# SPECTROSCOPIC ANALYSIS OF COUPLED CYANINE DYES IN DNA USING ABSORBANCE AND CIRCULAR DICHROISM

by

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A THESIS

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The fluorescent molecules cyanine-3 (Cy3) and cyanine-5 (Cy5) were inserted into opposing sides of both duplex and fork regions of the DNA backbone as probes for local conformation. The spectroscopic methods of absorbance and circular dichroism were used to observe the strength of exciton coupling within these dimers. Opposing Cy5 dyes in the duplex region of DNA showed strong exciton coupling, with the identification of H and J components in the spectra. The height and energies of the H and J component peaks are related to the conformation of these coupled dyes, suggesting that these results provide a useful basis for future conformational analysis of the duplex Cy5 dimer system. Cy3-Cy5 dimers showed similar signs of coupling, but H and J components were not identified. In the fork region of DNA, coupling of opposing Cy5 dyes was not apparent, suggesting that the dyes in the fork show weaker coupling and require more sensitive methods for conformational analysis.

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# Introduction

The study of life is a complex and intricate pursuit, largely due to the huge variation in life that exists on Earth. However, one thing that is nearly universal between these organisms is the coding of genetic information in DNA. As the cells that comprise living organisms grow and divide, DNA is used to pass on the information that the new cells need to survive. This information comes in the form of genes, which provide the blueprint for all the proteins in the cell. Proteins act as the cellular machinery, performing essential functions and helping the cell to grow and divide in its own time.

Genetic information in complex organisms like humans is carried in the form of chromosomes, large supercoils of DNA that are contained within the nucleus. Humans have 23 different chromosomes and normal human cells carry two copies of each – one from the mother and one from the father. This causes a problem when a cell divides because the two resulting cells must each contain a set of maternal and paternal chromosomes, and there only exists one of each. To succeed in cell growth and division, cells must have a way of making exact copies of each of their chromosomes. This is why cells go through the process of DNA replication, which results in two exact copies of each cellular chromosome.

Although DNA replication is an essential process for proliferating cells, we still lack a deep understanding of the process. However, the implication of DNA replication errors in numerous diseases, including Fanconi anemia, has increased interest in this field in recent years (Zeman and Cimpritch 2014). By improving our understanding of DNA replication, we may gain insight into treatments or ways to combat these genetic

diseases. Defects in DNA replication is an important aspect of all cancers, which are characterized by extremely fast growth and proliferation. Fostering a better understanding of the factors involved in DNA replication can lead to new treatments to specifically target these rapidly dividing cells.

While human cells have highly complex replication systems, they share many features with the simpler systems of some single-celled organisms and even some DNA viruses like the T4 bacteriophage (Miller et al 2003). In all cases, DNA replication is initiated via a protein binding event within a highly-specific sequence of DNA called the "replication origin." This initially bound protein subsequently "recruits" additional proteins that begin the process of replication. The initial binding step requires that the protein be present at the origin site, that the protein has adopted a favorable binding conformation, and that the DNA is in a proper conformation for binding such that the replication origin site is exposed to the protein.

Although these protein components vary between different replication systems, local DNA dynamics are important for them all, which is why characterizing these dynamics could have a huge impact in treating human disease and furthering biological research. In this study, we utilized "exciton coupled" fluorescent dyes as probes for DNA backbone structure. By using the relatively simple spectroscopic techniques of absorbance and circular dichroism (CD), we can gain useful information about the local conformation of the sugar-phosphate backbone of the DNA near potential protein-DNA binding sites.

**Background:**

This section of the dissertation is intended to familiarize readers with the foundation needed to understand exciton coupling and spectroscopy as a whole. Because much of the information presented herein is based on many decades of research, here is presented a brief overview of much of the presumed knowledge within this field. Further explanations of the vocabulary used are provided in the glossary at the end.

*Electrons and Spectroscopy:*

Electrons orbit the atomic nuclei of molecules at specific, quantized energy levels, which are called molecular orbitals. However, when a source of energy (like a photon of light) with the proper amount of energy interacts with the molecule, an electron can gain energy and move into an 'excited' molecular orbital with a higher energy, such that the electron is farther away from the nuclei. This excited state is unstable, and so the electron will eventually relax down to its original molecular orbital, or ground state, releasing the energy it absorbed in the process. Sometimes this energy is released as a photon of light. The excitation / emission process is never 100% efficient – some of the excited state energy can be dissipated as heat – so that the released photon has lower energy than the originally absorbed one. This property of a material is called fluorescence, and molecules that are strongly fluorescent we call fluorophores.

The color that we perceive light to be is related to the wavelength of the photons, with certain colors indicating less energy than others. A fluorescent chemical under a yellow light may glow red, but never blue since blue light contains more energy

than red light. By shining a yellow light on the molecule, and measuring the red light emitted, we can observe such fluorescence so long as the yellow light is not being detected. Similarly, we can also shine yellow light on the molecule and measure how much yellow light passes through, to see how much of the initial light the electrons of the molecule absorb. This measurement is known as the molecule's absorbance, and the wavelength of the absorbance as well as the intensity are related to the environment surrounding the molecules. Both measurements are examples of spectroscopy, which refers to the study of molecules using light. While fluorescence and absorbance are examples of two key measurements in the field of spectroscopy, there are many more examples, all of which are useful for revealing different information about molecules and their environments.

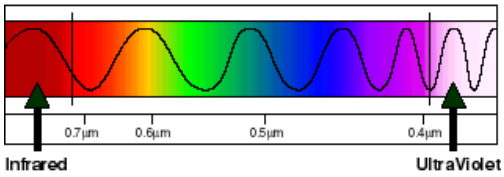



**Figure 1:** The visible region of light, showing the difference in wavelength between colors. The energy increases from left to right. **Source**: NASA

*Exciton Energy Transfer and Vibronic Coupling:*

When two fluorescent molecules are separated by a short distance from one another, their excited electrons can oscillate in a cooperative manner. Such a coupled pair of fluorophores can pass excited-state energy from one molecule to another through a process called exciton coupling. This means that when one molecule absorbs energy, the excited electron passes its energy to an electron of the coupled molecule, which may subsequently relax to its ground state and emit a photon. In some cases, the electronic excitation of a molecule may cause it to vibrate. This process of "electronic-vibrational coupling" can affect the way that energy is transferred between exciton coupled

molecules. When two molecules are coupled in this way, the nuclei of the two molecules vibrate in concert when they are electronically excited, much like two pendula attached by a spring. This phenomenon is referred to as vibronic coupling, because the interaction between the molecules affects both molecules' vibrational motion and electronic energy.

The electrons of coupled molecules can oscillate "in-phase," so that they move in the same direction at the same time, or "out of phase," so that they move in opposite directions (Perdomo et al 2012). Electrons of vibronically-coupled molecules with a large angle between them (near 90º) have both in-phase and out-of-phase oscillatory components. The in-phase oscillation has increased energy relative to the uncoupled molecules, and the out-of-phase oscillation has decreased energy. These in-phase and out-of-phase component oscillations are referred to as H- and J-like components, respectively (Spano 2009).

Because of these intermolecular electronic and intramolecular electronic-vibrational coupling interactions, vibronically coupled molecules have different properties than those of independent (uncoupled) molecules. We use spectroscopic measurements to reveal information about this coupling, including how strong it is and how much it is affecting the energy of the electrons. We can use this experimental information to characterize how two coupled molecules, which we refer to as a dimer, are positioned relative to one another. The relative orientation and distance separating the molecules of a dimer is called its "conformation."

*Spectroscopy in Dye-Labeled Duplex DNA:*

The properties of vibronically coupled dimers can provide a useful framework by which we can understand the local conformations of the sugar-phosphate backbones of DNA. DNA molecules are long chains of deoxyribose sugars. Each of these sugars is bonded to a DNA base: adenine, guanine, cytosine, or thymine (A, G, C, and T for short). Hydrogen bonding interactions between these bases leads to the formation of the classic double helix structure. The sequence of the bases is the key to DNA forming a stable hydrogen bonded structure, since A attracts to T and G attracts to C. When two individual single-stranded (ss) DNA molecules have sufficient matching or "complementary" sequences, they may form the double helix structure, which is referred to as duplex DNA.

It is the hydrogen bonding interactions between bases that determines the duplex structure. Hydrogen bonding occurs when one molecule with many electrons shares some of its electrons with another molecule that has few electron. This is not a chemical bond in the traditional sense because the molecules are not connected permanently, but there is a stabilizing (attractive) interaction between them.

Because the hydrogen bonding between the bases holds the DNA together, the bases in the duplex region generally stay lined up with one another to keep this hydrogen bonding intact. However, the DNA in our bodies does not just sit there. It is constantly being used by the protein machinery of our bodies to create new DNA or RNA molecules, which affects the expression of certain genes. Some of these proteins can attach to the outside of the DNA, but many must get in between the strands to carry out their function. This means that the hydrogen bonds must be broken and reformed as

the protein passes through the duplex region of DNA, and the mechanism of how this happens is relatively unknown. However, some studies have shown that DNA bases in the duplex region do not spend all their time lined up in the correct orientation for hydrogen bonding. For extremely short periods of time, bases in a duplex region that are close to a ss-ds DNA "junction" such as a replication fork may adopt a partially "open" conformation in a process called "DNA breathing" (Jose et al. 2009). This effect might affect the ability of proteins to bind in the DNA duplex, so a better understanding of this process could lead to many interesting new opportunities for research and medicine.

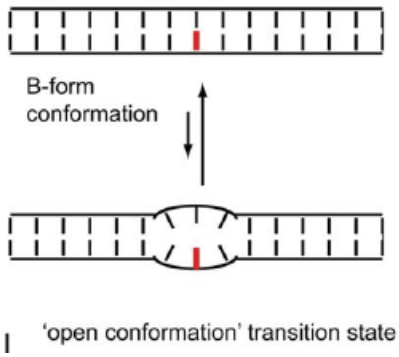



**Figure 2:** Illustration of the transition of DNA bases (black lines inside) from their normal hydrogen bonding to an 'open conformation.' This transition is thought to be one of the first steps in DNA melting. Source: von Hippel, 2013

Spectroscopy provides a useful framework for understanding the local conformation of the backbone of the DNA duplex. However, natural DNA is not suitable for direct spectroscopic study. Instead, we can "label" the DNA backbone using fluorescent molecules to observe conformational changes.

Cyanine-3 (Cy3) and cyanine-5 (Cy5) are useful molecules for spectroscopic visualization because they strongly absorb and emit light in the visible region of the electromagnetic spectrum. By labeling different regions of the DNA backbone with the Cy3 and Cy5 dyes, we can study the conformation of the DNA backbone near ss-ds forks and junctions.

