the parameters discussed throughout this entire section, the hypothesis can be drawn back to and understood fully.

If ethidium bromide is an intercalator dye and exhibits literature sourced characteristics, then it should intercalate into the major and minor grooves of the target ds-DNA. This will cause the ds-DNA to give a quantifiable emission output to the fluorimeter. This quantifiable emission

output will prove to the experimenter that the ethidium bromide is present either in the major groove or minor groove of the ds-DNA. Since it is known that TFOs bind specifically in the major groove of ds-DNA, the emission signal given off by the ethidium bromide should decrease by a large degree upon triplex formation and addition of the TFO. The emission signal should decrease due to the large amount of ethidium bromide that will be displaced from the major groove of the ds-DNA upon introduction of the TFO. The displaced ethidium bromide would return to the aqueous solution and be quenched, henceforth decreasing the emission signal. The purpose of this experiment will be to investigate how the concentration of the TFO probe effects the displacement of the ethidium bromide and the subsequent decrease in emission intensity upon displacement. This mechanism should maintain proportionality, meaning as the TFO concentration increases, the emission signal should decrease proportionally upon introduction of the TFO. TFO should follow Beer's Law of concentration proportionality with emission signal, hence the concentration study present within this experiment.

Methods

Reagents

Before experimental procedures can be discussed, the reagents used within this laboratory need to be identified. The first reagent to be discussed is ethidium bromide. The lot number of this reagent was MKCC7786 and no expiration date was given by the manufacturer, Sigma Life Science. The ethidium bromide used in this experiment was diluted to 200mM and

was used as the intercalating dye in the experiment. The next reagent to be discussed is the target ds-DNA. The lot number of this reagent was found to be 370717920 and the expiration date was given as January 26th, 2021. The manufacturer of this ds-DNA was found to be DNA-ID. This ds-DNA was diluted to 200µM and pre-annealed in 10mM HEPES buffer. This ds-DNA was used as the target strand for the intercalating dye and TFO strand. The sequence of the ds-DNA target was 5'-GGC GGC GGA AGA GGC GG-3' and 5'-CCG CCG CCT CTT CCG CGC CC-3'. The next reagent to be discussed is the triplex forming oligonucleotide probe. The GA-Motif was chosen for this experiment due to the lack of triplex formation criteria. The GA-motif only needs physiological pH and oligopurine and oligopyrimidine sequences to form triplexes, both of which the target ds-DNA chosen for this experiment contains. The lot number for this reagent was 365932237 and the expiration date was January 26th, 2021. The manufacturer of this TFO probe was DNA-IDT. This TFO probe was diluted to 200µM and was used as the displacement agent for the ethidium bromide. The next reagent to be discussed is the HEPES buffering agent. The lot number for this reagent was 17257-2500 and there was no expiration date given by the manufacturer, Acros Organics. The HEPES used in this experiment was diluted to 10mM and was used as a buffering agent. The next reagent to be discussed is the sodium chloride buffering agent. The lot number for this reagent was 088K0094 and no expiration date was given by the manufacturer, Sigma-Aldrich. The sodium chloride used in this experiment was diluted to 150mM and was used as a buffering agent. The next reagent to be discussed is the magnesium chloride binding enhancer. The lot number for this reagent was 1406032 and there was no expiration date given by the manufacturer, Sigma-Aldrich. The magnesium chloride used in this experiment was diluted to 10mM and was used as a ds-DNA binding enhancement motif as well

as a buffering agent. The final reagent to be discussed is the bleach cleaning agent. The bleach cleaning agent was manufactured by Clorox and contained 8.75% sodium hypochlorite. There was no expiration date on this reagent. The bleach used in this experiment was diluted to 0.1M and was used to clean the cuvettes after each run on the fluorimeter. Now that reagents have been discussed, the procedures carried out in this experiment can be elaborated upon.

Preparation of Buffer

The first procedure to be introduced in this experiment is the preparation of the buffer solution. The buffer solutions needs to be at a pH of 7.2-7.4 and needs to contain a HEPES concentration of 10mM, a NaCl concentration of 150mM, and a $MgCl_2$ concentration of 10mM. Calculations were ran to obtain the volumes needed to prepare this buffer solution based upon a final volume of 500mL of buffer. This volume of buffer lasted this entire experiment, as each cuvette to be investigated contained about 2.75mL of buffer on average. The calculations showed that with stock solutions of 1M for each reagent, the solution would need 75mL of NaCl, 5mL of HEPES, and 5mL of $MgCl_2$.The rest of the solution was diluted with deionized water for a volume of 415mL. Since this experiment takes place with ds-DNA, it is crucial that the pH of the buffer is in the 7.2-7.4 range. This was investigated with both pH paper and a pH meter to ensure the physiological conditions remained before each experiment was run.

Control Experiment One: Signal Increase Upon Addition of ds-DNA

The next procedure to be introduced is the signal increase upon addition of ds-DNA experiment, which is a two-part procedure. In this experiment, 2.985mL of buffer solution was added to a cleaned cuvette. Next, 15 µL of 200mM ethidium bromide was added to the cuvette. The cuvette was then placed in a dark environment at room temperature for five minutes. After the five minutes had passed, the cuvette was placed into the instrument and an excitation scan was taken. The emission setting was to be set at 605nm as that is the known wavelength that ethidium bromide emits light. The excitation scan gives the premium excitation wavelength for the sample in the cuvette. Once the excitation wavelength was determined from this scan, an emission scan was ran with the determined excitation wavelength. This data was then saved to a file and the next ethidium bromide sample was ran. A minimum of three samples are to be run.

Control Experiment One: Signal Increase Upon Addition of ds-DNA Part Two

The next procedure to discuss is the second part of the previous control experiment. This control experiment tests the increase in signal upon adding ds-DNA to the ethidium bromide intercalator dye solution. In this experiment, 15µL of 200mM ethidium bromide and 15µL of ds-DNA target were pipetted into a cuvette containing 2.970mL of buffer solution. The sample was then placed into a dark area at room temperature for five minutes and then ran using the fluorimeter. The excitation scan was conducted first, with the emission wavelength being set at 605nm. Once the excitation wavelength was determined from this scan,

an emission scan was ran with the determined excitation wavelength. This data was saved to a file and the next sample was ran. A minimum of three samples should be run.

