

LOCAL STRUCTURE AND CONFORMATIONAL DISORDER AT SINGLE-
STRANDED–DOUBLE-STRANDED DNA JUNCTIONS

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DISSERTATION ABSTRACT

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DNA functions as a stable repository for heritable information across generations. However, the structure of DNA within the cell must be dynamic, allowing for thermally induced fluctuations to facilitate the recognition and assembly of functional protein-DNA complexes. For example, the local conformations of the sugar-phosphate backbones near the replication fork junction are likely recognized by protein components during DNA replication. The presence of local backbone disorder (i.e., the absence of an ordered conformation) within duplex and ss-ds DNA junctions indicates a distribution of local backbone conformations that could, for example, facilitate kinetic competition between distinct protein regulatory factors. By fitting a theoretical model to experimental absorbance and circular dichroism (CD) spectra, the ensemble average conformations (relative orientation and distances) of Cy3 dimer probes within the DNA constructs was determined as a function of insertion site position and temperature. The results of our analyses were subsequently compared using increasingly complex models of exciton coupling between individual Cy3 labeling sites. To investigate local conformational disorder of the sugar-phosphate backbones as a function of temperature and proximity to protein binding sites, two-dimensional fluorescence spectroscopy (2DFS) was used, which allowed for direct

characterization of the local conformational disorder at the Cy3 labeling sites. The presence of local disorder at and near ss-ds DNA junctions suggests that these sites undergo rapid interconversion between different conformations, which were studied under varying DNA composition and buffer conditions, and with a novel technique, single molecule polarization sweep microscopy. The effect of assembling T4 bacteriophage helicase loading (gp59) and single strand binding protein (gp32) on the DNA ds-ss junction was examined and some of these conformations can function as secondary-structure motifs for interaction with protein complexes that bind to and assemble at these sites. This dissertation includes previously published and unpublished co-authored material.

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I am the mate and companion of people, all just as immortal and fathomless
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For my family

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Chapter I

Introduction

Chapter I serves as the introduction to the rest of the thesis. It briefly outlines a few of the major topics covered in subsequent chapters and is designed to give a non-expert adequate background to understand the material. Chapter II was published in the *Faraday Discussions* in 2019 with co-authors Justin Kittell, Loni Kringler, Amr Tamimi, Peter von Hippel, & Andrew Marcus. This chapter outlines our approach to computational simulations of circular dichroism and absorbance spectra pertaining to Cy3 labeled DNA. Chapter III was published in the *Journal of Chemical Physics* in 2022 with co-authors Justin Kittell, Peter von Hippel, & Andrew Marcus. This chapter describes the use of two-dimensional fluorescence spectroscopy to study conformational disorder at DNA ds-ss junctions. Chapter IV is an unpublished collaboration with Lulu Enkhbataar, Mohammed Sorour, Spiradoula Matsika, and Andrew Marcus. It examines potential improvements that can be made to the simulation of (iCy3)₂ labeled DNA structures with the inclusion of an atomistic partitioning of transition charges. Chapter V is an unpublished collaboration with Lulu Ehnkbataar, Jack Maurer, Patrick Herbert, Maya Pande, Peter von Hippel and Andrew Marcus. It examines how DNA base composition, salt conditions, and single strand length affect local DNA conformations, structural distributions, and the interconversion of structures on microsecond timescales. Chapter VI is an unpublished collaboration with Lulu Ehnkbataar, Tom Steinberg, Jack Maurer, Patrick Herbert, Peter von Hippel and Andrew Marcus. It discusses the use of (Cy3)₂-DNA constructs as probes of the protein-DNA complex formation found using T4 bacteriophage replisome proteins. Chapter VII is a concluding chapter which outlines some of the next logical steps for future investigators.

Background

DNA is a long chain biopolymer that is responsible for the information processing and reproduction for life on earth. It is therefore important that the structural integrity of DNA is maintained through subsequent generations and that the information content of DNA is accessible and replicated with high fidelity during cell division. The basic information content of DNA is provided by four nucleobases—adenine, thymine, guanine, and cytosine—which are

linked together by sugar-phosphate bonds to form long strands. The complementary hydrogen bonding behavior of adenine and thymine, as well as guanine and cytosine, allows single stranded DNA to come together to form duplex DNA, which can be generally thought of a double helix. The thermodynamic properties of this helix can be modified significantly by changes in temperature, buffer conditions, and nucleobase composition. While duplex DNA can adopt several conformers beyond the scope of Watson-Crick B-form DNA, notably A and Z forms, the biological activity of DNA in the cell as it is processed by proteins also leads to the emergence of many different unique topologies, as DNA must be unzipped and reorganized to carry out its function.

Much of this dissertation is based on the experimental results and theoretical calculations of spectroscopic measurements. As has been noted by countless philosophically minded chemists, the word *spectroscopy* is a Latin-Greek hybrid which shares the common etymological roots for the words for “spectrum” or “ghost”, and the suffix “-skopion” which is often translated as “to look at”. Hence, spectroscopic analysis aims to examine what we cannot easily see without the use of instrumentation. Many of the methods here outline *optical* spectroscopies, which employ regions of the electromagnetic spectrum we can physiologically perceive, however in far less detail, and with much lower sensitivity, than is required to make a proper assessment of a specimen’s detailed interactions with light. The beauty of these studies can, in part, be further appreciated by acknowledging the conformational specificity gained by using optical probes like Cy3 to study DNA structure. While native DNA nucleobases absorb ultraviolet radiation, and are commonly studied using UV-based spectroscopies, the implementation of a visible light absorbing molecule as a structural probe allows one to isolate information about specific regions of the DNA where the probe is inserted rather than the entire DNA strand.

A commonly used technique found in this dissertation is the measurement of absorbance as a function of wavelength. The wavelength of light is inversely related to the energy and frequency of the incoming photons which comprise the exciting light, and in this dissertation, we commonly adopt the use of wavenumbers (cm^{-1}) to describe the energy of these light particles. For some measurements, we have employed light sources that have a defined plane in space describing the axis of electromagnetic oscillation. Just as photons have a measurable energy, they share a polarization state, which can be held constant or constantly rotated. In the circular

dichroism measurements outlined below, we excite our specimens with two rotating polarized light sources, and ultimately report the difference in absorption found for light rotating clockwise and anticlockwise in the precession of its polarization state. When a photon is absorbed through an interaction between light and a molecule of interest, the energy can be reemitted through the process of fluorescence, which is the detection method of choice used for several of the methods below.

Finally, this work discusses in detail the results of multidimensional and ultrafast spectroscopies. In these experiments, ultrafast refers to the use of very short pulse durations (femtoseconds) in time to quickly interact with the labeled DNA samples, in order to probe the time evolution of excited states. Precise knowledge of the temporal properties of each ultrafast pulse, and its delay in time from any other incoming pulses, is necessary for the reconstruction of the spectral properties of the medium through which it passes, which is carried out through the Fourier transform. Multidimensional, namely two-dimensional, spectroscopies refer to ultrafast experiments which employ the choreographed interaction between the multiple pulses of light.

Chapter II

Measuring local conformations and conformational disorder of (Cy3)₂ dimer-labeled DNA fork junctions using absorbance and circular dichroism spectroscopy

This work was published in Volume 216 of the Faraday Discussions in 2019 as “Measuring local conformations and conformational disorder of (Cy3)₂ dimer-labeled DNA fork junctions using absorbance, circular dichroism and two-dimensional fluorescence spectroscopy”. Dylan Heussman, Loni Kringle and Amr Tamimi carried out the measurements. Dylan Heussman and Justin Kittell carried out the calculations. Peter von Hippel helped to design the experiments and provided editorial assistance. Andrew Marcus was the principal investigator for this work and provided editorial assistance and general feedback.

Introduction

While the structure of genomic DNA is generally pictured as the static B-form conformation of the Watson-Crick (W-C) duplex, many centrally important biological processes involve dynamic molecular events that require local regions of DNA to open spontaneously, thus allowing proteins to gain access to either single-stranded DNA base sequences, or to secondary structure motifs that depart from the stable W-C structure (2). For example, the elongation events of DNA replication involve the binding of proteins to non-sequence-specific positions at or near single-stranded (ss)—double stranded (ds) DNA junctions. To facilitate elementary biochemical steps of the elongation process, the sugar-phosphate backbone of DNA near ss-ds junctions likely fluctuates into a broad distribution of functionally relevant conformations to permit the proper binding of replication proteins. Thus, the nature and extent of conformational disorder at DNA junctions may be central to the molecular mechanisms of the binding and the subsequent function of the protein-DNA complexes involved.

The carbocyanine dye Cy3 is often used as a chromophore label for proteins and nucleic acids. Such fluorescently labeled biomolecular constructs are employed in diverse applications, from rapid screening of single-molecules to imaging of subcellular components (3-10). The Cy3

chromophore is comprised of an electronically conjugated trimethine group, which bridges two indole-like substituents (see Fig. 1A). The lowest energy $\pi \rightarrow \pi^*$ electronic transition occurs when the molecule is in its all-trans ground state configuration, with electric dipole transition moment (EDTM) polarized parallel to the long axis of the trimethine bridge. The absorbance spectrum of Cy3 labeled constructs exhibits a pronounced vibronic progression with energy spacing $\hbar\omega_0 \sim 1,100 \text{ cm}^{-1}$ (3). The presence of the vibronic progression indicates that the electronic transition is effectively coupled (as characterized by the Huang-Rhys parameter, $\lambda^2 \approx 0.55$) to at least one local vibrational mode of relatively high frequency (11-13), such as the Raman-active symmetric stretch of the trimethine bridge at $\sim 1,200 \text{ cm}^{-1}$ (14).

Cyanine chromophores can be chemically attached to a nucleic acid base or to an amino acid side chain via a flexible linker (3, 9). Cy3 and Cy5 are often used as a double-label pair to perform Förster resonance energy transfer (FRET) experiments (15). Such studies can provide information about inter-chromophore separation based on the resonant electronic coupling J between the optically excited Cy3 ‘donor’ and the unexcited Cy5 ‘acceptor.’ The relative fluorescence intensities of the Cy3 / Cy5 donor-acceptor pair depends on the magnitude of J , which is most sensitive to changes in inter-chromophore separation on the scale of the Förster radius $R_0 \sim 50 \text{ Å}$. FRET experiments are performed in the ‘weak-coupling regime’ (16), where the magnitude of J is small in comparison to the interactions between the chromophore and its local environment at room temperature ($\sim k_B T \approx 210 \text{ cm}^{-1}$) (11).

An alternative labeling scheme is to site-specifically incorporate the Cy3 chromophores ‘internally’ within the sugar-phosphate backbone of a single strand of DNA (see Fig. 1) (3, 8, 9). By annealing two complementary DNA strands, each containing a single internally-labeled Cy3 chromophore, a DNA construct can be formed with a (Cy3)₂ dimer (or a Cy3 monomer) at a predetermined position relative to a single-stranded (ss) – double-stranded (ds) DNA fork junction. The resulting chiral conformation of the (Cy3)₂ dimer is sterically constrained by the stabilizing interactions of the adjacently stacked bases within the DNA duplex. Conversely, the conformation of the (Cy3)₂ dimer can be destabilized by increasing the sample temperature or by positioning the probes close to the DNA fork junction where base stacking interactions are disrupted. Such internally-labeled Cy3 probes are thought to have only a minimal effect on the

local DNA structure, as their presence in relatively short oligomeric dsDNA constructs (~ 30 base pairs) does not significantly alter the denaturation temperature ($T_m = 65^\circ\text{C}$) (9).

Due to the relatively small inter-chromophore separations within internally-labeled $(\text{Cy3})_2$ dimer-DNA constructs ($R_{AB} \sim 5 \text{ \AA}$), the magnitude of the resonant coupling can be significantly greater than $k_B T$, approaching values comparable to the vibrational relaxation energy ($\lambda^2 \hbar \omega_0 \sim 600 \text{ cm}^{-1}$) (17). In this ‘intermediate-to-strong coupling regime’ (16), the $(\text{Cy3})_2$ dimer forms delocalized excitons comprised of symmetric and anti-symmetric superpositions of the electronic-vibrational product states of the component Cy3 monomers (18, 19). While FRET-based experiments can provide limited information about inter-chromophore separation, experiments that can resolve the relative intensity contributions from the symmetric and anti-symmetric excitons of internally-labeled $(\text{Cy3})_2$ dimer-DNA constructs can reveal detailed information about local dimer conformation and the ways in which fluctuations of the local environment broaden spectra and perturb the coupling. As we discuss further below, this information can be sensitively probed using absorbance, circular dichroism (CD), and two-dimensional fluorescence spectroscopy (2DFS) (17).

In previous work, we used absorbance and CD spectroscopy to study the monomer Cy3 and the dimer $(\text{Cy3})_2$ duplex DNA constructs depicted in Fig. 1A and 1B, respectively, over the temperature range $15\text{--}60^\circ\text{C}$ below the dsDNA denaturation transition ($T_m = 65^\circ\text{C}$) (17). We analyzed our results with the aid of the Holstein-Frenkel Hamiltonian model for an electronically coupled dimer of two-electronic-level molecules, each with their electronic transition coupled to a single vibrational (harmonic) mode (19). We found that the conformation of the $(\text{Cy3})_2$ -dsDNA construct undergoes systematic variation as a function of temperature, in terms of the inter-chromophore separation R_{AB} , the twist angle ϕ_{AB} (defined in Fig. 1D) and the degree of structural disorder. In the current work, we extend our approach to examine the local conformations of the $(\text{Cy3})_2$ dimer-labeled fork DNA construct depicted in Fig. 1C. DNA forks and other ss-ds DNA junctions are sites of interaction for proteins that participate in DNA replication, recombination and repair (20, 21). The local conformation of the sugar-phosphate backbone and conformational disorder at the fork junction is a consequence of DNA ‘breathing’ – i.e., thermally activated fluctuations of the folded secondary structure – that is a key factor affecting the assembly and stability of protein components (2). To characterize the local

conformations of the (Cy3)₂ dimer-labeled fork DNA construct, we introduce the structural parameter of the inter-chromophore tilt angle θ_{AB} (defined in Fig. 1D) to account for the disruption of hydrogen bonds and stacking of bases within the ss region adjacent to the (Cy3)₂ dimer. With the inclusion of this additional orientational parameter, we use the (Cy3)₂ dimer-labeled fork DNA construct as a test system to examine the reliability of the point-dipole approximation in estimating the resonant coupling strength.

In general, the resonant electronic coupling J is determined by integrating the Coulomb interactions between electric transition charge densities of the component monomers (11). Direct calculation of the atomic transition charge density by quantum chemical methods would provide the most accurate determination of the coupling strength, albeit at a significant computational cost (22). For expediency, the point-dipole approximation is often invoked, where the transition charge density is focused at a single point, and J is described as the interaction between two point-dipole moments. Of course, for inter-chromophore separations comparable to the monomer size, the point-dipole approximation cannot reflect the details of the transition charge density, which is extended across the length of the trimethine bridge and indole substituents of the Cy3 molecule. To examine the reliability of the point-dipole model we here implement an extended-dipole model (23), which includes a displacement parameter $\vec{l}$ to account for the length over which the transition charge q is distributed. In the extended dipole model, the displacement parameter is oriented parallel to the EDTM ($\vec{\mu}$), and the magnitudes of the transition charge and displacement satisfy $ql = \mu$ (22-24). We have performed our analyses for both the (Cy3)₂-ds DNA and (Cy3)₂-fork DNA constructs using both the point-dipole and extended dipole models. Our results indicate that both models yield very similar values for the structural parameters of these DNA constructs over the full range of temperatures studied.

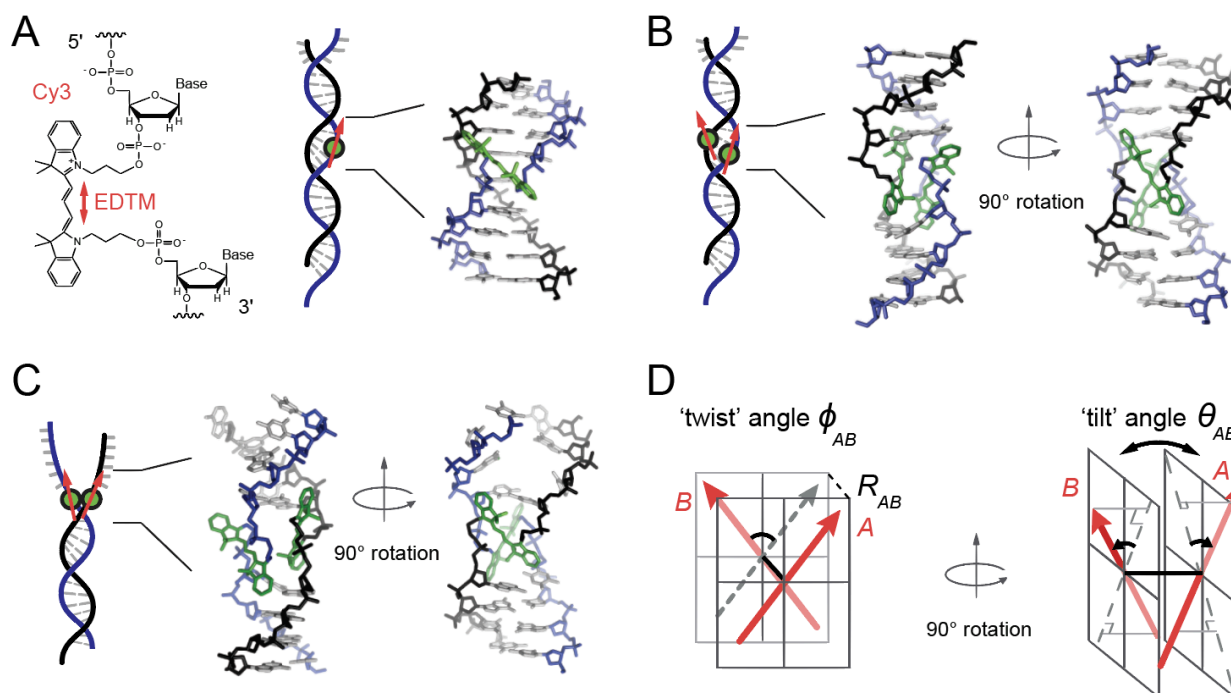


Figure 1. Model structure of the internally labeled Cy3 monomer and (Cy3)₂ dimer in dsDNA. **(A)** The structural formula of the internally labeled Cy3 chromophore is shown with its insertion linkages to the 3' and 5' segments of the sugar-phosphate backbone of ssDNA. A red double-headed arrow indicates the orientation of the electric dipole transition moment (EDTM), which lies parallel to the axis of the trimethine bridge in the all-trans configuration. **(B)** A (Cy3)₂ dimer-dsDNA construct is formed by annealing two complementary DNA strands, which each contain a site-specifically positioned Cy3 chromophore. Space-filling structural models performed using the Spartan program (Wavefunction, Inc.) suggest that the dimer exhibits the same approximate *D*₂ symmetry as right-handed (B-form) helical dsDNA (17). **(C)** A (Cy3)₂ dimer-fork DNA construct contains the dimer probe near the ss-ds DNA fork junction. The local conformation of the (Cy3)₂ dimer probe is expected to reflect the disruption of base-stacking interactions that occurs near the fork junction. In panels **(A – C)**, the sugar-phosphate backbones of the conjugate strands are shown in black and blue, the bases in gray, and the Cy3 chromophores in green. **(D)** The structural parameters that define the local conformation of the (Cy3)₂ dimer are the inter-chromophore separation vector $\mathbf{R}_{AB}$, the twist angle ϕ_{AB} , and the tilt angle θ_{AB} .

In addition to determining the average local conformations adopted by the backbone labels within the (Cy3)₂-dsDNA and (Cy3)₂-fork DNA constructs, we further examine structural disorder by interpreting inhomogeneous spectral line broadening as an indicator of this effect. Molecular dynamics simulations suggest that the Cy3 chromophore(s), when incorporated internally within the DNA duplex, may exist in a variety of conformations (25). This dispersion

is due to the coupling between the Cy3 probe chromophores and the fluctuating local DNA environment, which induces the transition energies of the Cy3 probes to occur over a broad spectral range.

Experimental Methods

Sample Preparation. We show in Table 1 the sequences and nomenclature of the internally labeled Cy3 monomer and (Cy3)₂ dimer DNA constructs used in this work. Oligonucleotide samples were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and used as received. For our absorbance and circular dichroism (CD) measurements, we prepared solutions with sample concentrations of 1 μ M and a standard aqueous buffer of 10 mM Tris, 100 mM NaCl, and 6 mM MgCl₂. We combined complementary oligonucleotide strands to form the Cy3 monomer- and (Cy3)₂ dimer-labeled DNA constructs, which contain both ds and ss regions. The Cy3 monomer-labeled constructs contained a thymine base (T) in the complementary strand position directly opposite to the Cy3 probe chromophore. For the ‘duplex’ constructs, the Cy3 probes are positioned deep within the double-stranded region of the DNA. For the ‘fork’ constructs, the Cy3 probes are positioned near the ss — ds DNA fork junction. Prior to the experiments, the sample solutions were annealed by heating to 95 °C for 3 minutes before they were allowed to slowly cool to room temperature.

Table 1. Base sequences and nomenclature for the Cy3 monomer and (Cy3)₂ dimer labeled DNA constructs used in these studies. The horizontal line indicates the regions of complementary base pairing.

DNA construct	Nucleotide base sequences
Cy3 monomer duplex DNA	3'-GTC AGT ATT ATA CGC TCy3C GCT AAT ATA TAC GTT TTT TTT TTT TTT TTT TTT TTT TTT T-5' 5'-CAG TCA TAA TAT GCG A T G CGA TTA TAT ATG CTT TTA CCA CTT TCA CTC ACG TGC TTA C-3'
(Cy3) ₂ dimer duplex DNA	3'-GTC AGT ATT ATA CGC TCy3C GCT AAT ATA TAC GTT TTT TTT TTT TTT TTT TTT TTT TTT T-5' 5'-CAG TCA TAA TAT GCGA Cy3G CGA TTA TAT ATG CTT TTA CCA CTT TCA CTC ACG TGC TTA C-3'
Cy3 monomer fork DNA	3'-GAG GGA GCA CAG CAG AGG TCA GTA TTA TAC GCT Cy3CG CTG GTA TAC CAC GTT T (x29)-5' 5'-CTC CCT CGT GTC GTC TCC AGT CAT AAT ATG CGA T AT GCT TTT ACC ACT TTC ACT CAG GTG CTT A-3'
(Cy3) ₂ dimer fork DNA	3'-GAG GGA GCA CAG CAG AGG TCA GTA TTA TAC GCT Cy3CG CTG GTA TAC CAC GTT T (x29)-5' 5'-CTC CCT CGT GTC GTC TCC AGT CAT AAT ATG CGA Cy3AT GCT TTT ACC ACT TTC ACT CAG GTG CTT A-3'

Absorbance and CD Measurements. We performed absorbance measurements using a Cary 3E UV-Vis spectrophotometer, and CD using a Jasco model J-720 CD spectrophotometer.

Temperature-dependent measurements were performed over the range 15 – 75 °C. Samples were held in a 1 cm quartz cuvette, and the temperature was controlled to within 0.1 °C using a Peltier thermoelectric heating block. We determined all spectra over the range 200 – 700 nm to examine the spectral region of the native bases (~275 nm), in addition to that of the Cy3 probe(s) (~540 nm). For all of the samples, we confirmed that the ds region adopted the anticipated Watson-Crick B-form conformation by examination of the ultraviolet absorbance and CD of the native bases (26).

In Fig. 2, we show absorbance and CD spectra at 25 °C for the Cy3 monomer- and dimer-labeled duplex DNA constructs, and the (Cy3)₂ dimer-labeled fork DNA construct. We note that the spectra of the Cy3 monomer fork DNA construct (not shown) are qualitatively similar to those of the corresponding duplex construct. The absorbance spectra of the Cy3 monomer duplex and fork DNA constructs exhibit a progression of vibronic features with the first (0–0) peak centered at 549 nm (18,280 cm⁻¹). The vibronic progression is still present in the spectrum of both the (Cy3)₂ dimer-labeled duplex DNA construct and the dimer-labeled fork DNA construct. However, individual vibronic features of the dimer are broadened relative to those of the monomer, and the ratio of the 0–0 to 1–0 vibronic peak intensities has decreased relative to that of the monomer [$I_{mon}^{(0-0)} / I_{mon}^{(1-0)} = 1.60$]. While the monomer CD signal is very weak (as expected), the CD of both the dimer duplex and fork constructs exhibit a progression of bisignate lineshapes (i.e. a change of sign within a given vibronic band), which is a signature of vibronic excitons in a chiral dimer (19, 27, 28). Furthermore, the CD spectra of the (Cy3)₂ dimer-duplex and fork DNA constructs have opposite signs, indicating that the two systems have opposite chiral symmetries.

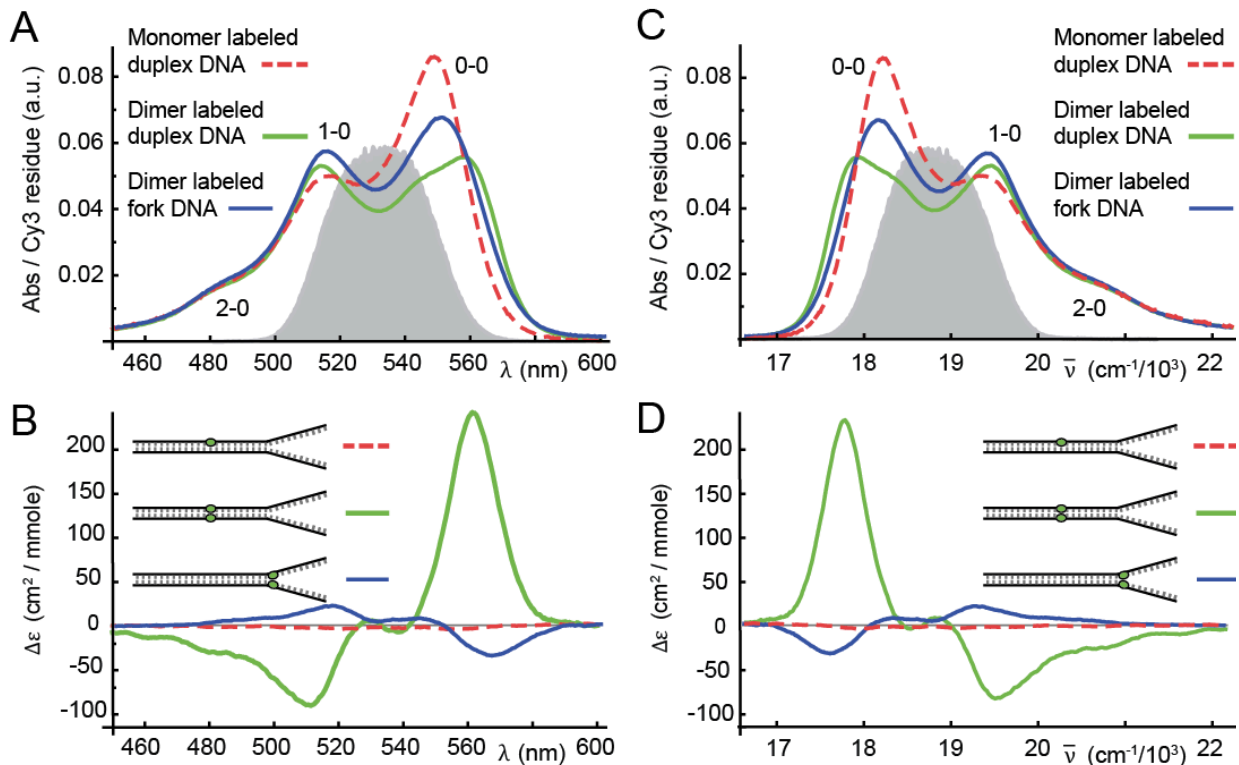


Figure 2. Room temperature (25 °C) absorbance (**A** & **C**) and CD (**B** & **D**) spectra for Cy3 monomer duplex (dashed red), (Cy3)₂ dimer duplex (solid green), and (Cy3)₂ dimer fork (solid blue) DNA constructs. The Here $\Delta\epsilon$ is the differential absorbance of left and right circular polarized light. Nucleotide sequences and placement of the chromophore probes are indicated in Table 1. The spectra are shown as a function of optical wavelength (**A** & **B**) and as a function of wavenumber (**C** & **D**). The vibronic features of the monomer absorption spectra are labeled $n_e - 0$, where n_e ($= 0, 1, 2$) indicates the vibrational occupancy of the electronically excited monomer. An example laser spectrum used for 2DFS experiments is shown in gray. The laser spectrum spans a region containing both the 0–0 and 1–0 vibronic sub-bands. 2DFS experiments will be described in detail in Chapter II.

Theoretical Modeling

Absorbance and CD Spectra. Building upon previous work (17), we implemented the Holstein-Frenkel (H-F) model to describe the electronic-vibrational structure of the (Cy3)₂ dimer probe, similar to the approach taken by others (19, 34–38). The H-F model accounts for the interactions between electronic and vibrational degrees of freedom internal to each chromophore, and the conformation-dependent resonant electronic coupling J between chromophores. The presence of

both types of interactions (excitonic and vibronic) gives rise to delocalized symmetric and anti-symmetric transitions of well-defined energies and polarizations, which feature prominently in absorbance and CD spectra. Because the positions and amplitudes of the spectroscopic features depend sensitively on the conformation of the (Cy3)₂ dimer, an optimization procedure that compares simulated to experimental spectra (17) allows us to extract structural information about the (Cy3)₂ dimer.

In the H-F model, each Cy3 monomer is treated as a two-electronic-level molecule with ground state $|g\rangle$, excited state $|e\rangle$, and transition energy ε_{eg} . The monomers are labeled A and B , with electric dipole transition moment (EDTM) $\vec{\mu}_{eg}^{A(B)}$. The electronic transition of each Cy3 monomer is coupled to a single harmonic mode with frequency ω_0 . The monomer Hamiltonian is given by

$$\hat{H}_{A(B)} = \varepsilon_{eg}|e\rangle_{A(B)}\langle e| + \hbar\omega_0\hat{b}_{A(B)}^\dagger\hat{b}_{A(B)} + \hbar\omega_0\{\lambda(\hat{b}_{A(B)}^\dagger + \hat{b}_{A(B)}) + \lambda^2\}|e\rangle_{A(B)}\langle e| \quad (1)$$

In Eq. (1), the electronic-vibrational coupling is characterized by the Huang-Rhys parameter $\lambda^2 = d^2\omega_0/2\hbar$, where d is the vibrational coordinate displacement of the electronic excited state potential minimum relative to the electronic ground state. The value of λ^2 is the number of vibrational quanta absorbed by the system upon optical excitation. The operator $\hat{b}_{A(B)}^\dagger$ ($\hat{b}_{A(B)}$) creates (annihilates) a vibrational excitation in the un-shifted electronic potential surface. The dimer Hamiltonian is

$$\hat{H}_{dim} = \hat{H}_A + \hat{H}_B + J\{|eg\rangle\langle ge| + |ge\rangle\langle eg|\} \quad (2)$$

where the final term couples the singly excited electronic states of the monomers. Here $|eg\rangle$ is the state in which monomer A is electronically excited and monomer B is unexcited, and $|ge\rangle$ is the state in which the A and B labels are interchanged. The resonant electronic coupling J depends on dimer conformation in terms of the Coulomb interaction between monomer site transition charge densities (11)

$$J = (4\pi\epsilon\epsilon_0)^{-1} \int_{-\infty}^{\infty} d\vec{r}_A \int_{-\infty}^{\infty} d\vec{r}_B \rho_A^{ge}(\vec{r}_A) \rho_B^{eg}(\vec{r}_B) / |\vec{R}_{AB}| \quad (3)$$

where $\rho_{A(B)}^{ge}(\vec{r}_{A(B)}) = \langle g |_{A(B)} \hat{\rho}(\vec{r}_{A(B)}) | e \rangle_{A(B)}$.

The delocalized excitons and corresponding energies of the coupled AB dimer are obtained by diagonalization of the Hamiltonian given by Eq. (2) (17). The singly excited states are symmetric (+) and anti-symmetric (−) superpositions of electronic-vibrational products $|e_{\pm}^{(\alpha)}\rangle = \sum_{n_e, n_g} c_{\pm}^{(\alpha)} [u_{n_e, n_g} |eg\rangle \pm u_{n_g, n_e} |ge\rangle]$. Here, the coefficients $c_{\pm}^{(\alpha)} u_{n_e, n_g}$ depend on the vibrational coordinates of monomers A and B , $n_{g(e)}$ ($= 0, 1, \dots$) are the number of vibrational excitations in the un-shifted (shifted) potential of an unexcited (excited) monomer, and α ($= 0, 1, \dots$) is an index in order of increasing state energy (19).

The absorbance spectrum is the sum of symmetric (+) and anti-symmetric (−) exciton features

$$\sigma_H(\varepsilon) = \sigma_{H+}(\varepsilon) + \sigma_{H-}(\varepsilon) \quad (4)$$

where $\sigma_{H\pm}(\varepsilon) = \sum_{\alpha} \left| \langle 0 | \vec{\mu}^{tot} | e_{\pm}^{(\alpha)} \rangle \right|^2 L_H(\varepsilon - \varepsilon_{\pm, \alpha})$, $\vec{\mu}^{tot} = \vec{\mu}_{eg}^A + \vec{\mu}_{eg}^B$ is the collective EDTM, and $L_H(\varepsilon) = \frac{1}{2} \Gamma_H / [\varepsilon^2 + (\frac{1}{2} \Gamma_H)^2]$ is a Lorentzian function that represents the homogeneous lineshape of the transition with eigen-energy $\varepsilon_{\pm, \alpha}$ and FWHM line width Γ_H . Similarly, the CD spectrum is the sum of symmetric and anti-symmetric rotational strengths

$$CD_H(\varepsilon) = \sum_{\alpha} \left[RS_{H+}^{(\alpha)} L_H(\varepsilon - \varepsilon_{+, \alpha}) + RS_{H-}^{(\alpha)} L_H(\varepsilon - \varepsilon_{-, \alpha}) \right] \quad (5)$$

where $RS_{\pm}^{(\alpha)} = \frac{\varepsilon_{eg}}{4\hbar c |\vec{\mu}_{eg}|^2} \langle 0 | \vec{\mu}_{eg}^A | e_{\pm}^{(\alpha)} \rangle \times \langle e_{\pm}^{(\alpha)} | \vec{\mu}_{eg}^B | 0 \rangle \cdot \vec{R}_{AB}$. In the above expressions, we have defined the ground electronic-vibrational state of the AB dimer $|0\rangle = |gg\rangle$. Because the dimer conformation is subject to disorder due to DNA breathing, the homogeneous absorbance and CD line shapes are convolved with an inhomogeneous distribution function $G_I(\varepsilon_{\pm, \alpha}) = \exp \left[-(\varepsilon_{\pm, \alpha} - \bar{\varepsilon}_{\pm, \alpha})^2 / 2\sigma_I^2 \right]$, which is centered at the average transition energy $\bar{\varepsilon}_{\pm, \alpha}$ and has standard deviation σ_I . The final expressions for the absorbance and CD spectra are given by the

Voigt profiles $\sigma(\varepsilon) = \int_{-\infty}^{\infty} d\varepsilon' \sigma_H(\varepsilon - \varepsilon') G_I(\varepsilon')$ and $CD(\varepsilon) = \int_{-\infty}^{\infty} d\varepsilon' CD_H(\varepsilon - \varepsilon') G_I(\varepsilon')$, respectively.

Estimation of the Resonant Electronic Coupling. In the point-dipole approximation, the electronic coupling is given by (30)

$$J = |\mu_{eg}|^2 (4\pi\epsilon\epsilon_0)^{-1} |\vec{R}_{AB}|^{-3} [\hat{d}_A \cdot \hat{d}_B - 3(\hat{d}_A \cdot \hat{R}_{AB})(\hat{R}_{AB} \cdot \hat{d}_B)] \quad (14)$$

where $\hat{d}_A$ and $\hat{d}_B$ are unit vectors that specify the monomer EDTM directions (see Fig. 1D). The point-dipole approximation provides a reasonable value for J when the inter-chromophore separation is much greater than the molecular size (11). In our previous studies, we estimated the long-axis dimension of the Cy3 chromophore to be ~ 14 Å and the inter-chromophore separation to be $R_{AB} \sim 6$ Å (17). In our current work, we have carried out additional analyses using the extended-dipole model (23, 24), which includes a one-dimensional displacement parameter $\vec{l}$ to account for the finite size of the chromophore. Each dipole is represented as two point charges of equal magnitude and opposite sign ($\pm q$) separated by the distance l . The direction of $\vec{l}$ is the same as that of the monomer EDTM. The resonant coupling between monomers A and B in the extended-dipole model is

$$J = |\mu_{eg}|^2 (4\pi\epsilon\epsilon_0)^{-1} l^{-2} \left[\frac{1}{R_{AB}^{++}} - \frac{1}{R_{AB}^{-+}} - \frac{1}{R_{AB}^{+-}} + \frac{1}{R_{AB}^{--}} \right] \quad (15)$$

where $ql = \mu_{eg}$ and the distances between point charges are $R_{AB}^{\pm\pm} = [R_{AB} \pm l(\hat{d}_A - \hat{d}_B)/2]$, $R_{AB}^{-+} = [R_{AB} - l(\hat{d}_A + \hat{d}_B)/2]$, and $R_{AB}^{+-} = [R_{AB} + l(\hat{d}_A + \hat{d}_B)/2]$ (24). For all of the calculations that follow, we used the measured EDTM value $\mu_{eg} = 12.8$ D, which we determined by integration of the absorbance lineshapes of the Cy3 monomer duplex and fork DNA constructs (17). We estimated $l = 7$ Å by assuming the same value that was used by Knoester and co-workers to model the coupling between carbocyanine dyes of similar structure to Cy3 (24). The above values imply $q = 0.38e$ (with e the charge of an electron). In both extended- and

point-dipole models, the value of J depends on structural parameters that specify the Cy3 dimer conformation – i.e., the inter-chromophore separation R_{AB} , the twist angle ϕ_{AB} , and the tilt angle θ_{AB} (see Fig. 1D).