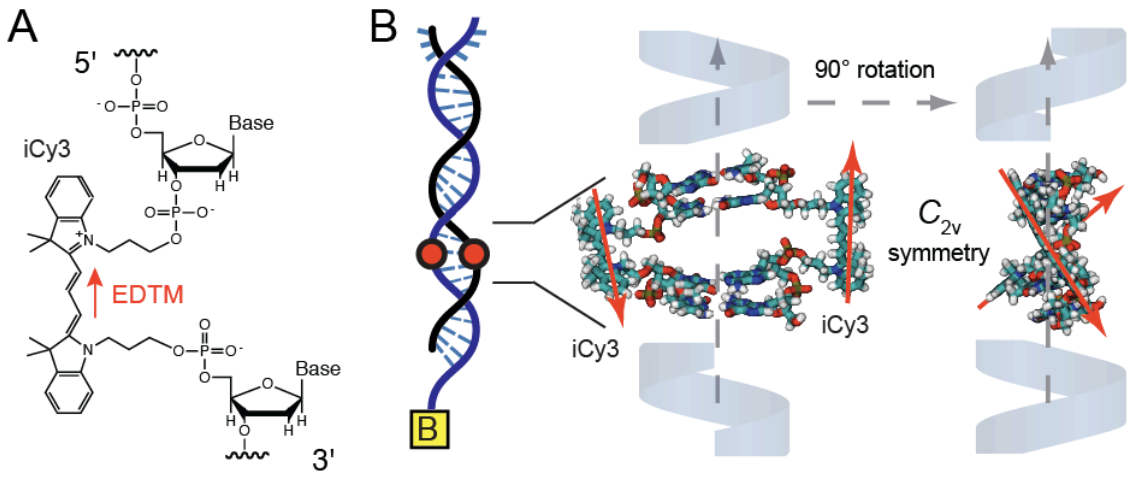



**Figure 3:** Diagram depicting the fluorescent probe "labeling" of DNA. (A) Chemical bond depiction of cyanine-3 dye insertion into the DNA backbone. The arrow indicates the electronic dipole transition moment (EDTM). (B) Within a DNA duplex with Watson-Crick base pairs, the EDTM of dyes on complementary strands form an angle near 90º. Exciton-coupled dimers oriented in this way have both H and J components which are observable using linear spectroscopy.

**Previous Research and Rationale:**

In previous experiments, the conformation of two exciton-coupled molecules was solved using mathematical analyses and theoretical modeling of experimental data obtained from a form of spectroscopy called two-dimensional fluorescence spectroscopy (2DFS) (Perdomo-Ortiz et al 2012). 2DFS is a *nonlinear* spectroscopic method that uses ultrafast (femtosecond = $10^{-15}$ sec) pulses of light to excite the coupled molecules and observe the emission of light. While linear spectroscopic methods determine the energy levels and transition strengths of molecular systems that are initially in their ground electronic states, nonlinear spectroscopies can provide additional information since they probe the optical transitions that can be accessed after the system has been "prepared" in an initially excited state. The 2DFS method provides information about the relationships between the various electronic-vibrational states that are excited by a sequence of four ultrafast optical pulses. The resulting 2DFS spectrum

is a two-dimensional contour plot that contains detailed information about the energy states of the coupled molecular dimer.

While 2DFS can provide high-level information about a coupled molecular dimer, the method is technically challenging to perform in its current implementation. 2DFS requires a finely tuned high-powered laser apparatus, which is used to excite the sample with spectrally broad phase-coherent light pulses. The laboratory must be isolated from environmental vibrations and other sources of noise, which can degrade the accuracy of these measurements. Such technical challenges can limit the utility of the 2DFS method for screening large numbers of samples. Nevertheless, 2DFS experiments are useful for building upon the linear spectroscopic properties of coupled molecular dimers.

The most important linear spectroscopic method that can provide suitable information about coupled molecular dimers is circular dichroism spectroscopy. Circular dichroism (CD) is a form of spectroscopy that looks at how molecules within a strong electrical field can absorb circularly polarized light. Light is often described as a transverse electromagnetic wave that oscillates in a direction perpendicular to its propagation. For light that is emitted from most thermal sources, such as an incandescent bulb or the sun, the oscillation direction is random. Light with random oscillation direction is referred to as “incoherent” or “non-polarized.” However, certain devices can restrict the ways in which the direction of the wave oscillation can be oriented as the waves leave a light source, producing what is called polarized light. In the case of circular dichroism, the waves oscillate in a spiral as they propagate through space. This spiral can be either “right-handed” or “left-handed” (imagine climbing a

spiral staircase and gripping the outer railing. The hand you use describes the spiral). A coupled molecular dimer oriented so that the spiral passes directly through both monomer subunits will absorb more of the light while a dimer oriented so that the spiral can only pass through one of the subunits will absorb less light (Lakowicz 2006).

Because coupled molecular dimers in different conformations absorb different amounts of light, CD spectroscopy can reveal information about how the coupled dimer is shaped. However, this information typically is not sensitive enough to reveal the exact conformation of the coupled molecules: there are often multiple conformations that could explain the same CD signals. For highly-coupled fluorescent molecules though, the differences in energy caused by H and J oscillation components can create separate, inverted peaks in the CD spectrum. The out-of-phase oscillation of the J-like component lowers the energy of the electrons in the excited state, while the in-phase interactions of the H increases the energy of the excited state (Kistler et al 2011). Because the position and relative amounts of these split energy transitions depend on the coupling between the molecules, if we can characterize the H and J components of coupled fluorophores, this will serve to characterize how the molecules interact and how they are oriented relative to one another in space.

**Central Questions and Goals:**

The major goal of my research is to use temperature dependent absorbance and CD measurements to characterize the conformations of cyanine dye probes that are inserted into the DNA backbone. Altering the temperature allows us to observe how spectral features (hereafter referred to as “peaks”) that are due to optical transitions of the coupled dimer change when the two strands separate and the coupling between the

fluorophores is diminished. We hope to use these results to identify H and J component peaks, as well as any other information that characterizes the coupling between the probes. This information may allow us to draw conclusions about the relative orientation of the fluorescent probes in various regions of DNA and at different temperatures. Furthermore, if these spectroscopic techniques are capable of characterizing coupled fluorophore-DNA systems in general, they may provide a new avenue for computational analysis. This might relax the need for complicated techniques like 2DFS for some systems, and provide a foundation for future research in this growing field.

## Materials and Methods

Fluorescent Cy3 and Cy5 dyes inserted into various regions of the backbone were used to analyze DNA conformation. T4 bacteriophage DNA with these substitutions was purchased from an industrial source. All DNA strands are between 50 and 70 bases long, with varying regions of complementarity to other strands. We annealed these strands to assemble various DNA duplexes, some with the fluorophores inside a double-stranded region, at a junction between the duplex and within a single-stranded DNA region. Within these regions different combinations of Cy3 and Cy5 insertions were used to vary the vibronic coupling and provide variation in the data. Table I below shows the various combinations of DNA used for these experiments. Sample codes are based on a letter (D or F) describing where in the DNA the probes are located and two numbers describing the probe on the first strand and the probe on the second strand respectively. The number 3 indicates Cy3 dye, while 5 indicates Cy5 dye and 0 indicates an unlabeled strand.

Table I: DNA construct names and sequences

| Code | Description | Sequence |
|---|---|---|
| D3-5 | Cy3-Cy5 duplex dimer | 3′- GTC AGT ATT ATA CGC T3C GCT AAT ATA CGA CGT TTT TTT TTT TTT TTT TTT TTT TTT TTT T<br>5′- CAG TCA TAA TAT GCG A5G CGA TTA TAT ATG CTT TTA CCA CTT TCA CTC ACG TGC TTA C –3′ |
| D5-3 | Cy5-Cy3 duplex dimer | 3′- GTC AGT ATT ATA CGC T5C GCT AAT ATA CGA CGT TTT TTT TTT TTT TTT TTT TTT TTT TTT T<br>5′- CAG TCA TAA TAT GCG A3G CGA TTA TAT ATG CTT TTA CCA CTT TCA CTC ACG TGC TTA C –3′ |

| | | |
|---|---|---|
| D5-5 | Cy5-Cy5 duplex dimer | 3′- GTC AGT ATT ATA CGC T5C GCT AAT ATA CGA CGT TTT TTT TTT TTT TTT TTT TTT TTT TTT T<br>5′- CAG TCA TAA TAT GCG A5G CGA TTA TAT ATG CTT TTA CCA CTT TCA CTC ACG TGC TTA C -3′ |
| D5-0 | Cy5 duplex monomer | 3′- GTC AGT ATT ATA CGC T5C GCT AAT ATA CGA CGT TTT TTT TTT TTT TTT TTT TTT TTT TTT T<br>5′- CAG TCA TAA TAT GCG ATG CGA TTA TAT ATG CTT TTA CCA CTT TCA CTC ACG TGC TTA C -3′ |
| F5-5 | Cy5-Cy5 fork dimer | 3′- GAG GGA GCA CAG CAG GGA TCA GTA TTA TAC GCT 5CG CTG GTA TAC CAC GTT TTT TTT TTT TTT TTT TTT<br>5′- CTC CCT CGT GTC GTC TCC AGT CAT AAT ATG CGA 5AT GCT TTT ACC ACT TTC ACT CAC GTG CTT A -3′ |

Each combination of DNA was made by adding small volumes of the stock solutions of single-stranded DNA in 10 mM Tris, 100 mM NaCl and 6 mM $MgCl_2$ buffer. The strands are individually stored in the freezer to prevent damage or degradation; however, this can lead to significant changes in concentration over time. To combat this, we determined the concentration of each single-stranded DNA solution before making each sample. First, we took 2 µL of the solution and mixed it with 998 µL buffer. We measured the absorption of the diluted solution at 260 nm. The company IDT (Integrated DNA Technology) provided extinction coefficients for each of these DNA strands, which we used to calculate the concentration of the diluted solutions using Beer's Law (Equation I below).

$$c = \frac{A}{\varepsilon l} \tag{I}$$

From this, we back-calculated the concentration of the stock solution by multiplying by 500 (the dilution factor). Once we knew the molar concentration of the stock solution, we took the product of our desired volume and concentration of the strand and divided it by the stock concentration to determine the volume of stock DNA that we needed to add.

Once the necessary volume of each strand was prepared, we added buffer until we reached the desired total volume. At this point, the new samples were placed into a heat block and heated up to between 90 and 95ºC. The heat block was then turned off and allowed to cool to room temperature. Samples were left to sit for at least 3 hours, giving adequate time for the complementary parts of the DNA strands to anneal into a duplex. Samples were not allowed to sit out for longer than 72 hours before being returned to the freezer where they were stored. Frozen samples were thawed to hand temperature, centrifuged at 10000 rpm, and once again heated to between 90 and 95ºC in the heat block and allowed to cool on the counter for at least 3 hours. We made 400 nM samples of these double-stranded DNA constructs to collect absorption spectra using a Cary UV-vis spectrophotometer. Absorption data were taken from 700 to 200 nm wavelength, slightly into the UV range. Samples were stored in the freezer between experiments, and were thawed, centrifuged and reannealed before data collection. We took a buffer scan with the same parameters as the baseline for the data. In our first experiments, temperature control was reliant on the block temperature reading on the spectrophotometer. We later improved upon this control by using an in-cell thermistor.