Control Experiment Two: Signal Decrease Upon Addition of TFO

The final control experiment to discuss is the control TFO experiment, a two-part process. The first part of this experiment consisted of adding 15µL of 200mM ethidium bromide and 15µL of ds-DNA target into a cuvette containing 2.955mL of buffer solution. This solution was placed within a dark space and incubated at room temperature for five minutes. The excitation scan was then conducted, with the emission wavelength being set at 605nm. Once the excitation wavelength was determined from this scan, an emission scan was ran with the determined excitation wavelength. This data was saved to a file and the sample was removed from the instrument. This data file is known as the emission before displacement. The second part of this process consisted of adding 15µL of 200µM GA-Motif TFO into the same cuvette that was previously measured. This new solution was incubated at room temperature in the dark for ten minutes. The excitation scan was then conducted, with the emission wavelength being set at 605nm. Once the excitation wavelength was determined from this scan, an emission scan was ran with the determined excitation wavelength. This data was then saved to a file, with this file being known as the emission after displacement.

Concentration Variation of TFO Experiment

The final experimental procedure to discuss revolves around the concentration study of TFO and involves two parts. In the first part of this experiment, 15µL of 200mM ethidium bromide and 15µL of ds-DNA target should be pipetted into a cuvette containing 2.984mL of buffer. This solution was stored in a dark place for five minutes and incubated at room temperature. After five minutes, the excitation scan was conducted, with the emission wavelength being set at 605nm. Once the excitation wavelength was determined from this scan, an emission scan was ran with the determined excitation wavelength. This data file is known as the emission before displacement of ethidium bromide. The second part of this experiment involves pipetting one microliter of GA-Motif TFO into the solution prepared in the first part of this experiment. This solution was then incubated for ten minutes in a dark place at room temperature. The excitation scan was then conducted, with the emission wavelength being set at 605nm. Once the excitation wavelength was determined from this scan, an emission scan was ran with the determined excitation wavelength. This data file is known as the emission after displacement of ethidium bromide. This experiment is to be conducted for ten different concentrations of GA-Motif TFO, with this example containing the preparation methods for 0.07µM TFO.

Cuvette Cleaning Procedure

The final procedure to discuss in the methods section is that of the cuvette cleaning procedure. Since ds-DNA was used in this experiment, bleach was able to be used as a cleaning agent since it destroys organic matter effectively. The cleaning solution was prepared in measurements of 50mL bleach and 450mL deionized water. The cuvettes were placed in the bleach solution for thirty seconds and then rinsed ten times with distilled water and ten times with deionized water.

Instrumentation

The experimental method of fluorescence spectroscopy is the cornerstone of data collection present within this experiment. Fluorescence spectroscopy is the method of analyzing the chemical phenomenon of luminescence.[32] Luminescence can be defined as the emission of light that does not derive energy from the temperature of the emitting body. Luminescence can be caused by many factors including chemical, biochemical, or crystallographic changes, as well as the motions of subatomic particles, or radiation induced excitation of an electronic system. Luminescence is an umbrella term that could pertain to phosphorescence, fluorescence, and bioluminescence.[32] For the sake of this document, only fluorescence will be described, as it is the predominant phenomenon being investigated by this experiment. The factors causing

luminescence in this experiment will pertain to chemical changes as well as radiation induced by an electronic system, as this experiment investigates such phenomenon.

Fluorescence Phenomenon and Fluorophores

Within this experiment, fluorescence spectroscopy is used to investigate the property of fluorescence exhibited by the implementation of an intercalation dye into ds-DNA. Fluorescence can be defined as a molecular phenomenon in which a substance radiates light almost immediately upon being excited with light from an outside source.[32,49] The process of a molecule absorbing energy from a light source and transcending to a higher energy state is called absorption. The relaxation of this excited energy state back to a lower energy state gives off a measurable amount of energy which can be quantified. This relaxation process and the quantifiable measurement obtained from it is known as emission.[32] When a molecule absorbs infrared, ultraviolet, or visible light, the emission given off upon relaxation is known as photoluminescence and is the backbone of all emission data gathered with this practice. Within this experiment the emission measurement is crucial in the data gathering procedure.

Molecules that exhibit fluorescence behavior are known as fluorophores and are very diverse in the way they exhibit their fluorescence properties.[32,49] Fluorophores absorb energy from a distinct excitation wavelength chosen by the experimenter. For most fluorophores, this distinct wavelength is typically in the ultraviolet to visible color range. The absorbed light will then be emitted through relaxation of the fluorophore at a lower energy and higher wavelength value.[24] Since the emitted wavelength of light is longer than the excitation wavelength of light

the emission is known to be red shifted with respect to the excitation wavelength.[25] These two statements are true of nearly all fluorophores, but there are different mechanisms by which this absorption and emission can occur.