In Fig. 3, we compare the results of calculations for the resonant electronic coupling based on the point-dipole model (shown in green) versus the extended-dipole model (shown in blue). In these calculations, we have set the inter-chromophore separation $R_{AB} \sim 5 \text{ \AA}$, which is close to the value obtained from our previous analyses for the (Cy3)₂ dimer duplex DNA construct (17). In Figs. 3A – 3C, the twist angle ϕ_{AB} is varied for fixed values of the tilt angle $\theta_{AB} = 0, 60$ and 100° , respectively. For small θ_{AB} , the point-dipole and extended-dipole models exhibit qualitatively similar sinusoidal dependences on ϕ_{AB} . However, the point-dipole approximation generally overestimates the magnitude of the coupling strength for arbitrary angles. The effect of the finite size of the chromophores, which is qualitatively captured by the extended-dipole model, is revealed for the case of a side-by-side geometry with $\phi_{AB} = 0^\circ$, and subsequently varying the tilt angle (see Fig. 3D). Here the extended-dipole model exhibits divergences at $\theta_{AB} = 90$ and 270° , which are due to the repulsive close-encounters between like charges, and a local minimum at the head-to-tail geometry of $\theta_{AB} = 180^\circ$, which is dominated by the attractive interaction between opposite charges. This ‘finite-size effect’ is entirely neglected by the point-dipole model, which varies smoothly with tilt angle, and reaches a local maximum at $\theta_{AB} = 180^\circ$. The stability of the head-to-tail conformation is most significant for increasing values of ϕ_{AB} (see Figs. 3E and 3F), in which case the repulsive barriers that are present for small values of ϕ_{AB} vanish. The above comparison indicates that, in principle, very different results might be obtained from analyses that employ the point-dipole model versus the physically more realistic extended-dipole model. Nevertheless, as we discuss further below, the results of our optimization analysis for the (Cy3)₂ dimer DNA constructs reveal that the two models produce very similar values for the conformational parameters, thus suggesting that the states accessible to the dimer are those for which the two models agree.

Multi-Parameter Optimization Procedure. To efficiently explore the space of structural parameters needed to model the absorbance, CD and 2DFS of the (Cy3)₂ dimer DNA constructs, we implemented an automated multi-variable regression analysis. The procedure is similar to one we have used in the past (17, 30, 39, 41), in which a random search algorithm generates an initial

set of input parameters, and commercial software (KNITRO) (42) is used to refine the corresponding solutions. For each set of input trial parameters, we calculate a linear least-squares target function χ^2 , which guides the selection of parameter values for subsequent iterations. The optimized solutions correspond to minimization of the target function. Error bars associated with the optimized parameters were determined by a 1% deviation of the target function from its minimized value. The results of our optimization analysis of the dimer spectra are presented in Table II and Table III, and discussed further below.

Comparison Between Point-Dipole and Extended-Dipole Models. We performed optimization calculations to simulate the absorbance and CD spectra of the Cy3 labeled DNA constructs (see Table I). For these calculations, we used values that were previously established for the monomer EDTM, $\mu_{eg} = 12.8$ D, and the homogeneous line width, $\Gamma_H = 186$ cm⁻¹ (17). For calculations of the Cy3 monomer duplex and fork DNA constructs, we applied the monomer Hamiltonian given by Eq. (1) to our temperature-dependent data, and thereby determined optimized values for the monomer electronic transition energy ε_{eg} , the Huang-Rhys parameter λ^2 , the vibrational frequency ω_0 , and the spectral inhomogeneity for the monomer specified by the Gaussian standard deviation $\sigma_{I,mon}$. The results of this analysis for the Cy3 monomer duplex and Cy3 monomer fork DNA constructs are listed in Table SI and Table SII of the SI section, respectively. For both duplex and fork DNA constructs, the Cy3 monomer (intra-chromophore) parameters ε_{eg} , λ^2 , and ω_0 do not exhibit significant temperature dependences, while the monomer inhomogeneity parameter $\sigma_{I,mon}$ increases with temperature.

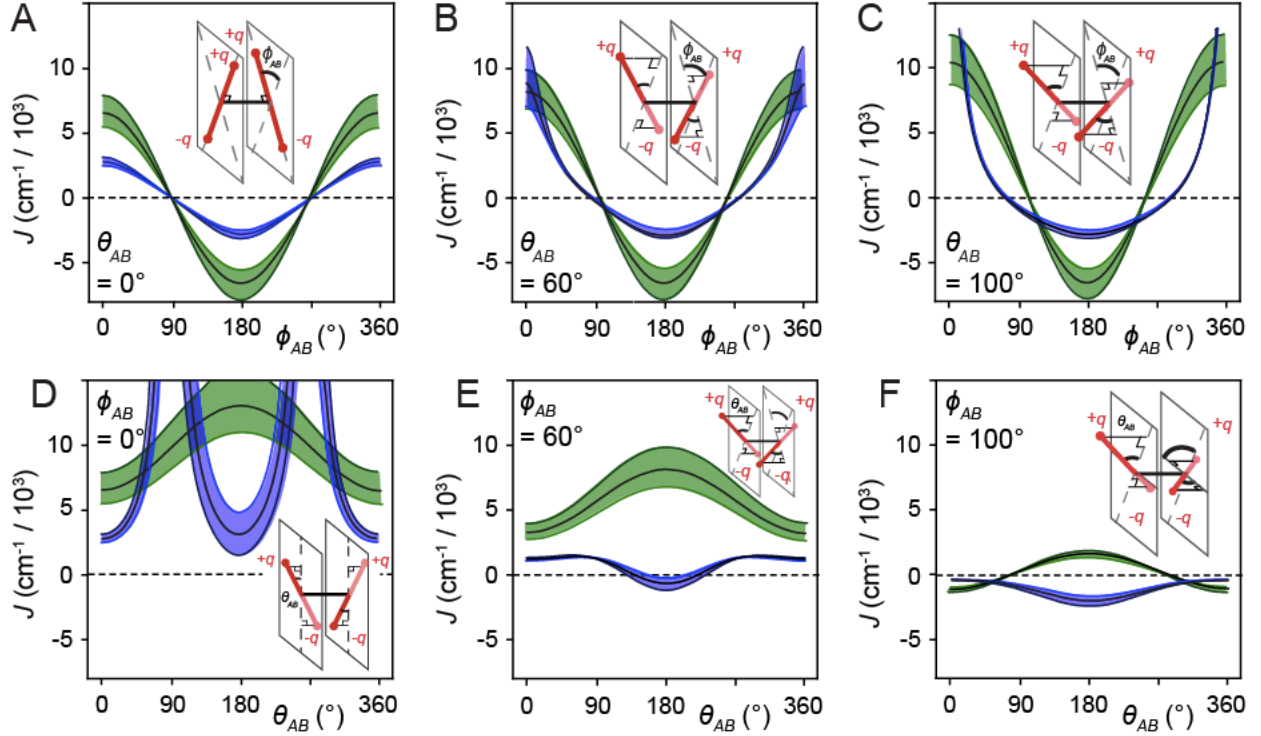


Figure 3. Calculations of the resonant electronic coupling J based on the point-dipole (shown in green) and extended-dipole (shown in blue) models. In the top row, J is plotted as a function of the twist angle ϕ_{AB} for tilt $\theta_{AB} = 0^\circ$ (A), 60° (B), and 100° (C), and in the bottom row as a function of θ_{AB} for $\phi_{AB} = 0^\circ$ (D), 60° (E), and 100° (F). In these calculations, we have used the following values for the transition dipole moment $\mu_{eg} = 12.8$ D, the extension length $l = 7$ Å, the transition charge $q = 0.38e$, and the inter-chromophore separation $R_{AB} = 5 \pm 0.3$ Å. The error bars in J correspond to the upper and lower values adopted for $R_{AB} = 5 \pm 0.3$ Å.

Table 2. (Next Page) Structural parameters of the (Cy3)₂ dimer DNA duplex construct at various temperatures using, alternatively, the point-dipole and extended-dipole models for the resonant coupling J . The reported values are based on optimized fits of the Holstein-Frenkel Hamiltonian model to absorbance and circular dichroism spectra. The calculations use as inputs the EDTM $\mu_{eg} = 12.8$ D, the homogeneous line width $\Gamma_H = 186$ cm⁻¹ (17), and for each temperature, the electronic transition energy ε_{eg} , the vibrational mode frequency ω_0 , and the Huang-Rhys parameter λ^2 obtained from analyses of the Cy3 monomer DNA construct absorbance spectra (see Table SI and Table SII of the SI section). For the extended dipole model calculations, the extension length $l = 7.1$ Å and the effective charge $q = 0.38e$ were used. The parameters listed are the resonant coupling strength J , the inter-chromophore twist angle ϕ_{AB} , the inter-chromophore tilt angle θ_{AB} , the inter-chromophore separation R_{AB} , and the standard deviation of the Gaussian inhomogeneous disorder function $\sigma_{I,dim}$. Structural parameters are presented at temperatures below the melting transition at 65°C , for which the dimer model may be reasonably applied. Error bars were calculated based on a 1% deviation of the target function from its optimized value.

Point Dipole Model					
T (°C)	J (cm ⁻¹)	ϕ_{AB} (°)	θ_{AB} (°)	R_{AB} (Å)	$\sigma_{I,dim}$ (cm ⁻¹)
15	527 +32/-11	82.9 ± 0.14	1.52 +6.2/-9.2	5.8 ± 0.04	292 ± 5.3
25	512 +33/-11	79.9 ± 0.22	7.02 +4.4/-18	6.6 ± 0.05	302 ± 5.1
35	495 +34/-12	76.0 ± 0.32	0.00 ± 10.5	7.4 ± 0.06	313 ± 5.2
45	482 +35/-13	72.0 ± 0.44	6.90 +6.7/-20	8.1 ± 0.07	325 ± 5.2
55	465 +33/-13	70.2 ± 0.48	9.28 +5.8/-24	8.4 ± 0.07	336 ± 5.0
65	361 +40/-10	73.5 ± 0.5	0.71 +14/-16	8.6 ± 0.10	336 ± 8.2

Extended Dipole Model					
T (°C)	J (cm ⁻¹)	ϕ_{AB} (°)	θ_{AB} (°)	R_{AB} (Å)	$\sigma_{I,dim}$ (cm ⁻¹)
15	532 +24/-38	80.7 +0.2/-0.1	18.1 +2.9/-2.1	3.7 ± 0.1	289 ± 5.2
25	514 +14/-29	79.6 ± 0.21	10.2 +6.4/-27	4.4 ± 0.07	302 ± 5.1
35	496 +26/-19	76.0 ± 0.32	3.0 +13/-19	5.5 ± 0.08	315 ± 5.5
45	483 +32/-16	71.9 ± 0.43	7.7 +8.5/-24	6.4 ± 0.08	325 ± 5.2
55	467 +31/-15	70.4 ± 0.46	7.0 +8.4/-22	6.8 ± 0.09	336 ± 5.0
65	354 +34/-17	73.7 ± 0.6	5.4 +11/-22	7.1 ± 0.13	353 ± 5.1

Table 3. Structural parameters of the (Cy3)₂ dimer DNA fork construct at various temperatures using, alternatively, the point-dipole and extended-dipole models to determine the resonant coupling J . The parameters and conditions for performing these calculations are the same as those described in Table II.

Point Dipole Model					
T (°C)	J (cm ⁻¹)	ϕ_{AB} (°)	θ_{AB} (°)	R_{AB} (Å)	$\sigma_{I,dim}$ (cm ⁻¹)
15	-538 +74/-65	103 ± 0.48	44.1 +2.7/-2.5	6.4 ± 0.10	356 ± 12
25	-488 +86/-91	101 ± 0.45	44.2 +2.9/-3.0	6.1 ± 0.13	364 ± 13
35	-390 +64/-68	101 ± 0.41	44.7 +2.6/-2.5	6.5 ± 0.13	362 ± 10
45	-351 +58/-62	98 ± 0.16	54.0 +1.0/-1.0	5.0 ± 0.10	364 ± 7.0
55	366 +29/-28	-11 ± 7.9	73.0 +2.3/-2.4	14.4 ± 0.23	347 ± 7.0

Table 3, continued

T (°C)	J (cm ⁻¹)	ϕ_{AB} (°)	θ_{AB} (°)	R_{AB} (Å)	$\sigma_{I,dim}$ (cm ⁻¹)
65	380 +34/-34	-23 ± 3.4	69.5 +2.5/-2.6	13.9 ± 0.25	414 ± 8.1

Extended Dipole Model					
T (°C)	J (cm ⁻¹)	ϕ_{AB} (°)	θ_{AB} (°)	R_{AB} (Å)	$\sigma_{I,dim}$ (cm ⁻¹)
15	-537 +68/-79	101 ± 0.67	47.3 +4.2/-4.5	5.3 ± 0.1	355 ± 12
25	-489 +84/-91	101 ± 0.82	44.2 +5.4/-6.5	5.4 ± 0.07	364 ± 13
35	-390 +62/-69	101 ± 0.74	44.4 +6.1/-7.4	6.1 ± 0.08	364 ± 10
45	-352 +57/-61	96 ± 0.52	58.2 +3.2/-3.5	5.6 ± 0.08	365 ± 9.5
55	366 +33/-31	-11 ± 7.3	73.0 +2.2/-2.3	14.4 ± 0.09	349 ± 7.5
65	384 +40/-38	-23 ± 3.2	69.6 +2.4/-2.5	13.7 ± 0.13	407 ± 8.8

We next performed calculations for the (Cy3)₂ dimer duplex and fork DNA constructs based on the dimer Hamiltonian given by Eq. (2). For each temperature, we used as inputs the corresponding monomer parameters listed in Table SI and Table SII of the SI section. We thus determined optimized values for the (Cy3)₂ inter-chromophore separation R_{AB} , the inter-chromophore twist angle ϕ_{AB} , the inter-chromophore tilt angle θ_{AB} , and the spectral inhomogeneity parameter for the dimer, $\sigma_{I,dim}$. As described above, we performed these calculations using, alternatively, the point-dipole [Eq. (14)] and the extended-dipole [Eq. (15)] model for the resonant coupling. In Fig. S1 of the SI section, we directly compare the results of our optimizations using either method to experimental spectra taken at 15 °C, for both the (Cy3)₂ dimer duplex and fork DNA constructs. We find that equally favorable agreement between experimental and simulated spectra can be achieved using either the point-dipole or the extended-dipole models. Moreover, both models produce similar values for the optimized parameters J , ϕ_{AB} , θ_{AB} , and $\sigma_{I,dim}$, while the extended-dipole model generally produces smaller values for inter-chromophore separation R_{AB} than does the point-dipole model. This behavior is consistent with the relatively small values of the tilt angle θ_{AB} for both the duplex and fork DNA constructs, where the primary difference between the point-dipole and extended-dipole models is the overestimation of the resonant coupling by the point-dipole model (see Fig. 3). In Table II

and Table III, we list the results of our optimization analyses for the (Cy3)₂ dimer duplex and fork DNA constructs, respectively. In the discussion that follows, we focus on the results of the extended-dipole model, which are qualitatively the same as those of the point-dipole model.

Discussion of Results

Determination of (Cy3)₂ Dimer Conformation and Conformational Disorder using

Absorbance and CD Spectroscopy. We studied the temperature-dependence of the absorbance and CD spectra of the (Cy3)₂ dimer-labeled duplex and fork DNA constructs. In Fig. 4, we present experimental absorbance and CD spectra of these samples at representative temperatures. Overlaid with the experimental data are simulated symmetric (+, shown in blue) and anti-symmetric (−, shown in red) exciton components resulting from our optimization procedure, and which are based on the extended-dipole model. The agreement between experiment and theory is very good over the full range of temperatures that we studied. In Table II and Table III, we list as a function of temperature the optimized values of the inter-chromophore structural parameters (i.e., J , ϕ_{AB} , θ_{AB} , R_{AB} , and $\sigma_{I,dim}$) for the (Cy3)₂ dimer-labeled duplex and fork DNA constructs, respectively. We note that for both of these dimer-labeled DNA constructs at temperatures above the melting transition ($T_m \approx 65$ °C), the spectra appear indistinguishable from those of the corresponding Cy3 monomer DNA constructs, signifying the complete separation between the conjugated single DNA strands.

Our temperature-dependent analyses of the absorbance and CD spectra shown in Fig. 4 reveal that for both (Cy3)₂ dimer-labeled duplex and fork DNA constructs, the individual vibronic bands are well separated into symmetric and anti-symmetric exciton components with energy spacings that depend on the coupling strength J . For both types of DNA constructs, each exciton component contributes with comparable intensities to the absorbance spectra [as described by Eq. (4)], which – as we discuss further below – is due to the occurrence in both species of the nearly orthogonal twist angles ϕ_{AB} . Moreover, we see from our analyses that the pronounced bisignate line shapes within individual vibronic bands of the CD spectra are due to the opposite sign contributions of the symmetric and anti-symmetric excitons [see Eq. (5)].

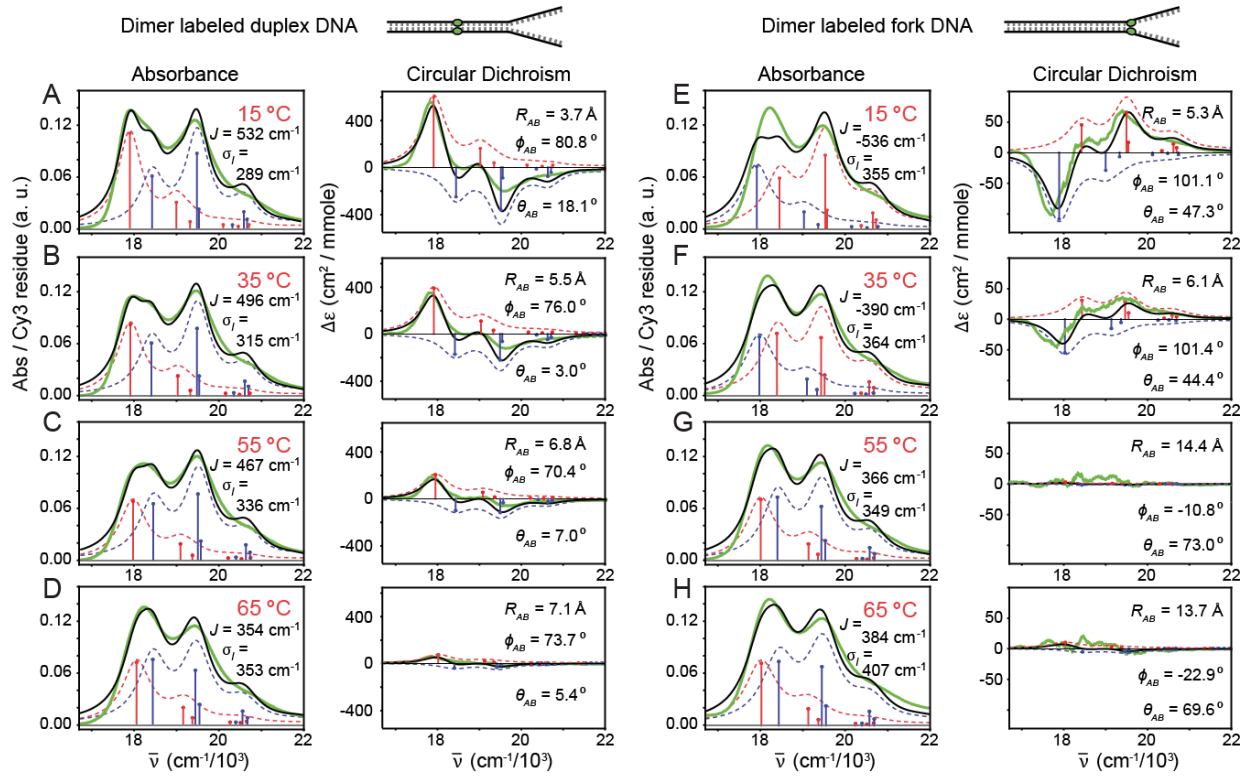


Figure 4. Temperature-dependent absorbance and CD spectra for $(\text{Cy3})_2$ dimer labeled duplex DNA construct (A-D), and for the $(\text{Cy3})_2$ dimer labeled fork DNA construct (E-H). Experimental spectra are shown in solid green, and the simulated total lineshapes (inhomogeneous-plus-homogeneous) are shown in solid black. The optimized parameters shown in the insets were obtained using the extended-dipole model for the resonant coupling. Symmetric and anti-symmetric transitions determined from the model are shown as blue and red sticks, respectively. Symmetric and anti-symmetric contributions to the inhomogeneous lineshapes are shown as dashed blue and red curves, respectively.

We first discuss our results for the $(\text{Cy3})_2$ dimer-labeled duplex DNA construct (see Fig. 4A) at 15 °C. The optimized values for the structural parameters are $J = 532 \text{ cm}^{-1}$, $\phi_{AB} = 80.7^\circ$, $\theta_{AB} = 18.1^\circ$, $R_{AB} = 3.7 \text{ Å}$, and $\sigma_{I,dim} = 289 \text{ cm}^{-1}$. These values for R_{AB} , ϕ_{AB} , and θ_{AB} are consistent with space-filling models for the local conformation of the $(\text{Cy3})_2$ dimer depicted in Fig. 1B, which shows the two Cy3 monomers positioned closely within the DNA duplex with an approximately coplanar and orthogonal relative orientation. Furthermore, this dimer conformation reflects the anticipated structure of the opposing segments of the sugar-phosphate backbone deep within the duplex region of the DNA construct. The value of the resonant coupling strength J is positive, indicating that the symmetric (anti-symmetric) exciton manifold

is blue- (red-) shifted relative to the transition energy of the uncoupled monomers. The positive sign of J corresponds to the right-handed chirality of the dimer (with $\phi_{AB} < 90^\circ$), which manifests as a positive Cotton effect in the CD spectrum (43). For both duplex and fork constructs, the magnitude of J is greater than that of the spectral inhomogeneity $\sigma_{I,dim}$, which is a necessary condition for the dimer to support delocalized excitons (11). Because the coupling strength is comparable to the intramolecular vibrational relaxation energy (i.e. $J \sim \lambda^2 \hbar \omega_0 = 602 \text{ cm}^{-1}$, where we have used $\lambda^2 = 0.54$ and $\hbar \omega_0 = 1,116 \text{ cm}^{-1}$), the dimer exists in the intermediate-to-strong exciton-coupling regime.

The results for the (Cy3)₂ dimer-labeled duplex DNA construct discussed above serve to confirm our general expectations for the right-handed helical structure of B-form DNA. In contrast, much less is known about local conformations of the sugar-phosphate backbone near DNA fork junctions. It is therefore interesting to compare our results for the two different (Cy3)₂ dimer-labeled DNA constructs. The optimized structural parameters for the dimer-labeled DNA fork construct at 15 °C (see Fig. 4E) are $J = -537 \text{ cm}^{-1}$, $\phi_{AB} = 101^\circ$, $\theta_{AB} = 47.3^\circ$, $R_{AB} = 5.3 \text{ \AA}$, and $\sigma_{I,dim} = 355 \text{ cm}^{-1}$. Here the values for R_{AB} , ϕ_{AB} , and θ_{AB} are consistent with the local conformation of the (Cy3)₂ dimer depicted in Fig. 1C, for which the two Cy3 monomers are positioned closely at the DNA fork junction with a non-coplanar and nearly orthogonal orientation. The increased value of the tilt angle θ_{AB} reflects the loss of cylindrical symmetry at the fork junction. While the magnitude of the resonant coupling J is nearly the same for the fork and duplex constructs, the sign of the coupling is negative for the DNA fork construct. This indicates that the symmetric (anti-symmetric) exciton manifold is red- (blue-) shifted relative to the transition energy of the uncoupled monomers. Thus, near the DNA fork junction the local backbone segments labeled by the (Cy3)₂ dimer has adopted a left-handed chiral symmetry (i.e. $\phi_{AB} > 90^\circ$) such that the CD exhibits a negative Cotton effect. The spectral inhomogeneity for the dimer-labeled fork DNA construct is greater than that of the duplex. This latter finding is consistent with the notion that the distribution of backbone conformations near the DNA fork junction may be broadened due to biologically significant ‘breathing’ fluctuations, which uniquely occur at this position (2).

As the temperature was increased over the range 15 – 65 °C, (see Fig. 4) the splittings between the symmetric and anti-symmetric exciton components decreased continuously, as did

the finite amplitudes of the CD signal. The CD signal of the DNA fork construct decreased much more rapidly with increasing temperature than that of the DNA duplex, which is due to a loss of chiral symmetry at $\sim 55^\circ\text{C}$, well below the denaturation transition. For both the $(\text{Cy3})_2$ dimer-labeled DNA duplex and fork constructs, the temperature-dependent properties are correlated to systematic changes in the resonant coupling strength J . This is due to the temperature sensitivity of cooperative interactions between constituent nucleic acid bases (i.e., base stacking interactions, Watson-Crick hydrogen bonding, etc.), which stabilize the right-handed helical structure of the duplex DNA construct, and only partially stabilize the left-handed conformation that we observe at the DNA fork junction. The temperature-dependent disruption of local DNA secondary structure is reflected by systematic changes in the conformation of the $(\text{Cy3})_2$ dimer DNA duplex and DNA fork constructs characterized by the structural parameters listed in Table II and Table III, respectively, and are plotted in Fig. 5.

As shown in Fig. 5, the majority of structural parameters of the $(\text{Cy3})_2$ dimer-labeled DNA duplex and fork constructs vary continuously over the range of temperatures $15 - 55^\circ\text{C}$. As discussed above, our results are nearly indistinguishable using either the point-dipole (shown in green) or the extended-dipole (shown in blue) models for the resonant coupling strength J . For our remaining discussion, we focus on the values given by the extended-dipole model.

For the $(\text{Cy3})_2$ dimer-labeled duplex DNA construct, the inter-chromophore separation R_{AB} increases from $3.7 - 6.8 \text{ \AA}$, the inter-chromophore twist angle ϕ_{AB} decreases from $80.7 - 70.4^\circ$, the tilt angle θ_{AB} decreases relatively quickly from $18.1 - 7.0^\circ$, the resonant coupling J decreases from $532 - 467 \text{ cm}^{-1}$, and the spectral inhomogeneity parameter $\sigma_{I,dim}$ increases from $289 - 336 \text{ cm}^{-1}$. The spectral inhomogeneity is a measure of the disorder of the local DNA environment experienced by the chromophores. In Table SI of the SI, we list the spectral inhomogeneity for the Cy3 monomer-labeled duplex DNA construct as a function of temperature. The values of both parameters $\sigma_{I,mon}$ and $\sigma_{I,dim}$ increase with temperature, which suggests that for both the monomer and dimer labeled duplex DNA constructs, there exists a distribution of thermally populated conformational sub-states separated by thermal barriers, which are overcome with increasing temperature. The level of disorder appears to be greater in the monomer versus the dimer duplex DNA construct. The disorder parameter of the monomer $\sigma_{I,mon}$ increases monotonically with temperature over the range $15 - 45^\circ\text{C}$, and then undergoes

a gradual decrease over the range 45 – 65 °C to nearly the same value as that of the dimer $\sigma_{I,dim}$ at the melting transition. This is likely a reflection of the less favorable packing conditions of the Cy3 monomer within the duplex DNA construct, for which a single thymine base is positioned across from the Cy3 chromophore on the opposing DNA single-strand (see Table I).

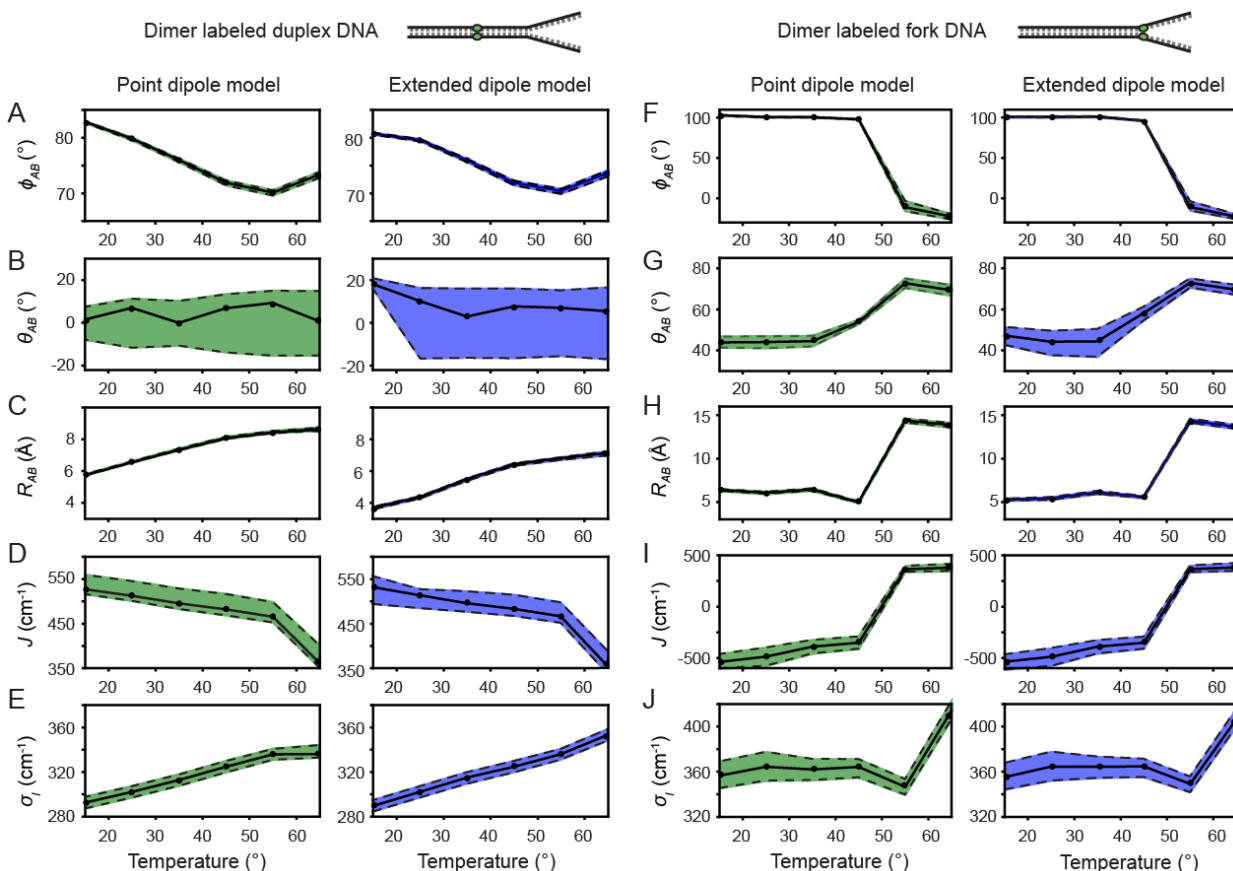


Figure 5. Temperature-dependent optimized parameters from (Cy3)₂ dimer absorption and CD spectra. Error bars were calculated based on a 1% deviation of the target function from its optimized value. The dashed line at 65 °C indicates the melting transition temperature T_m of the DNA constructs. **(A)** Inter-chromophore twist angle; **(B)** Resonant electronic coupling parameter; **(C)** Inter-chromophore separation; and **(D)** Spectral inhomogeneity parameter associated with the Cy3 monomer and the (Cy3)₂ dimer DNA constructs.

For the (Cy3)₂ dimer-labeled fork DNA construct, the inter-chromophore separation R_{AB} increases from 5.3 – 14.4 Å, the inter-chromophore twist angle ϕ_{AB} decreases from 101 to -11°, the tilt angle θ_{AB} increases from 47.3 – 73.0°, and the resonant coupling J increases from -537 – 366 cm⁻¹. An interesting property of the dimer-labeled fork DNA construct is that the spectral

inhomogeneity parameter $\sigma_{I,dim}$ remains essentially constant ($\sim 365 \text{ cm}^{-1}$) over the temperature range 15 – 55 °C, before abruptly increasing to 407 cm^{-1} at the denaturation temperature 65 °C. This weak temperature dependence of the inhomogeneity parameter suggests the absence of thermal barriers separating the broad distribution of conformational sub-states associated with the probe labels at the fork junction. It is interesting to compare the temperature-dependent inhomogeneity parameter of the Cy3 monomer-labeled DNA fork construct (see Table SII of the SI) to that of the $(\text{Cy3})_2$ dimer fork DNA construct. At 15 °C, we see that the value of $\sigma_{I,mon}$ is approximately equal to the value of $\sigma_{I,dim}$. However, over the temperature range that the dimer disorder parameter remains constant, the monomer disorder increases monotonically with temperature, approaching a value of 405 cm^{-1} , which is nearly equal to that of the dimer $\sigma_{I,dim}$ at the denaturation temperature 65 °C.

Conclusions

We studied the absorbance, circular dichroism (CD) and two-dimensional fluorescence (2DFS) spectra of a $(\text{Cy3})_2$ dimer that was site-specifically positioned within the sugar-phosphate backbone at the single-stranded (ss)—double-stranded (ds) DNA fork junction. We compared our results to those obtained from a $(\text{Cy3})_2$ dimer that was positioned deep within the DNA duplex. We adopted the Holstein-Frenkel (H-F) Hamiltonian model to characterize the symmetric and anti-symmetric excitons supported by the dimer over a range of temperatures below the denaturation transition of the dsDNA. From this analysis, we obtained a temperature-dependent parameterization of the average dimer conformation and the degree of conformational disorder (see Table III). We compared the results of our analyses using, alternatively, the point-dipole and the extended-dipole models, and we found that both models yield essentially the same temperature-dependent values for the conformational parameters.

At the lowest temperature we studied (15°C), the $(\text{Cy3})_2$ dimer-labeled DNA fork construct exhibits intermediate-to-strong resonant coupling ($J \sim -537 \text{ cm}^{-1}$), comparable in magnitude to the vibrational relaxation energy of the constituent monomers ($\lambda^2 \hbar \omega_0 \sim 600 \text{ cm}^{-1}$). Under these conditions, the dimer can support delocalized excitons composed of symmetric and anti-symmetric superpositions of electronic-vibrational product states. The delocalized electronic structure is a consequence of the $(\text{Cy3})_2$ dimer being held within the sugar-phosphate backbone

at a very small inter-chromophore separation ($R_{AB} = 5.3 \text{ \AA}$) and a nearly orthogonal inter-chromophore twist angle. The conformation of the $(\text{Cy3})_2$ dimer at the DNA fork junction lacks cylindrical symmetry with tilt angle $\theta_{AB} = 47.3^\circ$ and inter-chromophore twist angle $\phi_{AB} = 101^\circ$, which markedly deviates from the Watson-Crick B-form structure that we observe in the DNA duplex.

As the temperature is increased towards the ds – ss DNA denaturation temperature ($T_m = 65^\circ\text{C}$), the resonant electronic coupling strength of the $(\text{Cy3})_2$ dimer-labeled DNA fork construct gradually decreases in magnitude over a $\sim 200 \text{ cm}^{-1}$ range, and undergoes a sign inversion at $\sim 55^\circ\text{C}$. The Hamiltonian parameters characteristic of the Cy3 monomer-labeled DNA fork construct (i.e. the transition energy ε_{eg} , the Huang-Rhys electronic-vibrational coupling parameter λ^2 , and the vibrational frequency ω_0) remain approximately independent of temperature (see Table SI of the SI). This behavior is due to the sensitivity of the local secondary structure of the $(\text{Cy3})_2$ dimer-labeled fork (and duplex) DNA construct(s) to temperature, which affects the inter-chromophore separation, twist angle, and tilt angle, but not the electronic-vibrational properties internal to each Cy3 monomer.