Similar samples were made for taking circular dichroism data, however the concentration of the DNA was increased to 2.0 μM, enough to be comparable to literature values (Widom, 2013). These samples were stored and prepared the same way as the absorption samples. To improve our signal to noise ratio, we took 5 accumulations at each temperature, with an 8-second integration time, 2.0 nm bandwidth and a 50 nm/min scan speed. A buffer scan with the same parameters was used as the baseline. An in-cell thermistor was used for temperature control in all

experiments. To ensure homogeneity, we added a magnetic stir bar and a delay time of 1200 seconds after reaching the target temperature before starting data collection. The target temperature was reached when the temperature stayed within +/- 0.3 ºC for 10 s.

Once the data has been successfully collected, we will use mathematical relationships and simulated spectra to model the possible conformations of the fluorophores within each DNA construct, as was done in the previous study using 2DFS data (alongside absorbance and CD) (Perdomo-Ortiz, 2012). We will be able to gauge the successfulness of the experiment based on how specific our modeled conformation is (how many others would be possible to fit the data) as well as how well this modeled conformation with its simulated data fits the experimental data we collect.

# Results

*Absorbance:*

Absorbance spectra clearly demonstrated the coupling between fluorophores. Figure 4 below shows a comparison between the coupled Cy5-Cy5 fluorophore DNA duplex and a duplex formed with only a single Cy5 inserted into one strand, and a site lacking a base on the opposing strand. The differences in the spectra are apparent, with the single Cy5 spectra nearly independent of temperature and the Cy5-Cy5 spectra changing systematically as the temperature is increased through the DNA duplex to ssDNA melting point. In the coupled Cy5-Cy5 dimer, we observe a pronounced peak at ~670 nm at low temperatures, which gradually blue-shifts with increasing temperature to ultimately resemble the non-coupled single Cy5 spectra when the temperature reaches ~65 ºC. Similarly, a small peak is observed near 590 nm in the coupled spectra that disappears at temperature ~65 ºC, again more closely resembling the single fluorophore spectra.

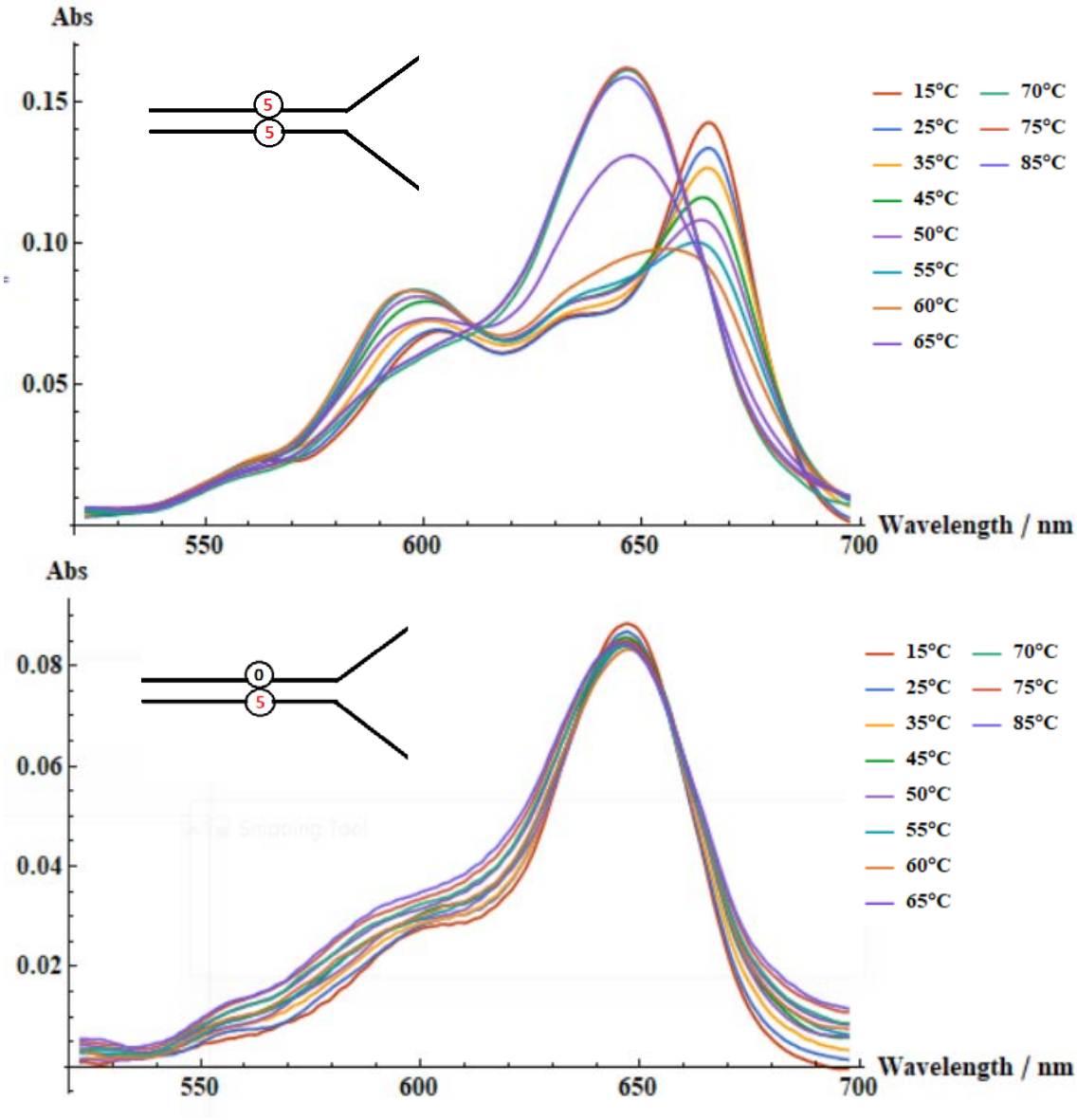



**Figure 4:** Comparison of the D5-5 (top) and D5-0 (bottom) absorbance spectra. The D5-5 has two Cy5 dyes directly opposite one another in the duplex, while the D5-0 has only one Cy5 dye in the same location and DNA context. The legend on the right represents the temperature of the solution for each measurement in ºC. The diagrams in each figure represent where in the DNA backbone each dye is located and which dye it is (5 = Cy5, 0 = no dye)

The existence of these features and their disappearance in the spectra near the melting point of the DNA duplex suggests that these peaks are the result of exciton

coupling, which we may assign to H- and J-type coupling. The transformation of the coupled spectra to closely resemble the single-fluorophore sample above the melting point further supports the conclusion that these features are due to the Cy5-Cy5 exciton coupling. When the DNA passes through the melting point, the duplex DNA strands separate to form single isolated strands in solution, thus pulling the fluorophores apart and eliminating the coupling between them.

We also measured the absorbance spectra of Cy3-Cy5 coupled duplexes. The spectra of the D3-5 and D5-3 constructs are shown in Figure 5 below. The same coupled energy splitting into H and J components is not observed, which makes sense because the energy levels of Cy3 and Cy5 are less alike than the two Cy5 molecules in the D5-5 duplex. More disparate energy levels have poorer energy splitting, so we already expect the effect of such coupling to be much less pronounced in these spectra. Further, the peaks of Cy3 and Cy5 slightly overlap with one another, which may obscure any small level of energy splitting.

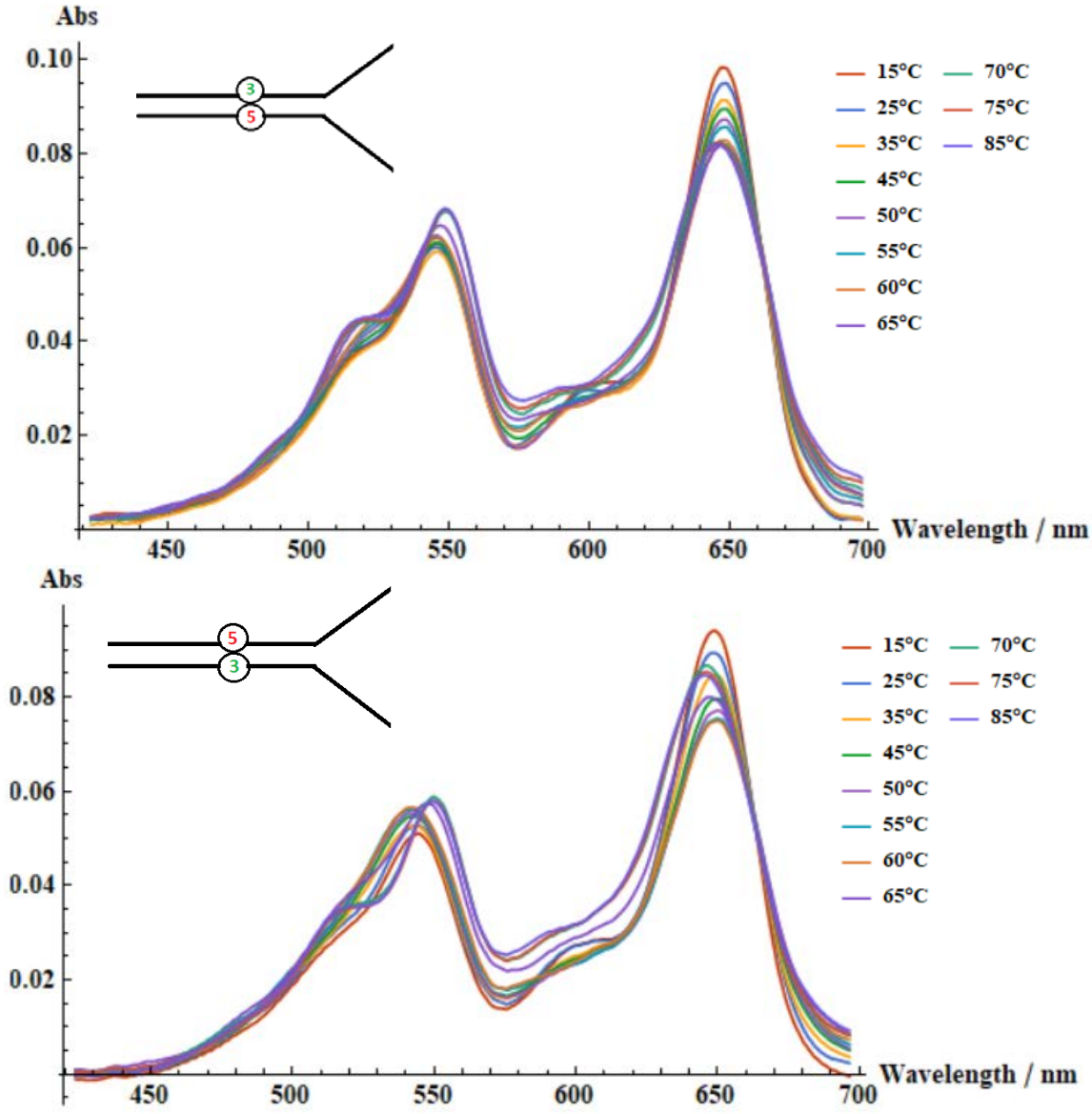



**Figure 5:** Absorbance spectra of the D3-5 (top) and D5-3 (bottom) DNA constructs. Both samples have opposing Cy3 and Cy5 fluorophores with the difference being which strand they are on. The legend on the right shows the temperature of the sample during each measurement in ºC. Likely due to both the difference in their energy levels and the overlapping of their peaks, neither construct shows clear signs of coupled energy splitting.