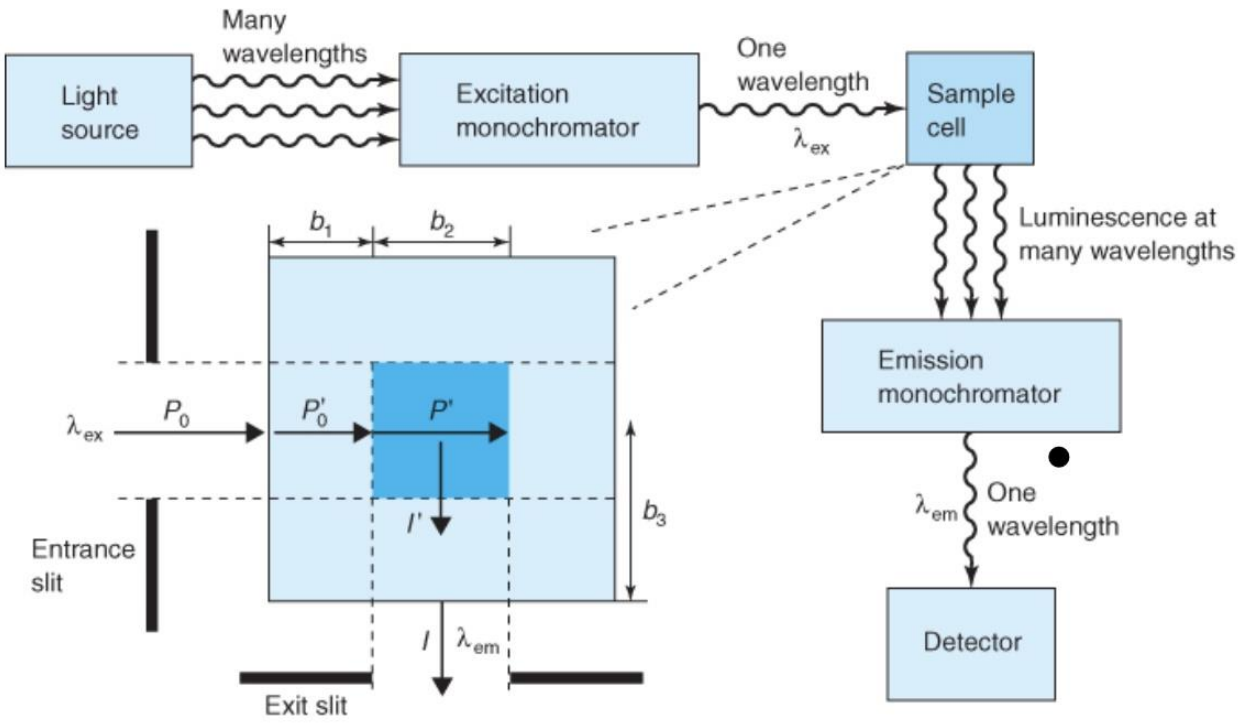
Some fluorophores, such as Green Fluorescent Protein, require an excitative light source at a specific wavelength to show its fluorescent properties. Green Fluorescent Protein absorbs ultraviolet light, transcends to a higher energy state, relaxes from that energy state, and then emits green light, which is lower in energy but higher in wavelength value than ultraviolet.[34] Other fluorophores, such as the ethidium bromide used in this experiment, can give off a quantifiable light emission by changing the chemical environment it is placed within.[44] The main ideas that should be focused upon in this section are the ideas of fluorescence, absorbance, emission, and excitation wavelengths, as these are the main ideas behind the instrumentation of a fluorimeter.

Fluorimeter Classification

The experimental method of fluorimetry uses an instrument known as a fluorimeter. Fluorimeters take advantage of the principles stated above, with emission being the primary measurement of interest when using this instrumentation.[25] The fluorimeter used within this experiment is the Shimadzu RF-6000 Spectro Fluorimeter. To discuss the process of taking an emission reading using a fluorimeter, the components of a fluorimeter must be discussed. Only the components found within the instrument used in this experiment will be discussed. A schematic of a typical fluorimeter can be seen below.

Figure Four

Schematic Diagram of Internal Fluorimeter Components[49]



-This figure shows the working mechanisms found within a fluorimeter. Each component of the fluorimeter is discussed below in detail, but the main components are the light source, monochromators, sample cell, and detector. The leftmost diagram shows the entrance of light into a monochromator.

Light Source

The first point of interest to discuss within this diagram is the light source. There are three possible light sources that fluorimeters utilize. First is LED lights. These light sources

are widely used to avoid photobleaching as the intensity of the light can be controlled electronically. The instrument used within this experiment does not use this form of light source due to the LEDs not being useful when investigating low wavelengths of light as well as the high upfront cost of an LED system. The next form of light source used by fluorimeters is Tungsten-Halogen lights. These light sources are used primarily for live cell imaging and because of their inexpensive nature. The instrument used within this experiment does not use this form of light source due to the intensity being too low to excite weak fluorophores. The final light source used by fluorimeters and the light source used in this experiment is a Xenon arc lamp. This light source was chosen to be used due to its ability to give high intensity peaks at wavelengths that commonly excite fluorophores and for its ability to see weak fluorescence signals. The downside to using this lamp is that it needs a special housing as it produces a large amount of heat.[49] The Xenon arc lamp is a crucial component of the instrument used within this report, however, the diffraction gratings within the monochromator that follow its path need to be discussed as well.

Monochromators

The second point of interest to discuss on the diagram is the topic of monochromators. A monochromator is defined as a device that is used to separate different wavelengths of light from the overall light spectrum.[26,27,49] Most fluorimeters have two distinct monochromators, an excitation monochromator and an emission monochromator. In the practice of fluorescence spectroscopy monochromators are used to determine the wavelength of light that will excite the sample and the wavelength of light that will reach the detector upon emission,

hence the need for two separate monochromators within the fluorimeter. Monochromators implement the usage of diffraction gratings to be able to split the light into desirable wavelengths.[26,27] Diffraction gratings are involved in complex calculations and formulas used to determine how they function, but the best way to think of diffraction gratings is to think of them like a comb. A large light, a xenon lamp in the case of this experiment, is shone behind the comb and the comb allows for certain light to pass through, namely where the gaps are located in between the comb teeth. On a fluorimeter, the gaps between the diffraction gratings, or comb teeth, are set to give the instrument operator complete control over what wavelength of light is allowed to hit the sample.[49] These gaps between the comb teeth are a perfect analogy for the gaps in a diffraction grating, however a diffraction grating's gaps are nanometers apart, whereas a comb's gaps are nearly an eighth of an inch apart.

Monochromators are needed to combat the large issue of stray light reaching both the sample and detector. Stray light can be defined as light outside of the parameters of the chosen wavelength that slip through the diffraction grating, light that is outside of the instrument, or light from another outside source. Monochromators are never perfect, but the instrument used in this experiment has been recently calibrated to combat the issue of stray light. Monochromators also aid in selecting the right wavelengths to excite a sample. Some molecules, such as Rhodamine 6G, have a specific wavelength of light that they absorb the best, 525nm for the compound in question. This wavelength of light allows for the molecule to become as excited as chemically possible. Without an excitation monochromator, this wavelength would never be able to be selected and the compound would not be able to live up to its energetic potential. Once the molecule is fully excited it will emit light. The light emitted is also at a very specific wavelength,

548nm for Rhodamine 6G. The emission monochromator is then employed to take an intensity reading from the sample but only at the 548nm wavelength.[28] If the experimenter were to choose a different wavelength, the detector would not pick up on the same intensity as the 548nm wavelength would give. Monochromators are incredibly important and sensitive parts of the fluorimeter and allow the experimenter to choose the parameters needed for investigation.