While the magnitude of spectral inhomogeneity ($\sim 365 \text{ cm}^{-1}$) is significant across the $15 - 65^\circ\text{C}$ temperature range, the effects of exciton delocalization within the $(\text{Cy3})_2$ dimer are not dominated by spectral inhomogeneity. The spectral inhomogeneity parameter of the Cy3 monomer-labeled fork DNA construct increases systematically over the $15 - 65^\circ\text{C}$ temperature range ($\sigma_{I,mon} = 355 - 398 \text{ cm}^{-1}$), signifying that the monomer probe experiences locally disordered, thermally activated regions of the DNA fork, well below the melting transition. However, the inhomogeneity parameter of the $(\text{Cy3})_2$ dimer-labeled fork DNA construct is relatively constant over the $15 - 55^\circ\text{C}$ temperature range ($\sigma_{I,dim} \sim 365 \text{ cm}^{-1}$) before abruptly increasing to 407 cm^{-1} at the denaturation temperature 65°C . Our observation of a weak temperature-dependence for the inhomogeneity parameter $\sigma_{I,dim}$ suggests that thermal activation does not play a significant role in populating a broad distribution of $(\text{Cy3})_2$ dimer conformational sub-states at the ss-ds DNA fork junction. This finding is consistent with the hypothesis that the sugar-phosphate backbone of DNA near ss-ds junctions fluctuates into a broad distribution of conformations to permit the proper binding of genome regulatory proteins.

Although the H-F model for the exciton-coupled (Cy3)₂ dimer is relatively simple as it assumes a single internal vibrational mode for each Cy3 monomer, the model appears to capture the essential features of the experimental absorbance, CD and 2DFS spectra. Temperature variation corresponds to systematic changes in local dimer conformation that allows the resonant coupling strength to be ‘tuned’ across the intermediate-to-strong exciton-coupling regime. The significant spectral inhomogeneity is due to the presence of local structural fluctuations of the DNA backbone and base stacking that influence the packing of the chromophore probes. Such local fluctuations of DNA are termed DNA ‘breathing,’ which likely play a functional role in the binding and assembly of gene regulatory proteins (2).

Bridge to Chapter III

The previous chapter demonstrated that the optical spectra of (iCy3)₂ dimer-labeled ds-ss DNA fork constructs can be reliably captured by Holstein-like models and under various approximations of the resonance interaction between the two iCy3 chromophore sites to construct models of the dimer conformation. The information about the conformation of the (iCy3)₂ dimer probes may thus be used to assess the local structure of the DNA sugar-phosphate backbones. Next, we look more closely at DNA labeled at and near ds-ss DNA junctions. In particular, 2D fluorescence spectroscopy (2DFS) will be used to examine the degree of structural disorder at the junction, which is likely important for productive DNA-protein interactions.

Chapter III

Using Two-Dimensional Fluorescence Spectroscopy to Study Structural Disorder at DNA ss-ds Junctions

This work was published in Volume 156 of the Journal of Physical Chemistry as “Temperature-dependent local conformations and conformational distributions of cyanine dimer labeled single-stranded–double-stranded DNA junctions by 2D fluorescence spectroscopy”. Dylan Heussman performed the measurements. Dylan Heussman and Justin Kittell carried out the analysis and associated calculations. Peter von Hippel helped to design the experiments and provided editorial assistance. Andrew Marcus was the principal investigator for this work and provided editorial assistance and general feedback.

Introduction

The Watson-Crick (W-C) B-form double-helix (1) is the most stable of the myriad structures that DNA can (and must) adopt in order to function both as a template for gene expression and as a vehicle for transmitting heredity. Under physiological conditions, double-stranded (ds) DNA exists primarily as a narrow, Boltzmann-weighted distribution of base-sequence-dependent conformations, for which the W-C structure represents an approximate free energy minimum. The molecular interactions that stabilize dsDNA includes cooperative stacking of adjacent nucleotide (nt) bases, internal strain that stacking induces in the sugar-phosphate backbones, W-C hydrogen bonding between opposite complementary strands, intra- and inter-chain repulsion between adjacent backbone phosphates, counterion condensation and orientation of polar water molecules within the nearest solvation layers at exposed DNA surfaces. All these interactions are subject to thermally-induced fluctuations (i.e., DNA ‘breathing’), which may lead to local segments adopting transiently unstable conformations over time scales spanning tens-of microseconds to several seconds (2). For example, on sub-second time scales, and depending on temperature relative to the overall melting temperature of the DNA duplex, the interior of local AT-rich regions of dsDNA may become exposed to the surrounding aqueous environment by spontaneously disrupting the W-C structure and forming open ‘bubble-like’ conformations. On the time scale of multiple seconds, segments of dsDNA may undergo higher

order sequence-dependent distortions of the local conformation, such as the formation of a local ‘bubble,’ ‘bend’ or ‘kink.’

The spontaneous formation of an unstable local dsDNA conformation is very likely a key initial step in the assembly mechanisms of complexes of gene regulatory proteins that recognize and bind to specific nt base sequences. In contrast, protein-DNA assembly mechanisms that occur largely independently of specific nucleotide base sequences must utilize the ability of the protein or protein complex to recognize certain secondary-structure motifs that can be adopted by the sugar-phosphate backbones of the DNA (3). For example, the assembly of DNA replication complexes involves the preferential binding of proteins to single-stranded (ss) – dsDNA forks and junctions (2). In principle, various types of DNA breathing near ss-dsDNA junctions may facilitate the interconversion between various unstable conformations, of which one or more may be expected to resemble that of the DNA framework within a stable protein-DNA complex. Such an unstable conformational species may serve as a ‘transition state’ for the interaction of DNA binding sites with replication proteins.

In this work, we present spectroscopic studies of the distributions of structural parameters that characterize the local conformations of the sugar-phosphate backbones at and near ss-dsDNA fork junctions. These experiments use DNA constructs in which the carbocyanine dye Cy3 has been covalently attached as a dimer pair within the sugar-phosphate backbones at specific positions relative to the ss-ds DNA junction (see Fig. 1). The Cy3 chromophore is often used as a fluorescent marker for DNA sequencing and other biotechnological applications due to its relatively high absorption cross-section and favorable fluorescence quantum yield (4). The Cy3 chromophore consists of a conjugated trimethine bridge that cojoins two indole-like substituents (see Fig. 1A). The lowest energy $\pi \rightarrow \pi^*$ electronic transition between ground state g and excited state e occurs when the molecule is in its *all-trans* ground state configuration.

The linear absorbance spectrum of the free Cy3 chromophore in solution, as well as when it is attached covalently to a nucleic acid, exhibits a pronounced vibronic progression, which can be simulated using a Holstein-Frenkel Hamiltonian with electronic transition energy $\varepsilon_{eg} = \sim 18,250 \text{ cm}^{-1}$, vibrational mode energy $\hbar\omega_0 = \sim 1100 \text{ cm}^{-1}$ and Huang-Rhys electronic-vibrational coupling parameter $\lambda^2 = \sim 0.55$ (5). The electric dipole transition moment (EDTM)

has magnitude $\mu_{eg} = \sim 12.8$ D and orientation that lies parallel to the Cy3 trimethine bridge (see Fig. 1A). Cy3 can be chemically attached ‘internally’ to the DNA with the ‘iCy3’ acting as a molecular bridge between bases as an extension of the sugar-phosphate backbone (6). When two complementary single strands of DNA with opposed iCy3 labeling positions are annealed, an (iCy3)₂ dimer probe is formed within the DNA duplex. If the sequence of nt bases at or near one side of the (iCy3)₂ dimer is non-complementary, the labeling location occurs at a ss-dsDNA fork junction, as shown schematically in Fig. 1B. iCy3 monomer-labeled ss-dsDNA constructs are similarly prepared with a thymine (T) base at the position opposite to the probe within the complementary strand.

Because of the relatively small separation between iCy3 chromophores within the (iCy3)₂ dimer-labeled ss-dsDNA fork constructs, the monomer EDTMs (labeled as sites *A* and *B* in Fig. 1C) can couple through a resonant electrostatic interaction *J*. This coupling gives rise to symmetric (+) and anti-symmetric (−) excitons with orthogonally polarized dipole moments $\mu_{\pm} = \frac{1}{\sqrt{2}}[\mu_A \pm \mu_B]$ and with relative magnitudes that depend on the local conformation of the (iCy3)₂ dimer probe. The symmetric and anti-symmetric excitons each consist of a manifold of delocalized electronic-vibrationally coupled states, which are superpositions of electronic-vibrational product states of the *A* and *B* monomer sites (7, 8). The absorbance and circular dichroism (CD) spectra of (iCy3)₂ dimer labeled ss-dsDNA constructs are well-described using the Holstein-Frenkel model, and can be used to determine local conformational parameters (5, 7). The structural parameters that characterize the dimer conformation are the ‘tilt’ angle θ_{AB} , the ‘twist’ angle ϕ_{AB} , and the separation R_{AB} . In our previous spectroscopic studies of (iCy3)₂ dimer labeled ss-dsDNA constructs, we calculated the resonant electrostatic coupling by treating the EDTMs as point dipoles (7). We later repeated these calculations using an ‘extended-dipole’ model (9, 10) that more accurately accounted for the extension of the transition charge density across the length of the molecule, and which yielded nearly identical results to those provided by the point-dipole approximation (5). We note that more accurate models of electrostatic coupling for Cy3, which are based on *ab initio* calculations of atomic transition charges, have become available and provide opportunities to test the validity of point-dipole and extended dipole models that can be found in the next chapter (11).

Here we focus on the distributions of structural parameters obtained from theoretical analyses of absorbance, CD, and two-dimensional fluorescence spectroscopy (2DFS) of (iCy3)₂ dimer labeled ss-ds DNA fork constructs. While absorbance and CD can be used to determine the mean structural parameters of the (iCy3)₂ dimer probes, 2DFS provides additional information about the distributions of these parameters. The underlying optical principles of 2DFS resemble those of 2D NMR (12, 13) and 2DIR, with the latter providing structural and dynamic information about local vibrational modes in proteins (14, 15), nucleic acids (16, 17) and biomolecular hydration shells (18). 2DIR has been used to distinguish sequence-dependent inter-base H-bonds in duplex DNA (16, 17) and the rearrangements of water molecules at or near the exposed surfaces of DNA strands (18). While these relatively fast processes likely contribute to nucleic acid stability and dynamics, they do not directly probe the DNA breathing fluctuations involved in protein recognition events (19, 20). In contrast, the signals detected by 2DFS on (iCy3)₂ dimer probe labeled ss-dsDNA constructs do directly monitor DNA backbone conformations and conformational disorder, which likely play a central role in protein recognition and binding events.

In the following experiments, we studied several different ss-dsDNA fork constructs in which we varied the (iCy3)₂ dimer labeling position, as shown in Fig. 1D, and for some of these constructs we varied the temperature. In contrast to the (iCy3)₂ dimer ss-dsDNA constructs, the linear spectra of the iCy3 monomer ss-dsDNA constructs are relatively insensitive to probe labeling position and temperature, as previously reported (5, 7). These findings suggest that for the (iCy3)₂ dimer ss-dsDNA constructs, the sensitivity of the homogeneous lineshapes to labeling position and temperature are due largely to variations of the coupling interaction J , which is sometimes referred to as ‘off-diagonal disorder’ in the reference frame of the monomer sites (15, 21).

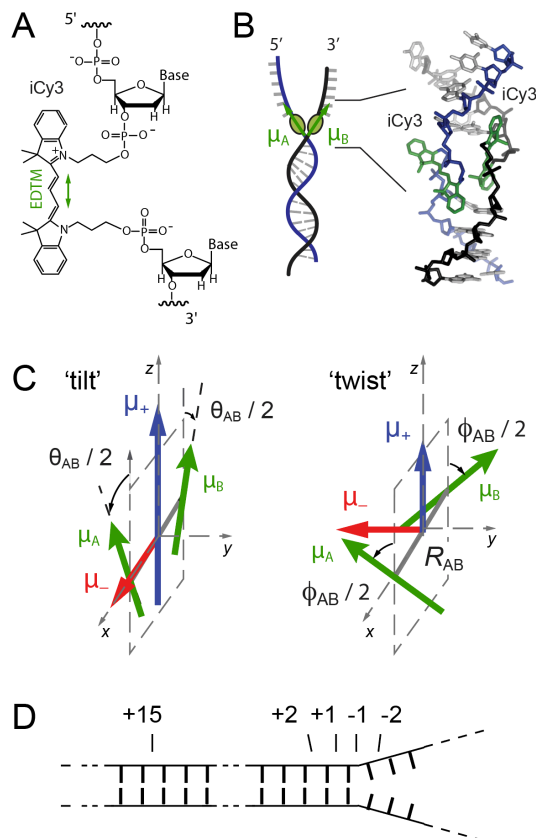


Figure 1. Labeling chemistry and nomenclature of the internal (iCy3)₂ dimer probes positioned within the sugar-phosphate backbones of model ss-dsDNA fork constructs. **(A)** The Lewis structure of the iCy3 chromophore is shown with its 3' and 5' linkages to the sugar-phosphate backbone of a local segment of ssDNA. The double-headed green arrow indicates the orientation of the electric dipole transition moment (EDTM). **(B)** An (iCy3)₂ dimer labeled DNA fork construct contains the dimer probe near the ss – ds DNA fork junction. The conformation of the (iCy3)₂ dimer probe reflects the local secondary structure of the sugar-phosphate backbones at the probe insertion site position. The sugar-phosphate backbones of the conjugate DNA strands are shown in black and blue, the bases in gray, and the iCy3 chromophores in green. **(C)** The structural parameters that define the local conformation of the (iCy3)₂ dimer probe are the inter-chromophore separation vector R_{AB} , the tilt angle θ_{AB} , and the twist angle ϕ_{AB} . The electrostatic coupling between the iCy3 chromophores gives rise to the anti-symmetric (–) and symmetric (+) excitons, which are indicated by the red and blue arrows, respectively, and whose magnitudes and transition energies depend on the structural parameters. **(D)** The insertion site position of the iCy3 dimer probe is indicated relative to the pseudo-fork junction using positive integers in the direction towards the double-stranded region, and negative integers in the direction towards the single-stranded region.

In our prior studies, we established that a combination of absorbance and CD spectra contain sufficient information to determine mean values of the structural parameters $\bar{R}_{AB}$, $\bar{\theta}_{AB}$ and $\bar{\phi}_{AB}$, in addition to an estimate of the inhomogeneous line broadening parameter σ_I (5, 7). Inhomogeneous line broadening is a direct measure of structural heterogeneity due to individual molecules of the sample exhibiting uniquely different homogeneous lineshapes, which depends on the local conformation of the (iCy3)₂ dimer probe. Our prior estimates of σ_I were obtained from a deconvolution of absorbance and CD spectra and were based on the value of the homogeneous line width $\Gamma_H = \sim 186 \text{ cm}^{-1}$. We determined the latter value from room temperature 2DFS experiments on iCy3 monomer and (iCy3)₂ dimer-labeled DNA constructs in which the probe labeling position was deep within the double-stranded region, and for which the laser bandwidth was $\Delta\lambda_L = \sim 16 \text{ nm}$ (7). In the current work, we perform a more accurate analysis of 2DFS data in which the laser bandwidth was $\Delta\lambda_L = \sim 33 \text{ nm}$. This increase in laser bandwidth permits us to determine simultaneously the homogeneous and inhomogeneous line shape parameters as a function of probe labeling position and temperature. In addition, we here extend our line shape analysis to model the distributions of the structural parameters R_{AB} , θ_{AB} , and ϕ_{AB} , because variation in these parameters ‘builds in’ the structural heterogeneity measured by our 2DFS experiments. In the analyses that follow, we have assumed that the distributions of structural parameters can be successfully modeled as Gaussians, which can be characterized using the standard deviations σ_R , σ_θ and σ_ϕ .

It is useful to briefly review here the general dependence of the homogeneous lineshapes of the absorbance, CD and 2DFS observables on the (iCy3)₂ dimer conformation and associated electrostatic couplings (5, 7). As previously mentioned, the optical transitions of an isolated iCy3 monomer probe are well-described using a simple Hamiltonian model based on a two-electronic level system coupled to a single vibrational (harmonic) mode. The iCy3 monomer has its lowest-lying optical transition centered at $\sim 18,250 \text{ cm}^{-1}$ (the ‘0-0’ line with no vibrational excitation) and sequentially higher lying optical transitions (e.g., ‘0-1’, ‘0-2’ with one and two vibrational excitations, respectively) spaced apart by the vibrational mode energy $\sim 1100 \text{ cm}^{-1}$. The absorbance spectrum of the iCy3 monomer therefore exhibits a vibronic progression with relative peak intensities determined by the associated Franck-Condon factors. The corresponding 2D fluorescence spectrum is a contour diagram that reflects the transition probability-weighted

correlations between successive optical transitions of the vibronic sub-bands. The 2D fluorescence spectrum thus exhibits peaks and cross-peaks associated with the optical transitions with relative intensities determined by both the Franck-Condon factors and the spectral overlaps between the optical transitions and the spectrum of the exciting laser. Because CD depends on the presence of chiral asymmetry of the transition dipole moments, the (achiral) iCy3 monomer exhibits an approximately featureless CD spectrum.

For the (iCy3)₂ dimer-labeled ss-dsDNA constructs studied in this work, the strength of the electrostatic interactions lies within the so-called ‘intermediate-coupling regime’ (22), so that the absorbance spectrum of the dimer has a similar shape to that of the monomer. However, the electrostatic interaction leads to each of the vibronic features (i.e., 0-0, 0-1, etc.) being split and additionally broadened into the symmetric (+) and anti-symmetric (−) exciton sub-bands. The relative peak intensities of the absorbance spectrum depend on (in addition to the above-mentioned factors that affect the iCy3 monomer) the local conformation of the dimer, which determines the magnitudes of the symmetric and anti-symmetric transition dipole moments $\mu_{\pm}$ (see Fig. 1C). The 2D fluorescence spectrum of the (iCy3)₂ dimer probe thus exhibits a higher density of peaks and cross-peaks, which are associated with the relatively high density of symmetric and anti-symmetric excitons, when compared to the spectrum of the iCy3 monomer. In contrast to the CD of the iCy3 monomer, which is featureless, the CD of the (iCy3)₂ dimer generally exhibits features characteristic of the chiral asymmetry of the exciton-coupled transition dipole moments.

Among the significant findings of this work is that the (iCy3)₂ dimer is a reliable probe of the local conformation of the sugar-phosphate backbones at and near ss-dsDNA fork junctions, which depends sensitively on the labeling site position and temperature. We studied the temperature-dependence of the local conformation of (iCy3)₂ dimer-labeled ss-dsDNA constructs, both at positions deep within the duplex region (+15) and near the ss-dsDNA fork junction (-1). We find that local conformations and conformational disorder of the sugar-phosphate backbones at the +15 position are minimized at room temperature (23°C) and change rapidly as the temperature is either raised or lowered away from room temperature under physiological salt conditions (100 mM NaCl, 6 mM MgCl₂), permitting the development of local conformations that deviate significantly from the W-C duplex DNA structure, such as bubbles,

bends and kinks. In contrast, local conformations, and conformational disorder of the ss-dsDNA junction at the -1 position do not vary significantly with increasing temperature, suggesting that the distribution of thermally accessible states is relatively narrow. Moreover, the mean local conformation and conformational disorder vary systematically with (iCy3)₂ dimer-labeling position (from +2 to -2, refer to Fig. 1D for probe labeling nomenclature). This transition is characterized by an increase in conformational disorder and a loss of cylindrical symmetry characterized by the mean tilt angle $\bar{\theta}_{AB}$, followed by a change in the local symmetry of the DNA backbones from right-handed to left-handed, as reflected by the mean twist angle $\bar{\phi}_{AB}$. Perhaps contrary to expectations, regions of the ss-dsDNA junction extending into the single strands appear to be relatively well-ordered.

Experimental Methods

Sample preparation

The sequences and nomenclature of the iCy3 monomer and (iCy3)₂ dimer labeled ss-ds DNA constructs used in our studies are shown in Table 1. Oligonucleotide samples were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and used as received. Solutions were prepared containing ~1 μ M oligonucleotide in 10 mM TRIS buffer with 100 mM NaCl and 6 mM MgCl₂. Complementary strands were combined in equimolar concentrations. The samples were heated to 95°C for 4 minutes and left to cool slowly on a heat block overnight prior to data collection. The annealed iCy3 monomer and (iCy3)₂ dimer labeled ss-ds DNA fork constructs contained both ds and ss DNA regions, with the probe labeling positions indicated by the nomenclature described in Fig. 1D. The iCy3 monomer labeled constructs contained a thymine base (T) in the complementary strand at the position directly opposite to the probe chromophore.

Table 1. (next page) Base sequences and nomenclature for the iCy3 monomer and (iCy3)₂ dimer ss-ds DNA fork constructs used in these studies. The horizontal lines indicate regions of complementary base pairing.

DNA construct	Nucleotide base sequence
+15 (iCy3) ₂ dimer	3'-GTC AGT ATT ATA CGC Tcy3C GCT AAT ATA TAC GTT TTT TTT TTT TTT TTT TTT TTT TTT T-5' 5'-CAG TCA TAA TAT GCG Acy3G CGA TTA TAT ATG CTT TTA CCA CTT TCA CTC ACG TGC TTA C-3'
+15 iCy3 monomer	3'-GTC AGT ATT ATA CGC Tcy3C GCT AAT ATA TAC GTT TTT TTT TTT TTT TTT TTT TTT TTT T-5' 5'-CAG TCA TAA TAT GCG A T G CGA TTA TAT ATG CTT TTA CCA CTT TCA CTC ACG TGC TTA C-3'
-1 (iCy3) ₂ dimer	3'-GAG GGA GCA CAG CAG AGG TCA GTA TTA TAC GCT Cy3CG CTG GTA TAC CAC GTT (T) ₂₈ -5' 5'-CTC CCT CGT GTC GTC TCC AGT CAT AAT ATG CGA Cy3AT GCT TTT ACC ACT TTC ACT CAG GTG CTT A-3'
-1 iCy3 monomer	3'-GAG GGA GCA CAG CAG AGG TCA GTA TTA TAC GCT Cy3CG CTG GTA TAC CAC GTT (T) ₂₈ -5' 5'-CTC CCT CGT GTC GTC TCC AGT CAT AAT ATG CGA T AT GCT TTT ACC ACT TTC ACT CAG GTG CTT A-3'
-2 (Cy3) ₂ dimer	3'-GAG GGA GCA CAG CAG AGG TCA GTA TTA TAC GCT Cy3CG CTG GTA TAC CAC GTT (T) ₂₈ -5' 5'-CTC CCT CGT GTC GTC TCC AGT CAT AAT ATG CGC Cy3AT ACT TTC GCC ACT TTC ACT CAC GTG CTT A-3'
+1 (Cy3) ₂ dimer	3'-GAG GGA GCA CAG CAG AGG TCA GTA TTA TAC GCT Cy3CG CTG GTA TAC CAC GTT (T) ₂₈ -5' 5'-CTC CCT CGT GTC GTC TCC AGT CAT AAT ATG CGA Cy3GT ACT TTC GCC ACT TTC ACT CAC GTG CTT A-3'
+2 (Cy3) ₂ dimer	3'-GAG GGA GCA CAG CAG AGG TCA GTA TTA TAC GCT Cy3CG CTG GTA TAC CAC GTT (T) ₂₈ -5' 5'-CTC CCT CGT GTC GTC TCC AGT CAT AAT ATG CGA Cy3GC ACT TTC GCC ACT TTC ACT CAC GTG CTT A-3'

Absorbance and circular dichroism (CD) measurements

We performed linear absorbance measurements using a Cary 3E UV-Vis spectrophotometer, and CD measurements with a JASCO model J-720 CD spectrophotometer. Series of temperature-dependent measurements were performed over the range 1 – 75°C. For all absorbance and CD measurements, the samples were housed in a 1 cm quartz cuvette. CD measurements over the temperature range 1 – 25°C were performed using a JASCO J-1500 CD spectrophotometer equipped with a Koolance EXOS liquid cooling system, which can operate reliably at near-freezing temperatures.

Two-dimensional fluorescence spectroscopy (2DFS)

Phase-modulation 2DFS experiments were performed on the iCy3 labeled ss-ds DNA fork constructs listed in Table I using methods and procedures described previously (5, 7, 23-26). The train of four collinear laser pulses used to excite the sample was centered on wavelength $\lambda_L = \sim 532$ nm ($\sim 18,800$ cm⁻¹), with bandwidth $\Delta\lambda_L = \sim 33$ nm ($\sim 1,100$ cm⁻¹). The pulses were generated using a custom-built non-collinear optical parametric amplifier (NOPA) that was pumped using a 140 kHz Ti:Sa regenerative amplifier (Coherent, RegA). The NOPA output was

divided into two paths using a 50/50 beam-splitter, and each beam was directed to a separate Mach-Zehnder interferometer (MZI). Acousto-optic Bragg cells, placed within the beam paths of each MZI, were used to apply a relative temporal phase sweep to the pulses exiting the MZI. Thus, the relative phase of pulses 1 and 2, and that of pulses 3 and 4, were swept continuously at the frequencies $\Omega_{21} = 5$ kHz and $\Omega_{43} = 8$ kHz, respectively. The relative paths of the pulses were varied using computer-controlled translation stages to step the time delay t_{21} between the first pair of pulses, and the delay t_{43} between the second pair of pulses. For all our measurements, the time delay t_{32} between the second and third pulse was set to zero. For each combination of time delays, the four pulses were used to excite resonant electronic transitions of the iCy3 probes, and the ensuing fluorescence was detected and demodulated simultaneously at the sum frequency $\Omega_{43} + \Omega_{21} = 13$ kHz and the difference frequency $\Omega_{43} - \Omega_{21} = 3$ kHz, which correspond, respectively, to the fourth-order non-rephasing (NRP) and rephasing (RP) signals (24, 26, 27).

The optical pulses were compressed using a quadruple-pass fused-silica prism pair to compensate for dispersive media in the optical paths preceding the sample. Pulse widths were characterized by placing a beta-barium borate (BBO) frequency doubling crystal at the sample position where a phase-modulated train of pulse pairs was incident. The frequency-doubled signal output was detected using a lock-in amplifier, which was referenced to the waveform signal used to modulate the relative phase of the pulses (23, 26). The pulse compressor was adjusted so that the full-width-at-half-maximum (FWHM) of the pulse-pulse autocorrelation, for each of the pulse pairs, was $\Delta\tau_L = \sim 28$ fs. We measured the laser bandwidth $\Delta\lambda_L = \sim 33$ nm ($\sim 1,100$ cm⁻¹) centered at $\lambda_L = \sim 532$ nm using an Ocean Optics spectrometer. The measured time-bandwidth product was $\Delta\tau_L \Delta\lambda_L c / \lambda_L^2 = \sim 0.98$, which is larger than the optimal value (0.44) for Fourier-transform limited Gaussian pulses and indicates the presence of some third-order dispersion that was not compensated by the prism compressor.

The laser pulse spectrum with the above spectral properties was reproducibly maintained and continuously monitored during each 2DFS measurement described in this work. Samples were housed in a 1 mm quartz cuvette that was mounted to a small aluminum heating block, which was itself placed in thermal contact with a copper block equipped with internally circulating cooling water. The temperature of the sample was maintained to within $\sim \pm 0.1$ °C using two thermoelectric chips, which were mounted directly to the aluminum block.

Fluorescence was detected at a 45° angle of incidence relative to the front face of the sample cuvette using a 5 mm collection lens and a 615 nm long-pass filter (Chroma, HQ615LP), which served to minimize scattered excitation light. A light stream of nitrogen was continuously flowed across the front face of the cuvette to prevent condensation of vapor for measurements performed at reduced temperatures.

Theoretical Modeling

Simulation of two-dimensional fluorescence spectra (2DFS)

We applied the H-F model to simulate our 2D fluorescence spectra according to methods developed previously and can be found in Chapter 1 (24, 27). The 2DFS signals are written in terms of the rephasing (RP) and non-rephasing (NRP) fourth-order response functions

$$S_{RP}(t_{21}, t_{32} = 0, t_{43}) \propto -(Q_{4a} + Q_{3a} + Q_{2b}^* - \Gamma_{2D} Q_{8b}^*) \quad (11)$$

and

$$S_{NRP}(t_{21}, t_{32} = 0, t_{43}) \propto -(Q_{5a}^* + Q_{2a} + Q_{3b}^* - \Gamma_{2D} Q_{7b}) \quad (12)$$

In Eqs. (11) and (12), the first two terms on the right-hand sides of the proportionalities represent, respectively, the ground state bleach (GSB) and stimulated emission (SE) contributions. The final two terms represent excited state absorption (ESA) contributions for the singly- and doubly-excited-state manifolds, respectively. The parameter Γ_{2D} represents the fluorescence quantum yield of the doubly-excited-state-manifold relative to that of the singly excited state manifold. The possible values of Γ_{2D} may range from 0 to 2, which has the effect of modifying the sign and magnitude of the ESA contributions relative to those of the GSB and SE. In our analyses of the 2D spectral lineshapes of the iCy3 monomer and (iCy3)₂ dimer ss-dsDNA constructs discussed below, we treated Γ_{2D} as one of three parameters (the others being the homogeneous and inhomogeneous line width parameters, Γ_H and σ_I , respectively) that were optimized to our experimental data. For all our calculations, we found that the optimized value for Γ_{2D} was ~ 0.3 (see Fig. S1 of the SI).

In writing the response functions, we designate $|v\rangle = |v_A v_B\rangle |gg\rangle$ as the dimer state with both monomers electronically unexcited and with vibrational quantum number $v = v_A + v_B$, such that, for example, state $|0\rangle$ is the electronic ground state with zero vibrational occupancy. The states $|e\rangle$ and $|e'\rangle$ represent any two of the symmetric and anti-symmetric excitons $|e_{\pm}^{(\beta)}\rangle$ within the singly-excited-state manifold, and the state $|f\rangle$ represents any one state within the doubly-excited-state manifold. When the effects of inhomogeneous broadening are included, the individual terms of the RP response functions can be written (30-32):

$$Q_{4a} = \sum_{v,e,e'} [\mu_{0e} \mu_{ev} \mu_{0e'} \mu_{ve'}]_{\mathbf{e}_1 \mathbf{e}_2 \mathbf{e}_3 \mathbf{e}_4} \alpha^2(\omega_{e'v}) \alpha^2(\omega_{e0}) e^{-\Gamma_H(t_{21}+t_{43}) - \frac{1}{2}\sigma_I^2(t_{21}-t_{43})^2 + i(\omega_{e'v}t_{43} - \omega_{e0}t_{21})} \quad (13)$$

$$Q_{3a} = \sum_{v,e,e'} [\mu_{0e} \mu_{e'0} \mu_{ev} \mu_{ve'}]_{\mathbf{e}_1 \mathbf{e}_2 \mathbf{e}_3 \mathbf{e}_4} \alpha^2(\omega_{e'v}) \alpha^2(\omega_{e0}) e^{-\Gamma_H(t_{21}+t_{43}) - \frac{1}{2}\sigma_I^2(t_{21}-t_{43})^2 + i(\omega_{e'v}t_{43} - \omega_{e0}t_{21})} \quad (14)$$

$$Q_{2b}^* = Q_{8b}^* = \sum_{e,e',f} [\mu_{0e} \mu_{e'0} \mu_{fe'} \mu_{ef}]_{\mathbf{e}_1 \mathbf{e}_2 \mathbf{e}_3 \mathbf{e}_4} \alpha^2(\omega_{fe}) \alpha^2(\omega_{e0}) e^{-\Gamma_H(t_{21}+t_{43}) - \frac{1}{2}\sigma_I^2(t_{21}-t_{43})^2 + i(\omega_{fe}t_{43} - \omega_{e0}t_{21})} \quad (15)$$

and for NRP:

$$Q_{5a}^* = \sum_{v,e,e'} [\mu_{e0} \mu_{ve} \mu_{e'v} \mu_{0e'}]_{\mathbf{e}_1 \mathbf{e}_2 \mathbf{e}_3 \mathbf{e}_4} \alpha^2(\omega_{e'0}) \alpha^2(\omega_{e0}) e^{-\Gamma_H(t_{21}+t_{43}) - \frac{1}{2}\sigma_I^2(t_{21}+t_{43})^2 + i(\omega_{e'0}t_{43} + \omega_{e0}t_{21})} \quad (16)$$

$$Q_{2a} = \sum_{v,e,e'} [\mu_{e0} \mu_{0e'} \mu_{e'v} \mu_{ve}]_{\mathbf{e}_1 \mathbf{e}_2 \mathbf{e}_3 \mathbf{e}_4} \alpha^2(\omega_{e'v}) \alpha^2(\omega_{e0}) e^{-\Gamma_H(t_{21}+t_{43}) - \frac{1}{2}\sigma_I^2(t_{21}+t_{43})^2 + i(\omega_{e'v}t_{43} + \omega_{e0}t_{21})} \quad (17)$$

$$Q_{3b}^* = Q_{7b} = \sum_{e,e',f} [\mu_{e0} \mu_{0e'} \mu_{fe'} \mu_{e'f}]_{\mathbf{e}_1 \mathbf{e}_2 \mathbf{e}_3 \mathbf{e}_4} \alpha^2(\omega_{fe}) \alpha^2(\omega_{e0}) e^{-\Gamma_H(t_{21}+t_{43}) - \frac{1}{2}\sigma_I^2(t_{21}+t_{43})^2 + i(\omega_{fe}t_{43} + \omega_{e0}t_{21})} \quad (18)$$

In Eqs (13) – (18), the factor $[\mu_{ab} \mu_{cd} \mu_{jk} \mu_{lm}]_{\mathbf{e}_1 \mathbf{e}_2 \mathbf{e}_3 \mathbf{e}_4}$ denotes the orientationally averaged four-point product, $\langle (\boldsymbol{\mu}_{ab} \cdot \mathbf{e}_1)(\boldsymbol{\mu}_{cd} \cdot \mathbf{e}_2)(\boldsymbol{\mu}_{jk} \cdot \mathbf{e}_3)(\boldsymbol{\mu}_{lm} \cdot \mathbf{e}_4) \rangle$, which accounts for the projections of the (iCy3)₂ dimer transition dipole moments onto the (parallel) plane polarizations of the four laser pulses and includes an average over the isotropic distribution of dimer orientations in

solution (33). The factor $\alpha^2(\omega_{ab})\alpha^2(\omega_{cd})$ is the product of the intensities of the laser at the transition frequencies ω_{ab} and ω_{cd} .

An important feature of the RP and NRP response functions is their distinct dependences on the homogeneous and inhomogeneous line width parameters, Γ_H and σ_I , respectively (31, 34) (see Fig. 2). The RP response functions [Eqs. (13) – (15)] contain the lineshape factor $\exp[-\Gamma_H(t_{21} + t_{43})]\exp[-\sigma_I^2(t_{21} - t_{43})^2/2]$, which decays exponentially at the homogeneous dephasing rate along the diagonal axis ($t_{21} + t_{43}$), and as a Gaussian envelope with inhomogeneous decay constant $\sigma_I^2/2$ along the anti-diagonal axis ($t_{21} - t_{43}$) (see Fig. 2A, top panel). In contrast, the NRP response functions [Eqs. (16) – (18)] contain the lineshape factor $\exp[-\Gamma_H(t_{21} + t_{43})]\exp[-\sigma_I^2(t_{21} + t_{43})^2/2]$, which decays along the diagonal axis with rate constants that depend on both the homogeneous and inhomogeneous parameters (see Fig. 2A, bottom panel). Examples of the RP and NRP response functions are shown in Fig. 2B. The RP and NRP 2D fluorescence spectra, which are functions of the optical frequencies $\bar{\nu}_{21}$ and $\bar{\nu}_{43}$, are obtained by performing two-dimensional Fourier transforms of the response functions with respect to the delay variables t_{21} and t_{43} given by Eqs. (11) and (12), respectively (see Fig. 2C).

In the limiting case for which spectral inhomogeneity greatly exceeds the homogeneous line width ($\sigma_I \gg \Gamma_H$), individual features of the RP 2D spectrum exhibit a Lorentzian lineshape in the direction of the anti-diagonal axis ($\bar{\nu}_{21} - \bar{\nu}_{43}$) and an inhomogeneously broadened lineshape in the direction of the diagonal axis ($\bar{\nu}_{21} + \bar{\nu}_{43}$) (31). In this limit, the homogeneous and inhomogeneous line width parameters can be determined directly from the RP 2D spectrum by fitting the anti-diagonal and diagonal cross-sections of the 2D spectral lineshape to model Lorentzian and Gaussian functions, respectively. However, in the more general case of moderate inhomogeneity ($\sigma_I \simeq \Gamma_H$), the homogeneous and inhomogeneous broadening mechanisms each contribute to the RP 2D lineshape in both the diagonal and anti-diagonal directions. In our analyses of the 2D spectral lineshapes of the iCy3 monomer and (iCy3)₂ dimer ss-dsDNA constructs presented below, we determined the homogeneous and inhomogeneous lineshape parameters by simultaneously fitting experimental RP and NRP 2DFS spectra to the numerical Fourier transforms of the model response functions given by Eq. (11) and (12). This approach provided an accurate description of the 2D spectra without imposing any assumed restrictions on the degree of inhomogeneity present.