All the absorbance measurements extended into the ultraviolet region to ensure similar concentrations of DNA between constructs. These spectra can be seen in Figure S1. We specifically considered the 260-nm peak, which we found to be highly

consistent between spectra. The 200-nm peak is also due to the DNA and as the signal extends into the non-linear region of the instrument differences between the spectra in this region can largely be discounted.

*Circular Dichroism:*

To supplement our absorbance data and help to identify H and J coupling features, we used circular dichroism spectroscopy (CD). CD is sensitive to chirality, with opposite orientations creating opposite signs in the spectra. In the context of H and J coupling, this means that the high and low energy features have opposing signs, making this coupling much easier to observe in CD spectra. Several CD spectra are shown in Figure 3 below. With no coupling, as in the D5-0 duplex, there is no signal in the fluorophore region of the spectra. In contrast, the D5-5, D5-3 and D3-5 samples all demonstrate interesting peaks due to the coupling between their fluorophores. As expected, these peaks have greater intensity in D5-5 and weaker in D5-3 and D3-5 due to the difference in the fluorophores' energy levels. Additionally, we see these coupled fluorophore peaks disappear as the temperature is raised above the melting point, confirming that this is an effect of spatial coupling between the fluorophores which is lost upon DNA melting. We extended these spectra into the UV to ensure that the chiral DNA was producing a consistent signal and that the machine was working properly. These spectra can be seen in Figure S2. As the temperature increased, these peaks decreased, which makes sense as the DNA structure and dipole becomes increasingly less rigid.

The coupled fluorophore (D5-5, D3-5 and D5-3) spectra all show distinct peaks within this region. The Cy3-Cy5 D5-3 and D3-5 samples yielded nearly the same

spectra. Both spectra have strong positive peaks near 650 nm, with a smaller positive peak around 600 nm and a negative peak around 550 nm.

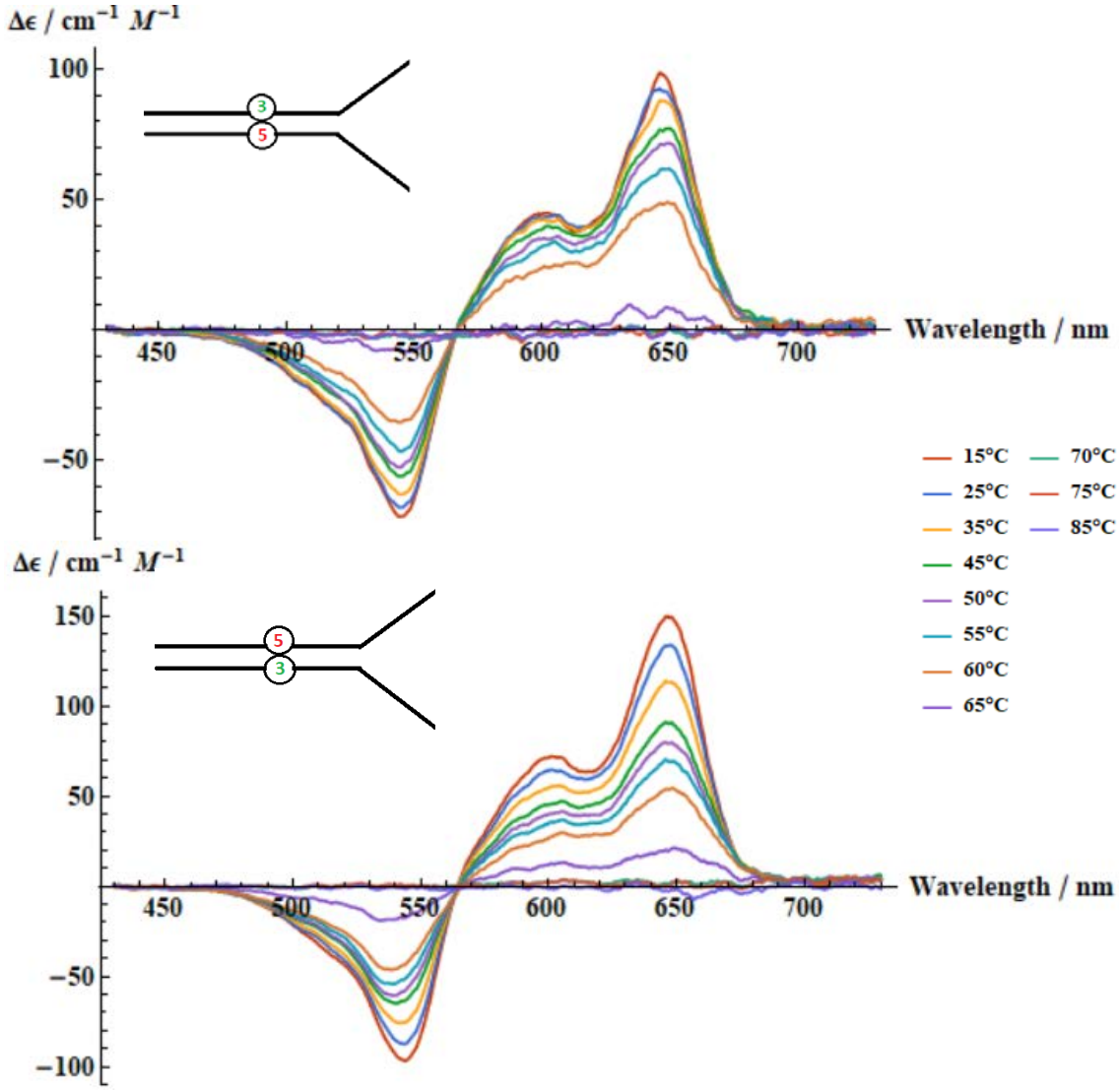



**Figure 6:** CD spectra of Cy3-Cy5 probed duplex DNA constructs. D3-5 (top) and D5-3 (bottom). Both samples have opposing Cy3 and Cy5 fluorophores with the difference being which strand they are on. The legend on the right shows the temperature of the sample during each measurement in ºC. These spectra demonstrate the same features, despite showing slightly different melting curves.

The D5-5 spectra stand out in comparison to the D5-3 and D3-5 peaks. The D5-5 spectra share no features with either the D5-3 or D3-5 spectra, suggesting that Cy3-Cy5 coupling behaves much differently than Cy5-Cy5 coupling. Some of these

differences may be explained by overlap of the Cy3 absorbance within the 550-nm region with the Cy5 absorbance peak, however the major factor is likely the weaker levels of coupling between two different fluorophores compared to the coupling between identical fluorophores. The weaker coupling between Cy3 and Cy5 may not be enough to demonstrate H and J features. However, the Cy5-Cy5 molecules in D5-5 are highly coupled, and notably the D5-5 spectra peaks transition from negative to positive around 650 nm, the same wavelength that shows the strongest peaks in the D5-3 and D3-5 spectra. Further, all notable features in this region in all three of these constructs disappear at high temperature.

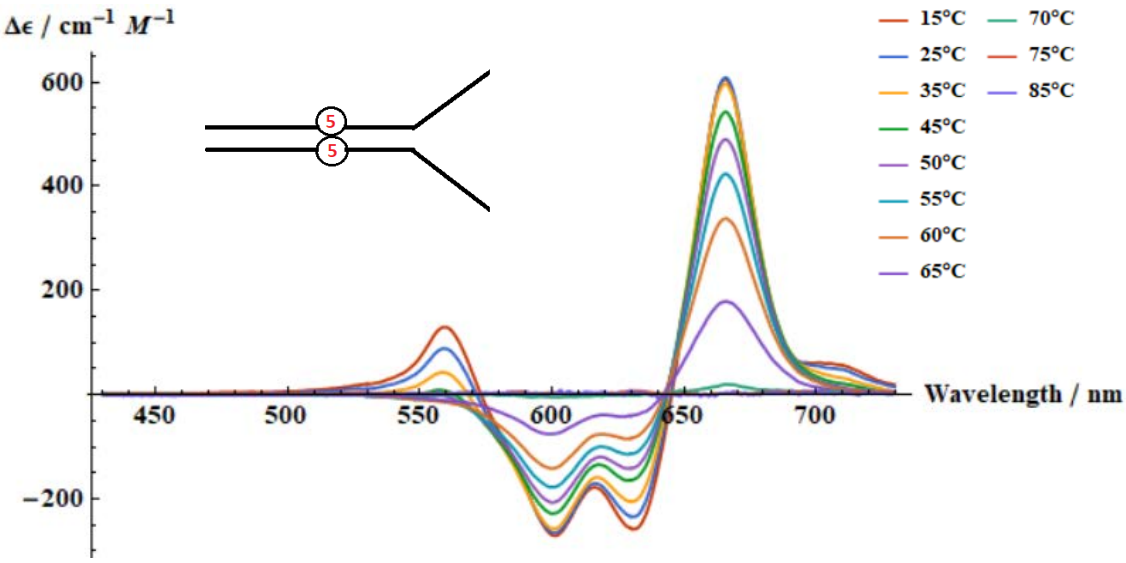



**Figure 7:** CD spectra of the D5-5 DNA construct with changing temperature. The legend on the right shows the temperature of the sample during each measurement in ºC. The highly coupled Cy5-Cy5 construct CD spectra show peaks at 670 nm, 630 nm, 600 nm and 560 nm. The transition between positive and negative peaks near 650 nm could be due to the sign inversion of H and J components in CD spectra. The diagram represents where in the DNA backbone each dye is located and which dye it is (5 = Cy5).

Similar duplex Cy5-Cy5 CD spectra were taken in a previous study, in which the researchers found most of the same peaks, except with a negative peak at 560 nm

instead of a positive one (Markova 2013). To confirm that the positive peak observed in this experiment was not an error of the measurement apparatus, a higher sensitivity CD scan was taken with the temperature fixed at 15 ºC. The results of this experiment are shown in Figure 8.