Sample Cell

The next portion of the diagram to be discussed is the sample cell. The types of samples placed within fluorimeters are liquid solutions held by a cuvette. The type of cuvette used during experimentation can vary based on the type of substance being analyzed. The most common form of cuvette that is used in fluorescence spectroscopy is made of quartz.[30] Quartz cuvettes are the type of cuvette used in this experiment as well. The cuvettes used in this experiment are made by Thorlabs and have a volume of three thousand and five hundred microliters. The cuvettes are classified as macro-fluorescence measuring equipment and four cuvettes were purchased to maintain measurement integrity upon each cuvette.[29] Upon filling the quartz cuvette with the substance to be analyzed, it is imperative that the cuvette be cleaned of any fingerprints or dust particles. Upon running the apparatus with a cuvette in the sample cell, the excitation wavelength from the first monochromator can enter the cell, excite the sample, and then the emission wavelength is measured by the detector after passing through the second monochromator. The detector is the final portion of the diagram to be discussed.

Detectors

Fluorimeters use incredibly sensitive detectors to give accurate emission spectra to the researcher. The detector used during this experiment was sold with the apparatus as a whole and is a Photomultiplier Detection System.[31] The mechanism of operation utilized by a Photomultiplier Detection System is outside of the range of this experiment, however they have been proven to give incredibly precise and accurate readings for other experiments apparent within the literature. The emission wavelength hits the detector and specific photon intensity is given back to the experimenter.[31] For more information regarding detectors and the methods in which the function by, further reading should be done on the Photomultiplier and Detection Systems patent. This is reference number 31 in the references section.

Results and Discussion

The results of this experiment will be portrayed in three separate parts. The first results to be discussed are that of the control experiment in which the increase in emission signal is investigated when ethidium bromide contacts ds-DNA. The next results that will be discussed are that of the second control experiment, TFO displacement of ethidium bromide intercalated into ds-DNA with set TFO concentration. The final results to be discussed are the results pertaining to the independent variable experiment, in which the TFO concentration is varied and the decrease in emission signal is investigated. Each section of results will have an

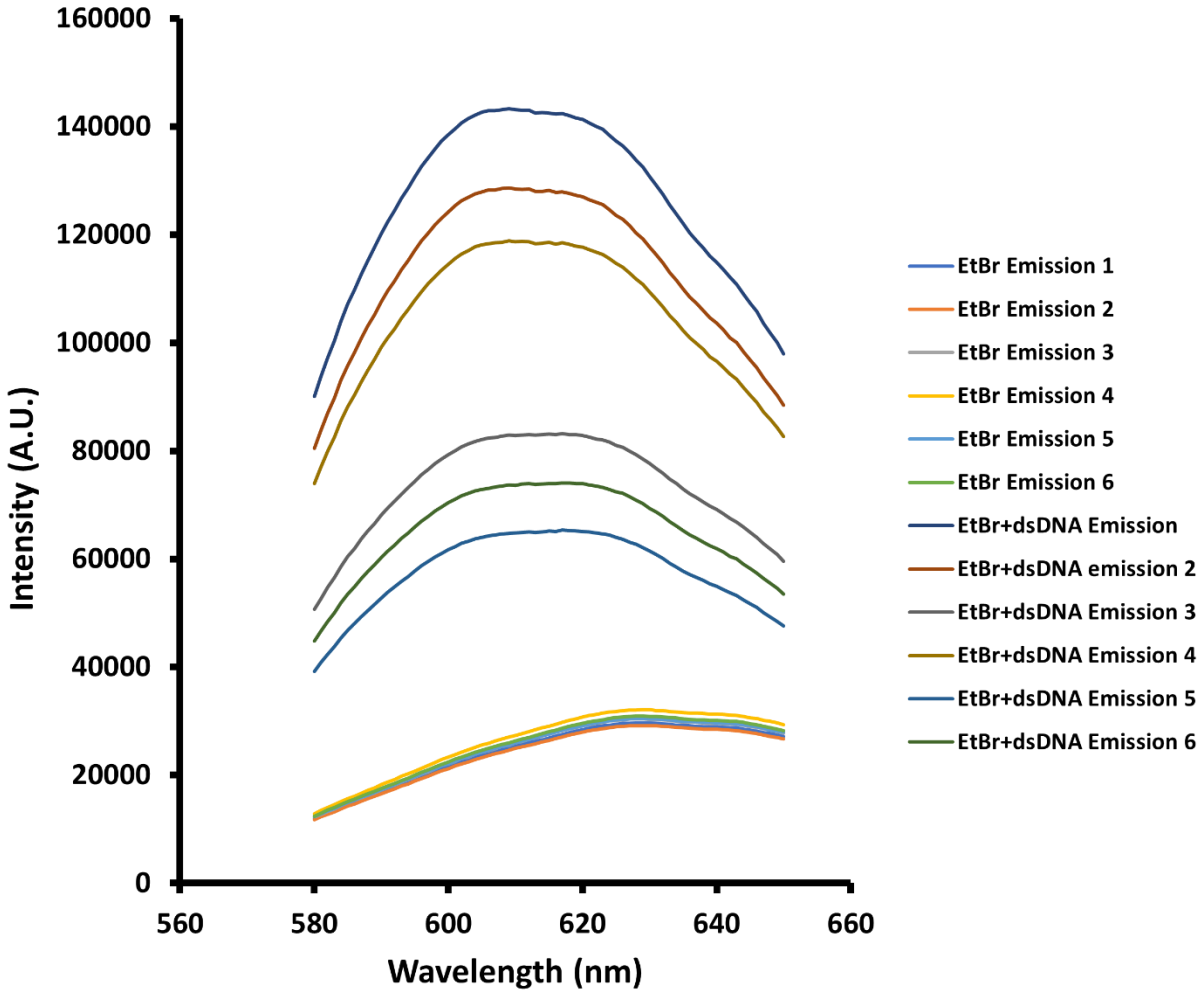
accompanying table or image to portray the data in a visual manner. First, the results of the control experiment of ethidium bromide contacting ds-DNA will be discussed.

Control Experiment One Results: Signal Increase Upon Addition of ds-DNA

The first results to be studied are that from the ethidium bromide and ds-DNA control experiment. In this experiment, ethidium bromide was pipetted into a buffer and an excitation and emission scan were ran. Next, ds-DNA was introduced to the aqueous solution and another excitation and emission scan was run. The difference in the emission signal was investigated. It was hypothesized that the emission signal would increase upon introduction of ds-DNA, as ethidium bromide would intercalate into the hydrophobic environment of the ds-DNA and be unquenched. This was seen to happen during this experiment and can be viewed in figure four. Even though the introduction of ds-DNA to ethidium bromide followed the hypothesis correctly, the accuracy of the measurements are questionable at best. This can be determined by viewing figure four. Figure four shows the ethidium bromide in free solution emission as the cluster of lines at the bottom of the figure. The separated lines above are the emission readings after ds-DNA is added. These lines should also be in a cluster, like the ethidium bromide in free solution emission readings. The spacing of these lines shows that there is significant standard deviation amongst the readings. The figure gives valuable information to experimenters; however the numerical values and a value-based bar graph are also to be discussed to draw conclusions.