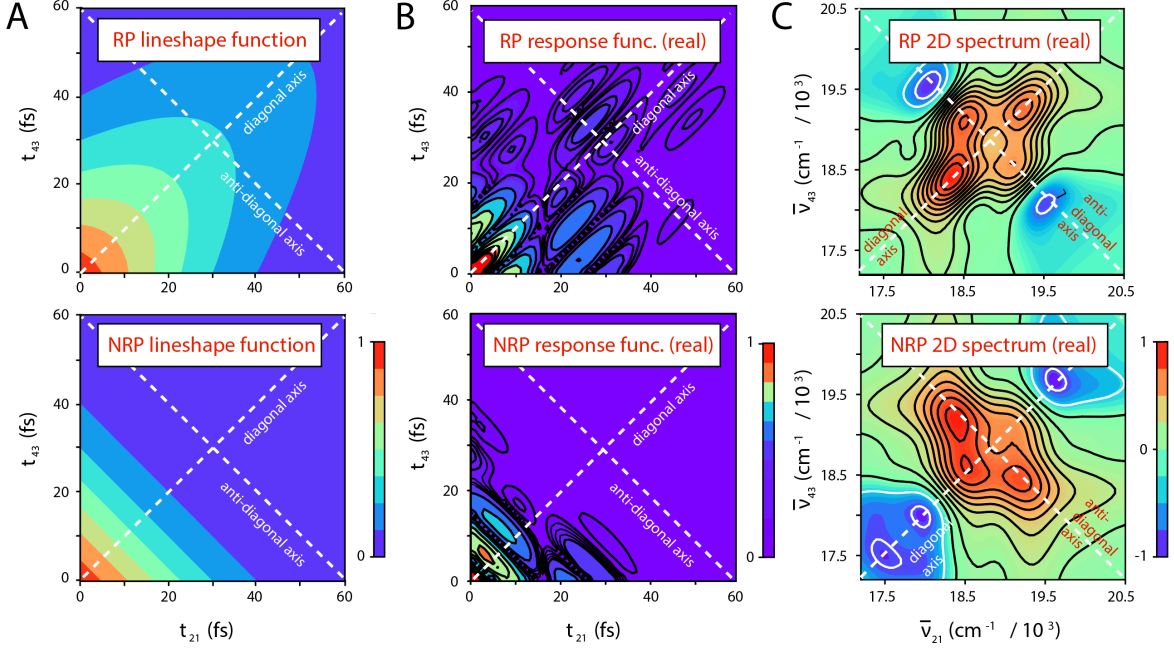


Figure 2. Example calculations of the 2DFS rephasing (RP) and non-rephasing (NRP) response functions and 2D spectra for the -1 iCy3 monomer ss-dsDNA construct at 25°C. The iCy3 monomer Hamiltonian parameters used in these calculations are given in Table S2 of the SI and include the values $\Gamma_H = 105 \text{ cm}^{-1}$ and $\sigma_I = 198 \text{ cm}^{-1}$. All functions are displayed as two-dimensional contour plots with diagonal and anti-diagonal axes indicated as white dashed lines. **(A)** The RP lineshape function (top panel), $\exp[-\Gamma_H(t_{21} + t_{43})]\exp[-\sigma_I^2(t_{21} - t_{43})^2/2]$, contains two independent factors, one representing an exponential (homogeneous) decay along the diagonal axis ($t_{21} + t_{43}$) and the other a Gaussian (inhomogeneous) decay along the anti-diagonal axis ($t_{21} - t_{43}$). The NRP lineshape function (bottom panel), $\exp[-\Gamma_H(t_{21} + t_{43}) - \sigma_I^2(t_{21} + t_{43})^2/2]$, depends on both the homogeneous and inhomogeneous line width parameters, which contribute to the decays along both diagonal and anti-diagonal axes. **(B)** The absolute value of the real parts of the RP response function $S_{RP}(t_{21}, t_{32} = 0, t_{43})$ [Eq. (11), top panel] and the NRP response function $S_{NRP}(t_{21}, t_{32} = 0, t_{43})$ [Eq. (12), bottom panel] contain the lineshape functions shown in panel A and the transition frequency phase factors given by Eqs. (13) – (18). **(C)** The RP 2D spectrum $\hat{S}_{RP}(\bar{\nu}_{21}, t_{32} = 0, \bar{\nu}_{43})$ (top panel) and the NRP spectrum $\hat{S}_{NRP}(\bar{\nu}_{21}, t_{32} = 0, \bar{\nu}_{43})$ are calculated by performing a two-dimensional Fourier transform of the response functions shown in panel B with respect to the time variables t_{21} and t_{43} .

Numerical optimization procedures

In previous work (5, 7), we characterized the iCy3 monomer absorbance spectrum using four independent parameters: the mean electronic transition energy $\bar{\epsilon}_{eg} \cong 18,250 \text{ cm}^{-1}$, the

Huang-Rhys vibronic coupling parameter $\lambda^2 \cong 0.57$, the vibrational frequency $\omega_0 \cong 1,100 \text{ cm}^{-1}$, and the spectral inhomogeneity parameter $\sigma_I \cong 300 \text{ cm}^{-1}$. We determined these values by performing a numerical optimization procedure in which we directly compared the simulated spectra [Eq. (3)] to experimental data. In our previous studies, we assumed constant values for the homogeneous FWHM line width $\Gamma_H = 186 \text{ cm}^{-1}$ and the monomer EDTM $\mu_{eg}^M = 12.8 D$, which we determined in separate experiments (7). We used these monomer parameters as inputs to our analyses of the absorbance and CD spectra of the (iCy3)₂ dimer ss-dsDNA constructs [Eqs. (9) and (10), respectively]. In our calculations of the dimer spectra, we included six vibrational levels in the monomer electronic-vibrational manifold of states to ensure numerical convergence (7). The dimer absorbance and CD spectra were thus used to obtain optimized values of the mean structural parameters $\bar{R}_{AB}$, $\bar{\phi}_{AB}$, and $\bar{\theta}_{AB}$, which determined the mean electrostatic coupling $\bar{J}$ [Eq. (6)].

We next used the set of optimized structural coordinates as inputs to our model analyses of the RP and NRP 2DFS data, which are described by Eqs. (11) and (12), respectively. The transition frequencies, ω_{ab} and ω_{cd} , appearing in the RP and NRP response functions [Eqs. (13) – (18)], in addition to the laser spectral overlap factors $\alpha^2(\omega_{ab})\alpha^2(\omega_{cd})$ and the transition-dipole orientation factors $[\mu_{ab}\mu_{cd}\mu_{jk}\mu_{lm}]_{\mathbf{e}_1\mathbf{e}_2\mathbf{e}_3\mathbf{e}_4}$, are all constants determined by the values of the mean structural coordinates. For our simulations of the 2DFS data, it was necessary to carry out the sums of Eqs. (13) – (18) over the space of transition pathways between the dimer ground electronic-vibrational state manifold (labeled $|v\rangle$, with dimension $6 \times 6 = 36$), the singly-excited electronic-vibrational state manifold (labeled $|e\rangle$ and $|e'\rangle$, with dimension $36 + 36 = 72$) and the doubly-excited electronic-vibrational state manifold ($|f\rangle$, with dimension 36). Nevertheless, the actual number of terms needed to simulate the response functions accurately is a small fraction of the total number of possible pathways, due to the resonance conditions imposed by the laser pulse spectrum [reflected by the factors $\alpha^2(\omega_{ab})\alpha^2(\omega_{cd})$]. In practice, for each response function the summation over transition pathways was calculated and stored as a two-dimensional interferogram that was multiplied by the lineshape function $\exp[-\Gamma_H(t_{21} + t_{43})]\exp[-\sigma_I^2(t_{21} - t_{43})^2/2]$ in the case of the RP response functions [Eqs. (13) – (15)], and the lineshape function $\exp[-\Gamma_H(t_{21} + t_{43})]\exp[-\sigma_I^2(t_{21} + t_{43})^2/2]$ in the case of the NRP response functions [Eqs. (16) – (18)]. We thus performed optimization analyses of our 2DFS

data to obtain accurate values for the homogeneous and inhomogeneous line width parameters, Γ_H and σ_I , respectively.

For our optimization calculations, we implemented an automated multi-variable regression analysis to efficiently explore the parameter space of the spectroscopic models. We have applied similar procedures in past studies (5, 7, 24, 27, 35-37), in which a random search algorithm was used to select an initial set of input parameters that were refined iteratively using commercial software (KNITRO) (38). For each set of input trial parameters, we calculated a linear least squares error function χ^2 , which was minimized to obtain the optimized solution. Thus, for our optimizations of the absorbance and CD spectra of the (iCy3)₂ dimer ss-dsDNA constructs, we minimized the function

$$\chi_{lin}^2(\bar{R}_{AB}, \bar{\phi}_{AB}, \bar{\theta}_{AB}, \sigma_I) = \chi_{abs}^2(\bar{R}_{AB}, \bar{\phi}_{AB}, \bar{\theta}_{AB}, \sigma_I) + \chi_{CD}^2(\bar{R}_{AB}, \bar{\phi}_{AB}, \bar{\theta}_{AB}, \sigma_I) \quad (19)$$

and for our optimizations of 2DFS data, we minimized the function

$$\chi_{2DFS}^2(\Gamma_H, \sigma_I) = \frac{1}{2} [\chi_{RP}^2(\Gamma_H, \sigma_I) + \chi_{NRP}^2(\Gamma_H, \sigma_I)] \quad (20)$$

We performed error-bar analyses of the optimized parameters, which we determined by a 1% deviation of the χ^2 function from its optimized value.

Modeling conformational heterogeneity of (iCy3)₂ dimer labeled ss-dsDNA constructs

As discussed in previous sections, the information provided by the linear absorbance and CD spectra of the (iCy3)₂ dimer permits us to determine the mean values of the conformational coordinates $\bar{R}_{AB}$, $\bar{\theta}_{AB}$ and $\bar{\phi}_{AB}$. By expanding the analysis to include 2DFS data, we determined additional information about the inhomogeneously broadened distribution of homogeneous lineshapes, which is due primarily to the variation of local (iCy3)₂ dimer conformations within the ensemble of ss-dsDNA molecules. To develop our interpretation of the inhomogeneous lineshape in terms of structural disorder, we assumed that the conformational coordinates, R_{AB} , θ_{AB} and ϕ_{AB} , can be treated as independent variables, and that their distributions can be described as a product of Gaussians

$$G_I(R_{AB}, \theta_{AB}, \phi_{AB}) = \frac{1}{(2\pi)^{\frac{3}{2}}\sigma_R\sigma_\theta\sigma_\phi} \exp\left[-\frac{(R_{AB} - \bar{R}_{AB})^2}{2\sigma_R^2}\right] \exp\left[-\frac{(\theta_{AB} - \bar{\theta}_{AB})^2}{2\sigma_\theta^2}\right] \exp\left[-\frac{(\phi_{AB} - \bar{\phi}_{AB})^2}{2\sigma_\phi^2}\right] \quad (21)$$

We emphasize that by assuming that the structural coordinates are independent variables – i.e., that possible covariance terms are negligible – Eq. (21) may only be used to determine estimates of the standard deviations.

To model our inhomogeneously broadened 2DFS data, we calculated a library of ‘homogeneous’ RP and NRP 2D fluorescence spectra spanning a range of equally spaced values for the conformational coordinates, and for which the homogeneous and inhomogeneous line width parameters were set equal – i.e., $\Gamma_H = \sigma_I = 100 \text{ cm}^{-1}$. In Fig. 3A, we show examples of simulated homogeneous RP and NRP 2D fluorescence spectra of the +2 (iCy3)₂ dimer ss-dsDNA construct corresponding to three different values of the mean twist angle $\bar{\phi}_{AB} = 80.6^\circ, 82.6^\circ$ and 84.6° , and for mean tilt angle $\bar{\theta}_{AB} = 5.1^\circ$ and mean separation $\bar{R}_{AB} = 2.8 \text{ \AA}$. From the library of homogeneous 2D spectral lineshapes, we simulated inhomogeneously broadened RP and NRP 2D spectra by numerically sampling the library according to the Gaussian distribution given by Eq. (21). We thus followed a procedure similar to that described in previous sections to iteratively calculate the linear least squares error function $\chi_{2DFS}^2(\sigma_R, \sigma_\theta, \sigma_\phi) = \frac{1}{2}[\chi_{RP}^2(\sigma_R, \sigma_\theta, \sigma_\phi) + \chi_{NRP}^2(\sigma_R, \sigma_\theta, \sigma_\phi)]$ between simulated and experimental spectra, which we minimized to obtain optimized values for the standard deviations of the conformational parameters, σ_R , σ_θ and σ_ϕ . Optimized values were obtained according to the definitions discussed below, which depended on the functional dependence of the error function on the standard deviation parameter.

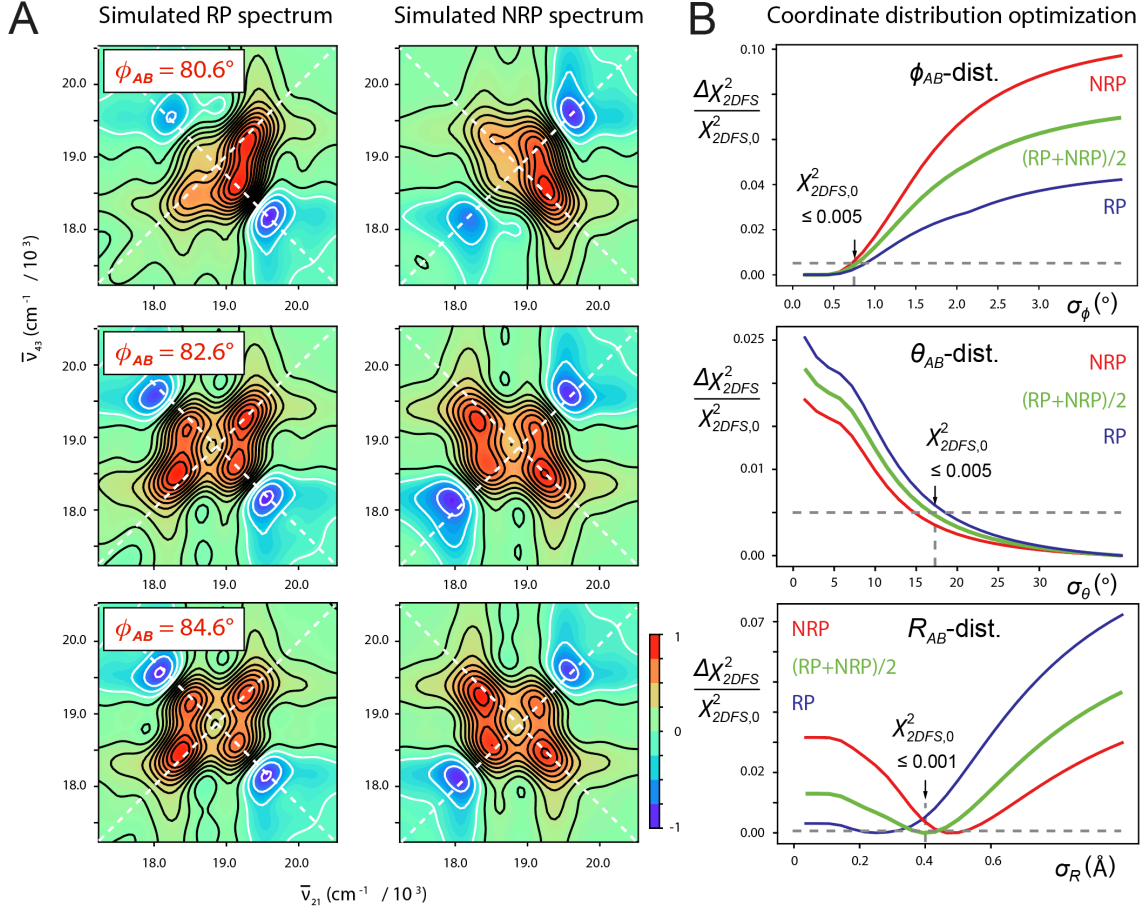


Figure 3. (A) Simulated RP and NRP ‘homogeneous’ 2D fluorescence spectra (real part) of the +2 (iCy3)₂ dimer ss-dsDNA construct for various values of the mean twist angle: $\bar{\phi}_{AB} = 80.6^{\circ}$ (top row), 82.6° (middle) and 84.6° (bottom), mean tilt angle $\bar{\theta}_{AB} = 5.1^{\circ}$, mean separation $\bar{R}_{AB} = 2.8 \text{ \AA}$, and homogeneous and inhomogeneous linewidth parameters $\Gamma_H = \sigma_I = 100 \text{ cm}^{-1}$. (B) Cross-sections of the relative deviation of the linear least squares error function, $\Delta\chi_{2DFS}^2(\sigma_R, \sigma_\theta, \sigma_\phi) / \chi_{2DFS,0}^2 = [\Delta\chi_{RP}^2(\sigma_R, \sigma_\theta, \sigma_\phi) + \Delta\chi_{NRP}^2(\sigma_R, \sigma_\theta, \sigma_\phi)] / 2\chi_{2DFS,0}^2$, are shown as functions of the standard deviations σ_ϕ (top), σ_θ (middle) and σ_R (bottom). Inhomogeneously broadened spectra of the +2 (iCy3)₂ dimer ss-dsDNA construct were simulated by numerically sampling the library of ‘homogeneous’ 2D fluorescence spectra according to the Gaussian distribution of structural coordinates given by Eq. (21). Error function cross-sections are shown plotted relative to their ‘optimized’ values, $\chi_{2DFS,0}^2$ (indicated by vertical arrows), which are defined as the 0.5% threshold for cases in which the function approached its minimum asymptotically (as do the σ_ϕ and σ_θ cross-sections), and the 0.1% threshold for cases in which the function exhibited a distinct minimum (as shown for the σ_R cross-section).

In Fig. 3B, we show example cross-sections of the relative deviation of the linear least squares error function $\Delta\chi_{2DFS}^2(\sigma_R, \sigma_\theta, \sigma_\phi)/\chi_{2DFS,0}^2$ and their RP and NRP contributions, $\Delta\chi_{RP}^2(\sigma_R, \sigma_\theta, \sigma_\phi)/\chi_{2DFS,0}^2$ and $\Delta\chi_{NRP}^2(\sigma_R, \sigma_\theta, \sigma_\phi)/\chi_{2DFS,0}^2$, respectively, with $\Delta\chi_{2DFS}^2 = \chi_{2DFS}^2 - \chi_{2DFS,0}^2$. These functions are plotted relative to their ‘optimized’ values, $\chi_{2DFS,0}^2$, which we defined as the 0.5% threshold (i.e., $\chi_{2DFS,0}^2 \leq 0.005$) in cases for which the minimum is approached asymptotically. In cases for which the function exhibited a distinct minimum, we defined the optimized value such that $\chi_{2DFS,0}^2 \leq 0.001$. For the error function plotted along the σ_ϕ -axis (Fig. 3B, top), we see that $\Delta\chi_{2DFS}^2$ approaches an asymptotic minimum for small values of the standard deviation and increases abruptly for $\sigma_\phi \geq 0.7^\circ$. This indicates that the distribution of the local twist angle parameter for the +2 (iCy3)₂ ss-dsDNA construct is relatively narrow, and that the optimized value for σ_ϕ is an upper bound. In contrast, the cross-section plotted along the σ_θ -axis (Fig. 3B, middle) decreases monotonically with increasing standard deviation, indicating that the optimized value $\sigma_\theta = 17^\circ$ is a lower bound. The average cross-section plotted along the R_{AB} -distribution axis (Fig. 3B, bottom) exhibits a distinct minimum with optimized value $\sigma_R = 0.4 \text{ \AA}$. In all the panels shown in Fig. 3B, threshold values are indicated by horizontal dashed lines.

Determination of the relative fluorescence quantum yield parameter Γ_{2D}

The value used for the parameter Γ_{2D} , which characterizes the relative fluorescence quantum yield of the doubly versus singly excited state populations [described by Eqs. (11) and (12)], is important for fitting 2DFS data to theoretical models (24, 27, 39, 40). To determine the value of Γ_{2D} , we performed an optimization analysis of our temperature-dependent 2DFS data for the (iCy3)₂ dimer labeled +15 (duplex) ss-dsDNA construct based on the H-F Hamiltonian model. In these calculations, we minimized the function χ_{2DFS}^2 described by Eq. (20) while varying the parameter Γ_{2D} . The procedure was carried out for data sets taken at five different temperatures (5, 15, 23, 35 and 45 °C). The remaining input parameters for the H-F model were obtained from the optimized fits to the linear absorbance and CD spectroscopic measurements taken at the same temperatures, as discussed further below. We show the results of these calculations in Fig. S1 of the SI. For each temperature, we observe a progression of the parameter Γ_{2D} that favors lower values except for 25 °C, for which the analysis is relatively

insensitive to the value of Γ_{2D} . We therefore adopted the value $\Gamma_{2D} = 0.3$ for all the (iCy3)₂ dimer calculations presented in the remainder of this work.

Results and Discussion

Local conformations and spectral inhomogeneity of the iCy3 monomer and (iCy3)₂ dimer ss-dsDNA constructs labeled at the +15 ‘duplex’ and -1 ‘fork’ positions

In previous studies, we examined the temperature-dependent absorbance and CD spectra of iCy3 monomer and (iCy3)₂ dimer labeled ss-dsDNA constructs, in which the chromophore probes were positioned either at the +15 position (deep within the duplex region) or at the -1 position relative to the ss-dsDNA fork junction (see Fig. 1C and Table 1) (5, 7). We found that the structural parameters and coupling strengths that characterized the absorbance and CD spectra of the (iCy3)₂ dimer ss-dsDNA constructs varied with probe labeling position and temperature. In Fig. 4, we compare our results for the room temperature (25 °C) CD, absorbance and 2DFS measurements of the iCy3 monomer and (iCy3)₂ dimer labeled +15 ‘duplex’ and -1 ‘fork’ ss-dsDNA constructs.

We first consider the CD, absorbance and 2D fluorescence spectra of the iCy3 monomer labeled +15 ‘duplex’ and -1 ‘fork’ ss-dsDNA constructs (see Figs. 4A and 4C, respectively), which are well-described using the monomer Hamiltonian [Eqs. (1) – (3)], as expected. Values obtained from model fits to the absorbance and CD spectra for the mean electronic transition energy ε_{eg} , electric dipole transition moment $\mu_{eg}^M = 12.8 D$, vibrational frequency ω_0 , and Huang-Rhys parameter λ^2 are shown in the insets. The laser spectrum used for the 2DFS experiments is shown (in gray) overlaid with the absorbance spectra (second row) and spans the spectral region containing the 0-0 and 0-1 vibronic transitions of the monomer (shown as green line segments). The signatures of these transitions are present in the experimental 2DFS data (RP spectra, third row). We simulated the 2DFS data (fourth row) using the monomer Hamiltonian and the optimized parameters obtained from the CD and absorbance spectra, as described in Sect. 3B. We note the good agreement between simulated and experimental 2D spectra shown in Fig. 4A and 4C, although in both cases the simulation predicts slightly higher cross-peak intensities and weaker inhomogeneous broadening than we observed experimentally. For the iCy3 monomer labeled ss-dsDNA constructs, we obtained the optimized values: $\Gamma_H = 145 \text{ cm}^{-1}$ and σ_I

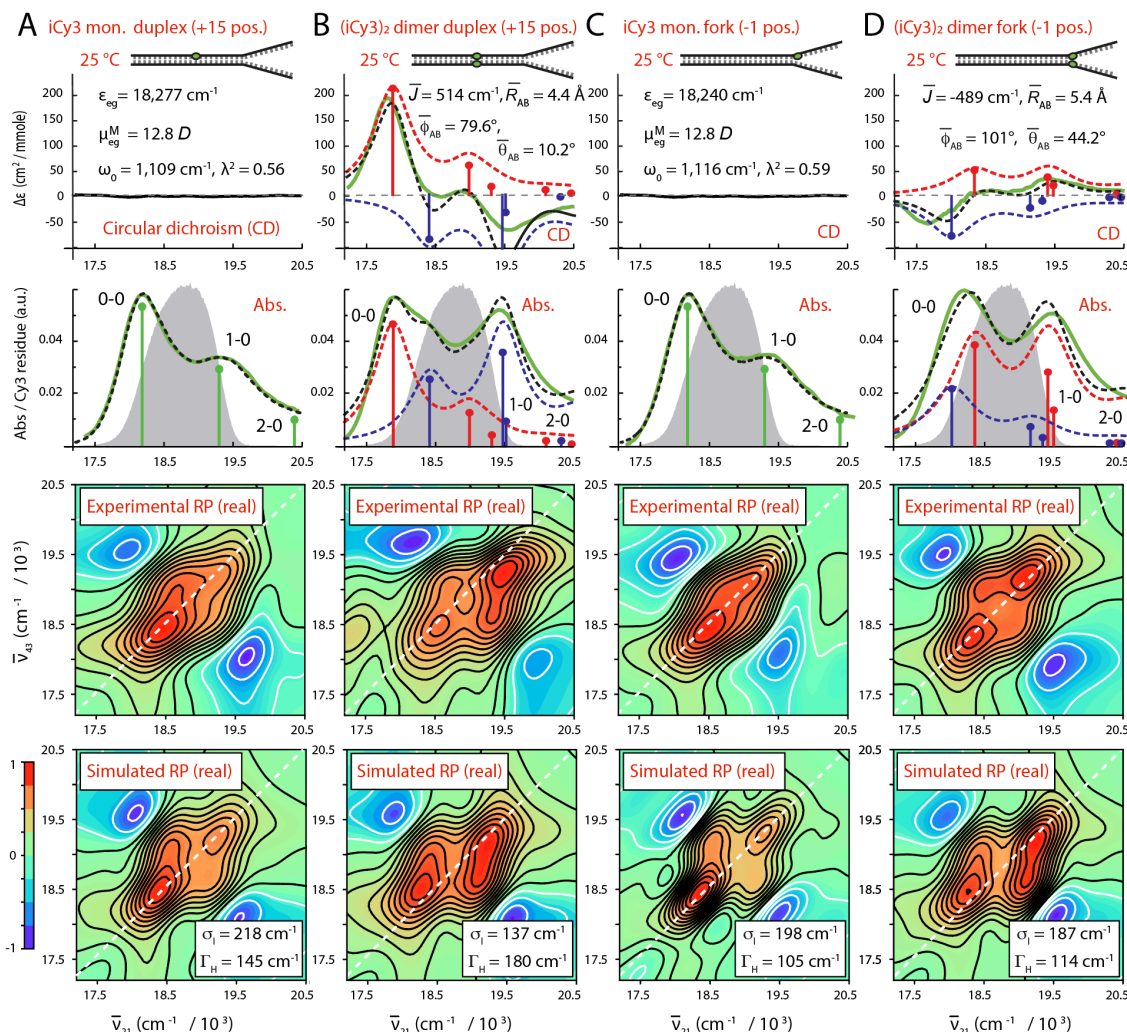
= 218 cm⁻¹ for the +15 ss-dsDNA construct, and $\Gamma_H = 105$ cm⁻¹ and $\sigma_I = 198$ cm⁻¹ for the -1 ss-dsDNA construct. Optimized values for the iCy3 monomer Hamiltonian parameters for the +15 and -1 ss-dsDNA constructs, and their associated error bars, are listed in Table S1 and Table S2 of the SI, respectively.

For the (iCy3)₂ dimer labeled +15 ‘duplex’ and -1 ‘fork’ ss-dsDNA constructs, the CD, absorbance and 2DFS data are well described using the H-F dimer Hamiltonian [Eqs. (4) – (10)] (see Fig. 4B and 4D, respectively). Mean values of the structural parameters and electronic coupling are shown in the insets. The simulated symmetric (+) and anti-symmetric (–) vibronic manifolds are shown overlaid with the experimental spectra as blue and red dashed curves, respectively. The symmetries of the CD spectra, and the corresponding signs of the electrostatic couplings $\bar{J}$, determine the handedness of the (iCy3)₂ dimer conformation. The optimization of the H-F model to the +15 ‘duplex’ ss-dsDNA construct provides values for the mean separation, twist and tilt angles $\bar{R}_{AB} = 4.4$ Å, $\bar{\phi}_{AB} = 79.6^\circ$ and $\bar{\theta}_{AB} = 10.2^\circ$, respectively, which indicates that the sugar-phosphate backbones adopt a right-handed, cylindrically symmetric local conformation at the +15 position consistent with the Watson-Crick B-form crystallographic structure of duplex DNA. In contrast, the optimized values obtained for the -1 ‘fork’ ss-dsDNA construct are $\bar{R}_{AB} = 5.4$ Å, $\bar{\phi}_{AB} = 101^\circ$ and $\bar{\theta}_{AB} = 44.2^\circ$, which indicates that the sugar-phosphate backbones at the -1 position adopt, on average, a left-handed, splayed open conformation. The experimental 2DFS data (third row) exhibit diagonal and off-diagonal peaks, which are due to optically resonant transitions involving the symmetric and anti-symmetric vibronic manifolds. The 2DFS data were simulated (fourth row) using the same optimized structural parameters obtained from the CD and absorbance spectra according to the procedure discussed in Sect. 3B. From our analyses of the 2DFS lineshapes we obtained optimized values of the homogeneous and inhomogeneous line width parameters for the (iCy3)₂ dimer labeled ss-dsDNA constructs: $\Gamma_H = 180$ cm⁻¹ and $\sigma_I = 137$ cm⁻¹ for the +15 ss-dsDNA construct, and $\Gamma_H = 114$ cm⁻¹ and $\sigma_I = 187$ cm⁻¹ for the -1 ss-dsDNA construct.

We note that the simulated and experimental 2DFS data shown in Fig. 4B and 4D are in good agreement. However, like the results we obtained for the iCy3 monomer-labeled constructs described above, the simulations for the (iCy3)₂ dimer-labeled constructs predict slightly higher cross-peak intensities than we observed experimentally. The most likely explanation for the

discrepancy is the presence of third-order dispersion in the laser spectrum, which is not accounted for in our modeling procedure. An additional simplification of our model is the assumption that the system is comprised solely of the exciton-coupled (iCy3)₂ dimer. While the dimer model is necessary to account for the CD spectrum of the -1 (iCy3)₂ ss-dsDNA construct, it is possible that a minor fraction of the dimer probes exist in an uncoupled (monomer-like) configuration. A slightly more complicated model, which describes the system as a heterogeneous mixture of (iCy3)₂ dimer and iCy3 monomer sub-populations would more realistically represent the experimental absorbance and 2DFS data. Nevertheless, we have found that including an iCy3 monomer ‘background’ contribution to the absorbance and 2DFS simulations is not by itself sufficient to explain the discrepancies we observe between simulated and experimental spectra. The third-order dispersion of the laser spectrum, which was maintained constant for all our measurements, is the most likely source of the additional line broadening that we observed in our 2DFS measurements.

Figure 4. (next page) Experimental and simulated spectroscopic measurements of iCy3 monomer and (iCy3)₂ dimer labeled +15 ‘duplex’ and -1 ‘fork’ ss-dsDNA constructs performed at room temperature (25 °C). (**A**) iCy3 monomer +15 ss-dsDNA construct; (**B**) (iCy3)₂ dimer +15 ss-dsDNA construct; (**C**) iCy3 monomer -1 ss-dsDNA construct; and (**D**) (iCy3)₂ dimer -1 ss-dsDNA construct. The experimental CD (top row) and absorbance spectra (second row) are shown (in green) overlaid with vibronic spectral features (black dashed curves) obtained from the optimized fits to the H-F model. For the monomer constructs (**A** and **C**), the vibronic features are shown in green, and for the dimer constructs (**B** and **D**) the symmetric (+) and anti-symmetric (−) excitons are shown in blue and red, respectively. Values of optimized parameters are shown in the insets of the corresponding panels. The laser spectrum, with center frequency $\bar{\nu}_L = 18,796 \text{ cm}^{-1}$ ($\lambda_L = 532 \text{ nm}$) and FWHM bandwidth $\Delta\bar{\nu}_L = 1,100 \text{ cm}^{-1}$ ($\Delta\lambda_L = \sim 33 \text{ nm}$), is shown (in gray) overlaid with the absorbance spectra and spans a region containing both the 0-0 and 1-0 vibronic sub-bands, as shown. Experimental RP spectra (third row) are compared to the optimized simulated RP spectra (fourth row). Simulated spectra are based on the structural parameters obtained from our optimization analyses of the CD and absorbance spectra, the fluorescence quantum yield parameter $\Gamma_{2D} = 0.3$, and the homogeneous and inhomogeneous line width parameters (Γ_H and σ_I , respectively), which are listed for the (iCy3)₂ dimer-labeled constructs in Table 2 and Table 3, and for the iCy3 monomer-labeled constructs in Table S1 and Table S2 of the SI.



The spectral inhomogeneity that we determined from our room temperature 2DFS lineshape analyses was generally larger for the iCy3 monomer labeled constructs than for the (iCy3)₂ dimer labeled constructs. This finding is especially evident for the +15 ss-dsDNA constructs and suggests that the local environment of the sugar-phosphate backbones of the iCy3 monomer probes is more disordered than that of the (iCy3)₂ dimer probes. We note that the (iCy3)₂ dimer ss-dsDNA constructs have the two monomer probes positioned directly opposite to one another in a symmetric manner, while the monomer-labeled ss-dsDNA constructs have a single thymine (T) base positioned on the conjugate strand directly opposite to the iCy3 probe. Monomer substitution may thus introduce a ‘defect site,’ which may be more disruptive to the local conformation and dynamics of the sugar-phosphate backbones than dimer substitution at the same probe labeling positions at room temperature. This finding is consistent with recent studies of the sensitivity of cyanine monomer substituted DNA constructs to the local

environment (41), which can influence fluorescence intensity, local mobility and photostability (6).

Temperature-dependent local conformations and spectral line width parameters of iCy3 monomer and (iCy3)₂ dimer ss-dsDNA constructs labeled at the +15 ‘duplex’ and -1 ‘fork’ positions

We carried out temperature-dependent absorbance, CD and 2DFS measurements of both the iCy3 monomer and the (iCy3)₂ dimer labeled +15 and -1 ss-dsDNA constructs. In previous work, we reported the results of our temperature-dependent studies of the absorbance and CD spectra of these constructs to determine optimized structural and spectroscopic parameters using the monomer and H-F Hamiltonian models (5, 7). Although these studies determined the mean values of the monomer and dimer Hamiltonian parameters, including the mean structural parameters $\bar{R}_{AB}$, $\bar{\phi}_{AB}$ and $\bar{\theta}_{AB}$, they could not provide accurate assessments for the homogeneous and inhomogeneous line widths as we report in the current study. Comparisons between our temperature-dependent experimental 2DFS data and optimized simulations for the monomer and dimer labeled +15 ‘duplex’ ss-dsDNA constructs are shown in Figs. S2 – S5 and Figs. S6 – S9 of the SI, respectively. Similar comparisons for the monomer and dimer labeled -1 ‘fork’ ss-dsDNA constructs are shown in Figs. S10 – S13 and Figs. S14 – S17 of the SI, respectively. In Table 2 and Table 3, we list for these same constructs the mean structural parameters obtained from the absorbance and CD spectra, and the associated line width parameters obtained from our analyses of the 2DFS data over the full range of temperatures studied. The optimized monomer Hamiltonian parameters for the +15 and -1 ss-dsDNA constructs are listed in Table S1 and Table S2 of the SI.

In Fig. 5, we illustrate the temperature-dependence of the absorbance (panel *A*), CD (panel *B*), experimental 2DFS data (panel *C*) and simulated 2DFS data (panel *D*) for the (iCy3)₂ dimer labeled +15 ss-dsDNA construct. The data presented in Fig. 5 and the corresponding parameters listed in Table 2 show that for this construct the mean coupling strength is maximized at 15°C ($\bar{J} = 532 \text{ cm}^{-1}$), and decreases systematically with increasing and decreasing temperature. At the lowest temperatures (1 – 25°C), the absorbance and CD spectra exhibit, respectively, the intensity borrowing and bi-signate lineshapes that are

characteristic of the vibronically coupled iCy3 dimer system (7, 8). The H-F analysis of the absorbance and CD data indicate that the sugar-phosphate backbones of the duplex adopt a progressively increasing mean tilt angle with decreasing temperature, while the mean twist angle does not change significantly (Table 2). Moreover, the 2DFS data at low temperatures show well-separated and relatively narrow peaks and cross-peaks, which indicate the presence of the delocalized symmetric (+) and anti-symmetric (−) excitons.

Table 2. Mean structural parameters and 2DFS line widths determined for the (Cy3)₂ dimer labeled +15 ‘duplex’ ss-dsDNA construct. The parameters determined from model analyses of linear absorbance and CD spectra are the mean resonant coupling $\bar{J}$, the mean twist angle $\bar{\phi}_{AB}$, the mean tilt angle $\bar{\theta}_{AB}$, and the mean interchromophore separation $\bar{R}_{AB}$. The parameters determined from the 2DFS data are the inhomogeneous and homogeneous line widths σ_I and Γ_H . Error bars were calculated based on a 1% deviation of the χ^2 function from its minimum (optimized) value.