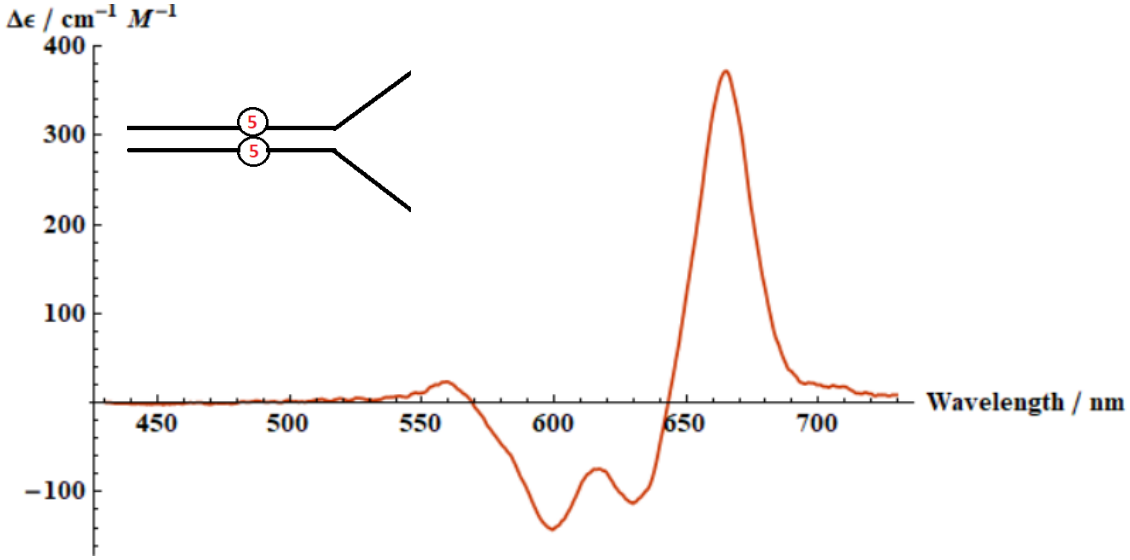



**Figure 8**: Additional CD spectrum of the D5-5 DNA construct. The temperature for this scan was held constant at 15 ºC. The positive peak at 560 nm is present but at a reduced magnitude compared to previous scans. The diagrams in each figure represent where in the DNA backbone each dye is located and which dye it is (5 = Cy5).

*Replication Fork Dimer:*

The previous experiments utilized fluorophores held deep within the duplex region of a cyanine labeled DNA construct, in which the fluorophores are strongly-coupled. Such strong coupling leads to better observation of these H and J exciton features. However, applying these same techniques to regions where the DNA transitions from a duplex to become single-stranded may help us relate these findings to biological processes involving protein binding. The F5-5 DNA construct contains two Cy5 molecules inserted into such a region of DNA, called a fork or fork junction.

We took absorbance and CD spectra of this construct to see how the spectra compare to our previous results. These spectra indicate that the CD is too small to be observed in these constructs, as shown in Figure 9.

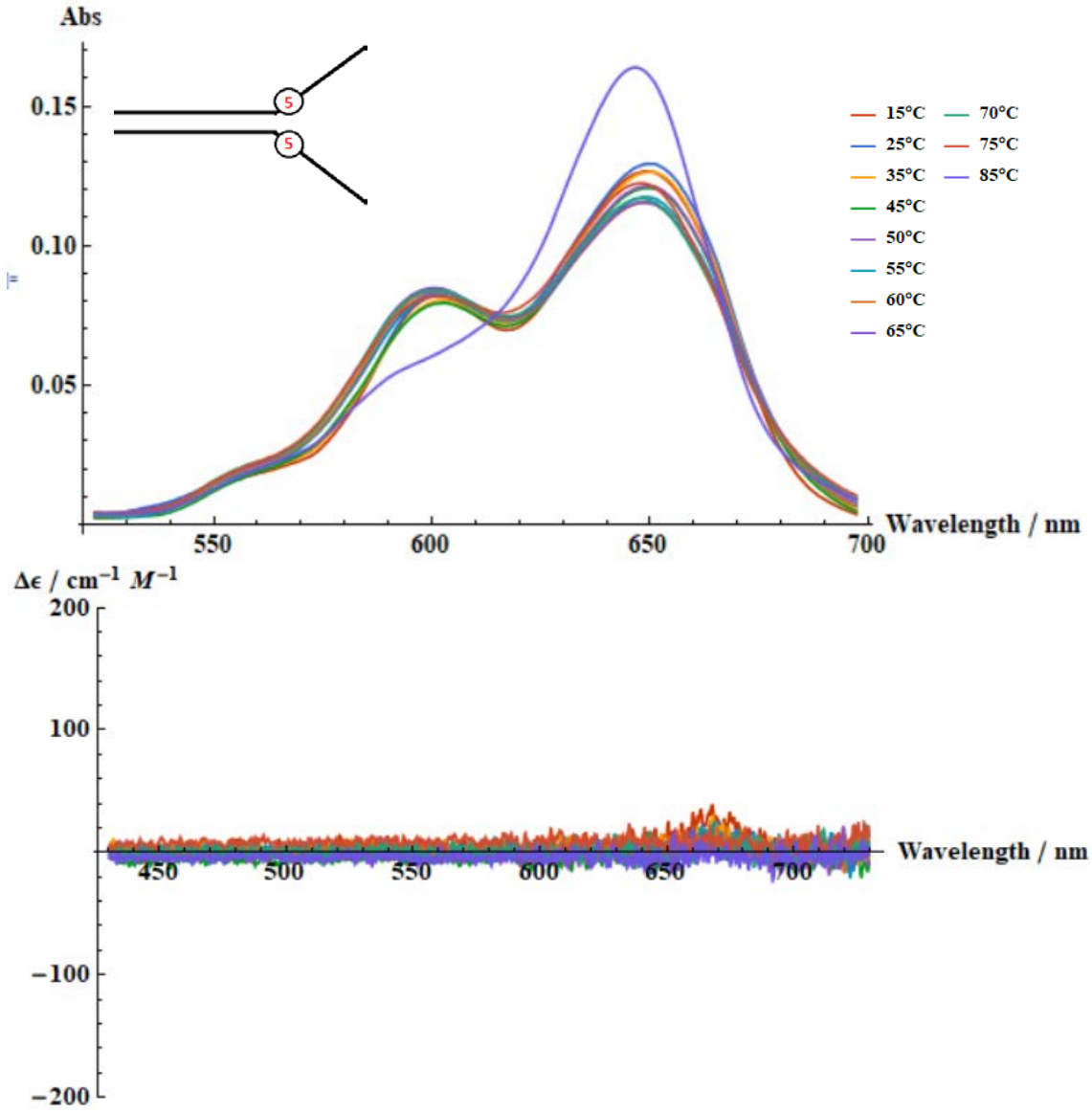



**Figure 9:** Spectra of the F5-5 DNA construct with changing temperature. The F5-5 construct contains two Cy5 fluorophores directly adjacent to the complementary duplex region of the DNA. The legend on the right indicates changing temperature in ºC. (A) Absorbance spectra for F5-5 show similar features to D5-5 but lack the small, disappearing peaks indicative of coupling. (B) CD spectra for F5-5 show no distinct features in the Cy5 fluorophore region. Although some features may be obscured by noise, any existing peaks are much smaller than observed in the previous D5-5 CD spectra.

## Discussion

The simple spectroscopic techniques of absorption and CD may be useful in characterizing the conformation of exciton-coupled systems within DNA. The absorption spectra for the highly-coupled D5-5 DNA construct, in which two Cy5 fluorophores are held in close proximity, showed interesting features when compared to the D5-0 system with only one uncoupled Cy5 molecule. Compared to the D5-0 system, the largest peak for D5-5 at low temperature is red-shifted, from 650 nm to 670 nm, with a very small peak appearing around 630 nm that is not present in the D5-0 spectra. While this peak may be present in D5-0 and just be obscured by the large peak at 650 nm, it is also possible that these two peaks that appear at low temperature represent the high and low energy H and J features of the exciton. Supporting this interpretation is the observation that at high temperature the D5-5 spectra shift to almost exactly reproduce the D5-0 spectra. This suggests that the proximity and coupling of these fluorophores in the duplex DNA is responsible for the existence of these features, and it is not just a difference between D5-5 and D5-0. Additionally, H and J components appear more strongly with greater levels of coupling. The lack of any split peak features in the D5-3 and D3-5 can be explained in this context because the energy levels of Cy3 and Cy5 are more disparate, and therefore have much weaker coupling than the degenerate energy levels of the two Cy5 molecules in D5-5.

H and J components are unique to strongly-coupled dimers. The out-of-phase oscillation of electrons in the dimer creates a peak at higher energy (J) and the in-phase oscillation creates a peak at lower energy (H). In absorbance measurements, these peaks are additive, and may combine to produce a single feature with two smaller peaks, as

we observe in the D5-5 data. This makes it difficult to identify H and J components of excitons using absorption data alone. However, circular dichroism (CD) allows us to identify H and J features because the two types of peaks acquire an opposite sign. This means that the peaks are subtractive, but because they are typically separated in wavelength due to the difference in their energies, one shows up negative while the other is positive (Kistler et al. 2011).

This is exactly what we observe in the D5-5 CD spectra. A prominent positive peak is found at around 670 nm, while a smaller negative peak is at 630 nm, the same positions that were implicated as H and J peaks in the absorption spectra. These features also disappeared entirely at high temperatures, which brings them to duplicate the uncoupled D5-0 spectra which showed no notable peaks outside of the DNA region (Figure S4). These peaks, then, are certainly due to fluorophore coupling, and their reduction with increased temperature and their eventual disappearance are consistent with attributing the features to the H and J components of the dimer.

Another group working with Cy5 dyes in DNA previously published temperature dependent circular dichroism data for Cy5 oligonucleotide dimers, which match the D5-5 CD data we took in this experiment except for the peak at 560 nm (Markova et al 2013). The published data reported a negative peak here, but both our initial measurements and a follow-up scan showed this CD peak as positive for D5-5. However, the difference in sign could be due to several parameters that differ between the experiments. Context around the coupled fluorophores affects their energy levels, and the exciton will tend toward the confirmation that is the lowest energy given this context. This context includes not only the DNA bases neighboring the fluorophores but

also the buffer surrounding the system. The authors which found this peak to be negative were using a phosphate buffer, rather than a Tris buffer. This difference in solution around the dimer could have changed their orientation and flipped the sign of this peak. Additionally, the two Cy5 fluorophores in this experiment bordered T and C on one strand and A and G bases on the other. The previously mentioned experiment used Cy5 molecules bordered by A and C on one strand and by T and G on the other. Stacking interactions between different neighboring bases can certainly alter the exciton coupling, which could have flipped the sign (and magnitude) of the 560-nm peak. The D5-5 construct was also nearly 60 nucleotide bases in length, while the oligonucleotides used previously were only 20 bases in length. It is possible that the conformation of the coupled fluorophores is altered by interactions with distant DNA, which would be impossible for the short oligonucleotides used in the previous experiment, resulting in a positive peak at 560 nm. Regardless, this difference in sign is an interesting finding, and provides an avenue for further experiments to confirm and explain this phenomenon.