Figure Five

Ethidium bromide and ds-DNA emission comparison



-This figure shows the emission intensity change upon adding ds-DNA to ethidium bromide. The bottom cluster of lines are the emission data for 15µL of 200mM ethidium bromide in 2.70 mL of a 150mM NaCl, 10mM $MgCl_2$, 10mM HEPES buffer. The clusters of data near the top of the figure show the emission signal when 15µL of 200µM ds-DNA is added to the previously

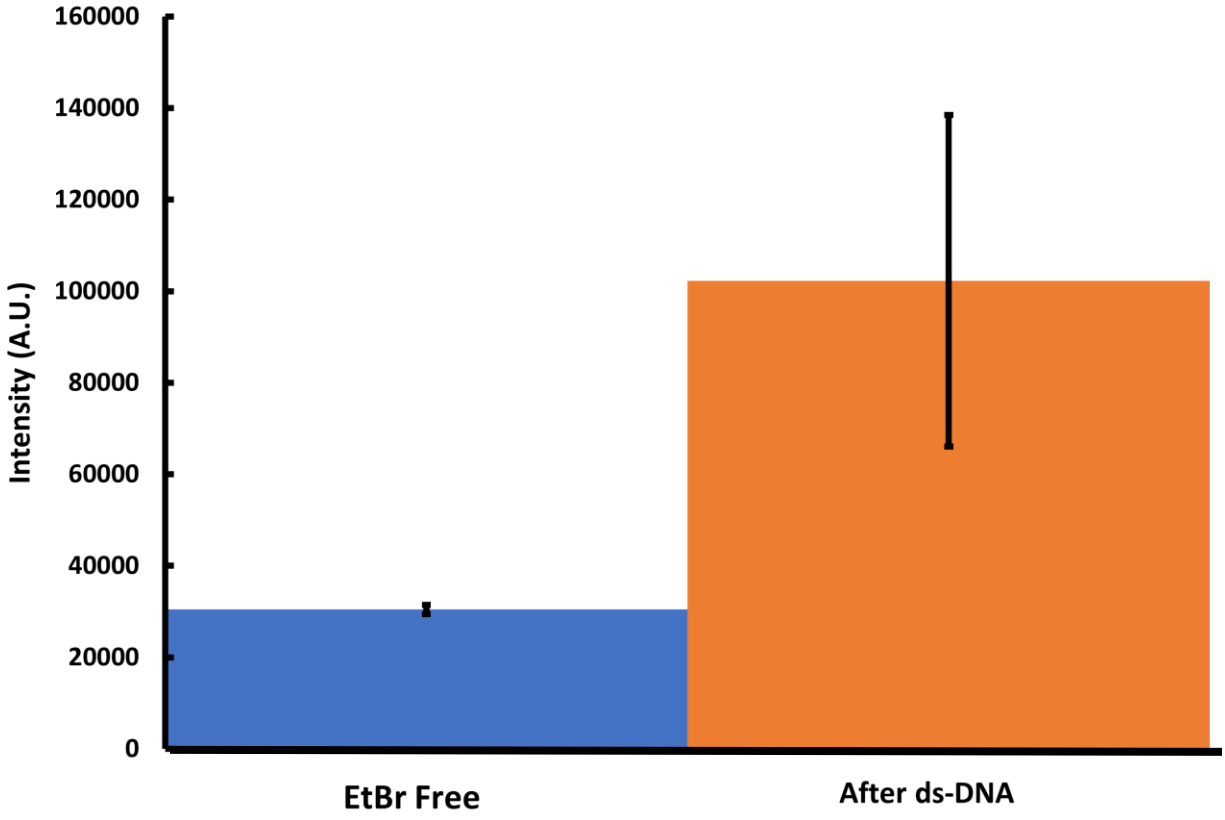
mentioned solution. The large change in intensity is a great discovery, as it proves that the ethidium bromide is intercalating the ds-DNA.

The success of the first control experiment cannot be validated until viewing the numerical figures for the experiment, as well as viewing the error bars on the value-based bar graph. It must be noted that each experiment contains a figure with error bars and numerical measurements with standard deviations. The error bars were created using the standard deviation and not by using the 95% confidence interval. The 95% confidence interval would require the error bars be the standard deviation multiplied by two, and since the error bars are already incredibly large the 95% confidence interval cannot apply. Table one can be seen showing the numerical values for the average relative intensity change and the average numerical intensity change. Both values also include the standard deviations of such measurements. From this table it can be concluded that the data obtained is not accurate, but conclusions may still be able to be drawn depending upon error bar analysis. Figure five shows a value-based bar graph showing the average intensities for both the ethidium bromide in free solution as well as when ds-DNA is introduced. The most important aspect of this bar-graph is the error bars. The error bars on this figure do not overlap, which means that most of the data collected between both runs are significantly different. This parameter of error bars typically signifies that the data is conclusive and safe conclusions can be drawn. The validation of conclusive results from the error bar analysis is contradictory to the standard deviations given by each measurement. With both factors in mind, this data is inconclusive regarding accuracy. However, the increase in signal upon the introduction of dsDNA in general has been proven to be true. Even though the accuracy

of this experiment is lacking, the principles of intercalation and hydrophobicity of the ds-DNA is correct according to the hypothesis.

Figure Six

Ethidium bromide free versus with dsDNA



-This figure shows the average maximum emission intensity for 15µL of ethidium bromide in 2.70mL of 150mM NaCl, 10mM $MgCl_2$, 10mM HEPES buffer solution. This figure also shows the average maximum intensity for the same solution with 15µL of 200µM ds-DNA added to it. as well as ethidium bromide after adding ds-DNA. The error bars on this figure show that the

data within the two bars are significantly different. This allows for conclusions to be drawn if the standard deviations of the measurements are low.

Table One:

Numerical values for changes in intensity

Average Relative Change in Intensity	Average Numerical Change in Intensity
237 ± 111 %	58,300 ± 36,200 A.U.