Optimized Parameters for (iCy3) ₂ Dimer +15 ‘Duplex’ ss-dsDNA Constructs						
From Absorbance and CD Optimization					From 2DFS Optimization	
T (°C)	$\bar{J}$ (cm ^{−1})	$\bar{\phi}_{AB}$ (°)	$\bar{\theta}_{AB}$ (°)	$\bar{R}_{AB}$ (Å)	σ_I (cm ^{−1})	Γ_H (cm ^{−1})
1	493	78.3+0.3/-0.1	44.2+1.4/-0.4	3.49+0.9/-0.6	121.5 +35.4/-32.4	105.1+22.2/-17.4
5	488	79.3+0.2/-0.1	39.3+0.7/-0.4	2.7+1.1/-0.4	212.7 +36.8/-31.0	109.5 +21.2/-17.9
15	532	80.7 +0.2/-0.1	18.1 +2.9/-2.1	3.7 ± 0.1	141.8 +34.3/ -33.4	149.4 +19.7/-18.7
23	514	79.6 ± 0.2	10.2 +6.4/-27	4.4 ± 0.1	136.7 +45.8/-40.1	180.4 +25.0/-20.3
35	496	76.0 ± 0.3	3.0 +13/-19	5.5 ± 0.1	253.2 +27.6/-25.3	91.8 +14.0/-14.4
45	483	71.9 ± 0.4	7.7 +8.5/-24	6.4 ± 0.1	268.4 +31.8/-26.4	78.5 +17.0/-12.0
55	467	70.4 ± 0.5	7.0 +8.4/-22	6.8 ± 0.1	253.2 +36.2/-33.9	60.8 +18.7/-13.6
65	354	73.7 ± 0.6	5.4 +11/-22	7.1 ± 0.1	217.8 +31.4/-25.2	56.3 +16.7/-14.1

The simulated 2DFS data shown in Fig. 5D, which assumes the optimized structural parameters obtained from the H-F analysis of the absorbance and CD, are in very good agreement with the experimental data at low temperatures (5 – 23°C) and provide an

accurate determination of the homogeneous and inhomogeneous lineshape parameters. We note the agreement is less good for the 1°C data (see Figs. S6 and S7 of the SI), which may be the result of experimental error introduced due to the proximity of the freezing point. As the temperature is increased (35 – 65°C), the absorbance and CD spectral lineshapes change systematically to reflect the decrease in the mean coupling strength. At the highest temperature for which the single strands of the duplex have fully separated (75°C) the absorbance and CD spectra resemble those of the iCy3 monomer substituted ss-dsDNA constructs. The spectral features of the 2DFS data, which are well-defined at low temperatures, become progressively less pronounced as the temperature is increased. We note an abrupt change in the experimental 2DFS lineshapes at temperatures above 23°C in which the intensities of the off-diagonal features decrease, and the exciton-split diagonal features appear to merge into a single diffuse feature. While our simulations predict the observed broadening of the 2DFS lineshapes with increasing temperature, they do not fully capture the merging of the diagonal peaks into a single diffuse feature. The discrepancies are likely due to minor shortcomings of our model, which does not account for laser pulse dispersion or contributions to the signal from the iCy3 monomer. Nevertheless, the simulated spectra do reflect the observed broadening of the 2D lineshapes for most temperatures that we studied, and thus provide valid estimates of the homogeneous and inhomogeneous line width parameters at these temperatures.

As mentioned previously, we simulated our 2DFS data for the iCy3 monomer and (iCy3)₂ dimer +15 ‘duplex’ and -1 ‘fork’ labeled ss-dsDNA constructs using the same temperature-dependent Hamiltonian parameters that we determined from analyses of absorbance and CD data (see Table 2 and Table 3) (5, 7). Our simulations of the 2DFS (iCy3)₂ dimer spectra exhibited peaks and cross-peaks with positions and relative intensities that matched closely our experimental results for the temperature range 5 - 45°C, and for the iCy3 monomer spectra over the range 5 – 65°C. We thus performed optimization calculations on our 2DFS data to determine the best fit homogeneous and inhomogeneous line width parameters, Γ_H and σ_I , respectively, as a function of temperature. These results are presented in Table 2 and Table 3, and the line width parameters are plotted in Fig. 6. Our simulations of the 2DFS data for the (iCy3)₂ dimer +15 ss-dsDNA ‘duplex’ construct do not entirely capture the observed merging of the exciton-split diagonal features at temperatures above 45°C. At these elevated temperatures

ranging through the melting point of the double-stranded region of the ss-dsDNA constructs ($T_m \sim 65^\circ\text{C}$), our simulations appear to underestimate the experimentally observed inhomogeneous line widths, as discussed above. Moreover, for the (iCy3)₂ dimer -1 ss-dsDNA ‘fork’ construct, the highest temperature that we investigated is 45°C , which is ~ 20 degrees lower than the duplex melting point. This is due to the relatively low CD signals of this ss-dsDNA construct at elevated temperatures, which prevented us from obtaining accurate values of the structural parameters above 45°C . As we discuss further below, the structural parameters of the (iCy3)₂ dimer -1 construct appear to have converged to plateau values at temperatures below 45°C .

Table 3. Mean structural parameters and 2DFS line widths determined for the (Cy3)₂ dimer labeled -1 ‘fork’ ss-dsDNA construct. The parameters determined from model analyses of spectroscopic data are the same as defined in Table 2.

Optimized Parameters for (iCy3) ₂ Dimer -1 ‘Fork’ ss-dsDNA Constructs						
From Absorbance and CD Optimization					From 2DFS Optimization	
$T (^\circ\text{C})$	$\bar{J} (\text{cm}^{-1})$	$\bar{\phi}_{AB} (^\circ)$	$\bar{\theta}_{AB} (^\circ)$	$\bar{R}_{AB} (\text{\AA})$	$\sigma_I (\text{cm}^{-1})$	$\Gamma_H (\text{cm}^{-1})$
1	-340	96.1 ± 0.3	$39.9 + 2.2/-2.4$	5.0 ± 0.1	$187.3 + 14.5/-15.3$	$118.35 + 12.2/-9.6$
5	-339	$97.5 + 0.2/-0.7$	$35.53 + 1.8/-7.6$	$5.3 + 0.2/-0.1$	$207.6 + 22.3/-19.6$	$105.1 + 15.1/-11.7$
15	-537	101 ± 0.7	$47.3 + 4.2/-4.5$	5.3 ± 0.1	$217.7 + 39.1/-37.8$	$87.3 + 17.1/-18.3$
23	-489	101 ± 0.8	$44.2 + 5.4/-6.5$	5.4 ± 0.1	$187.3 + 20.5/-22.0$	$113.9 + 13.6/-11.6$
35	-390	101 ± 0.7	$44.4 + 6.1/-7.4$	6.1 ± 0.1	$192.4 + 19.8/-18.1$	$113.9 + 13.8/-10.6$
45	-352	96 ± 0.5	$58.2 + 3.2/-3.5$	5.6 ± 0.1	$187.3 + 17.7/-15.0$	$113.9 + 11.8/-9.5$

In our previous studies we estimated the inhomogeneous line widths based solely on analyses of linear spectroscopic data, for which we assumed that the homogeneous line width was constant ($\Gamma_H = 186 \text{ cm}^{-1}$) for all temperatures and probe labeling positions (7). The results of those studies suggested that for both the iCy3 monomer and (iCy3)₂ dimer labeled +15 (duplex) ss-dsDNA constructs, the inhomogeneous line width parameter increased monotonically with temperature over the range $15 - 65^\circ\text{C}$. However, for the iCy3 monomer and (iCy3)₂ dimer

labeled -1 (fork) ss-dsDNA constructs, the inhomogeneous line width parameter remained relatively constant over this same temperature range.

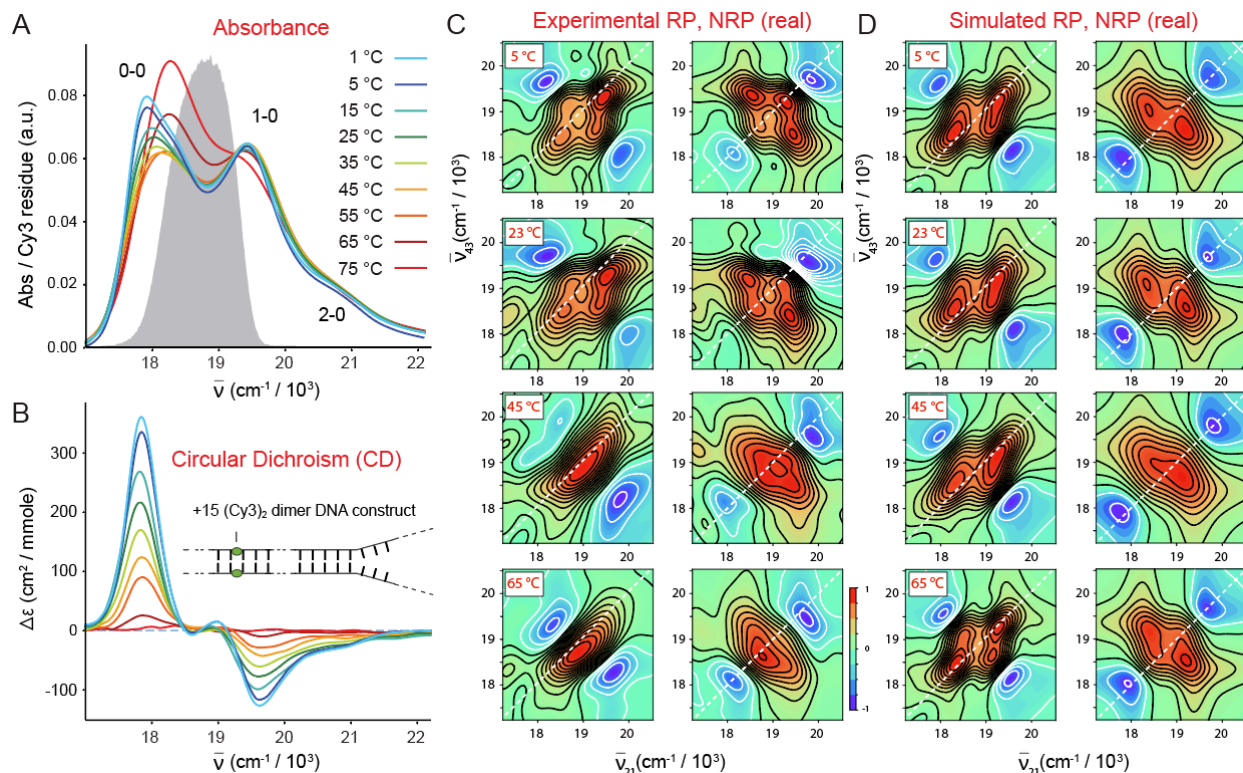


Figure 5. Temperature-dependent spectroscopic measurements of the (iCy3)₂ dimer +15 ss-dsDNA construct. (A) absorbance, (B) CD, (C) experimental 2DFS and (D) simulated 2DFS. The gray curve in panel (A) is the same laser spectrum as shown in Fig. 4. In panels (C) and (D), both RP (left columns) and NRP (right columns) 2D spectra are shown. Additional comparison between the experimental 2DFS data and the optimized simulated spectra for the (iCy3)₂ dimer +15 ss-dsDNA construct are presented in Figs. S6 – S9 of the SI.

We first consider the line width parameters corresponding to the iCy3 monomer labeled +15 ‘duplex’ (Fig. 6A) and the -1 ‘fork’ (Fig. 6C) ss-dsDNA constructs. Our results indicate that the two iCy3 monomer labeled constructs exhibit qualitatively similar temperature dependencies of the inhomogeneous line widths. At the lowest temperatures ($\sim 5 - 15^\circ\text{C}$) the inhomogeneous line width ($\sigma_I \sim 200 \text{ cm}^{-1}$) is significantly larger in magnitude than the homogeneous line width ($\Gamma_H \sim 100 - 140 \text{ cm}^{-1}$), which suggests there is significant conformational disorder of the sugar-phosphate backbones labeled by the iCy3 monomer probe in both the duplex region and at the fork -1 position. Furthermore, the inhomogeneous line widths undergo very little variation

over the temperature range 5 – 65°C, indicating a relatively constant level of conformational disorder of the sugar-phosphate backbones as the temperature is increased towards the melting point.

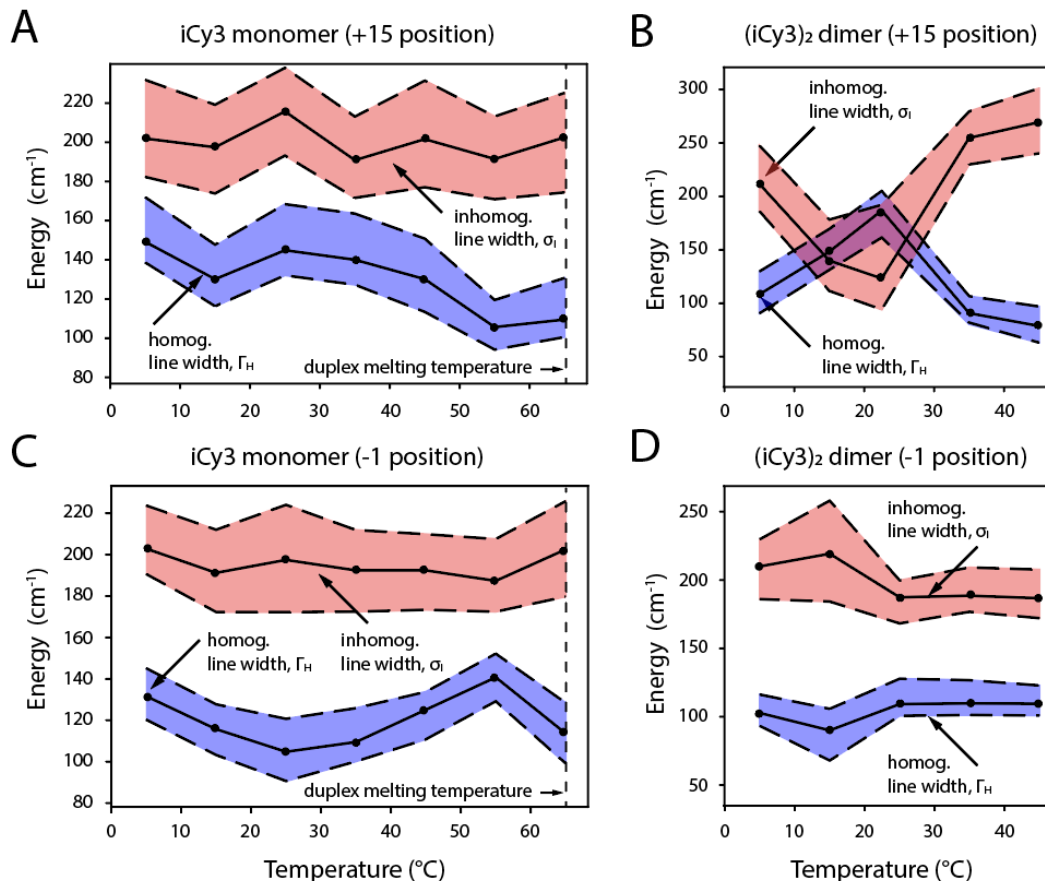


Figure 6. Optimized homogeneous and inhomogeneous line width parameters as a function of temperature obtained from 2DFS lineshape analyses. (A) iCy3 monomer +15 ss-dsDNA construct; (B) (iCy3)₂ dimer +15 ss-dsDNA construct; (C) iCy3 monomer -1 ss-dsDNA construct; and (D) (iCy3)₂ dimer -1 ss-dsDNA construct. Shaded regions bounded by dashed lines indicate error bars, which were calculated based on a 1% deviation of the χ^2_{2DFS} function [Eq. (20)] from its minimum value. The vertical dashed line at 65°C indicates the melting temperature T_m of the duplex regions of the DNA constructs. Direct comparisons between experimental and optimized simulated 2DFS data are presented in Figs. S2 – S17 of the SI.

In Fig. 6, we present the results of our 2DFS lineshape analysis, which provides a far more detailed picture of the temperature- and position-dependent behavior of the homogeneous

and inhomogeneous line width parameters (shown as blue- and teal-shaded regions, respectively). The optimized values of the line width parameters are presented as points and the shaded regions bounded by dashed lines indicate error bars, which are based on a 1% deviation of the χ^2_{2DFS} function [see Eq. (20)] from its optimized value. As mentioned previously, our results at 1°C appear to behave as ‘outliers’ from the remaining temperature-dependent data shown over the range $\sim 5 - 65^\circ\text{C}$, which we next discuss.

The homogeneous line widths of both iCy3 monomer labeled ss-dsDNA constructs exhibit more complicated temperature-dependent behavior. In the case of the iCy3 monomer labeled +15 ‘duplex’ ss-dsDNA construct, the value of the homogeneous line width decreases rapidly from $\Gamma_H \sim 190 \text{ cm}^{-1}$ to $\sim 130 \text{ cm}^{-1}$ over the temperature range $1 - 15^\circ\text{C}$, followed by a slight increase from $\Gamma_H \sim 130 \text{ cm}^{-1}$ to $\sim 145 \text{ cm}^{-1}$ over the range $15 - 25^\circ\text{C}$, and a gradual decrease from $\Gamma_H \sim 145 \text{ cm}^{-1}$ to $\sim 105 \text{ cm}^{-1}$ over the range $25 - 55^\circ\text{C}$. The highest temperature of 65°C corresponds to the melting point of the duplex region, for which 50% of the single strands are expected to be completely separated. At the melting point we observe a significant reduction of the homogeneous line width ($\Gamma_H \sim 114 \text{ cm}^{-1}$) in comparison to the value we obtained for the same constructs at room temperature ($\Gamma_H \sim 145 \text{ cm}^{-1}$). In contrast, for the iCy3 monomer labeled -1 ‘fork’ ss-dsDNA construct, the homogeneous line width decreases gradually from $\Gamma_H \sim 132 \text{ cm}^{-1}$ to $\sim 105 \text{ cm}^{-1}$ over the range $5 - 25^\circ\text{C}$, followed by an increase from $\Gamma_H \sim 105 \text{ cm}^{-1}$ to $\sim 140 \text{ cm}^{-1}$ over the range $25 - 55^\circ\text{C}$. It is interesting to note that while the homogeneous line width of the iCy3 monomer +15 ss-dsDNA construct is maximized at room temperature ($\Gamma_H \sim 145 \text{ cm}^{-1}$), the value of the homogeneous line width of the -1 ‘fork’ ss-dsDNA construct is minimized at room temperature ($\Gamma_H \sim 105 \text{ cm}^{-1}$).

The homogeneous line width is related to the total dephasing time (T_2) according to $T_2 = (\pi c \Gamma_H)^{-1}$ ($\approx 100 \text{ fs}$ for $\Gamma_H \approx 100 \text{ cm}^{-1}$). The total dephasing time can be written in terms of the population relaxation time (T_1) and the pure dephasing time (T'_2), according to $(T_2)^{-1} = (2T_1)^{-1} + (T'_2)^{-1}$ (34). The value of T_1 can be estimated from the room temperature fluorescence lifetime $\tau_F \sim 162 \text{ ps}$ (4). Although the fluorescence lifetime of iCy3 labeled DNA constructs can vary with temperature due to, for example, thermally activated photoisomerization (4, 42-44), such processes are many orders of magnitude slower than the

tens-of-femtosecond time scales of pure dephasing. The homogeneous line width is thus dominated by pure dephasing, which depends on interactions between the electronic transitions and the phonon bath. This suggests that we may interpret the temperature-dependent variation of the homogeneous line width in terms of changes to the iCy3 probe's direct interactions with its local environment, which is comprised primarily by the sugar-phosphate backbones.

We next turn to the homogeneous and inhomogeneous line width parameters of the (iCy3)₂ dimer labeled +15 'duplex' (Fig. 6B) and -1 'fork' (Fig. 6D) ss-dsDNA constructs. These data show that the 2D spectral lineshapes of the (iCy3)₂ dimer labeled duplex and fork ss-dsDNA constructs exhibit strikingly different and more complex temperature-dependent behavior than the iCy3 monomer labeled ss-dsDNA constructs discussed above. The results shown in Fig. 6B were obtained from our analysis of 2DFS data of the (iCy3)₂ dimer labeled +15 duplex ss-dsDNA construct shown in Figs. 5C and 5D and Figs. S6 – S9 of the SI. In this case, both the homogeneous and inhomogeneous lineshape parameters undergo sensitive temperature-dependent variations. For the temperature range 5 – 23°C, the inhomogeneous line width decreases rapidly from the value $\sigma_I \sim 213 \text{ cm}^{-1}$ to $\sigma_I \sim 137 \text{ cm}^{-1}$. The room temperature value of the inhomogeneous line width appears to be a local minimum, since increasing the temperature from 23 – 35°C leads to a rapid increase to the value, $\sigma_I \sim 253 \text{ cm}^{-1}$, suggesting a dramatic increase of local conformational disorder just above room temperature. This relatively high value of the inhomogeneous line width parameter persists ($\sigma_I > \sim 250 \text{ cm}^{-1}$) over the temperature range 35 – 45°C. In contrast, the homogeneous line width parameter increases over the range 5 – 23°C to its maximum value $\Gamma_H \sim 180 \text{ cm}^{-1}$, followed by an abrupt decrease to $\Gamma_H \sim 92 \text{ cm}^{-1}$ over the range 23 – 35°C. This relatively low value of the homogeneous line width persists ($\Gamma_H < \sim 70 \text{ cm}^{-1}$) over the temperature range 35 – 45°C.

We note that the homogenous and inhomogeneous line width parameters appear to depend on temperature in a reciprocal manner, with extremum values attained at room temperature. Evidently at room temperature, the local conformation of the (iCy3)₂ dimer probes at the +15 position, which is deep within the duplex region of the ss-dsDNA construct, is minimally disordered such that the probes interact uniformly with their local environments to maximize the electronic dephasing rate. The room temperature condition

appears to be unique. As the temperature is raised or lowered from 23°C, the local conformational disorder increases abruptly, while the mean coupling strength between the electronic transitions of the probe chromophores and the phonon bath decreases. These results suggest that the W-C local conformation of the (iCy3)₂ dimer labeled sugar-phosphate backbones at sites deep within the duplex region is the optimally stable structure at 23°C, and that the distribution of conformations broadens substantially at temperatures just above or below room temperature. These findings indicate that the activation barriers for thermally induced breathing at these positions are readily surmounted just above room temperature. At the same time, decreasing the temperature below 23°C destabilizes the W-C local conformation in the duplex region, like ‘cold denaturation’ in proteins (45-47).

We next discuss the temperature-dependent optimized line width parameters for the (iCy3)₂ dimer labeled -1 ‘fork’ ss-dsDNA construct, shown in Fig. 6D. Like the (iCy3)₂ dimer labeled +15 duplex construct, the homogeneous and inhomogeneous line widths appear to vary with temperature in a reciprocal manner. However, in this case the principal variation occurs over the temperature range 5 – 23°C for which the inhomogeneous line width increases from $\sigma_I \sim 208 \text{ cm}^{-1}$ to $\sigma_I \sim 218 \text{ cm}^{-1}$, and then decreases to $\sigma_I \sim 187 \text{ cm}^{-1}$. Over this temperature range, the homogeneous line width initially decreases from $\Gamma_H \sim 105 \text{ cm}^{-1}$ to $\Gamma_H \sim 87 \text{ cm}^{-1}$, followed by an increase to $\Gamma_H \sim 114 \text{ cm}^{-1}$. Upon further increasing the temperature above 23°C, the inhomogeneous and homogeneous line width parameters do not undergo significant additional changes, suggesting that – unlike the duplex labeled ss-dsDNA constructs – the distribution of local conformations of the (iCy3)₂ labeled fork construct is not broadened by thermally activated processes near room temperature.

Local conformations and spectral line width parameters of (iCy3)₂ dimer ss-dsDNA constructs labeled at the +2, +1, -1, and -2-positions

We next performed room temperature absorbance, CD and 2DFS experiments on (iCy3)₂ dimer labeled ss-dsDNA constructs in which the dimer probe position was systematically varied across the ss-dsDNA fork junction. The results of these studies are summarized in Fig. 7, in which columns A – D correspond to the probe label positions: +2, +1, -1, and -2, respectively.

The corresponding optimized values for the mean structural parameters and homogeneous and inhomogeneous line widths are listed in Table 4. Comparisons between the experimental and optimized simulated 2DFS data are presented in Figs. S18 – S21 of the SI, respectively. In the top two rows of Fig. 7, the experimental CD and absorbance spectra (green curves) are shown overlaid with simulated spectra resulting from our H-F model analyses. The color schemes are the same as those used in Fig. 4 above. Experimental and simulated 2DFS data using laser spectrum with FWHM $\Delta\bar{\nu}_L = 1,100 \text{ cm}^{-1}$ ($\Delta\lambda_L = \sim 33 \text{ nm}$, overlaid with absorbance spectra in gray) are shown in the third and fourth rows, respectively.

From our H-F model analyses of the absorbance and CD data, we see that the mean electrostatic coupling $\bar{J}$ undergoes a sign inversion as the (iCy3)₂ dimer probe position is changed from the +1 to -1 position across the ss-dsDNA junction. This is accompanied by non-continuous changes of the local conformational coordinates: the mean interchromophore separation $\bar{R}_{AB}$ [2.8 Å (+2), 5.8 Å (+1), 4.3 Å (-1) and 4.4 Å (-2)], the mean twist angle $\bar{\phi}_{AB}$ [84° (+2), 77° (+1), 96° (-1) and 97° (-2)], and the mean tilt angle $\bar{\theta}_{AB}$ [6.6° (+2), 48° (+1), 26° (-1) and 27° (-2)]. The sign inversion of the electrostatic coupling between the +1 and -1 positions indicates that the conformation of the (iCy3)₂ dimer probe, and presumably that of the sugar-phosphate backbones labeled at these sites, changes from right-handed to left-handed. This change of handedness is correlated to a change of the mean twist angle $\bar{\phi}_{AB}$ from values that are less than 90° (right-handed) to values that are greater than 90° (left-handed). In addition, the mean tilt angle $\bar{\theta}_{AB}$ undergoes an abrupt increase from 7° to 48° between the +2 and +1 positions, followed by a decrease to $\sim 27^\circ$ at the -1 and -2 positions. This indicates that the local conformation of the sugar-phosphate backbones within the ss-dsDNA fork constructs undergo an abrupt loss of cylindrical symmetry at the interface between the +2 and +1 positions. However, some of the cylindrical symmetry is recovered at the -1 and -2 positions.

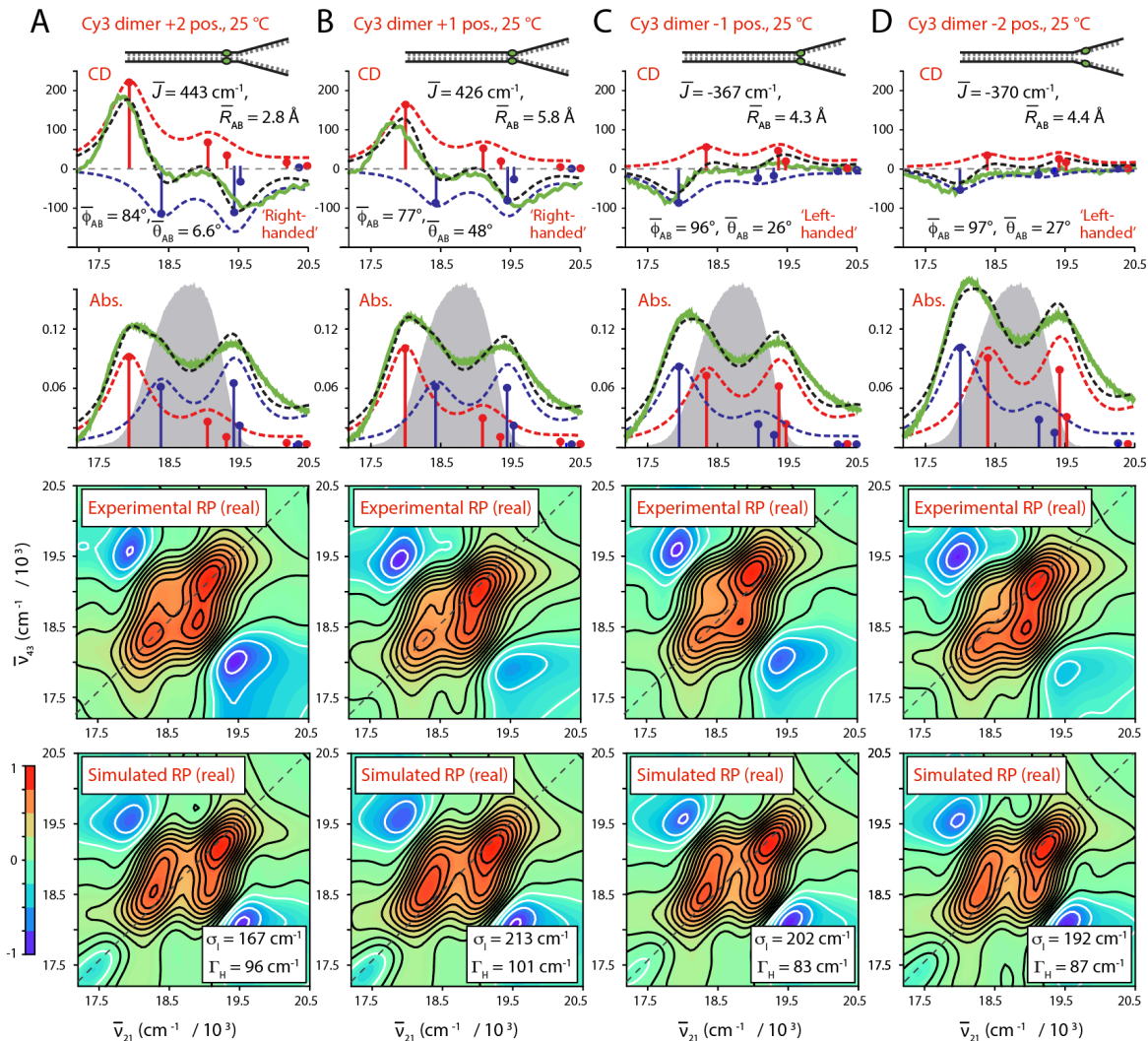


Figure 7. Experimental and simulated spectroscopic measurements performed at room temperature (25 °C) for (iCy3)₂ dimer ss-dsDNA constructs as a function of probe labeling position. (A) +2; (B) +1; (C) -1; and (D) -2. The experimental CD (top row) and absorbance spectra (second row) are shown (in green) overlaid with vibronic spectral features (black dashed curves) obtained from optimized fits to the H-F model. The symmetric (+) and anti-symmetric (−) excitons are shown in blue and red, respectively. Values of the optimized parameters are shown in the insets of the corresponding panels. The laser spectrum (in gray) is shown overlaid with the absorbance spectrum and is the same as in Fig. 4. Experimental RP spectra (third row) are compared to the optimized simulated RP spectra (fourth row). Simulated spectra are based on the structural parameters obtained from our optimization analyses of the CD and absorbance spectra, the fluorescence quantum yield parameter $\Gamma_{2D} = 0.3$, and the homogeneous and inhomogeneous line width parameters (Γ_H and σ_I , respectively) listed in Table 4. Comparisons between experimental and optimized simulated 2DFS data are presented in Figs. S18 – S21 of the SI.

Table 4. Conformational parameters and 2D spectral line widths determined from H-F model analyses of absorbance, CD and 2DFS of (iCy3)₂ dimer labeled ss-dsDNA fork constructs.

Construct	$\bar{J}$ (cm ⁻¹)	$\bar{\phi}_{AB}$ (°)	$\bar{\theta}_{AB}$ (°)	$\bar{R}_{AB}$ (Å)	σ_I (cm ⁻¹)	Γ_H (cm ⁻¹)
+2	443	84.2 ± 0.1	6.6 ± 32	2.8 ± 0.1	167.1 +21.8/-16.6	96.2 +14.8/-10
+1	426	77.0 ± 0.3	48.5 ± 3.3	5.8 ± 0.1	212.6 +28.2/-22.8	100.6 +18.2/-12.9
-1	-367	96.0 ± 0.2	26.4 ± 2.5	4.3 ± 0.1	202.5 +20.3/-18.0	82.9 +11.2/-12.4
-2	-420	97.3 ± 1.8	27.1 ± 54	4.4 ± 0.7	192.4 +20.0/-16.2	87.3 +13.6/-9.6

The simulated and experimental 2DFS measurements shown in Fig. 7 are in very good agreement, although for the -1 and -2 positions the simulated cross-peak intensities are slightly greater than those observed experimentally. Our analyses of the 2DFS spectral lineshapes show how local conformational disorder of the (iCy3)₂ dimer probes, in addition to interactions between the electronic transitions and the phonon bath, depend on the probe labeling position. In Fig. 8A, we plot the position-dependent values of the inhomogeneous and homogeneous line width parameters. For the +2 ss-dsDNA fork construct, the inhomogeneous line width $\sigma_I \sim 167$ cm⁻¹, and the homogeneous line width $\Gamma_H \sim 96$ cm⁻¹. The value for the inhomogeneous line width is significantly larger than the one we obtained for the +15 ss-dsDNA construct ($\sigma_I \sim 137$ cm⁻¹), while the value for the homogeneous line width is smaller (compare to 23°C point of Fig. 6B), indicating a higher degree of conformational disorder at the +2 position relative to +15. When the probe labeling position is changed to +1, the inhomogeneous line width parameter increases: $\sigma_I \sim 213$ cm⁻¹, while the homogeneous line width undergoes only a slight increase: $\Gamma_H \sim 101$ cm⁻¹. This finding suggests that although the sugar-phosphate backbones at the +1 position maintain the right-handed local conformations seen at the +2 and +15 positions (characteristic of the B-form double-helix), the distribution of local conformations is further broadened at the +1 position in comparison to the +2 position. When the probe labeling position is changed to -1, we see that both the inhomogeneous and homogeneous line width parameters decrease to the values $\sigma_I \sim 202$ cm⁻¹ and $\Gamma_H \sim 83$ cm⁻¹. These values do not change significantly when the probe labeling sites are

shifted to the -2 position: $\sigma_I \sim 192 \text{ cm}^{-1}$ and $\Gamma_H \sim 87 \text{ cm}^{-1}$. These findings suggest that the distribution of local conformations decrease slightly at the -1 and -2 positions relative to the +1 position. Thus, the abrupt change in average local conformation that we observe across the +2 to +1 ss-dsDNA junction (from right-handed cylindrically symmetric to right-handed cylindrically asymmetric) is accompanied by the appearance of local conformational disorder at the +1 position. An additional change in the average local conformation occurs across the +1 to -1 positions (from right-handed to left-handed) for which the local conformational disorder persists for positions extending into the single-stranded region of the ss-dsDNA constructs.

Distribution of local conformational parameters of (iCy3)₂ dimer ss-dsDNA constructs labeled at the +2, +1, -1, and -2-positions

We used the 2DFS data shown in Fig. 7 and Figs. S18 – S22 of the SI to model the distributions of conformational parameters according to the method outlined in Sect. 3D. The results of these studies are summarized in Table 5 and are shown together with the optimized values for the inhomogeneous and homogeneous line width parameters in Fig. 8. In Figs. 8B – 8D, the optimized values for the mean conformational parameters, which we determined from our analyses of absorbance and CD spectra, are presented as points. Shaded regions represent the optimized Gaussian widths of the corresponding distributions, which we determined by minimizing the least squares error functions χ^2_{2DFS} shown in Fig. S23 of the SI. In Fig. 8A, the inhomogeneous and homogeneous line width parameters and 1% deviation error bars are presented, which we determined according to the 2D lineshape analysis presented in previous sections.

In Fig. 8B, we plot the position-dependence of the mean twist angle $\bar{\phi}_{AB}$ and standard deviation σ_ϕ . For the +2 ss-dsDNA construct, the mean twist angle has the value $\bar{\phi}_{AB} = 84.2^\circ$, which is very close to that obtained for the +15 position (79.6°). The value we determined for the standard deviation at this position is relatively small, $\sigma_\phi = \sim 0.7^\circ$. As the position is changed to +1, the twist angle decreases to $\bar{\phi}_{AB} = 77.0^\circ$ with standard deviation $\sigma_\phi = \sim 1.7^\circ$. When the position is changed to -1, the mean twist angle undergoes a significant increase to $\bar{\phi}_{AB} = 96.0^\circ$

(from right-handed to left-handed) with standard deviation $\sigma_\phi = \sim 1.1^\circ$. The values for both the mean twist angle and standard deviation do not change significantly when the probe labeling position is changed from -1 to -2. From these results, we conclude that while the mean twist angle undergoes significant changes as the probe labeling position is varied across the ss-dsDNA fork junction, the distribution of twist angles remains relatively narrow ($\sigma_\phi < \sim 2^\circ$) for these positions.