Overall, evaluation of the data for D5-5 demonstrates the presence of H and J components in both the absorption and CD spectra, and suggests that this method may be generalized to characterize the H and J components of other highly-coupled exciton system. The magnitude of these H and J peaks as well as their difference in energy from the typical absorption peak of Cy5 (650 nm) provide useful information for computational determination of dimer conformation. If this strategy can be applied more generally, it will allow us to characterize the conformations of biologically relevant regions of DNA as well. However, success in identifying H and J components using absorption and CD did not extend to less coupled systems.

For the Cy3-Cy5 systems, D5-3 and D3-5, the fluorophores were both held within the duplex region of the DNA. Being held so closely together, the molecules are coupled, however the disparity between the energy levels of the two different fluorophores leads to less overlap in their orbitals and therefore the overall coupling between them is weaker than in between Cy5 and Cy5 in the D5-5 system.

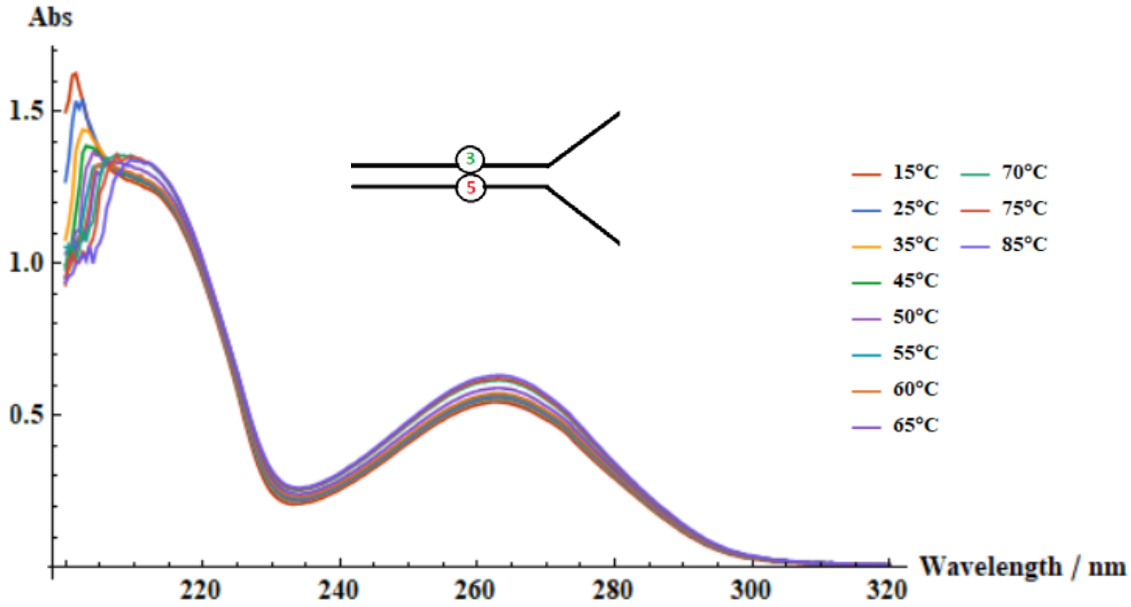
The absorption spectra for these weaker coupled systems are nearly identical. Both D5-3 and D3-5 show major peaks at 650 nm and 550 nm, the major absorption peaks for Cy5 and Cy3 respectively. There are interesting high energy features to the left of each of these peaks, including a small peak at 600 nm, which seems to diminish slightly at high temperature and appears as a positive peak in the CD spectra. This peak in the CD spectra transitions to a negative 540-nm peak, which corresponds to a small shift in the D3-5 and D5-3 absorption at low temperature to high. This feature is similar to that which we interpreted as H and J components in the D5-5 spectra. However, the fact that the D5-0 and D5-5 both had a similar absorption peak at 600 nm and that D5-5 also showed a negative CD peak at 600 nm suggests that this may not be due to H and J components at all. We may instead attribute this to the 0-1 energy transition for Cy5 gaining an electric dipole and CD signal when coupled to another fluorophore. In any case, the absorption and CD data for this system did not allow us to identify H and J components in the D5-3 or D3-5 system.

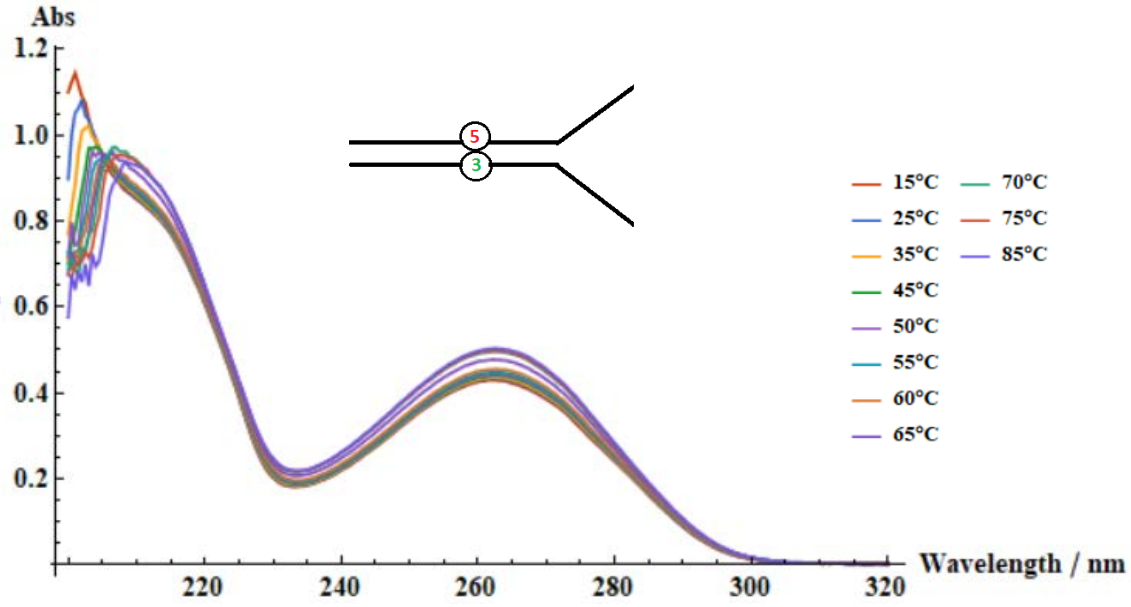
The F5-5 DNA construct also did not demonstrate any H and J coupling, and did not show significant CD signal in the fluorophore region for any temperature, just like the CD signal for the uncoupled D5-0. F5-5 is a construct with two Cy5 molecules placed within the replication fork instead of within the duplex. Because their energy

levels are degenerate, they should have strong coupling if they are being held in close proximity, however the lack of signal and uncoupled behavior suggest that the issue may be that the Cy5 molecules are not being held tightly enough to couple and form an exciton. The fork region is no longer held within the duplex, so it is not unexpected that the molecules would not be held closely enough to show a CD signal, however it is an interesting result. Some proteins, like helicases in DNA replication, must preferentially bind a DNA fork junction. The similarity of the F5-5 CD signal to that of D5-0 suggests that the local environment and dynamics of the fork junction are like and might even mimic single-stranded DNA. This question provides an interesting facet for future research in fork dynamics.

In total, although absorption and CD determination of H and J components does not work for all coupled systems, we demonstrated that it works for tightly-coupled degenerate systems, like the D5-5 DNA construct. The information about the H and J components, including the magnitude of energy splitting and the relative amounts of H and J behavior, provides an experimental insight into the local conformation of the dimer. Obtaining this information about the system may allow for computational determination of the relative conformation of the exciton coupled Cy5-Cy5 dimer from the absorption and CD data alone. Such a calculation is beyond the scope of this dissertation but provides an excellent field for future investigation. H and J component determination through absorption and CD spectra works for highly-coupled exciton systems, and can be utilized for other similar systems in the future.