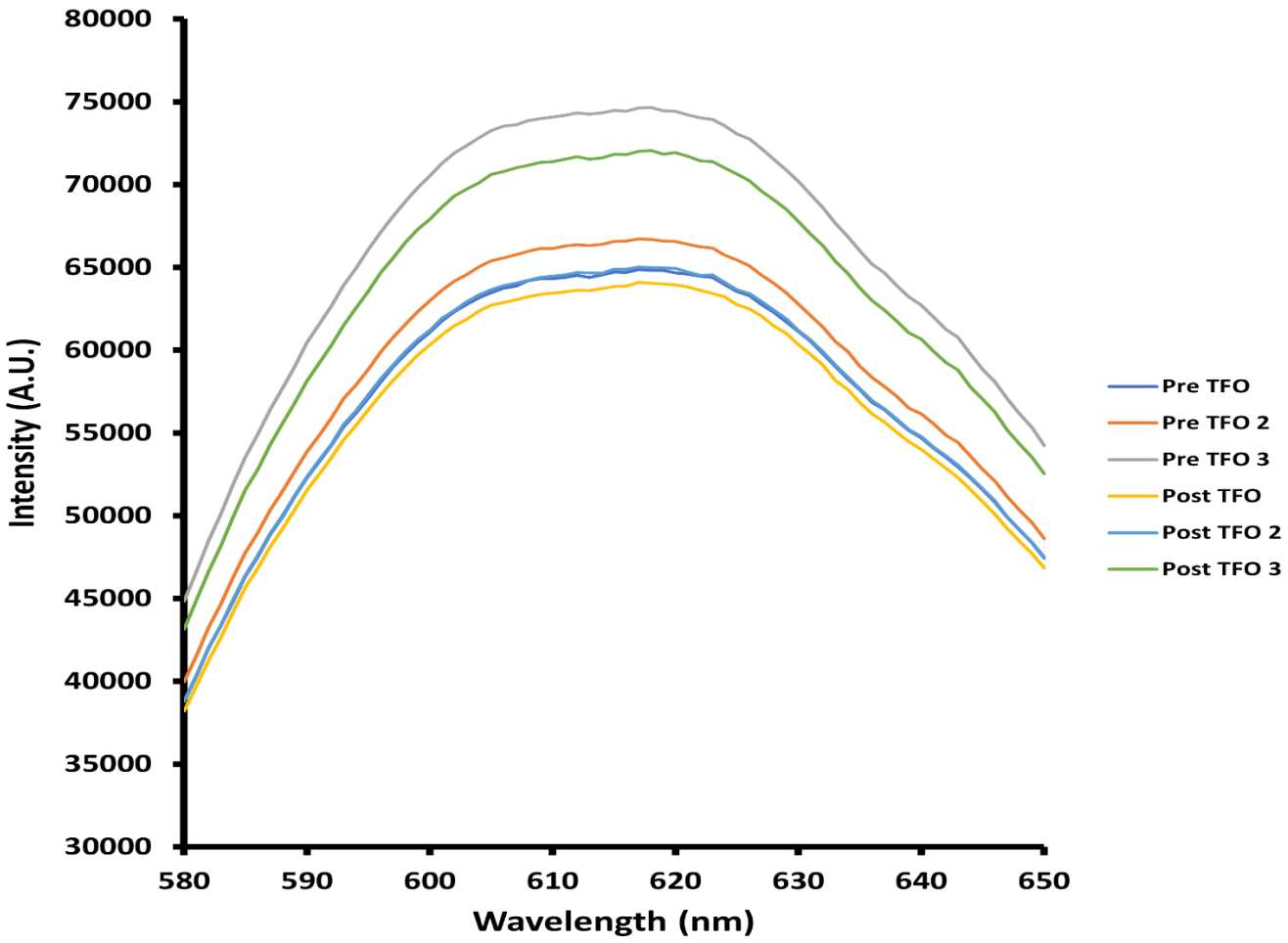
Control Experiment Two Results: Signal Decrease Upon Addition of TFO

The second results to be studied pertain to the TFO displacement control experiment. This experiment is like the first experiment discussed, as emission and excitation data are taken for an ethidium bromide and dsDNA sample in an aqueous cuvette. After these measurements have been taken, the GA-Motif TFO probe is introduced to the cuvette and the emission and excitation scans are taken again. The difference in emission scans are then analyzed to determine if displacement occurs. Displacement can be proven to occur if the emission signal decreases after adding TFO. It was hypothesized that upon addition of GA-motif TFO the emission signal of the solution would decrease. This hypothesis exists because the ethidium bromide would be displaced in the major groove by the TFO, expelled into a quenching environment, and the signal would decrease overall. This phenomenon was seen to occur and can be viewed in figure six. Figure six shows this phenomenon precisely, as the lines on the figure show the samples and how they reacted to the addition of TFO. Every sample had a quantifiable

decrease in signal. While there is a significant decrease in signal upon the addition of the GA-Motif TFO, the lines on figure six have a large spread and therefore shows that there is a large standard deviation within the data. To analyze the data with more accuracy the numerical values for the change in emission signal, as well as the relative change in emission signal for the change in emission signals need to be investigated.

Figure Seven:

TFO displacement control experiment



-This figure is an exhibit of the TFO control experiment. The lines labeled “pre-TFO” are emission data for a solution of 15µL of 200µM ds-DNA, 15µL of 200mM ethidium bromide, and 2.55mL of 10Mm HEPES, 10mM $MgCl_2$ and 150mM NaCl buffer solution. The lines labeled “post TFO” are emission data for the same solution after 15µL of 200µM Ga-Motif TFO has been added. Each time TFO was added a decrease in emission signal was seen.

The success of the second control experiment cannot be validated until the numerical figures of the emission intensities and the error bars on the value-related bar graphs are analyzed. Table two shows the average relative change in intensity as well as the average numerical change in intensity. Both measurements also contain standard deviations. This table shows that the average relative intensity has a low standard deviation, as well as the average numerical change in intensity. Since both measurements have low standard deviations it would be easy to assume that this data is accurate, however the error bars for the value-based bar graphs also need to be analyzed. Figure seven shows the value-based bar graph, as well as the error bars associated with the measurements. Figure seven shows that the error bars for both measurements overlap severely. When error bars overlap at a level of this magnitude, it is safe to conclude that these measurements are inaccurate and that the data obtained is not significantly different. In other words, the dependent and independent variable share too many of the same measurements for accurate conclusions to be drawn. The takeaway from this control experiment is that the GA-Motif of TFO does cause a signal decrease, however the measurements obtained are too inaccurate to give a quantifiable numerical emission decrease upon introduction of TFO.