We next consider the position-dependent behavior of the mean tilt-angle $\bar{\theta}_{AB}$ and its standard deviation σ_θ , as shown in Fig. 8C. For the +2 labeled ss-dsDNA construct, the mean tilt angle is $\bar{\theta}_{AB} = 6.6^\circ$ and the standard deviation is $\sigma_\theta = \sim 17^\circ$, indicating that there is a significant degree of conformational disorder in the tilt angle parameter at this position, although the sugar-phosphate backbones occupy, on average, a cylindrically symmetric local conformation. When the label position is changed to +1, the mean tilt angle increases dramatically to $\bar{\theta}_{AB} = 48.5^\circ$ and the standard deviation decreases slightly to $\sigma_\theta = \sim 7^\circ$. This indicates that the cylindrical symmetry of the sugar-phosphate backbones, which is normally found in the duplex region, is no longer present at the +1 position and that the conformational disorder of the tilt angle has decreased slightly. When the label position is changed to -1, the mean tilt angle decreases significantly to $\bar{\theta}_{AB} = 26.4^\circ$ and the standard deviation decreases to $\sigma_\theta = \sim 3.5^\circ$. When the label position is changed from -1 to -2, the mean tilt angle does not change significantly ($\bar{\theta}_{AB} = 27.1^\circ$), although the standard deviation increases to $\sigma_\theta = \sim 6^\circ$.

Table 5. Standard deviations of conformational parameters determined from H-F model analyses of absorbance, CD and 2DFS data of (iCy3)₂ dimer labeled ss-dsDNA fork constructs. The optimized values were obtained by minimizing the least squares error function χ^2_{2DFS} described in Sect. 3D, which are shown in Fig. S23 of the SI.

Construct	σ_ϕ (°)	σ_θ (°)	σ_R (Å)
+2	0.8	17	0.4
+1	1.8	7.0	0.28
-1	1.1	4.0	0.22
-2	1.0	5.5	0.32

In Fig. 8D, we show the position-dependent behavior of the mean interchromophore separation $\bar{R}_{AB}$. For the +2 labeled construct, the mean separation is $\bar{R}_{AB} = 2.8 \text{ \AA}$ and the standard deviation is $\sigma_R = \sim 0.4 \text{ \AA}$. We acknowledge that this value for the mean separation is unrealistically small as it lies below the van der Waals contact distance of $\sim 3.5 \text{ \AA}$. This inconsistency is likely due to a breakdown of our H-F model analysis that underestimates the coupling strength $\bar{J}$ for small interchromophore separations (11). When the probe label position is changed to +1, the mean separation decreases slightly to $\bar{R}_{AB} = 5.8 \text{ \AA}$ and the standard deviation decreases slightly to $\sigma_R = \sim 0.3 \text{ \AA}$. However, when the position is changed from +1 to -1, the mean separation decreases significantly to $\bar{R}_{AB} = 4.3 \text{ \AA}$ and the standard deviation decreases to $\sigma_R = \sim 0.2 \text{ \AA}$. When the position is changed further from -1 to -2, the mean separation does not change significantly ($\bar{R}_{AB} = 4.4 \text{ \AA}$) and the standard deviation is relatively unchanged: $\sigma_R = \sim 0.2 \text{ \AA}$.

It is interesting to compare our results for the distributions of local conformational parameters, as summarized in Figs. 8B – 8D, to the optimized values we obtained for the homogeneous and inhomogeneous line widths shown in Fig. 8A. The position-dependencies of the line width parameters were discussed in the previous section and show that there is relatively little variation in the inhomogeneous line width σ_I as the probe label position is changed from the +2 to -2 positions across the ss-dsDNA junction. Although the mean structural coordinates vary sensitively across the ss-dsDNA junction, the standard deviations of the structural coordinates that we obtained from our 2DFS lineshape analysis indicate that the distributions of conformational coordinates at these positions remain relatively narrow at room temperature.

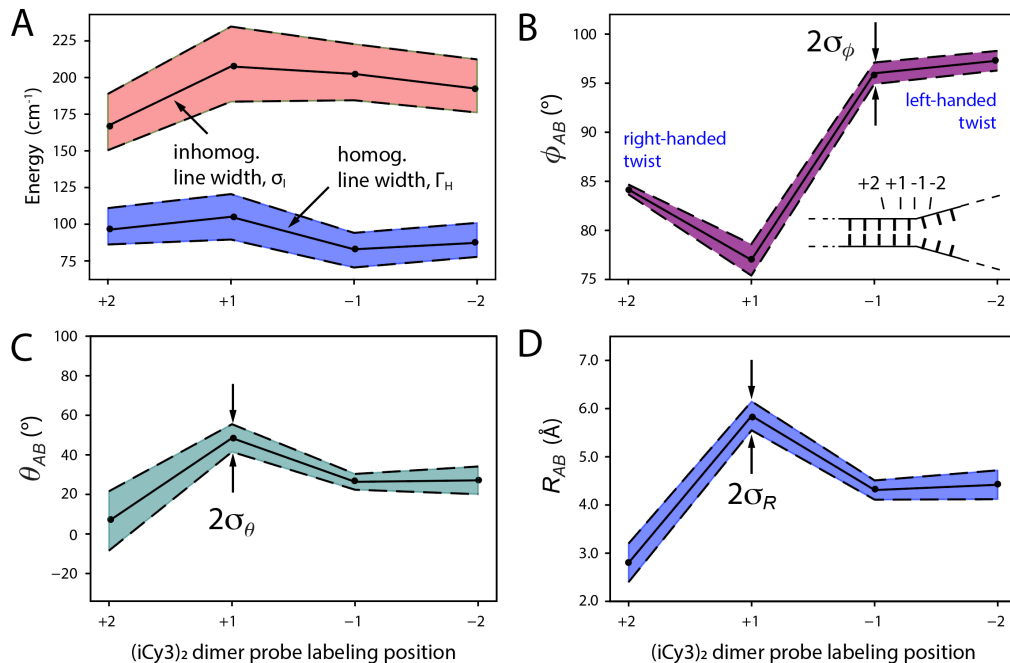


Figure 8. Optimized spectral line width and structural parameters of (iCy3)₂ dimer labeled ss-dsDNA constructs for varying label position obtained from 2DFS lineshape analyses. **(A)** homogeneous and inhomogeneous line width parameters. **(B)** Mean twist angle $\bar{\phi}_{AB}$; **(C)** mean tilt angle $\bar{\theta}_{AB}$; and **(D)** mean inter-chromophore separation $\bar{R}_{AB}$. In panel **A**, the shaded regions bounded by dashed lines indicate error bars, which were calculated based on a 1% deviation of the χ^2_{2DFS} function [Eq. (20)] from its minimized value. In panels **(B – D)**, the shaded regions bounded by dashed lines indicate the standard deviations of the structural parameter distributions obtained from analyses of the 2DFS spectral lineshapes, which are listed in Table 5. These values were obtained by minimizing the least squares error functions shown in Fig. S23 of the SI.

Conclusions

The conservation of base sequence integrity within genomic DNA is critical to maintaining well-regulated gene expression and replication. However, the structure of DNA within the cell must be dynamic, allowing for thermally induced fluctuations (i.e., DNA ‘breathing’) to facilitate productive interactions, including binding-site recognition and the assembly of functional protein-DNA complexes. For example, at ss-dsDNA fork junctions, transient local conformation fluctuations of the sugar-phosphate backbones are likely transition

states for the formation of a stable helicase-primase (primosome) sub-assembly during DNA replication, with the existence of multiple DNA conformers working to facilitate competition between different protein regulatory factors and replisome proteins.

In this work, we have probed the average local conformations and the degree of conformational disorder at and near model ss-dsDNA replication fork junctions through site-specific internal labeling with two cyanine dyes (iCy3) rigidly inserted within the sugar-phosphate backbones at opposite positions within complementary single strands. We performed linear (absorbance and CD) and nonlinear (2DFS) spectroscopic studies of iCy3 monomer and (iCy3)₂ dimer-labeled ss-dsDNA constructs as a function of temperature and probe label position (see Table 1). Our analyses of the absorbance spectra of the iCy3 monomer-labeled ss-dsDNA constructs indicate that the monomer Hamiltonian parameters (i.e., the mean electronic transition energy $\bar{\epsilon}_{eg}$, the Huang-Rhys vibronic coupling parameter λ^2 , and the vibrational frequency ω_0) are largely insensitive to temperature and probe label position (see Table S1 and Table S2 of the SI). In contrast, the absorbance and CD spectra of (Cy3)₂ dimer-labeled ss-dsDNA constructs respond sensitively to changing temperature and probe label position, from which we observed systematic changes of the optimized dimer Hamiltonian parameters (i.e., the mean resonant coupling $\bar{J}$, the mean twist angle $\bar{\phi}_{AB}$, the mean tilt angle $\bar{\theta}_{AB}$, and the mean interchromophore separation $\bar{R}_{AB}$, see Tables 2 – 4). Our 2DFS experiments have provided information about the local conformational disorder of the Cy3 probes at the probe label positions, from which we found that the Cy3 monomer-labeled ss-dsDNA constructs are significantly more disordered than the corresponding (Cy3)₂ dimer-labeled constructs at room temperature (see Fig. 6). The relatively high disorder that we observed in the iCy3 monomer-labeled ss-dsDNA constructs is likely due to a mismatch of the W-C base pairing in the vicinity of the probe labeling site. In the iCy3 monomer-labeled ss-dsDNA constructs, a single thymine (T) base was positioned directly opposite to the iCy3 probe on the conjugate strand to act as a spacer. Nevertheless, normal W-C pairing is likely perturbed by the incorrect spacing between complementary bases introduced by the presence of the single iCy3 monomer probe. In contrast, we found that the (iCy3)₂ dimer-labeled ss-dsDNA constructs are minimally disordered at room temperature and physiological buffer salt conditions, and their conformation-dependent spectroscopic properties appear to reflect the site-specific local conformations of the sugar-phosphate backbones at positions relatively close to the ss-dsDNA fork junction.

From our temperature-dependent studies of the (iCy3)₂ dimer labeled ss-dsDNA +15 ‘duplex’ construct, we found that local conformations of the sugar-phosphate backbones deep within the double-strand region occupy a minimally disordered, right-handed B-form conformation at room temperature (23°C). The B-form conformation is destabilized when the temperature is either raised or lowered from room temperature (see Fig. 6B and Table 2). These observations are consistent with the notion that the room temperature stability of the B-form conformation results from a nearly equal balance between opposing thermodynamic forces (entropy-enthalpy compensation), and that small departures from room temperature (in either the positive or negative direction) alter the free energy landscape and serve to populate non-B-form conformations. Such a picture is sometimes invoked in ‘DNA breathing and trapping models,’ in which non-canonical local conformations of the DNA framework are transiently populated under physiological conditions and function as activated states required for protein-DNA complex assembly (2, 37, 48).

Our temperature-dependent studies of the (iCy3)₂ dimer-labeled ss-dsDNA -1 ‘fork’ construct showed that the average local conformation of the sugar-phosphate backbones at this probe position is left-handed and relatively disordered (in comparison to the +15 position) at room temperature. Increasing the temperature above 23°C did not significantly change the average local conformation or conformational disorder at the -1 position, suggesting that the free energy landscape in this region of the construct is relatively insensitive to increasing temperature. However, like our observation for the +15 duplex ss-dsDNA construct, decreasing temperature below 23°C did lead to a significant increase of the conformational disorder at the -1 position. These observations suggest, as has been studied in protein systems, that the concept of ‘cold denaturation’ (defined as positions in the phase diagram for the folding-unfolding transition of the protein where changes in temperature in either direction decreases the stability of the folded form) might be productively applied to investigations of the stability of ss-dsDNA transitions as well (46, 47, 49-51). Future studies of nucleic acid stability, using the 2DFS approach described in the current work to investigate conformational disorder at nucleic acid positions of possible physiological interest, may help to shed new light on the underlying molecular mechanisms of some of the central processes of genome expression.

The results of our position-dependent studies of the (iCy3)₂ dimer labeled ss-dsDNA constructs at room temperature provide detailed information about the local conformations of the sugar-phosphate backbones at positions across the ss-dsDNA fork junction and are summarized in Fig. 9. The mean local conformations of the sugar-phosphate backbones are right-handed (with mean twist angle, $\bar{\phi}_{AB} < 90^\circ$) for positive integer positions, and left-handed ($\bar{\phi}_{AB} > 90^\circ$) for negative integer positions. Local conformations deep within the duplex region are cylindrically symmetric (with mean tilt angle $\bar{\theta}_{AB} \approx 5 - 10^\circ$) and minimally disordered (with inhomogeneous line width, $\sigma_I = 137 \text{ cm}^{-1}$). The disorder increases significantly for positive positions approaching the ss-dsDNA fork junction ($\sigma_I = 167 \text{ cm}^{-1}$ at the +2 position). At the +1 position, we observe an abrupt loss of cylindrical symmetry ($\bar{\theta}_{AB} = 48^\circ$), which coincides with an additional gain in conformational disorder ($\sigma_I = 213 \text{ cm}^{-1}$). The left-handed conformations at the -1 and -2 positions exhibit somewhat smaller mean tilt angles ($\bar{\theta}_{AB} = 27^\circ$ and 26° , respectively), and decreasing conformational disorder ($\sigma_I = 202 \text{ cm}^{-1}$ and 192 cm^{-1} , respectively), suggesting that the peak perturbation to secondary structure within the ss-dsDNA fork junction occurs at the +1 position. Our 2DFS experiments provide additional information about the standard deviations of the distributions of conformational coordinate (see Fig. 8 and Table 5). Perhaps surprisingly, our analyses indicate that the distributions of conformational parameters at positions traversing the ss-dsDNA fork junction are narrow, suggesting that regions of the junction extending into the single strands are relatively well-ordered. We emphasize that while the above conclusions are based on the interpretation of ensemble spectroscopic measurements, future single-molecule experiments performed on (iCy3)₂ dimer ss-ds DNA constructs can, in principle, probe directly the individual conformational states that underlie the distributions reported here.

Our findings provide a detailed picture of the variation of local conformation and conformational disorder of the sugar-phosphate backbones at and near the ss-dsDNA fork junction. The relatively narrow distributions of local conformations at such key positions implies that the number of possible states that mediate protein binding may be rather limited and suggests a possible structural framework for understanding the roles of transient DNA junction conformations in driving the processes of DNA-protein complex assembly and function.

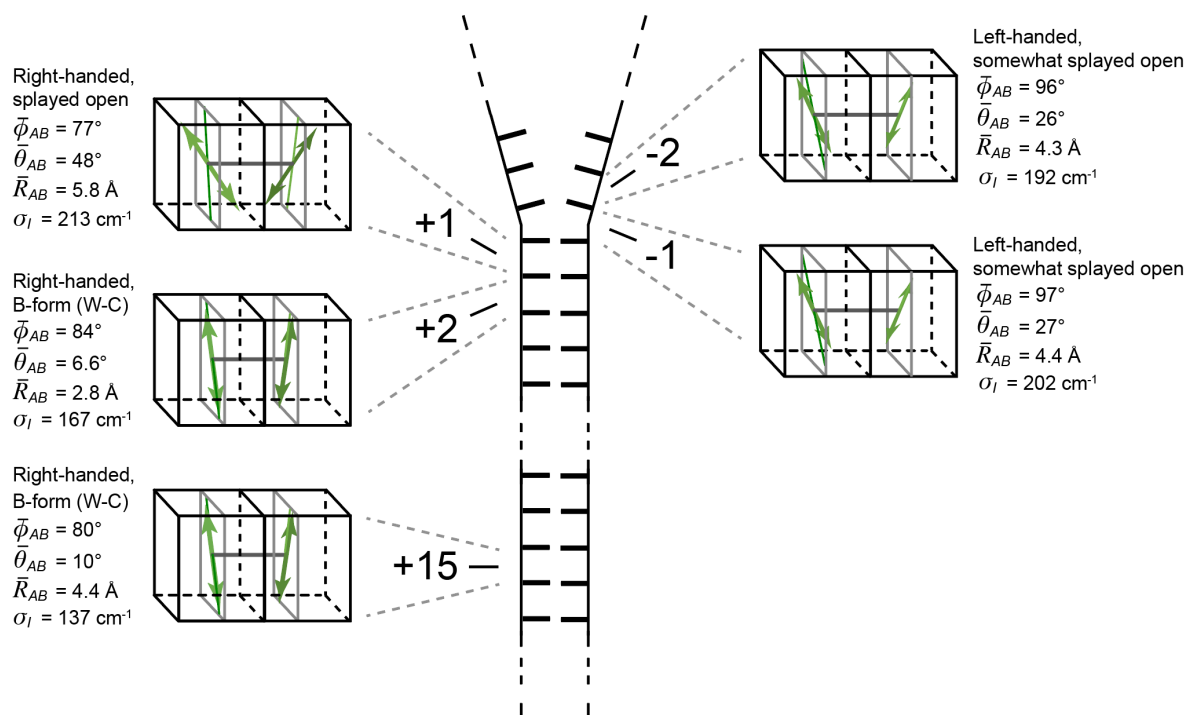


Figure 9. Schematic illustration of the average local conformations of the (iCy3)₂ dimer labeled ss-dsDNA junction at positions +15, +2, +1, -1 and -2 at room temperature.

Bridge to Chapter IV

After analyzing labeled DNA duplexes and fork junctions labeled at the junction using 2DFS, it is evident that there are structural motifs and disorder parameters that are characteristic of positions as the labels are moved across the ss-ds DNA junction. Next, we revisit the structural parameters found for duplex DNA and ds-ss fork junctions using a novel transition charge density methodology, which more accurately captures the Coulomb interaction of atomistically partitioned transition charges than point-dipole or extended-dipole models. The transition charge density approach provides additional information about dimer conformation, and aids in the elimination of unphysical structures that produce overlaps between distinct atomic sites.

Chapter IV

Experimental Validation of Transition Charge Density Models for Excitonically-coupled Cy3 Dimer-labeled DNA Constructs

This chapter is taken from “Experimental validation of transition charge density models for excitonically-coupled Cy3 dimer-labeled DNA constructs”, a manuscript in preparation for journal submission. Dylan Heussman carried out the calculations and performed the experiments. Lulu Enhkbataar carried out calculations under my direction. Mohammed Sorour (Temple University) provided the computational assignment of partial atomistic transition charges on Cy3 dyes. Spiridoula Matsika (Temple University) helped to design the study and provided general feedback. Andrew Marcus is the primary investigator for this work and provided editorial assistance.

Introduction

The chemical properties of biological macromolecules rely on their ability to undergo specific and non-specific interactions at binding interfaces, which help to guide their assembly into multi-subunit complexes. Understanding the physical-chemical basis of these interactions is an area of active research. For example, understanding how local conformational fluctuations of the nucleobases and the sugar-phosphate backbones of DNA facilitate binding to proteins can provide key insights about the functional mechanisms of the macromolecular machines central to genome maintenance and expression. Although established structural tools such as NMR and x-ray crystallography provide detailed molecular-level information about stable protein-nucleic acid complexes at high concentrations, such methods are not well-suited to directly probe site-specific protein-DNA interactions at the micromolar-to-nanomolar concentrations at which these complexes typically assemble and function.

An approach that is well suited for studies at physiological concentrations is to perform spectroscopic experiments on a DNA molecule that has been site-specifically labeled with fluorescent optical probes. For example, the fluorescent dyes Cy3 and Cy5 can be chemically attached to a nucleic acid base or to the distal end of a nucleic acid chain using a flexible linker. Such fluorescently labeled DNA constructs are commercially available for fluorescence

microscopy, gene sequencing technologies, and other bio-analytical applications (1). Measurements based on Förster resonant energy transfer (FRET) are regularly used to determine structural changes of a DNA strand labeled with a Cy3 ‘donor’ and a Cy5 ‘acceptor’ on the length scale of a few nanometers (2). Alternatively, Cy3 can be attached ‘internally’ (termed ‘iCy3’) within the framework of a DNA strand using phosphoramidite chemistry to create an exciton-coupled (iCy3)₂ dimer-labeled ss-dsDNA construct (see Fig. 1) (3).

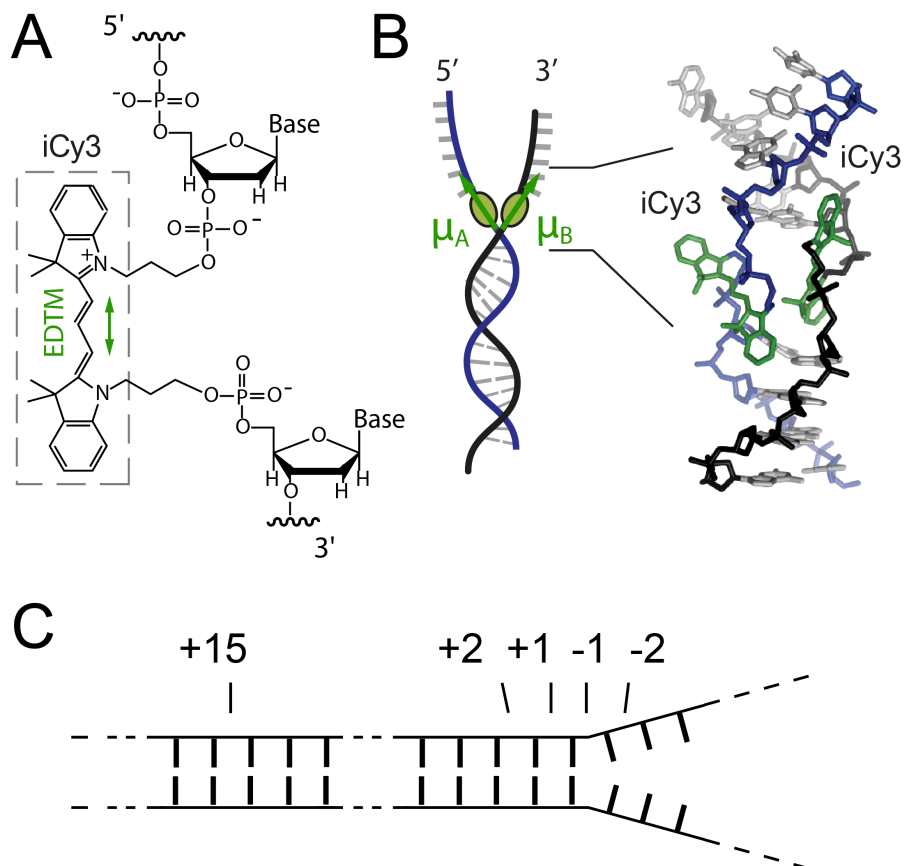


Figure 1. (A) The Lewis structure of the iCy3 chromophore is shown with its 3' and 5' linkages to the sugar-phosphate backbone of a local segment of ssDNA. The electric dipole transition moment (EDTM, indicated by green double-headed arrow) is orientated parallel to the all-trans trimethine bridge of the iCy3 chromophore. (B) An (iCy3)₂ dimer-labeled DNA fork construct contains the dimer probe near the ss-dsDNA fork junction. The local secondary structure of the sugar-phosphate backbones at the probe insertion site position is reflected by the dimer probe conformation. The sugar-phosphate backbones of the conjugate DNA strands are shown in black and blue, the bases are shown in gray, and the iCy3 chromophores are shown in green. (C) The position of the dimer probe is indicated relative to the pseudo-fork junction using positive (negative) integers in the direction toward the double- (single-) stranded region.

An internally attached iCy3 within a DNA single-strand acts as a molecular bridge between bases and as an extension of the sugar-phosphate backbone between adjacent nucleotides (Fig. 1A) (3). By annealing two complementary strands of DNA with opposite iCy3 labeling positions, an exciton-coupled (iCy3)₂ dimer probe (with monomers labeled *A* and *B*) can be formed at a predetermined position within a model DNA fork construct (see Fig. 1B). The annealed DNA construct contains both double-stranded (ds) and single-stranded (ss) DNA regions, and the (iCy3)₂ dimer probe can be selectively inserted relative to the ss-ds DNA fork junction (see Fig. 1C for probe-labelling nomenclature). The closely spaced monomers of the (iCy3)₂ dimer probe can couple through an electrostatic interaction *J* (4-6). The value of *J* and the resulting spectroscopic properties of the (iCy3)₂ dimer-labeled DNA construct depend sensitively on the relative orientation and spacing between the iCy3 monomers on the length scale of a few Angstroms. Such cyanine dimer-labeled DNA constructs have been used, for example, to study electronically excited-state dynamics in coupled chromophore networks (7).

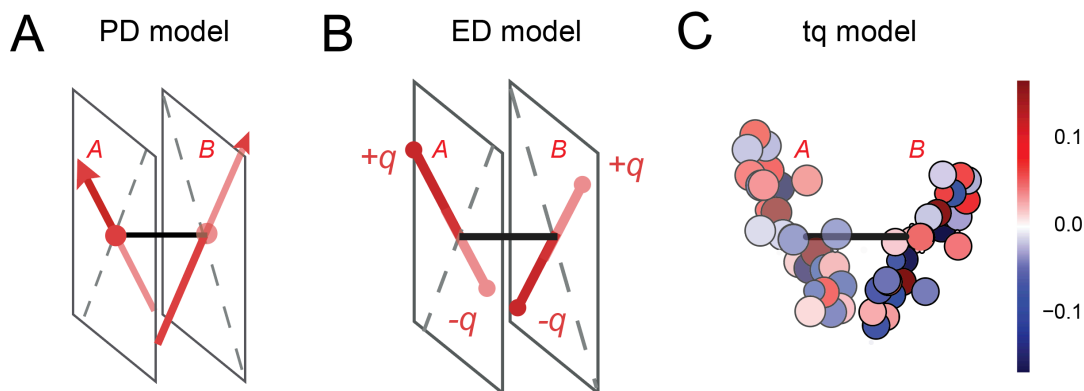


Figure 2. Transition charge density models for the estimation of the electrostatic coupling of (iCy3)₂ dimer-labeled ss-dsDNA constructs. (A) Point-dipole (PD) model. (B) Extended-dipole (ED) model. (C) Transition charge (TQ) density model. See text for further explanation.

The value of *J* can be calculated using quantum chemical models of the monomer electronic transition charge density. In previous work, we studied the mean local conformations and conformational disorder of (iCy3)₂ ss-dsDNA constructs for specific probe insertion-site positions by comparing experimental optical spectra [i.e., absorbance, circular dichroism (CD) and two-dimensional fluorescence spectroscopy (2DFS)] to simulated spectra in which we

applied the ‘point-dipole’ (PD) and the ‘extended-dipole’ (ED) models to calculate J (4-6). For intermolecular separations smaller than the iCy3 monomer dimension, the PD model unrealistically assumes that the transition charge density is focused on a point localized to the molecular center-of-mass (see Fig. 2A). The finite dimension of the molecule is more realistically accounted for by the ED model, which represents the transition charge density as a line segment that is oriented parallel to the transition dipole moment with equal and opposite charges on its endpoints (Fig. 2B). While the results of our prior studies suggest that the PD and ED models can provide self-consistent and reliable structural interpretations of optical spectra for (iCy3)₂ dimer-labeled ss-dsDNA constructs, a firm understanding of the strengths and limitations of these models has not been fully established. In the current work, we apply an atomistically-detailed electrostatic coupling model that is based on *ab initio* calculations of the ‘transition-charge’ (TQ) density to calculate the optical spectra of (iCy3)₂ ss-dsDNA constructs. The TQ model more realistically describes the transition charge density of the iCy3 monomer in comparison to the PD and ED models by assigning individual Mulliken transition charges to the atomic coordinates (8). (Fig. 2C).

Our modeling permits us to define, in addition to θ_{AB} , ϕ_{AB} and R_{AB} , the ‘roll’ angle η_{AB} (Fig. 3C), the vertical ‘shear’ displacement δ_{AB} (Fig. 3D) and the horizontal ‘shift’ displacement ξ_{AB} (Fig. 3E). For a specific set of conformational parameters, and depending on the coupling model, we calculate the value of J and simulate the optical spectra. We thus obtain ‘optimized values’ of the conformational coordinates by applying the above procedure iteratively in combination with a nonlinear least squares minimization algorithm. In addition, we imposed the constraints associated with the approximate van der Waals radii of the component atoms such that physically impossible conformations within the parameter space are ruled out. Thus, by comparing our results based on the PD, ED and TQ models, we may assess the accuracy of the conformational parameters so obtained.

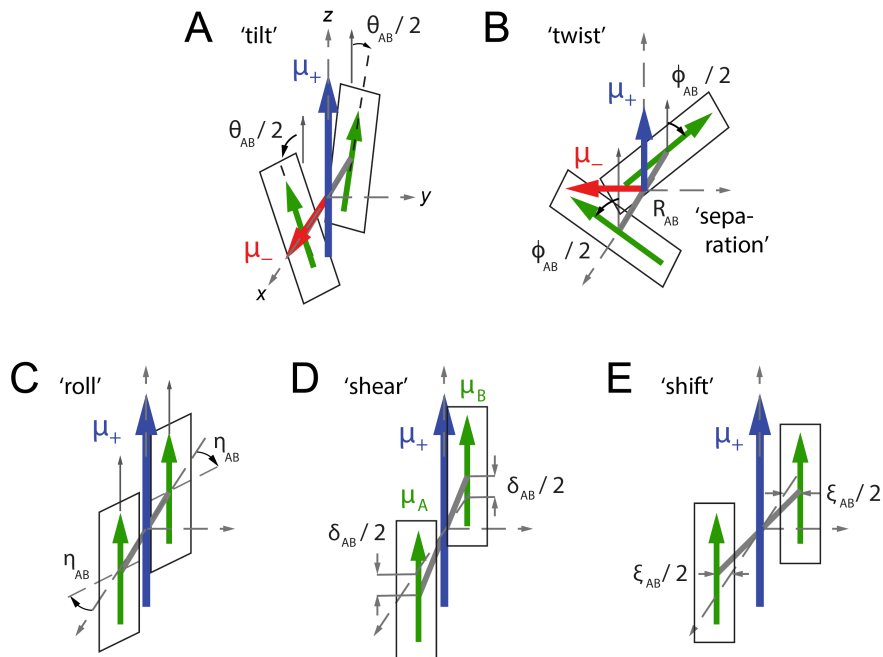


Figure 3. Conformational parameters used for the electrostatic coupling models studied in this work. Green arrows indicate the monomer EDTMs (labeled *A* and *B*), and rectangles indicate the π -conjugation planes of the iCy3 trimethine groups (as shown in Fig. 1A). The electrostatic coupling between the iCy3 chromophores gives rise to symmetric (+) and anti-symmetric (−) excitons, which are indicated by blue and red arrows, respectively. (A) ‘Tilt’ angle θ_{AB} . (B) ‘Twist’ angle ϕ_{AB} and inter-chromophore center-of-mass separation R_{AB} . (C) ‘Roll’ angle η_{AB} . (D) Vertical ‘shear’ displacement δ_{AB} . (E) Horizontal ‘shift’ displacement ξ_{AB} .

We find that all three models provide self-consistent and accurate information about the orientational parameters (i.e., the ‘tilt’ angle θ_{AB} and the ‘twist’ angle ϕ_{AB}) of (iCy3)₂ dimer-labeled ss-dsDNA constructs. However, reliable information about the ‘roll’ (or stacking) angle between the iCy3 monomers is only available using the atomistically-detailed TQ model. As we discuss in further detail below, we find that for (iCy3)₂ dimer probes positioned deep within the double-stranded region (at the +15 position), the trimethine chromophores adopt a ‘stacked’ face-to-face conformation. For (iCy3)₂ dimer probes positioned near the ss-dsDNA fork junction (at the +1 position) there is a characteristic increase in the tilt that is consistent with our previous results. Upon generating atomic coordinates for the (iCy3)₂ dimers based on the optimized conformational parameters we obtained from our analysis of optical spectra, we are able to construct three-dimensional structural models of the (iCy3)₂ dimer-labeled DNA constructs to assess the implications of the proposed models on the local DNA base structure immediately surrounding the chromophore probes.

Theoretical Background

The Cy3 chromophore consists of a conjugated trimethine bridge, which cojoins two indole-like groups (Fig. 1A). The absorbance spectrum of Cy3 in solution, or when it is attached internally (as iCy3) to DNA, is minimally affected by its environment, and exhibits a pronounced vibronic progression (4). The lowest energy ($\pi \rightarrow \pi^*$) electronic transition occurs when the molecule is in its *all-trans* ground state configuration (9). Like many π -conjugated molecules, Cy3 exhibits numerous Franck-Condon-active modes, which range in energy from tens-to-several hundred wavenumbers. Nevertheless, the homogeneous spectral lineshape of the molecule in solution is broadened due to rapid electronic dephasing and thermal occupation of low energy vibrational levels. The lowest energy electronic transition from ground state $|g\rangle$ to excited state $|e\rangle$ is predominantly coupled to one low-frequency ($\sim 30\text{ cm}^{-1}$) bending mode along the trimethine bridge, in combination with a cluster of relatively high-frequency modes in the vicinity of $\sim 1,300\text{ cm}^{-1}$ [Sorour 2022]. The spectrum of the iCy3 monomer can be simulated using a relatively simple quantum mechanical Hamiltonian, where the $|g\rangle \rightarrow |e\rangle$ transition (with energy $\varepsilon_{eg} = \sim 18,250\text{ cm}^{-1}$) is coupled to a single ‘effective’ harmonic mode (with energy $\hbar\omega_0 = \sim 1,100\text{ cm}^{-1}$). Full theoretical modeling of the absorbance and circular dichroism spectra can be located in Chapter II.

Theoretical Considerations Motivating the Transition Charge (TQ) Approach

There are fundamental difficulties associated with constructing simple electrostatic models for the interaction between separate transition charge densities localized to distinct molecular sites. For large conjugated molecules, the transition charge density is extended over distances comparable to the inter-site separation of closely spaced dimers, so that the point-dipole approximation is expected to be unreliable. In these cases, the point-dipole model invokes an unphysical representation of how complex perturbations to electron density affects neighboring molecules in close proximity. This is a clearly recognizable concern for the treatment of multi-chromophore systems as interacting point dipoles. A related issue involves the shortcomings of any electrostatic coupling model when applied to systems of tightly packed molecules approaching their van der Waals contacts. In such systems, individual molecules no longer

maintain their local wavefunction character, and the system as a whole must be treated collectively (10). As noted by Kasha, the applicability of perturbation-based approaches diminishes at small intermolecular distances due to contributions from exchange, overlap, and charge-transfer processes (11). There have been several attempts to address this issue, as can be found in various diabaticization-based methods in which the resonance interaction energy, J , is decomposed into short and long-range contributions (12).

Ultimately, there is a trade-off between the accuracy of the conformational parameters that can be obtained from the modeling of optical spectra and the level of approximation used for the transition charge density. We find that models that include more detailed information about the transition charge density act to reduce the Coulombic ‘penalties’ for smaller inter-chromophore separations (13). Our work intends to tease out the importance of some of the above considerations within regimes where overly simplistic approximations are thought to fall short of capturing the actual molecular behavior. The electrostatic interactions of (iCy3)₂ dimer-labeled ss-ds DNA constructs are on the order of the vibrational relaxation energy ($\sim 500 \text{ cm}^{-1}$) and thus fall within the so-called ‘intermediate coupling regime,’ as defined by Förster [Förster 1965].” As such, the magnitude and sign of the coupling strength is expected to depend sensitively on dimer conformation. Although the behavior of the resonant coupling with respect to different transition charge density models has been evaluated theoretically (5,13), there has not yet been an extensive analysis to test the agreement of these models with experimental data taken under various environmental conditions.

Calculations of the Electrostatic Interaction of (iCy3)₂ Dimers for Increasingly Complex Transition Charge Density Models

To compare transition charge density models of varying levels of complexity, it is useful to define the transition charge displacement, $\vec{\mu}$, which is conserved for all models. The magnitude of the iCy3 monomer electric transition dipole moment (μ) can be found experimentally, or by *ab initio* methods. Our value of μ ($= 12.8 \text{ D}$) was determined by integrating the spectral lineshape of an iCy3 monomer-labeled ss-ds DNA construct in aqueous buffer solution at room temperature (23 °C). Experimental determination of μ permits us to account for possible changes to the absorptive properties of the monomer arising from

electrostatic interactions with the local solution environment – i.e., the DNA polyelectrolyte scaffolding in physiological salt buffer solution (100mM NaCl, 6 mM MgCl₂, 10 mM Tris).

Under the point-dipole (PD) approximation, the magnitude of the transition charge displacement vector is equal to μ . However, an equivalent value can be achieved using the extended dipole (ED) and atomic transition charge (TQ) models by scaling the associated transition charges. Under the ED model, this results in two point charges of opposite magnitude q separated by the molecular length l (7 Å), such that $ql = \mu$. The resulting point charges are of magnitude $q = \pm 0.38e$. In applying the atomistically detailed TQ model, Mulliken transition charges are uniformly scaled such that $\sum_i q_i \vec{r}_i = \vec{\mu}$. We thus establish an equivalent value of μ employed in each of the three methods. The values used for the transition charges in the TQ model, in addition to a thorough description of their calculation, can be found in [Sorour *et al.*, 2022], which uses density functional theory (DFT) with the B3LYP hybrid functional and 6-31G(d) basis set.