# Supplementary Figures



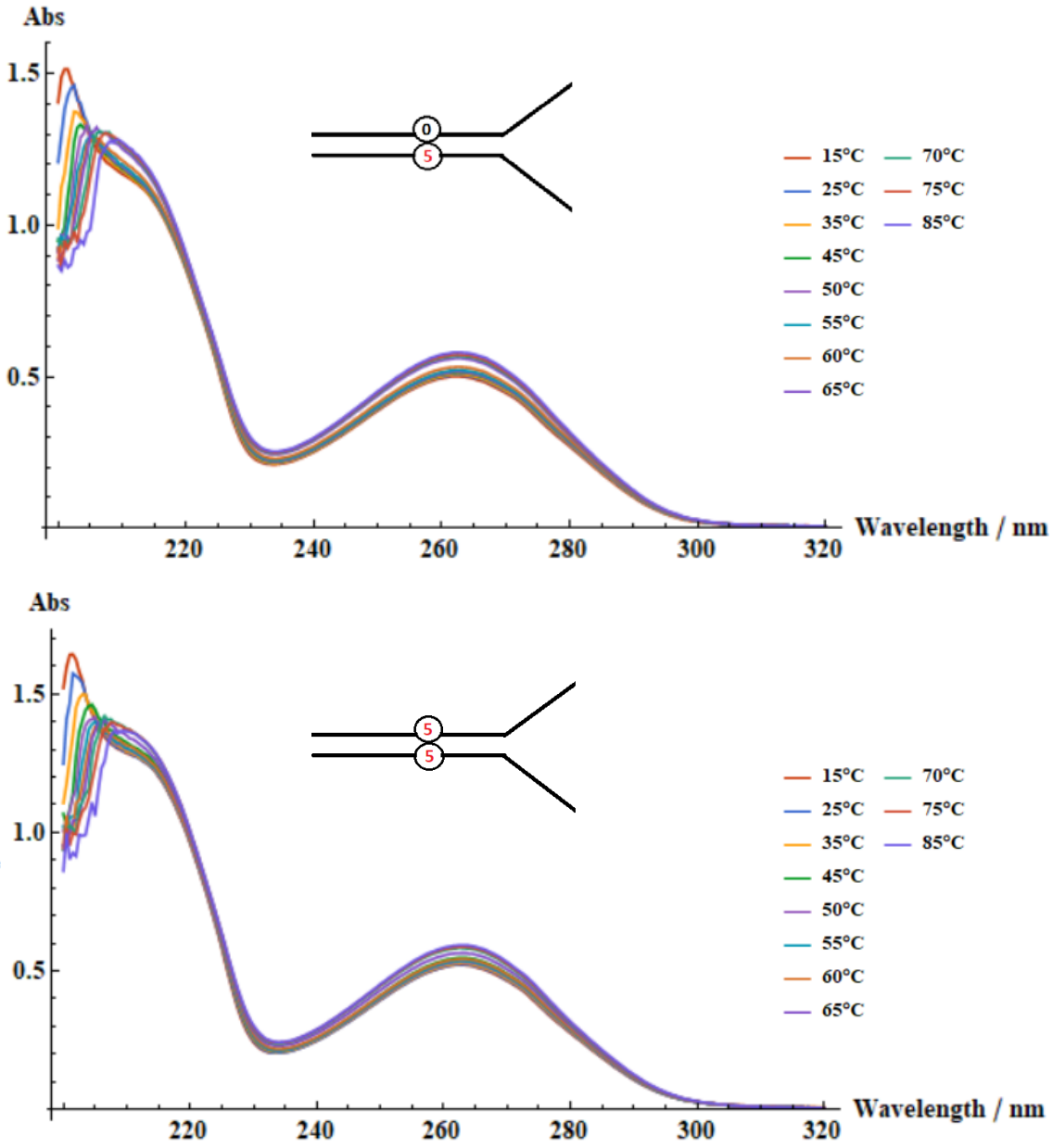



**Figure S1**: Nucleotide region absorbance with changing temperature (D3-5, D5-3, D5-0, D5-5 in order of appearance). The temperature at which each spectrum was collected in ºC is shown in the legend on the right.

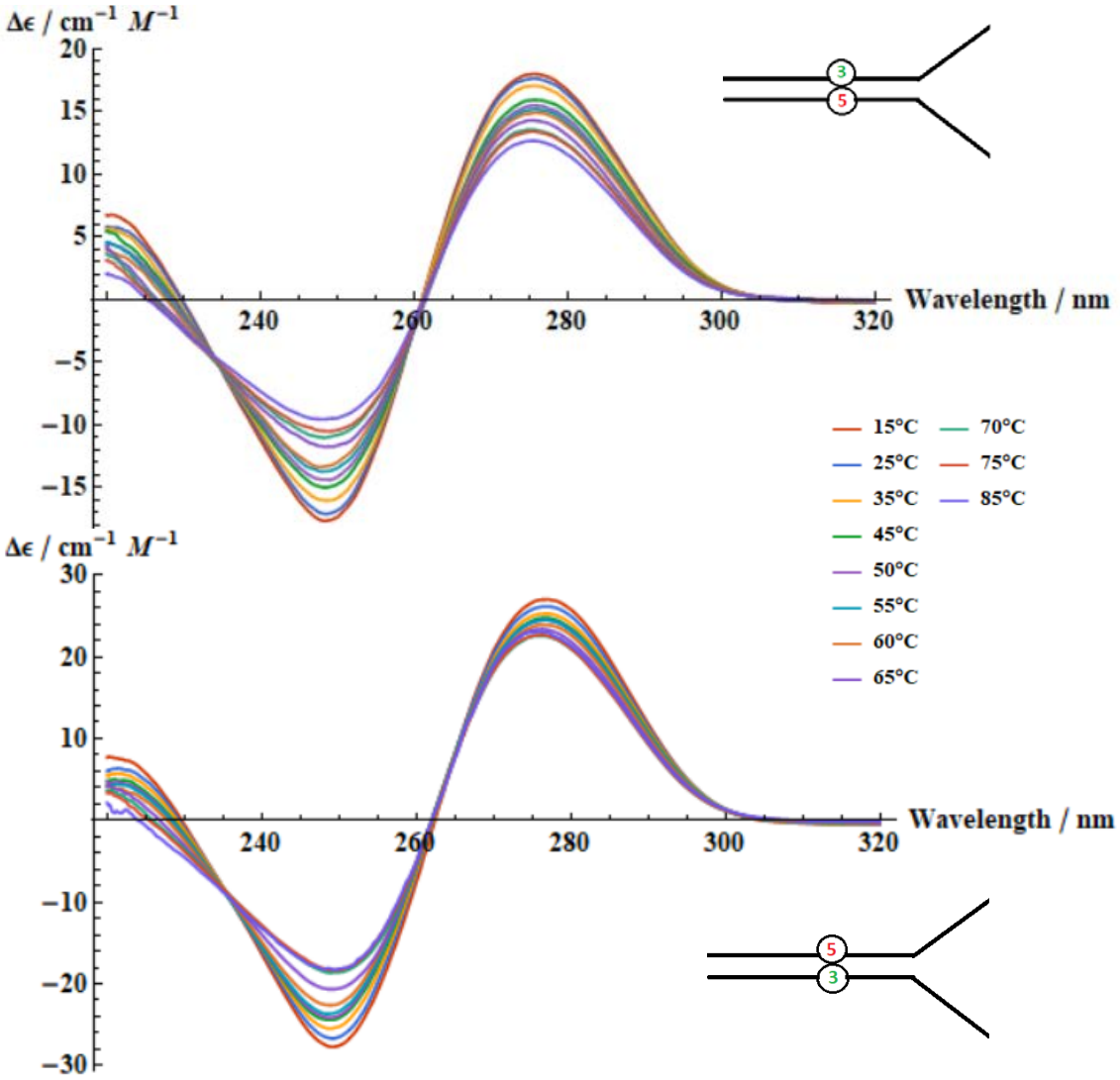



**Figure S2**: D3-5 and D5-3 CD in the nucleotide region with changing temperature. The temperature at which each spectrum was collected in ºC is shown in the legend on the right.

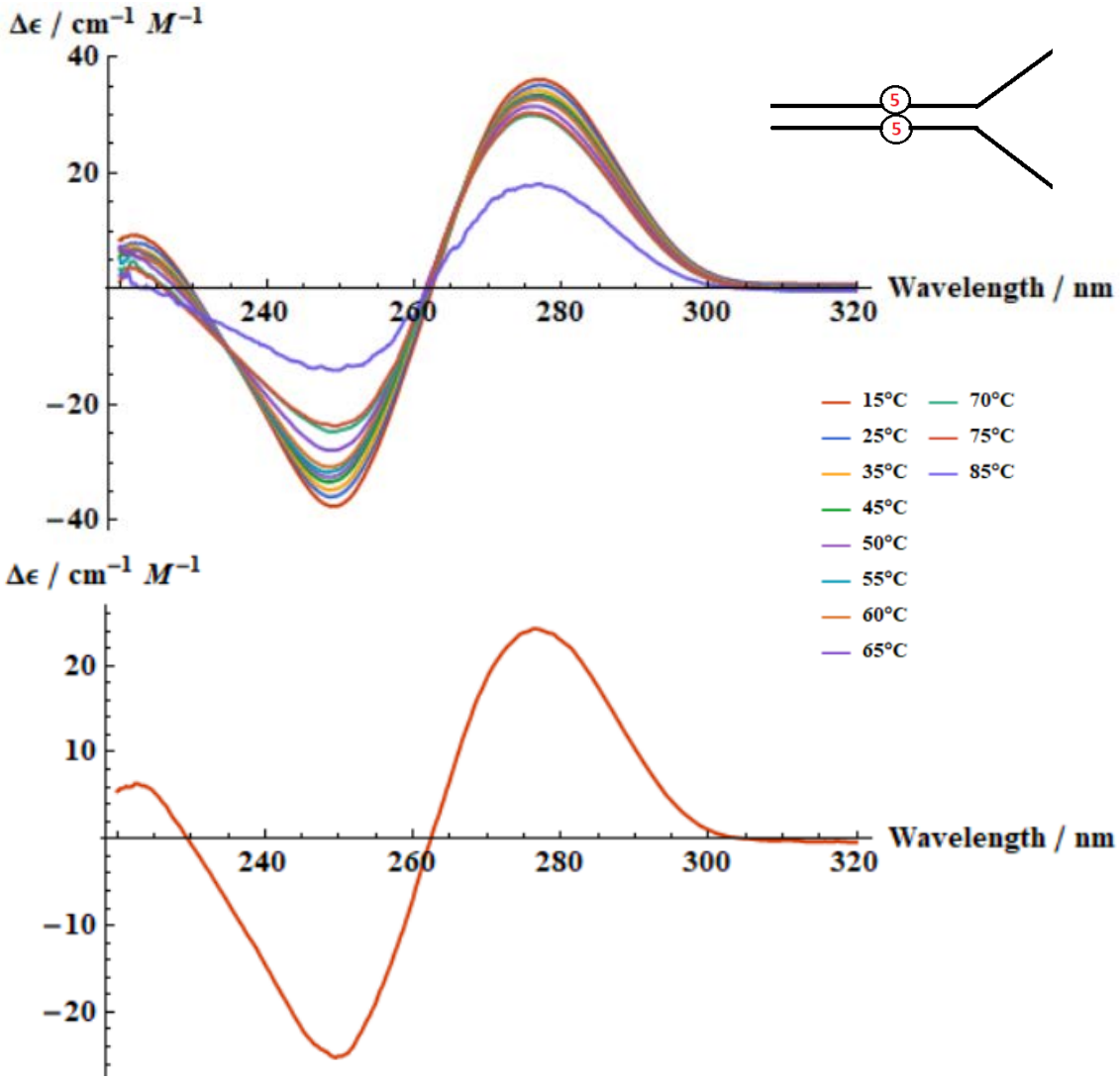



**Figure S3**: Nucleotide region of the temperature-dependent D5-5 CD spectra (top) and the follow-up D5-5 scan at 15 ºC (bottom). The legend describes the temperature at which the spectrum was collected in ºC.