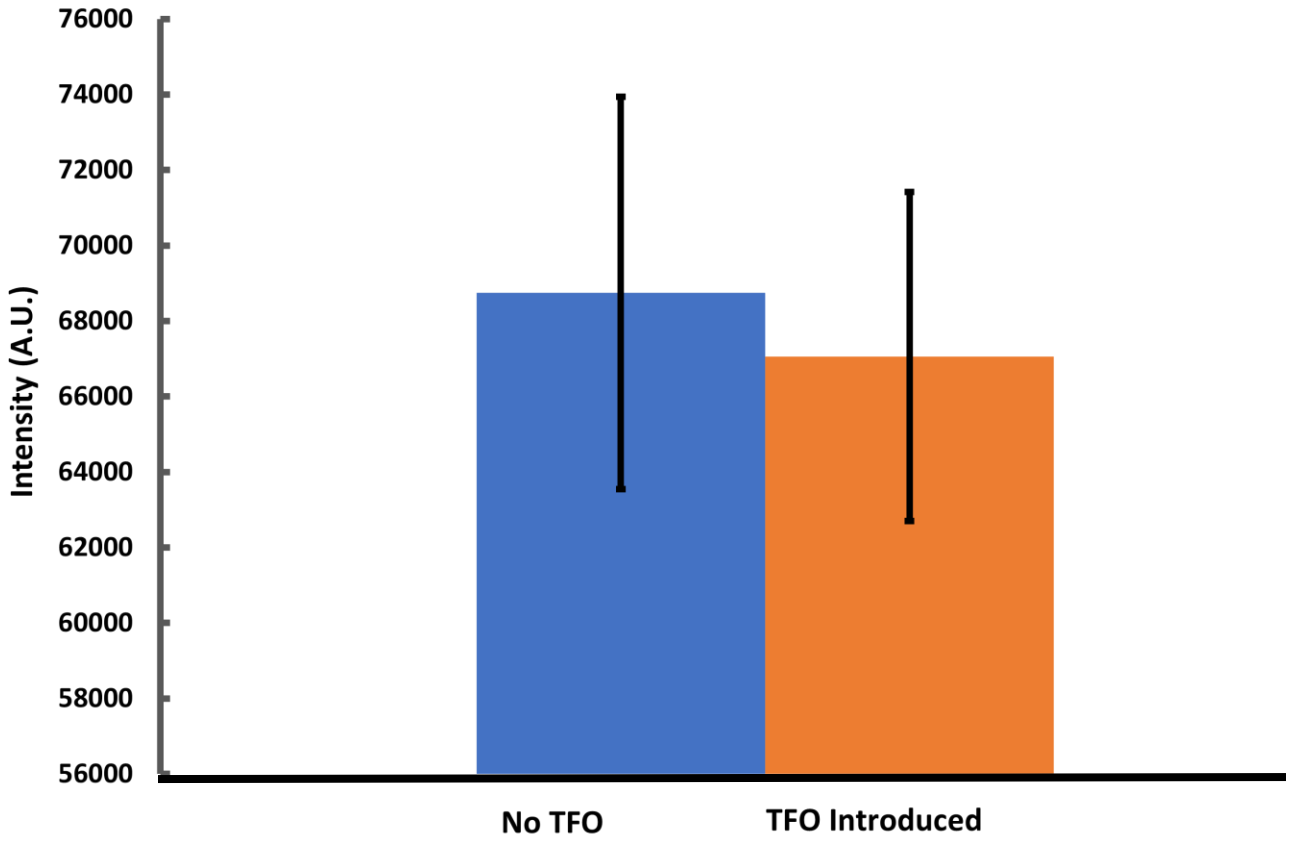
Table Two:

Numerical values for TFO control experiment

Average Relative Change in Intensity	Average Numerical Change in Intensity
2.40 ± 1.14 % Emission Decrease	1,680 ± 905 A.U. Decrease

Figure Eight:

Value-based bar graph on TFO control experiment



-This figure is an exhibit of the TFO control experiment. No TFO indicates that the sample consists of 15µL of 200µM ds-DNA, 15µL of 200mM ethidium bromide, and 2.55mL of 10Mm HEPES, 10mM $MgCl_2$ and 150mM NaCl buffer solution. TFO introduced indicates that this is the same sample with 15µL of 200µM Ga-Motif TFO that has been added. Each bar is the average maximum emission intensity for each of the runs for the control experiment. Since the error bars overlap, it can be concluded that this data is not significantly different and no concrete conclusions can be made.