In this work, the conformation of two iCy3 chromophores is described using a relative coordinate system formed by the EDTM vectors of each monomer ($\vec{\mu}_A$ and $\vec{\mu}_B$). Our EDTM coordinate system has been described previously in terms of the relative tilt angle (θ_{AB}), the twist (ϕ_{AB}), and the inter-chromophore separation (R_{AB}) (Figure 2). Changes to the roll angle (η_{AB} , applied symmetrically or asymmetrically), become electrostatically meaningful under the TQ model, where there is a three-dimensional representation of the transition charges that are sensitive to the degree of stacking of the chromophores. For our TQ calculations, $\eta_{AB} = 0$ denotes iCy3 molecules that are vertically displaced edge-to-edge with deviations from the edge-to-edge configuration indicated by the reported roll angle. With the additional inclusion of longitudinal and horizontal displacement, or shear and shift (δ_{AB} and ξ_{AB} , respectively), these coordinates allow for a complete description of the chromophores in space. However, we consider linear translations of the chromophores (shear and shift) to be largely limited by symmetry constraints and the natural pitch of the DNA. Here we limit the shear and shift displacements to less than 5 Å in either direction; these translational transformations do not affect the relative orientational parameters (θ_{AB} , ϕ_{AB} and η_{AB}) describing the (iCy3)₂ dimer conformation. The orientational transformations applied to the chromophore coordinates are achieved by an ordered series of matrix operations.

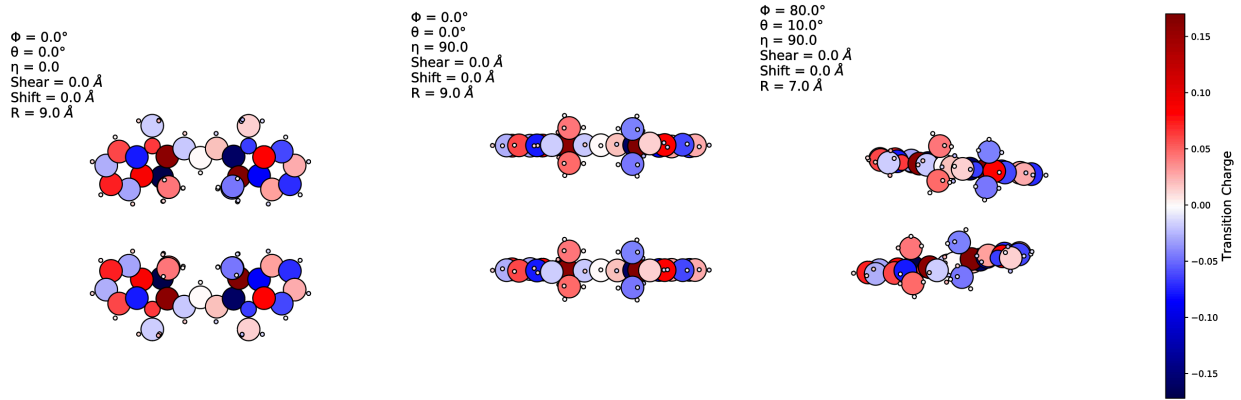


Figure 4. Distribution of atomistic transition charge on the Cy3 monomers shown within dimer configurations under a subset of geometric transformations.

Calculations of the resonance interaction between chromophores grounded in the electrostatic abstraction all seek to capture the fundamental interaction between transition charge densities, ρ , upon chromophores (Eqn. 1), in which the overlap integral found in Equation 1.3 describes the interaction between the two migrating charge densities. Under the treatment of the iCy3 transition dipole moment as a point-dipole (PD), we can estimate the resonant interaction as

$$J = |\mu_{eg}|^2 (4\pi\epsilon\epsilon_0)^{-1} |\hat{R}_{COM}|^{-3} [\hat{d}_A \cdot \hat{d}_B - 3(\hat{d}_A \cdot \hat{R}_{COM})(\hat{R}_{COM} \cdot \hat{d}_B)] \quad (2)$$

in which R_{COM} is calculated as $\sqrt{R_{AB}^2 + \delta_{AB}^2 + \xi_{AB}^2}$. Extended dipole (ED) and atomic transition charge (TQ) Coulomb interactions can be effectively modeled using the equations:

$$J_{AB}^{ED} = |\mu_{eg}|^2 (4\pi\epsilon\epsilon_0)^{-1} l^{-2} \left[\frac{1}{R_{AB}^{++}} - \frac{1}{R_{AB}^{+-}} - \frac{1}{R_{AB}^{-+}} + \frac{1}{R_{AB}^{--}} \right] \quad (3a)$$

$$J_{AB}^{TQ} \cong \sum_a^{N_A} \sum_b^{M_B} \frac{q_a q_b}{|R_a^A - R_b^B|} \quad (3b)$$

In practice, the ED model consists of an abridged version of the TQ model, in which the charges are placed at the head and tail ends of a theoretical molecule (i.e., a line segment) of transition charge distribution length, l . The calculation of Coulombic interactions can be carried

out between atomic transition charges or extended dipole charges, q . R_a^i defines the location of atom (or extended dipole charge) of chromophore i , containing N atoms, or two extended dipole charges. The coupling strength J is calculated pairwise between each transition charge on chromophore i with the transition charges on neighboring chromophore j .

Theoretical Modeling

Model-Dependent Characterization of the Resonance Interaction

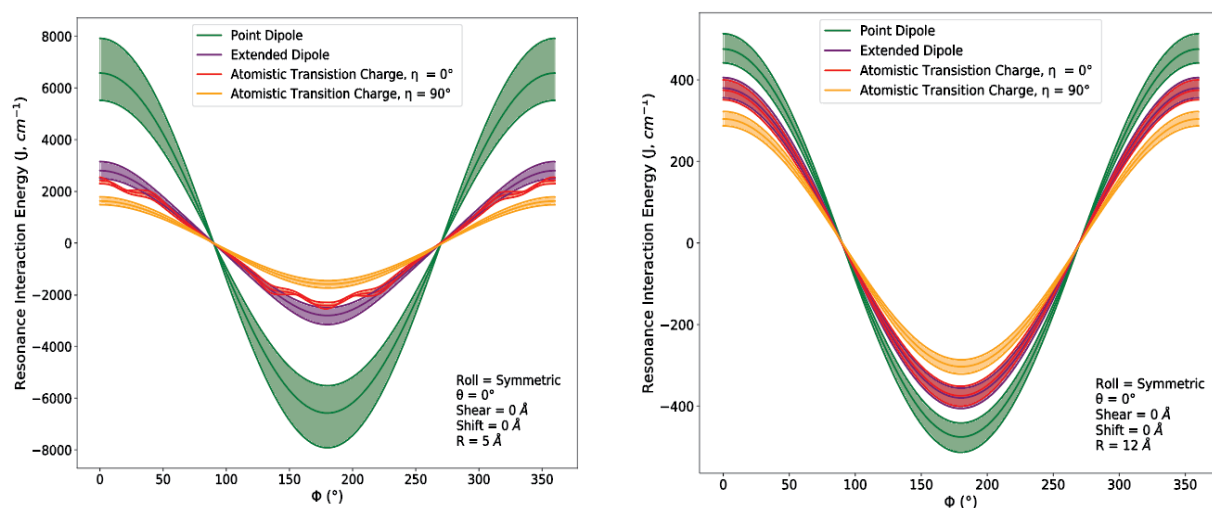


Figure 5. The resonant coupling interaction energy dependence on ϕ_{AB} and η_{AB} under the PD, ED, and TQ approximations. Changes to the roll angle η_{AB} do not alter the resonant coupling under these treatments, with the coupling energies found under the PD approximations to be systematically larger in magnitude than the ED model. (Left panel) At small intermolecular separations (5 Å), significant ϕ_{AB} -dependent atomic charge overlap leads to modulation of the electrostatic coupling for edge-to-edge ($\eta_{AB} = 0^\circ$) stacked conformations, which is reduced by face-to-face stacking. (Right panel) At larger inter-chromophore separation (12 Å), the TQ approximation ($\eta_{AB} = 0^\circ$) and ED approximations exhibit nearly identical ϕ_{AB} -dependences (and are overlaid in the plot). However, face-to-face stacking ($\eta_{AB} = 90^\circ$) decreases the overall magnitude of the coupling for all values of ϕ_{AB} .

The overall trends found for the coupling J under the TQ approximation as a function of the twist angle ϕ_{AB} and roll angle η_{AB} are shown in Figure 5. The value of J undergoes a sign inversion as the twist angle ϕ_{AB} is varied across 90° , in which case the net interaction is dominated by repulsion between like charges. At small inter-chromophore separations, the PD and ED models tend to overestimate the coupling strength. Variation of the roll angle η_{AB} acts to

modulate the coupling found for a particular twist angle through modifications to the face-to-face stacking of the chromophores, thereby altering the length scales over which component atomistic Coulomb interactions occur (see Figure 6). Importantly, the possibility of varying the roll parameter increases the availability of sterically allowed conformations, as visualized by the emergence of accessible regions of the conformational space, which are visualized as transparent regions in Figure 6. This permits the realization of physically meaningful candidate conformations that are separated by distances smaller than the diameter of the Watson-Crick B-helix DNA (~ 20 Å). These results suggest refinements to some of the (iCy3)₂ dimer-labeled DNA conformations that we had previously reported, which exhibited a separation of less than 6 Å. The refined structures take into account that the available conformational space is quite limited at near contact separation distances in the absence of any shift or shear transformations applied. The tilt of the chromophores also acts to bring select transition charges closer to one another, and directly change the magnitude of the coupling interaction. Nevertheless, these effects lead to comparatively minor changes to the overall coupling strength and chirality of the (iCy3)₂ dimer (5,6).

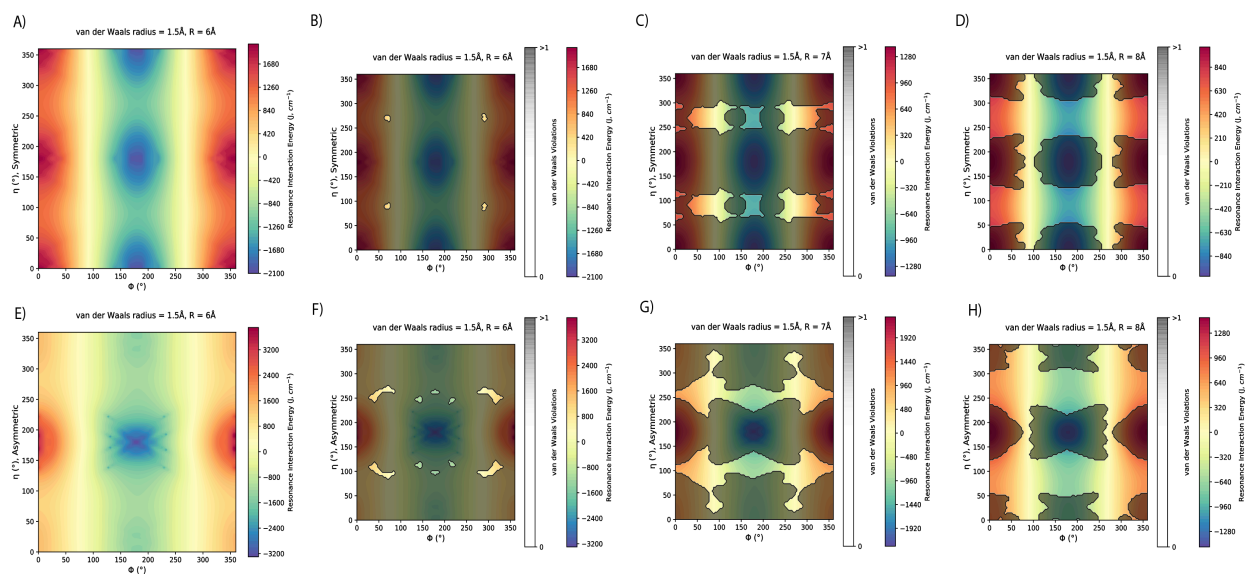


Figure 6. The ϕ_{AB} and η_{AB} -dependence of the electrostatic coupling J under a symmetric (top row) and asymmetric (bottom row) roll transformations. The first column (A, E) shows the electrostatic coupling calculation with van der Waals overlap effects omitted. Coupling energies for inter-chromophore separations $R_{AB} = 6$ Å (A & B, E & F), 7 Å (C, G), and 8 Å (D, H) are shown with a semi-transparent mask to indicate regions of the conformational space that generate sterically unphysical molecular overlaps, which are disallowed by the finite van der Waals radii of 1.5 Å.

The shift and shear displacements (δ_{AB} and ξ_{AB} , respectively), which are also common parameters used in the interpretation of DNA helix deformations, act to remove the overlap of the monomers and lower the symmetry and coupling strength between the iCy3 chromophores. While we consider variations of these parameters to be fundamentally restricted by the constraints of hydrogen bonding and base stacking, the application of small deviations can help to reduce steric overlap and introduce small changes to the coupling under the various electrostatic interaction models. The dependences of the coupling strength on δ_{AB} and ξ_{AB} under the TQ approximation are plotted in Figure 7.

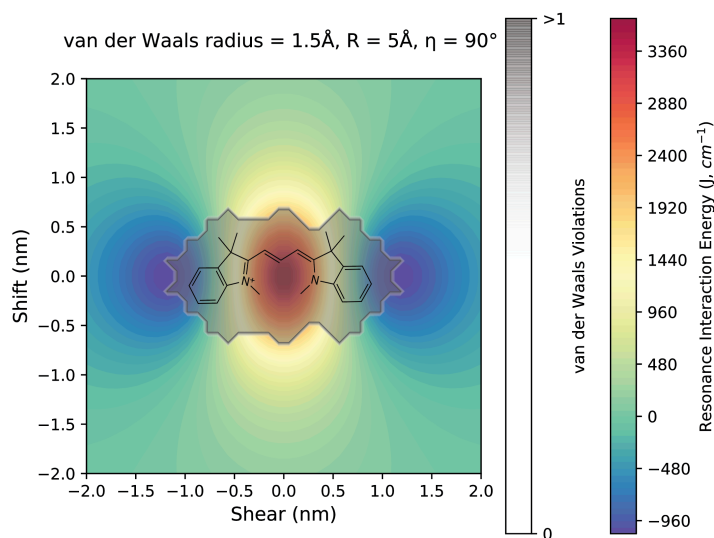


Figure 7. Electrostatic coupling as a function of shift (δ_{AB}) and shear (ξ_{AB}) displacements at separation $R_{AB} = 5 \text{ Å}$ and roll angle $\eta_{AB} = 90^\circ$ (face-to-face stacking). In the absence of twist or tilt ($\phi_{AB} = \theta_{AB} = 0$), variations of the shear displacement produce a sign-inversion of J , as like transition charges are brought closer to one another. Pure shift and shear displacements at small separations ($R_{AB} = 5 \text{ Å}$) map out relevant steric violations, which indicate the cutoff of van der Waals radii of individual iCy3 chromophores.

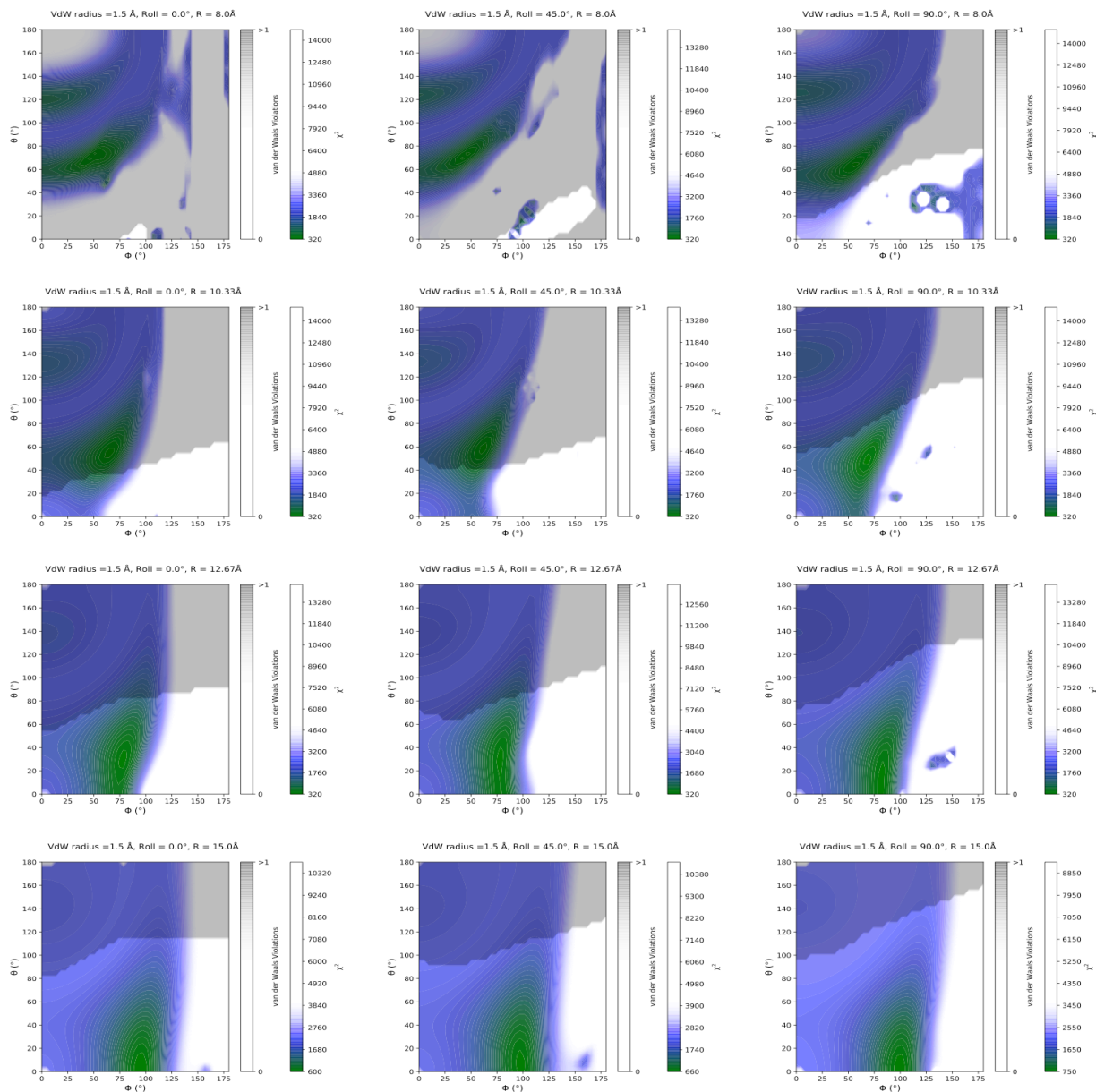
Simulation of Absorbance and Circular Dichroism Spectra for (iCy3)₂ Dimers in DNA Using Atomistic Transition Charges

The procedure to calculate circular dichroism (CD) and absorbance spectra in terms of dimer conformation is treated in Chapter 1. As shown in Figure 8, the optimization procedure for structure selection can be misleading when models are employed that do not account for the

detailed locations of the atoms of the interacting iCy3 chromophores. In this figure, we have plotted the χ^2 best fit values against two conformational parameters, the relative twist angle ϕ_{AB} and the tilt angle θ_{AB} . Each successive row represents an increasing value of the separation distance R_{AB} , from 8 to 15 Å, and each successive column represents an increasing value of the roll angle η_{AB} , varied symmetrically from 0°, 45°, and 90°. The green regions represent the lowest values of χ^2 found as a function of ϕ_{AB} and θ_{AB} for fits to the duplex +15 experimental data, and for fixed values of R_{AB} and η_{AB} ; a gray mask is applied over this surface to indicate regions that are conformationally inaccessible based on an assumed value of the van der Waals radius of 1.5 Å.

This analysis reveals that relatively large values of the tilt angle ($\theta_{AB} > 80^\circ$) cannot be achieved in (iCy3)₂ dimer-labeled ss-dsDNA systems and that (iCy3)₂ dimer-labeled duplex DNA likely features a twist angle ϕ_{AB} spanning a range of values between 55° – 85°. Importantly, a systematic increase in the local χ^2 minima for structures with inter-chromophore separation greater than 12 Å (bottom row) indicates that the optimal structure for this construct does not correspond to value of R_{AB} greater than ~14 Å. Additionally, steric overlap restrictions appear to reduce the availability of structures with R_{AB} less than ~7 Å.

Figure 8. (next page) The ϕ_{AB} and θ_{AB} -dependence of χ^2 determined for the (iCy3)₂ dimer-labeled duplex DNA construct at 15°C. Green-shaded regions indicate the globally-minimized best fit values of ϕ_{AB} and θ_{AB} subject to specified values of η_{AB} and R_{AB} , while gray-shaded regions are conformationally inaccessible. Shear and shift parameters are not translated from the origin. As the inter-chromophore separation is increased (moving from top to bottom), and as the roll angle is brought closer to a face-to-face configuration with $\eta_{AB} = 90^\circ$ (antisymmetric, moving from left to right), the accessible conformational space increases. Many local minima of the χ^2 surface are inaccessible due to the steric interaction of the iCy3 chromophores. Spectral amplitudes are re-optimized at each calculated point on the χ^2 surface.



Improvements to the Parameter Optimization Algorithm

To determine a unique set of conformational parameters corresponding to a global χ^2 minimum in agreement with experimental data, we employ, in tandem, a brute force grid search and a stochastic differential evolution algorithm to generate candidate solutions within the structural parameter space. For both methods, the top structural candidates are subsequently optimized via a gradient-based minimization routine. These optimizations explore a nine-

dimensional parameter space (three angular transformations, three linear spatial translations, two spectral amplitude coefficients, and one inhomogeneous lineshape broadening parameter). We have found that the generation of candidate solutions under a differential evolution algorithm procedure produces globally optimized solutions with improved computational efficiency in comparison to gradient-based χ^2 minimization approaches. Candidate solutions are rejected when they violate the specified van der Waals overlap by returning the χ^2 associated with the calculated iCy3 monomer spectra. Because candidate structures that violate the van der Waals overlap criteria can lead to discontinuities in the χ^2 surface under minimization, it is important to maintain a combination of stochastic and gradient-based approaches when sampling the χ^2 function. A rigorous explanation of the differential evolution algorithm that we employed is given in Storn and Price, 1997 (14).

For all three of the electrostatic coupling models that we considered, the detailed atomic coordinates of the iCy3 chromophores are an important consideration for the final structure determination. We include the three-dimensional characteristics of Cy3 molecules for the PD and ED models by applying the same structural transformations of atomic coordinates to dimer configurations with the same value of the EDTM, $\mu = 12.8$ D. While the simulated spectra are relatively insensitive to some of the structural parameters in the fitting procedure (e.g., the roll angle η_{AB}), this permits us to eliminate structures that do not represent physically meaningful conformations (i.e., conformations that exhibit atomic overlap). Thus, the uncertainties associated with the values determined for the roll angle η_{AB} under the PD and ED models are determined exclusively by the range of rotation sterically possible in accordance with the atomic van der Waals radii of 1.5 Å.

Discussion of Results

Comparison Between PD, ED and TQ Models

There is a remarkable consistency between the conformational parameters obtained from our optimization procedures by employing the three different electrostatic coupling models, which each represents a different level of course-grained approximation to the true transition charge density. The structural coordinates of iCy3 dimers in DNA can generally be well

described by simplified mathematical models of the EDTMs of the constituent iCy3 monomers. When the iCy3 chromophores are rigidly inserted into the sugar-phosphate backbones of ss-dsDNA fork constructs, the (iCy3)₂ dimer adopts configurations that are similarly well-characterized by each of the three transition charge density models (PD, ED and TQ), as summarized in Tables 1 and 2). However, the results of our analyses suggest that different approximations of the transition charge density can potentially lead to diverging results when applied to systems with relatively large or small inter-chromophore separations R_{AB} . We have addressed the latter concern by including impenetrable atomic van der Waals radii within our optimization, and the former by establishing an equivalency between the monomer EDTMs as sums over the total displaced transition charges.

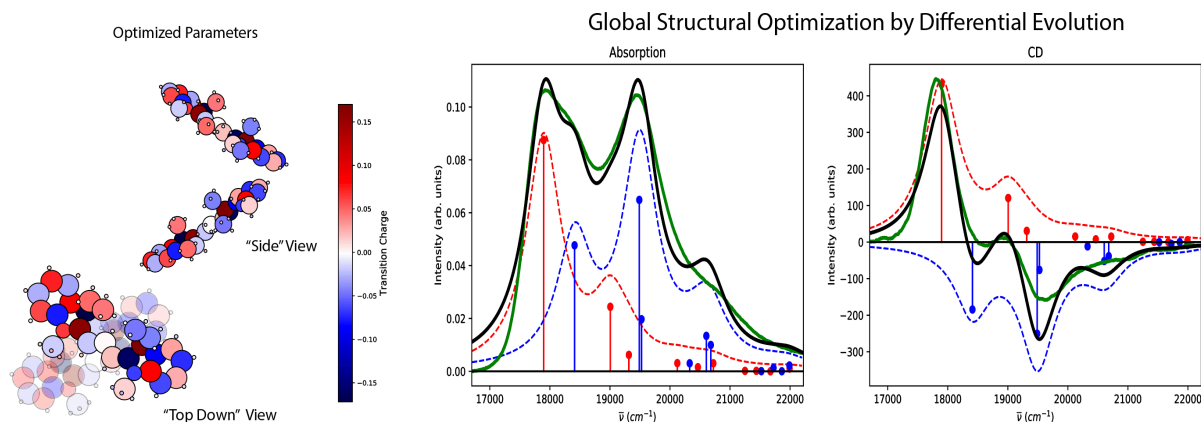


Figure 9. Example of atomic coordinates determined from a structural optimization of the mixed base sequence (iCy3)₂ dimer-labeled duplex DNA construct at 25°C (*left*), and comparison between the corresponding simulated and experimental absorbance and CD spectra of the system using the TQ model with symmetric roll angle $\eta_{AB} = 95^\circ$ (*right*).

TQ Analysis Reveals Face-to-Face Stacking Behavior and Herringbone Conformation of the (iCy3)₂ dimer in the DNA Duplex

When the (iCy3)₂ dimer-labeled duplex DNA construct (with mixed base sequence, 25°C) was analyzed to determine the optimized structural parameters using the TQ model (see Table 1), we found that the dimer adopts a ‘herringbone’ conformation with a smaller twist angle ($\phi_{AB} = \sim 56^\circ$) and larger tilt angle ($\theta_{AB} \sim 60^\circ$) than previously reported. This result differs from

the that of earlier calculations, which found a twist angle $\phi_{AB} = \sim 80^\circ$ and a minimal tilt angle $\theta_{AB} \sim 0$. It is important to note that the positions of the atoms, and their van der Waals overlaps, were not actively monitored in previous optimizations, which led to unphysically small inter-chromophore separations. If small inter-chromophore separations ($\sim 3 - 6 \text{ \AA}$) are not methodically discounted, the inherent differences between the three transition charge density models can lead to significant discrepancies between the optimized structures, and these conformations cannot be reconciled with three dimensional models of dimer conformation without generating significant steric overlap. An additional shortcoming of less sophisticated approaches to these simulations is the inability to restrict values of the tilt angle θ_{AB} as a parametric function of the other structural parameters. For example, as can be seen in Figure 8, at small separation distances ($R_{AB} < \sim 10 \text{ \AA}$) it is important to restrict the possible values of θ_{AB} to avoid sterically overlapping configurations, as discussed in Chapter II. However, when a larger separation distance ($R_{AB} > \sim 10 \text{ \AA}$) is required to reduce overlap between chromophore sites, the value of θ_{AB} must be increased to adjust the electrostatic coupling, in which case the value of θ_{AB} should not be restricted unnecessarily. This compensatory effect can be observed to some extent for all transition charge density models applied to the (iCy3)₂ dimer-labeled duplex DNA construct.

Table 1. Conformational coordinates for the +15 dimer under the transition charge, extended dipole and point dipole models.

	Model	ϕ_{AB} ($^\circ$)	θ_{AB} ($^\circ$)	η_{AB} ($^\circ$)	δ_{AB} ($\text{\AA}$)	ξ_{AB} ($\text{\AA}$)	R_{AB} ($\text{\AA}$)	σ (cm^{-1})	J (cm^{-1})
Sym. Roll	TQ	59.78 +.83/-4.00	55.97 +4.62/-6.9	94.9 +3.69/-18.79	-1.09 +0.08/-5.2	-2.06 +.98/- .12	9.11 +0.06/- .36	319.88 +14/-17	514.80
	ED	78.43 +.69/3.11	17.86 +31.1/-22.7	107.14 + 48.6/-74.00	1.35 +0.26/- 1.44	3.93 +5.0/0.172	8.89 +0.07/- .42	320.7+14/- 18	514.47
	PD	77.71+.29/ -1.6	20.97 +2.67/-4.3	87.25 +3.47/-18.95	-.97 +.039/- .224	1.57 +.036/- .216	7.56+.039/- .21	320.2 +15/- 18	514.43
Asym. Roll	TQ	59.97 +1.05/-4.73	55.77 + 4.02/-0.604	75.54 +34.38/-16.72	-1.94 +.11/- .72	1.37 +0.20/- 2.72	9.91+.0599/ -.339	319.9+15/- 18	514.69

Table 1, continued

	Model	ϕ_{AB} (°)	θ_{AB} (°)	η_{AB} (°)	δ_{AB} (Å)	ξ_{AB} (Å)	R_{AB} (Å)	σ (cm ⁻¹)	J (cm ⁻¹)
	ED	75.18 +1.03/-4.0	29.32 +7.97/- 1.23	71.84 +/-∞	-2.04 +0.21/-1.94	3.28 +4.19/- 0.22	10.42 +0.06/-0.37	320.02 +14.8/-17.8	514.6
	PD	71.53 +1.19/- 4.59	38.91 + 4.90/-0.72	85.19 +53.3/- 73.54	-2.59 +0.72/-3.80	-.428 +1.18/- 0.296	10.05 +0.048/- 0.26	319.66 +15.0/-17.5	514.82

A unique conclusion that can be made from these calculations is that the roll angle η_{AB} adopts a $\sim 90^\circ$ angle for (iCy3)₂ dimer-labeled duplex DNA constructs, indicating face-to-face stacking of the chromophores. The TQ model permits this determination of the optimized value of η_{AB} by increasing the sensitivity of the electrostatic coupling J through the detailed Coulombic interactions between partial atomic transition charges. It is perhaps somewhat surprising that the optimized value of η_{AB} obtained under the TQ approximation can also be assigned using the PD and ED models by simply applying a Boolean gate to exclude sterically overlapping structures. Nevertheless, the associated error bars for roll parameters obtained in this way are quite large and should only be used to loosely generate candidate (iCy3)₂ structures under the PD and ED models.

When comparing the PD, ED and TQ models, there is a clear increase in the electrostatic coupling J for decreasing separation R_{AB} (see Figure 5). However, there is no clear trend found for the optimized results under the three different models, especially with the inclusion of small shear and shift displacements. While the calculated coupling strengths are quite similar for optimizations performed under the three models, the results of each model correspond to only marginally different dimer structures. Our analysis suggests the existence of two possible conformations for the (iCy3)₂ dimer-labeled duplex DNA construct at 25°C with $\phi_{AB} = 60\text{-}65^\circ$ and $\phi_{AB} = 80^\circ$, indicating a degeneracy between structural solutions when employing our full suite of conformational parameters.

Analyses of (iCy3)₂ dimer-labeled ds-ss DNA Fork Constructs

The analysis of (iCy3)₂ dimer-labeled ss-dsDNA fork constructs labeled at positions near the junction indicates that the twist angle ϕ_{AB} varies from $53.6^\circ - 98.3^\circ$ as the labelling site is varied from the -1 to the +1 position, which is consistent between all three transition charge density models of the electrostatic coupling (see Table 2). This finding supports the results reported in earlier chapters describing structures found for regions at and near the ds-ss DNA fork junction, in which we observed a change in the local twist angle from 'right-handed' ($\phi_{AB} < 90^\circ$) to 'left-handed' ($\phi_{AB} > 90^\circ$) as the (iCy3)₂ dimer-labelling position was varied across the ss-dsDNA junction from the duplex towards the single-stranded region. In addition, we find that the tilt angle θ_{AB} increases as the dimer probe position is varied from the +2 to +1 position, from 53.7 to 74.5° . This increase in the value of θ_{AB} is also consistent with the results discussed in previous chapters. In this study, we observe a decrease in the tilt angle θ_{AB} as the (iCy3)₂ dimer probe position is varied across the junction towards the single-stranded region, which was not reported in our previous analyses. However, in previous work the assigned uncertainty in the tilt angle θ_{AB} was comparatively large because the optimization procedure did not properly exclude sterically overlapping structures. Finally, the roll parameter η_{AB} does not indicate the presence of stacked face-to-face conformations for any of the ss-dsDNA constructs labeled near the junction. In contrast, the (iCy3)₂ dimer-labeled DNA construct with probes deep in the duplex region adopts an approximately stacked conformation with $\eta_{AB} = \sim 90^\circ$ under the symmetric transformation. For the (iCy3)₂ dimer probes near the ss-dsDNA fork junction, the value of $\eta_{AB} = \sim 130^\circ$, indicating an approximately edge-to-edge conformation. The optimized values of the structural coordinates that we obtained for the (iCy3)₂ dimer-labeled ss-dsDNA constructs at and near the ds-ss DNA junction using an asymmetric roll transformation are given in the Appendix.

Table 2. (next page) Conformational coordinates for labeled dimer sites at and around ss-ds DNA junctions, simulated under the transition charge, extended dipole and point dipole models and a symmetric roll.

Position	Model (Symmetric)	ϕ_{AB} (°)	θ_{AB} (°)	η_{AB} (°)	δ_{AB} (Å)	ξ_{AB} (Å)	R_{AB} (Å)	σ (cm ⁻¹)	J (cm ⁻¹)
+2	TQ	68.2 +1.2 /-1.2	53.73 +1.12/-1.17	107.17+15.2/ -8.9	-1.76 +0.14/- 0.14	-1.74 +0.40/-0.31	9.41 +0.12/- 0.12	298.9 +11.8/- 11.6	439.06
	ED	71.4 +1.5/- 1.6	49.1 +1.5/- 1.6	114.8 +251/ -78	-2.0 +0.3/ -0.3	1.7 +0.3/- 0.3	11.9 +0.1/ -0.1	298.7 +12/- 11.4	439.11
	PD	61.01 +1.7/- 1.7	61.97+1.38 /-1.43	102.88 +66.6/-56.5	-0.18 +0.21/- 0.21	-1.67 +0.27/- 0.24	11.58 +0.11/- 0.11	298.7 + 12.0/- 11.4	439.12
+1	TQ	53.65 +1.5/-1.5	74.5 +1.16/- 1.2	100.7 +7.7/- 6.4	0.47 +0.15/- 0.14	-1.9 +0.3/- 0.3	10.81 +0.15/- 0.14	301.43 +11.4/- 11.0	421.3
	ED	84.38 +0.8/- 0.8	33.09 +4.8/ -4.8	121.6 +27.3/ -77.1	2.13 +0.23/- 0.21	3.08 +0.16/- 0.15	8.1 +0.15/ -0.14	301.4 +11.4/- 11.0	421.32
	PD	56.9 +2.24/- 2.25	72.24 +1.33/- 1.37	103.5 +65.1/ -57.1	-0.14 +0.23/- 0.24	-2.8 +0.17/- 0.17	11.87 +0.12/- 0.12	301.4 + 11.4/- 11.0	421.34
-1	TQ	98.3 +0.6/- 1.2	6.13 +10.2/- 1.9	126.72 +0.15/-3.7	-0.2 +0.77/- 0.04	4.93 +0.08/-0.38	7.025 +0.11/- 0.01	315.7 +11.6/- 11.1	-358.8
	ED	96.1 +1.1/- 1.1	24.3 +1.6/- 1.6	120.2 +27.2/-53.6	-1.6 +0.1/ -0.1	- 2.54+0.07/- 0.07	8.7 +0.1/- 0.1	315.1 +12.1/- 10.6	-359.1
	PD	97.03 +1.03/- 1.06	18.9 +3.6/- 5.1	128.68 +16.2/-96.4	0.55 +1.7/- 0.3	4.48 +0.35/- 0.29	8.507 +0.1/-0.1	315.9 +11.5/- 11.3	-358.7
-2	TQ	98.79 +0.72/- 1.18	3.2 +18.3/- 17.8	129.3 +0.23/ -3.63	0.39 +0.38/- 0.04	4.98 +0.14/-0.34	7.05 +0.11/- 0.02	312.4 +11.8/- 10.7	-362.4
	ED	98.1 +1.6/- 1.6	13.4 +7/- 32	96.4 +/-∞	0.12 +2.3/-0.8	-3.6 +0.16/- 0.16	11.4 +0.14/- 0.14	312.7 +11.4/- 11	-362.3
	PD	97.52 +0.9/- 0.9	18.48 +4.5/ -3.3	111.5 +40.9/ -62.4	2.13 +0.22/- 0.28	3.66 +0.27/- 0.25	8.22 +0.09/- 0.09	312.85 +11.4/- 11.0	-362.2

Local Structural Models Inform Neighboring Three-Dimensional DNA Structure

While symmetric and asymmetric rolling of the chromophores does not significantly change the optimized structures, the relative roll angle does have an effect on the ability of the

chromophores to connect to neighboring sugar-phosphate groups with minimal strain on bond lengths, bond angles, and steric overlap. The atomic coordinates gathered from our full transition charge models can be used to construct theoretical structures of the local DNA bases and sugar-phosphate backbones surrounding the (iCy3)₂ dimer probe. The computer generation of (iCy3)₂ dimer-labeled DNA structures permits us to re-evaluate the proposed optimized solution for its ability to fit sterically within the DNA framework with minimal deformation, as it is thought that the iCy3 chromophores introduce little disruption to the local hydrogen bonding behavior or general stability of the DNA to form the thermodynamically stable B-helix. The hypothesis that the dimers are minimally invasive is supported by thermal denaturation measurements of the melting temperature, native base spectroscopy, and by systematic spectroscopic measurements of these systems performed as a function of temperature, label position, and DNA base composition. Based on our current and previous analyses, it appears likely that the (iCy3)₂ dimer probes rotate with *symmetric*, as opposed to asymmetric, rolling character at the labelling positions to accommodate the probe linkages to the DNA sugar-phosphate backbones.