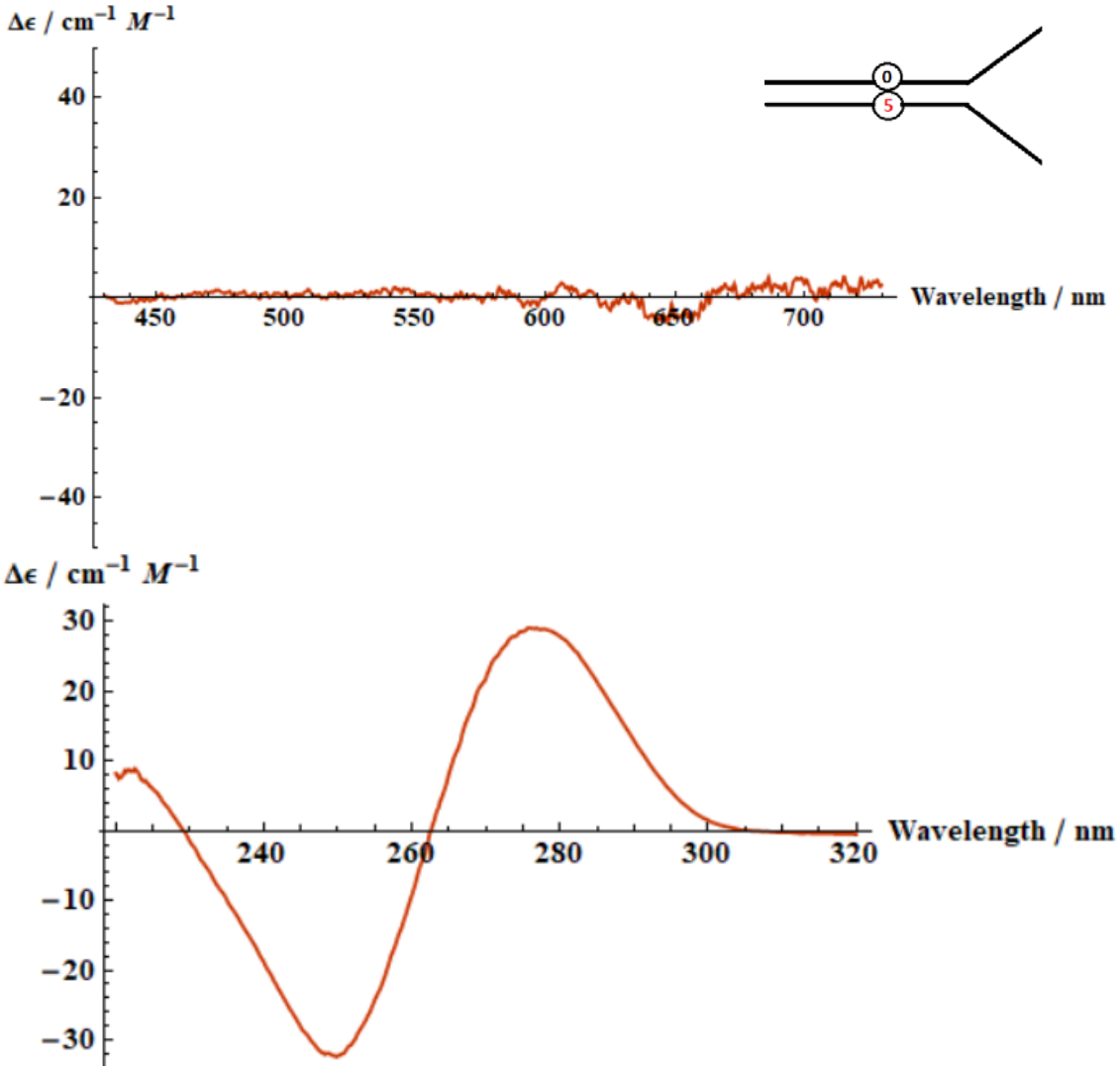



**Figure S4**: CD spectra for D5-0 DNA at 25 ºC in both the fluorophore region (top) and the DNA region (bottom). The Cy5 monomer in D5-0 is uncoupled, and so as expected it shows no peaks in the fluorophore region. The peaks in the DNA region for these data mimic what we observed for the other DNA constructs and support the validity of these results.

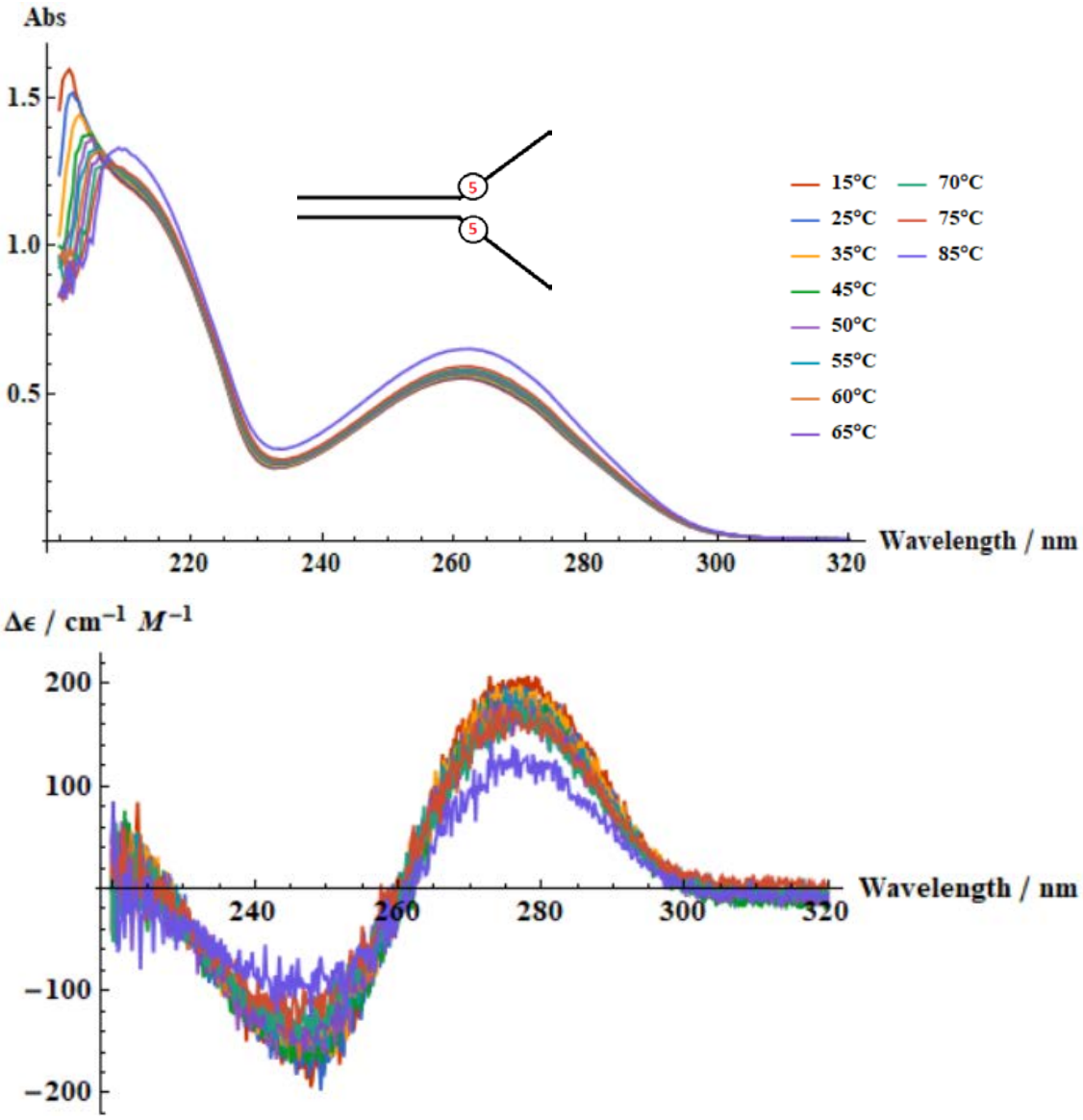



**Figure S5:** Nucleotide region absorbance (top) and CD spectra (bottom) for F5-5 with changing temperature. The key to the right indicates the temperature at which each spectrum was collected in ºC. The absorbance spectra show the same general shape and trend as seen in the duplex-labeled DNA spectra. The CD spectra show the characteristic positive and negative peaks of DNA, matching the other duplex-labeled spectra despite showing a higher level of noise.

# Glossary

**Absorption**: A measure of the amount of light that is absorbed by the electrons of a molecule. Usually taken as a spectrum across a range of wavelengths.

**Chemical bond**: An attractive interaction between atoms that causes the atoms to share their electrons. This is different from interactions like hydrogen bonding and exciton coupling because chemical bonding alters electron orbitals.

**Circular Dichroism**: A measure of how well a sample absorbs circularly polarized light, which depends on both the environment and the orientation of the molecules in the solution. Molecules with opposite orientations will produce alternately positive and negative peaks in the spectrum, while there is no signal for randomly oriented molecular dipoles.

**Conformation**: The relative positions and orientations of molecules compared to one another.

**Dimer**: A pair of molecules that are held together by an attractive interaction.

**DNA bases**: Adenine, cytosine, guanine, or thymine (A, C, G, or T). These bases are attached to the DNA backbone and can form hydrogen bonds with their complementary base on another strand of DNA to form the classic double helix. Adenine hydrogen bonds with thymine and guanine hydrogen bonds with cytosine.

**DNA melting**: The process by which the hydrogen bonds between DNA bases are broken and a duplex region of DNA becomes single-stranded. Not related to a change between solid and liquid states.

**Exciton coupling**: A form of interaction between molecules that causes the energy from an electronic excitation in one molecule to be transferred to the other molecule. For some systems, exciton coupling can lead to vibronic coupling.

**Electrons**: Particles that orbit the center of every atom and carry a negative charge. The exact paths of these electrons cannot be described classically, but they are limited to quantized energy levels and can only move into a new energy level if they absorb exactly the necessary amount of energy.

**Emission**: The release of light from electrons as they move from an excited state to a lower energy state.

**Energy**: The ability to do work or cause a change. For electrons, more energy means orbiting farther from the nucleus (center of the atom). For light, more energy means waves with greater frequency and smaller wavelength.

**Fluorescence**: Property of a molecule that causes it to release energy absorbed by electrons as photons of light. These photons always contain less energy than was originally absorbed. The amount and wavelength at which a sample fluoresces can be measured spectroscopically and is the basis for many advanced techniques (like 2DFS).

**Fluorophore**: A molecule that fluoresces strongly with a measurable emission.

**H and J components**: In-phase and out-of-phase oscillations of the electrons of coupled molecules. Both components are present in exciton-coupled dimers with a large (near 90º) angle between them. The relative levels of these components in a sample of excitons describes the strength of coupling between molecules.

**Hydrogen bond**: An attractive interaction by which a part of a molecule with a lot of electrons is attracted to a part of another molecule lacking electrons. This is one of the major interactions that hold bases together in duplex DNA.

**Spectra**: Graph showing how a spectroscopy measurement changes over a range of wavelengths.

**Spectroscopy**: Experimental analysis of the interaction of matter with light.

**Visible Region**: The range of wavelengths of light that are visible to the human eye, about 800 nanometers (nm) to 300 nm. Longer wavelengths correspond to more reddish light and lower energy, while lower wavelengths correspond to more blueish light and higher energy. Green light is in-between.

**Vibronic coupling**: Interaction between two exciton-coupled molecules which causes their nuclei to vibrate together when excited. This is analogous to two pendula connected by a spring, in that when one pendulum begins swinging, the other will begin moving with it due to their interaction.

**Wavelength**: Describes the distance between the crests of the light wave. The smaller this distance, the greater the energy contained by the photon.

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