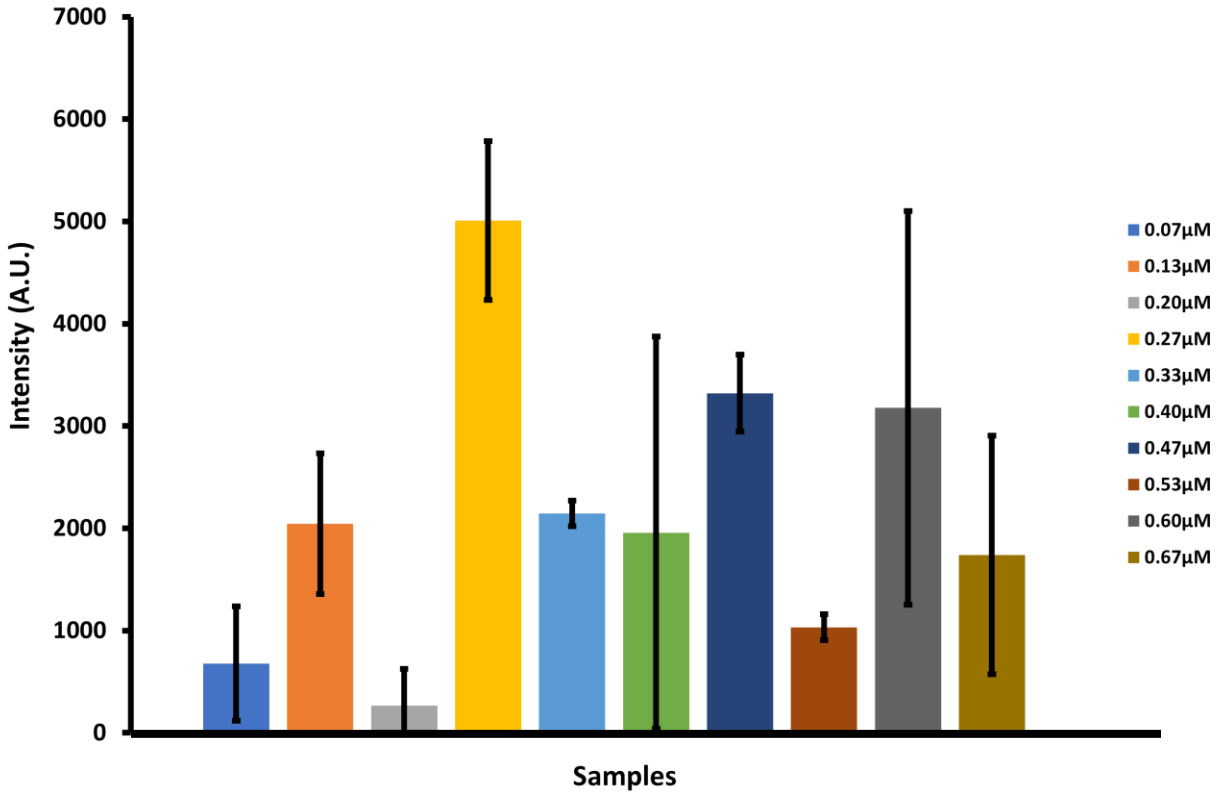
Concentration Variation of TFO Experiment

The final results to be investigated pertain to the concentration variance of GA-Motif TFO experiment. In this experiment, a sample containing ethidium bromide and ds-DNA weas ran in the fluorimeter and the excitation and emission spectra was obtained. Upon obtaining this spectra, a concentration of GA-Motif TFO was placed within the aqueous solution and the excitation and emission spectra are taken once again. This process was repeated ten times with ten different concentrations of GA-Motif TFO ranging from 0.07µM to 0.67µM. The hypothesis regarding this experiment is that as the concentration of GA-Motif TFO increases, the emission signal after adding the TFO will be less than that of the initial solution and respective initial signal. The hypothesis also states that the signal decrease will be proportional to the amount of TFO used and that the TFO should follow Beer's Law of absorbance and concentration proportionality. The experiment was conducted on ten different concentrations and figure eight was obtained. Figure eight shows the signal decrease in intensity for each of the concentrations

studied. This figure is the primary form of data to be reported in this document, as the table of average relative change in intensity and average numerical change in intensity would be too cumbersome to analyze. Figure eight shows that upon the addition of GA-Motif TFO the emission signal decreased. This is the only conclusion that can be drawn from this data, as each point of data has an error bar that overlaps with at least one other error bar from another data point. This means that each data point is not significantly different from at least one other data point in the set and accurate conclusions cannot be drawn regarding this data. It may be concluded however that GA-Motif TFO does not exhibit behavior that pertains to Beer's Law. If this were the case, figure eight would look like a staircase. Though this experiment was as inconclusive as the rest of the control experiments, it proves promising that GA-Motif TFO does cause displacement of ethidium bromide, however this displacement cannot be quantified numerically, and it has been concluded that the displacement does not follow Beer's Law on a very small concentration scale.

Figure Nine:

Concentration study of signal decrease upon TFO addition



-This figure shows the concentration study regarding TFO concentration. The ten concentrations used can be seen on the right side of the figure. The figure shows the average signal decrease when the differing concentrations of TFO were added to a 15µL of 200µM ds-DNA, 15µL of 200mM ethidium bromide, and 2.55mL of 10Mm HEPES, 10mM $MgCl_2$ and 150mM NaCl buffer solution. The results seen from this figure are negligible due to the error bars overlapping

for nearly all of the measurements. The best way to combat this issue has been concluded to be to increase the overall concentration of the TFO being studied.

Conclusion

Through using the method of fluorimetry and displacement assays, it cannot be concluded if GA-Motif TFO follows proportional signal decrease with proportional concentration increase. This shows that GA-Motif TFO may not follow Beer's Law of concentration proportionality. It must be noted however that the concentrations studied in this experiment are very low. There was not a sufficient amount of reagents to conduct a higher concentration study of TFO. I believe this to be the largest flaw of this experiment, as I believe that at higher concentrations of TFO there would be a proportionality between concentration and signal decrease. The literature also partially corroborates this statement. While the relationship between the proportionality of concentration and decrease in emission signal was proven inconclusive for GA-Motif TFO, the TFO did displace some of the ethidium bromide within the ds-DNA major groove as indicated by figure eight. This leads this experiment into stages of further investigation in the future. The control experiments also gave inconclusive data. While the ds-DNA caused the ethidium bromide to intercalate and increase the emission signal drastically, the standard deviations of these measurements were far from accurate. It was promising to see the hypothesis proven correct, as ethidium bromide intercalation into the hydrophobic environment did cause the emission signal to increase, however the data obtained was inaccurate and a quantifiable measurement of how much the signal increases upon addition of ethidium bromide and ds-DNA cannot be had. The second control experiment follows a

similar narrative. While GA-Motif TFO does cause a decrease in emission signal upon displacing ethidium bromide, it is impossible to quantify numerically due to the poor error bar and standard deviation issues associated with the data.

While surface level conclusions can be made about this experiment, no accurate and quantifiable conclusions can be made. This experiment proved that ethidium bromide will intercalate ds-DNA in the major groove and increase the emission signal drastically. This experiment proved that upon an addition of GA-Motif TFO to an ethidium bromide intercalated ds-DNA strand, the ethidium bromide will be displaced from the major groove and replaced by TFO to form a triplex and decrease the emission signal. While this experiment was able to prove these conclusions, no quantifiable amount of decrease in emission signal or increase in emission signal was able to be had. This is most likely due to the minute concentrations of GA-Motif TFO studied.

Future work on this project should be directed towards using a higher concentration of TFO and further studying its relationship with Beer's Law. This experiment would be perfect for an undergraduate researcher, as the chemicals are easy to obtain and the instrument is user friendly. Future work should also be concerned with quantifying the amount of increase and decrease in emission signal upon addition of ethidium bromide and upon addition of TFO respectively.

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