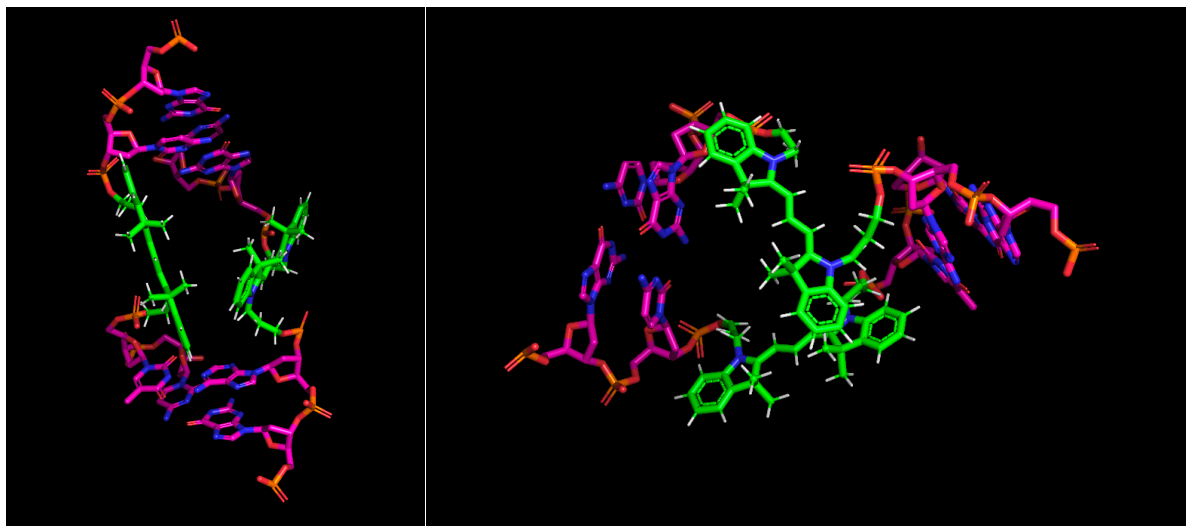


Figure 10. Proposed physical structures for +15 (iCy3)₂-DNA at room temperature using the structure parameters retrieved from the TQ optimized fitting routine under a symmetric roll.

As shown in Figures 10 and 11, we have generated physically realistic (iCy3)₂ conformations with inter-chromophore separation $R_{AB} = 10 \text{ \AA}$ that fit inside the local DNA surroundings. These models, which are based solely on optimizations to the CD and absorbance measurements, exhibit few steric clashes. This type of modeling permits us to directly compare

optimized conformations that are determined by spectral analysis to molecular dynamics (MD) simulations, which attempts to minimize the free energy of the local DNA structure and takes into account steric overlap. It may be possible to further constrain these structural models by including as constraints additional experimental data, such as polarization-sweep single molecule spectroscopy (PS-SMS). By directly observing thermally-induced transitions between stable conformational states, it should be possible to assign conformations to the multiple minima of the free energy surface that governs the equilibrium and dynamic properties of the system.

Theoretical Implications of iCy3 Dye Face-to-Face Stacking

As has been noted in several earlier works, purely electrostatic approximations of intermolecular couplings cannot address intermolecular couplings derived from interactions outside of the scope of strict Coulomb coupling between singlet-singlet Förster-like energy transfer states. The structural optimizations shown from the TQ-based spectral fitting routine indicate that the dimer chromophores probes of the (iCy3)₂ ss-dsDNA constructs form stacked (face-to-face) conformations within duplex DNA, potentially introducing overlap between neighboring HOMO-LUMO orbitals which protrude from the faces of the indole ring groups. However, the effects of charge transfer and orbital overlap are generally thought to be comparatively small for Cy3 chromophores, which has a relatively short π -orbital conjugation length, a relatively large EDTM, and an excited singlet electronic state that is well separated from other excited electronic states (15,16,17). Orbital overlap effects are generally minimal for chromophore pairs that have large average intermolecular crossing angles describing constituent transition moments (15). Symmetric changes to the roll angle within our coordinate system do not permit typical ‘face-to-edge’ or ‘herringbone’ packing motifs typically observed for many chromophore aggregates, which may also act to limit direct face-to-face packing.

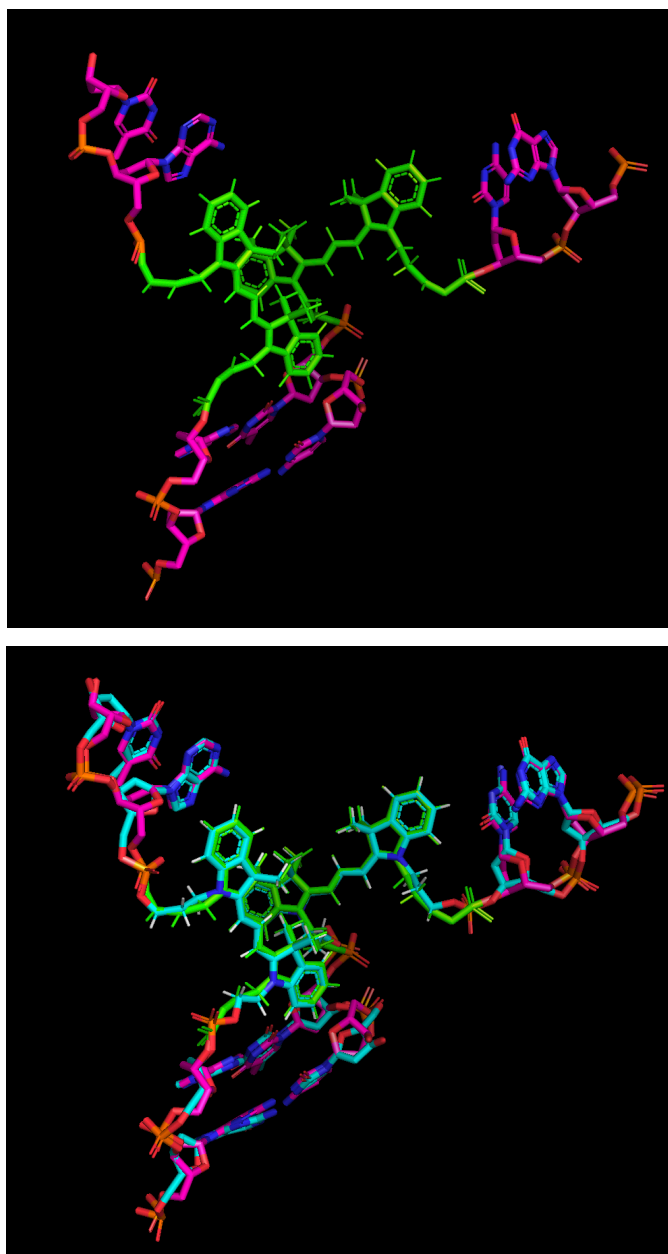


Figure 11. Proposed physical structures for -1 (iCy3)₂-DNA at room temperature using the structural parameters retrieved from the TQ optimized fitting routine under a symmetric roll (*top*). Proposed physical structures for -1 (iCy3)₂-DNA at room temperature using the structural parameters retrieved from the TQ optimized fitting routine under a symmetric roll overlaid with structure after optimizing the geometry of and lengths of chemical bonds (*bottom*).

While changes to the shift and shear displacements alter the pairwise Coulomb interactions between Mulliken partial transition charges in our TQ model, these purely longitudinal translations may also act to modify electron and hole transfer integrals between closely spaced, interfacial stacked chromophores. The effect of shift and shear displacement may

thus modify the Coulomb interaction (16) when an exchange coupling term is included in the system Hamiltonian. The exchange integral for large chromophores falls off very quickly with separation distance and does not appear to affect the solutions to our refined structural optimizations. By including the effects of orbital overlap, it may be possible to explain poorly captured spectral changes observed in 2DFS measurements of dimers at temperatures above room temperature (Chapter III), especially those in constrained (duplex DNA) conditions. Increasing temperature below the thermal denaturation point of duplex DNA may increase the collision rate between chromophores, which facilitates exchange-like interactions. Our structural optimizations disregard any physical constraints imparted by the attached oligonucleotides that may dictate local stacking structure, and do not account for attractive intermolecular forces experienced between the chromophores, or additional interactions between the chromophores and the neighboring bases. Inclusion of these forces and properly accounting for molecular orbital overlap may influence structural solutions and explain deviations from molecular dynamics trajectories for the (iCy3)₂ dimer-labeled ss-dsDNA constructs and similar systems.

Conclusions

Applying accurate theoretical models for the electrostatic couplings within a molecular dimer is a key step for simulating its optical properties and analyzing its optical spectra. By employing the TQ model, which assigns individual Mulliken transition charges to the atomic coordinates of the Cy3 chromophores, we compared simulated to experimental absorbance and circular dichroism (CD) spectra of ‘internally-labeled’ (iCy3)₂ dimer-labeled DNA fork constructs for which the iCy3 probes are held rigidly within the sugar-phosphate backbones at distinct positions relative to the ss-dsDNA fork junction. We find that the PD, ED and TQ models provide complementary and comparable information about the conformational parameters of the (iCy3)₂ dimer-labeled DNA fork constructs. However, the atomistically detailed TQ model can be used to better distinguish dimer conformations in which the ‘roll angle,’ ‘shear’ and ‘shift’ displacements between the planar trimethine groups of the iCy3 monomers are varied. We find that for (iCy3)₂ dimer probes positioned deep within the DNA duplex, the trimethine chromophores adopt a ‘stacked’ face-to-face conformation, while for

(iCy3)₂ dimer probes positioned near the DNA fork junction there is less preference for face-to-face stacking.

In future experiments, we hope to use these techniques to study the effects of protein binding on local DNA structure. The local ds-ss DNA junction is likely composed of a structural ensemble, driven by natural ‘breathing’ modes at physiological temperatures, and works to facilitate kinetic competition between regulatory factors and to recruit nonspecific DNA binding proteins at the ds-ss junction. Quantitative structural examination of these processes appears possible with the aid of small fluorescent sugar-phosphate backbone ‘analogs’ like iCy3. Inclusion of iCy3 probes at ds –ss may retain sufficient structural cues for the successful binding of many non-specific replisome proteins. With minimal disruption to neighboring DNA structure, and to protein-DNA complex formation, there is an indication that (iCy3)₂-DNA geometries may be useful in characterizing DNA replication-associated protein signaling processes, or DNA-protein bound states. However, the reliable extraction of these structures may require increasingly accurate models of Cy3 resonance interaction energies and physically-motivated distribution of transition charge.

Bridge to Chapter V

After examining how refinements to our computational model affect the results of our structural analyses, we next investigate how chemical modifications of (iCy3)₂ dimer-labeled ss-dsDNA constructs, such as base sequence composition and buffer conditions, influence the local conformations of the sugar-phosphate backbones. We compare the results of our bulk measurements to a novel single molecule methodology to gain insight into the rates of conformational exchange at the ss-ds DNA fork junction.

Chapter V

The Effects of Base Sequence Composition, Length, and Isostabilizing Salts on Local Conformational Distributions and Dynamics of (iCy3)₂-Labeled ss-ds DNA Fork Constructs

The experiments and composition of this section was prepared by myself with the help of Lulu Enhkbataar and Maya Pande, who carried out experiments under my direction. Jack Maurer, Pat Herbert and Dylan Heussman designed the single molecule polarization sweep experiments and analysis protocol. The design of experiments was aided by Peter von Hippel and Andrew Marcus, who is the principal investigator for this work and provided some editorial assistance.

Introduction

The specific nucleobases that comprise the DNA polymer are responsible for differentially altering its thermodynamic stability. To an approximate level of accuracy, scientists can predict the stability (ΔG°) and the melting behavior (ΔH°) of any DNA duplex structure from knowledge of its primary base sequence (1). In general, GC base pairs tend to increase the melting temperature of DNA when compared to AT base pairs, due in part to the additional Watson-Crick (WC) hydrogen bonds formed between GC nucleobases. While complementary hydrogen bonding provides the specificity for the annealing of complementary single strands, the formation of WC hydrogen bonds affords little enthalpic stability to the DNA duplex relative to the single strands, which would otherwise form hydrogen bonds with the aqueous solvent. In general, the greater stabilizing effect of the CG base pair does not result from its larger enthalpic contribution, but from its more negative entropic contribution in comparison with that of the AT base pair (3). DNA ‘breathing’ fluctuations, which are thermally driven excursions from the conformational free energy minima, act to introduce structural variety and transiently expose the bases to the exterior surroundings for sequence recognition by proteins (2), the rates of which can also be modulated by DNA composition. Base stacking, the stabilizing interactions between the nucleobase surfaces, provide the driving force that maintains the double

helix structure and an organizational force that likely populates transient macrostates in the ss-region (4). It is clear nature has designed an excellent vehicle for protecting the information content of DNA, tucking the nucleobases away inside a sugar-phosphate backbone, however it must allow some periodic exposure of the bases, at rates low enough as to not invite unwanted chemical modification and the accumulation of mutations.

To study the difference in structure and melting behavior as a function of base composition, we examined several (iCy3)₂-DNA constructs with altered base composition (Table 1) neighboring the iCy3 probes. In addition to studying (iCy3)₂-DNA under physiological buffer conditions (100 mM NaCl, 6 mM MgCl₂, 10mM TRIS), we studied the spectroscopic effects of isostabilizing salts on our (iCy3)₂-DNA constructs (5-6). Isostabilizing salts are a category of molecules that are thought to differentially stabilize (or destabilize) duplex DNA based on a preferential interaction with specific nucleobases. The structural effects of these molecules may also be of interest as reagents for improving biochemical assays. Generally, isostabilizing salts are comprised of small, charged molecules that can form close physical contacts with the DNA. In this study, we examine the effects of three different isostabilizing molecules to elucidate the effects that these small molecules might have on the local DNA structure, as interpreted by iCy3 dimer probes covalently inserted into the DNA sugar-phosphate backbone.

The small molecule additives studied here include tetraethylammonium chloride (TEA), tetramethylammonium chloride (TMA), and betaine monohydrate. Here the prefix “iso” refers to the equalizing effects that the salt additives impart onto the relative stabilities of DNA comprised of GC-rich vs. AT-rich base pairs, with iso-stabilizing (destabilizing) molecules tending to increase (decrease) the melting point (denaturation) temperature and increasing the cooperativity through which the melting process occurs (5-6). These molecules do not exhibit their isostabilizing effects at all concentrations and must be present at a relatively high molarity to influence the melting behavior of the DNA in a manner that can be interpreted as an isostabilizing effect. Note, we use the term “isostabilizing” to refer to all these molecular additives regardless of their observed effect of increasing or decreasing the DNA melting behavior. The concentrations at which the isostabilizing effects can be observed were previously determined to be 3.3 M for TMA, 5.5 M for betaine, and 2.4 M for TEA (5-6). However, there has been some evidence that the TMA does not universally neutralize DNA sequence dependent

stability, and exhibits melting temperature effects that are concentration and sequence dependent (8). In addition to the molecules studied here, there have been isostabilizing effects ascribed to other small molecules, such as choline (7). While TMA and TEA carry a positive charge in solution, the small molecule betaine is a zwitterion, and does not alter the ionic strength of the DNA/buffer solution.

The underlying molecular mechanism for DNA isostabilization imparted by these molecules is not well understood. However, molecular dynamics (MD) and NMR studies suggest that TMA ions are preferentially localized in the minor groove of DNA at AT base pairs, and that TMA ions exhibit less preference for the major groove than other alkali and alkali earth metal ions (7). Investigating the effects of isostabilizing salts using the iCy3 dimer probes provides an interesting possibility to study how the probable conformation and structural disorder implied by melting point measurements is related to the structure and local heterogeneity of DNA composition revealed spectroscopic measurements of site-specifically labeled DNA constructs. If DNA constructs of a particular base pair composition exhibit a propensity to undergo structural fluctuations specific to the local DNA sequence, the presence of isostabilizing salts may modify these fluctuations, and thus permit us to characterize the pathways by which base sequence-dependent fluctuations mediate protein binding events.

In addition to studying DNA sequence and isostabilizing effects in the DNA duplex region, we also examine how changes to the base composition, isostabilizing salt concentration and single-strand length of ds-ss DNA junction-containing constructs alters the average local structure at and near the ds-ss junction, and how these results suggest the presence of a multimodal *distribution* of conformationally distinct structures whose average is detected in ensemble measurements. We then briefly examine the (iCy3)₂ labeling position-dependent results of a novel technique, polarization-sweep single molecule spectroscopy (PS-SMS), which provides evidence for the existence of rapidly interconverting conformational states. Our preliminary results using PS-SMS experiments support the hypothesis that DNA junctions are comprised of several identifiable and marginally stable conformational states at the ds-ss junction, which undergo structural interconversion at microsecond to millisecond timescales. The results of our ensemble spectroscopic measurements indicate that isostabilizing salts, and changes to the DNA base sequence, act to preferentially alter the relative stability of certain DNA conformers within the distribution of available backbone structures. These systems can be

further studied by single molecule methods to retrieve the relative stability of and rates of interconversion between conformers, and to examine how stabilizing additives and base sequence might influence the kinetics governing conformational exchange.

Discussion of Results

By observing the temperature dependence of circular dichroism (CD) and absorbance measurements of DNA constructs with different base sequences adjacent to the (iCy3)₂ dimer probes, it is evident that the coupling between the iCy3 dyes is disrupted at a significantly lower temperature when the probes are flanked by AT base pairs in comparison to GC base pairs (see Figure 1). The compositions of these constructs are listed in Table 1. In our measurements, the structure of the AT-rich (iCy3)₂ dimer-labeled DNA construct appears to dissipate completely at temperatures below 55 °C, while the structure of the GC-rich DNA construct is maintained at temperatures approaching 75 °C. These results are consistent with the standard melting behavior of duplex DNA comprised solely of AT-rich or GC-rich base pairs in the absence of the (iCy3)₂ dimer chromophore probes. The AT-rich (iCy3)₂ dimer-labeled DNA construct ‘breathes open’ and exposes its internal bases at ambient temperatures to a greater extent than does the GC-rich DNA construct, which ultimately leads to the denaturation of the two DNA strands at lower temperatures. Calculations of the melting temperature, T_m , for the duplex regions of the DNA using the rudimentary formula:

$$T_m = 64.9 + 41(yG + zC - 16.4)/(wA + xT + yG + zC),$$

with the lower-case coefficients representing the mole fraction of each of the component nucleobases, suggest that the anticipated melting temperatures are approximately 75.3 °C for the GC-rich DNA construct and 43.8 °C for the AT-rich DNA construct. The value of T_m indicates the estimated temperature at which 50% of the WC base pairing is disrupted. The DNA melting process is highly cooperative near its transition temperature, and the degree of cooperativity is enhanced when the nucleobase composition is not equally balanced between AT-rich and GC-rich regions. This is consistent with our observation of a rapid change in the CD signal of the AT-rich DNA construct over the temperature range 45 °C – 55 °C (see Figure 1).

Table 1. Base Pair Sequences of (iCy3)₂ Dimer-Labeled ss-ds DNA Fork Constructs

DNA construct	Nucleotide base sequence
+10 (iCy3) ₂ GC dimer	3'-GTC AGT CGC GGC CCG G/iCy3/CC GGG CCG CCC CAC GTT TTT TTT TTT TTT TTT TTT TTT TTT-5' 5'-CAG TCA GCG CCG CGC C/iCy3/GG CCC GGC GGA TGC TTT TAC CAC TTT CAC TCA CGT GCT TA-3'
+10 (iCy3) ₂ AT dimer	3'-GTC AGT ATT ATA TAA T/iCy3/AT ATA ATA TAC CAC GTT TTT TTT TTT TTT TTT TTT TTT TTT-5' 5'-CAG TCA TAA TAT ATT A/iCy3/TA TAT TAT ATA TGC TTT TAC CAC TTT CAC TCA CGT GCT TAC-3'
+1 (iCy3) ₂ GC dimer	3'-GAG GGA GCA CAG CAG AGG TCA GTA TTA TAC GCG /iCy3/CGC TGG TAT ACC ACG (T) ₂₈ -5' 5'-CTC CCT CGT GTC GTC TCC AGT CAT AAT ATG CGC /iCy3/GTA CTT TCG CCA CTT TCA CTC ACG TGC TTA-3'
+1 (iCy3) ₂ AT dimer	3'-GAG GGA GCA CAG CAG AGG TCA GTA TTA TAC GCT /iCy3/AGC TGG TAT ACC ACG (T) ₂₈ -5' 5'-CTC CCT CGT GTC GTC TCC AGT CAT AAT ATG CGA /iCy3/TTA CTT TCG CCA CTT TCA CTC ACG TGC TTA

While the AT-rich (iCy3)₂ dimer-labeled duplex DNA construct melts at a significantly lower temperature than the GC-rich DNA construct, the local backbone structure appears to be generally consistent across the full range of temperatures investigated, conserving the structure previously observed (associated with the B-helix) for (iCy3)₂ dimer-labeled duplex DNA constructs of mixed base sequence (Chapters II-IV). The thermodynamic stability of DNA is known to depend on its local base sequence. However, the local base sequence does not significantly perturb the structure of the B-helix, especially in duplex regions far removed from any emergent secondary structural topologies, such as those present at and near ss-ds DNA junctions, which may serve as nonspecific recognition sites for protein-DNA binding events. In Table 2, a comparison is made between the temperature-dependent structural parameters determined from the analysis of these constructs under the TQ electrostatic coupling model. Both constructs maintain a ‘right-handed’ average conformation through their melting temperature. It is interesting to note that the GC-rich sequence exhibits a smaller CD amplitude at the lowest temperature studied, which may indicate the presence of a relatively unstable minority conformational species, such as one that exhibits an achiral conformation that does not contribute to the CD amplitude, or a ‘left-handed’ species that acts to cancel the amplitude of the ‘right-handed’ majority species. Alternatively, the larger AT rich CD signal might indicate a lowering of barrier to support the existence of additional right-handed conformations. A discussion of the behavior of mixed-base DNA sequences is given in Chapter II, Figure 5, which displays a

melting behavior that can be described as an intermediate between AT and GC neighboring homopolymers.

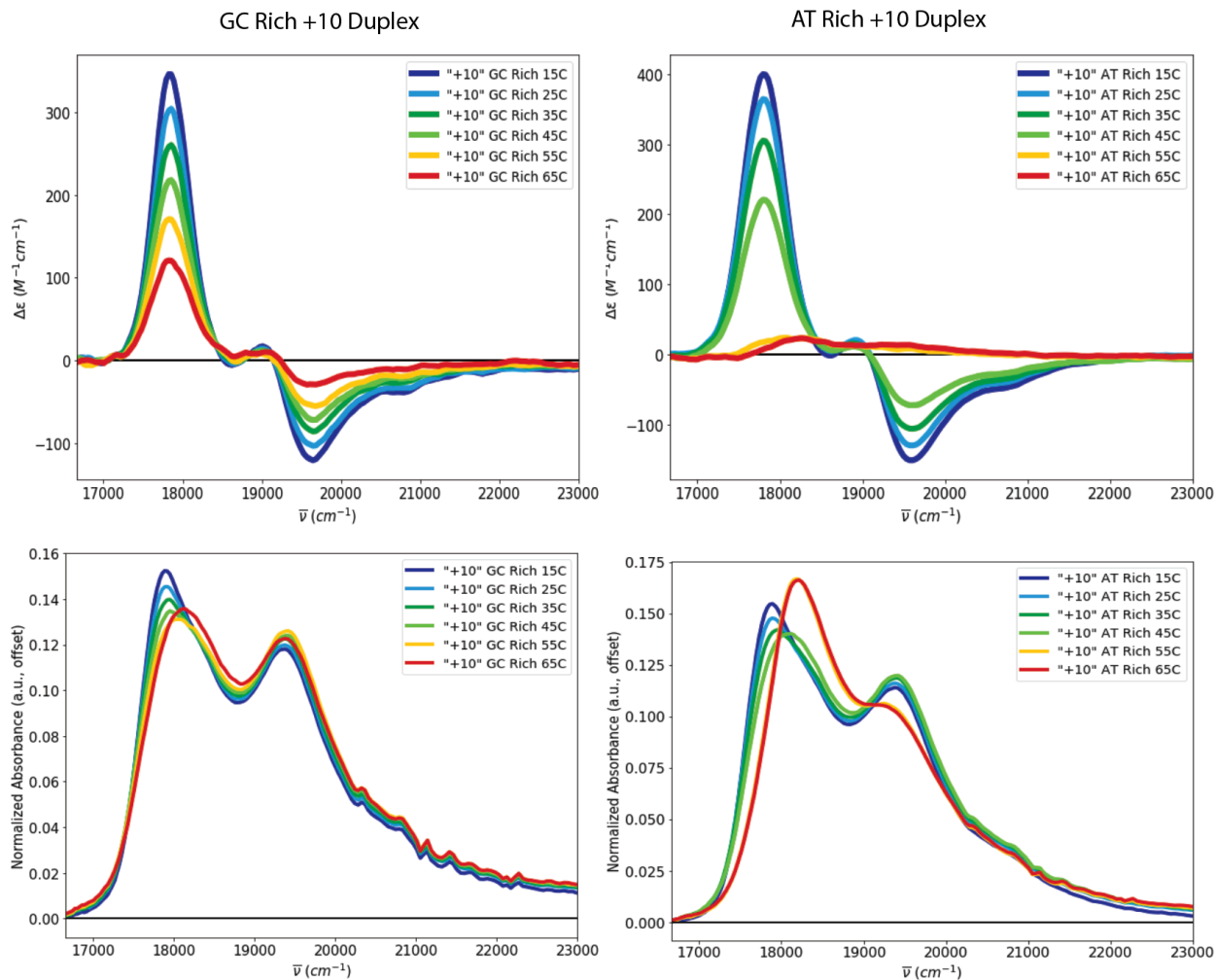


Figure 1. Temperature- and DNA composition-dependent CD and absorbance measurements for the GC-rich (iCy3)₂ dimer-labeled DNA construct (*left*) and the AT-rich (iCy3)₂ dimer-labeled DNA construct (*right*). The (iCy3)₂ dimer probe is inserted at the +10 position.

The temperature-dependent structural trends outlined in Table S1 provide additional evidence that the nature and stability of the (iCy3)₂ coupling is imparted by the local DNA scaffold, and no appreciable stability is conferred by favorable interactions between the iC3 chromophores. At elevated temperature the sugar-phosphate backbones maintain the average B-helix structure. However, the nucleation of bubbles along the DNA acts to decrease the average coupling

strength and increase the conformational disorder as function of temperature until the DNA has melted, which is a constant trend found for all ‘duplex’ constructs we have examined. If our measurements had revealed no sensitivity to the changing neighboring base composition at varying temperatures, it may indicate that the local structure is largely determined by preferential contacts made between the iCy3 chromophores themselves. Our results indicate that the conformations adopted by the (iCy3)₂ dimer probe are reflective of the local conformations of the DNA sugar-phosphate backbones and display similar base-sequence dependent temperature sensitivities.

Iso-destabilizing salts through the lens of iCy3 Dimers in DNA: Betaine and TEA

As shown in Figure 2, betaine and TEA act to reduce the melting point temperature of the (iCy3)₂-DNA constructs of mixed-base sequences. Upon examining the melting behavior as determined by the change in CD amplitude at 565 nm, it is evident that TEA and betaine also increase the cooperativity of melting by decreasing the temperature range over which the CD amplitude decreases. The composition of the mixed-base sequence DNA construct is given in Table 1.1. When comparing mixed-base sequence +15 labeled (iCy3)₂-DNA under isostabilizing conditions and AT-rich construct under physiological conditions to the +15 labeled (iCy3)₂-DNA under physiological conditions the increase in cooperativity is apparent given the increase in slope m throughout the melting regime, from $m = 0.03 - 0.05$. The slope of melting curves is often characteristically shallow for large, mixed base sequences of DNA, as the WC hydrogen bonds of G-C and A-T base pairs dissociate and unstack at different temperatures. This enhancement in cooperativity under iso-destabilizing conditions has been reported when analyzing high molecular weight calf thymus DNA by tracking changes in the hypochromicity of native DNA at 260 nm (6).

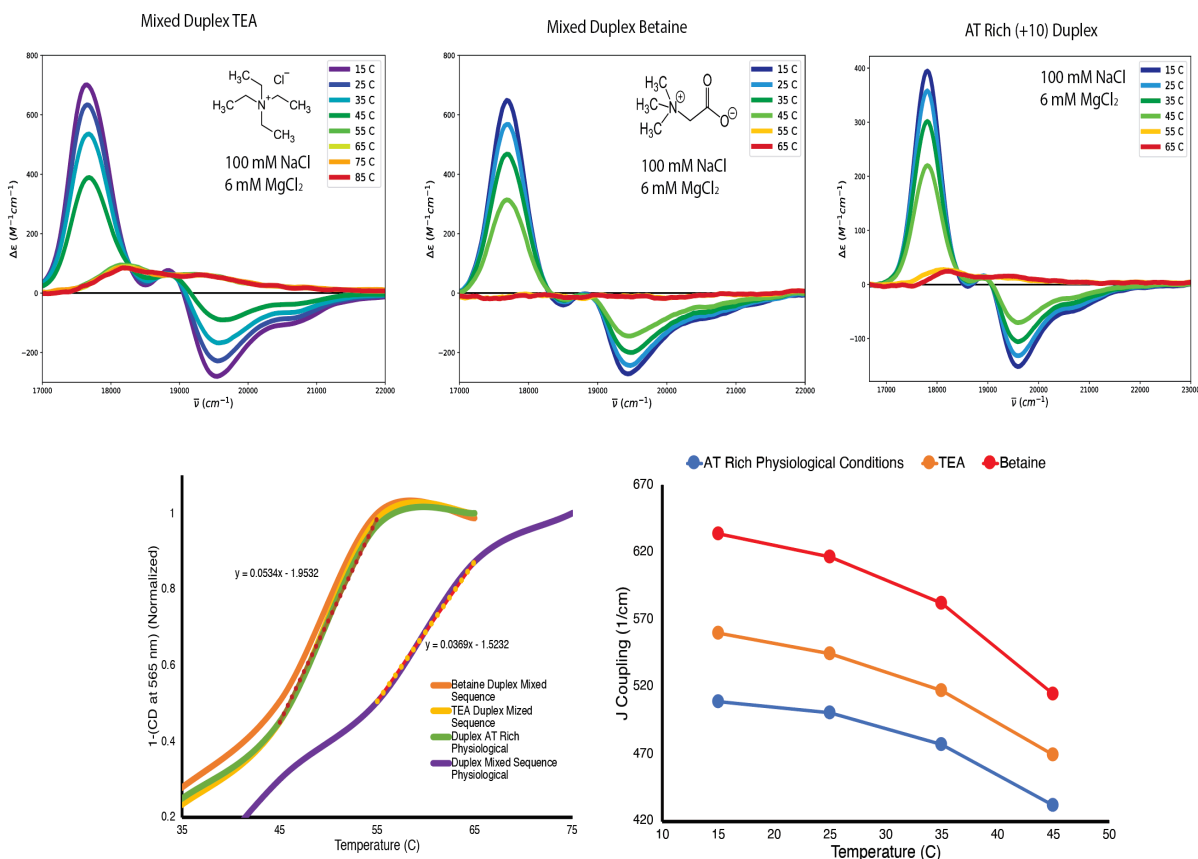


Figure 2. The temperature-dependent changes to the CD signal of +15 mixed base composition DNA sequences under (A) 2.4 M tetraethylammonium chloride (TEA), (B) 5.5 M betaine and (C) normal physiological conditions (100mM NaCl, 6mM 6 $MgCl_2$, 10 mM TRIS). Isostabilizing buffers were prepared with 10mM Tris and 6mM $MgCl_2$. (D) The melting profile of (iCy3)₂-DNA for each construct as determined by the change in the CD amplitude at 565 nm (17699 cm^{-1}). The change in the CD amplitude was normalized over the melting range of each construct (15 °C – 65 °C for TEA, betaine containing and AT-rich constructs; 35 °C – 75 °C for mixed-base sequence +15 DNA). (E) The temperature dependence of the resonant coupling under each condition.

While a sudden change in the hypochromicity of DNA bases in the UV region reflects the cooperative ‘lifting off’ of bases from one another, thus reducing the degree of base stacking, the change in (iCy3)₂-DNA CD is indicative of an abrupt reduction of the electrostatic coupling of the (iCy3)₂ dimer probe induced by the melting of neighboring bases. Prior to the disruption of the (iCy3)₂ electrostatic coupling, the DNA maintains an average ‘right-handed’ structure characteristic of B-form DNA, which is consistent with the conserved behavior of the native

DNA UV CD spectrum under iso-destabilizing salt conditions at room temperature (6). As seen in Figure 2, the temperature-dependent electrostatic coupling under iso-destabilizing salt conditions falls off more quickly than under physiological buffer salt conditions, which follows a strictly linear temperature-dependence (see Figure 1.5). A non-zero background is seen at temperatures above the melting point for the TEA and AT-rich samples, which was not observed for other iCy3-DNA samples.

Our temperature-dependent analysis of the (iCy3)₂-DNA under iso-stabilizing salt conditions reveals that the right-handed structure with $\phi_{AB} < 90^\circ$ is retained (Table 2), even under the extreme changes to the ionic environment induced by the presence of iso-stabilizing salts, which illustrates the robust stability of duplex DNA. An important observation is the increased amplitude of the CD signal in the presence of iso-stabilizing salts in comparison to physiological conditions at a given temperature. The enhanced CD amplitude does not alter the conformational parameters resulting from our optimizations, which regard the (iCy3)₂ dimer-labeled DNA system as a single average structure. However, an increase in the CD amplitude (in the absence of an increase in the coupling J) suggests the presence of achiral species under physiological conditions that do not contribute to the CD signal. These changes may reveal a reduction in the heterogeneity of the ensemble of conformational species that we are not sensitive to through the analysis of absorbance and CD data. However, they may lead to errors in the results obtained from simultaneously fitting the CD and absorbance spectra, which assumes that the system can be represented as a single conformational species that contributes equally to the absorbance and CD signals. Fewer members of the ensemble with achiral or uncoupled orientation could indicate a fundamental change to the heights of the energy barriers associated with the chiral to achiral transition, or a lowering of the free energy of the B-form ‘right-handed’ structure under iso-destabilizing salt conditions.

Table 2. (next page) Temperature-dependent fits for the mixed base sequence +15 DNA under iso-destabilizing conditions modeled using a TQ coupling calculation and a symmetric roll angle, η_{AB} .

	Temp	ϕ_{AB} (°)	θ_{AB} (°)	η_{AB} (°)	δ_{AB} (Å)	ξ_{AB} (Å)	R_{AB} (Å)	σ (cm ⁻¹)	J (cm ⁻¹)
Betaine	15	77.4 +2.2/ - 2.2	70.4 +1.0/ - 1.0	99 +5.9/ - 1.7	-3.7 +0.2/ - 0.2	-0.3 +0.3/ - 0.7	10.44 +0.1/ -0.1	304.4 +18/ -15	633.3
	25	75.4 +2.3/ -0	69.4 +0/ -1	100.15 +18/ - 0.02	-3.82 +0.2/ - 0.004	0.685 +0.01/ - 2	10.5 + 0.1/ - 0.004	319 +17/ -16	616.3
	35	74.3 +1.3/ - 1.3	64.2 +1.1/ - 1.1	103.2 +7/ -5	-1.87 +0.1/ - 0.1	-0.56 +0.8/ - 0.7	9.03 +0.1/ -0.1	337.3 +17/ -16	582
	45	61.75 +2.6/ - 2.6	70.95 +1.3/ - 1.3	90.76 +36/ - 12.5	-2.6 +0.3/ - 0.3	-1.89 +0.4/ - 0.3	11.24 +0.1/ -0.1	363.1 +16.5/ - 15.7	514.7
TEA	15	75.5 +1.8/ - 0.1	61.3 +0.02/ - 1.1	111.25 +0.1/ - 25.2	-3.83 +0.2/ - 0.008	-1.25 +2.2/ - 0.5	10.14 +0.1/ - 0.004	351.2 +16.4/ - 15.4	559.5
	25	55.6 +2.3/ - 2.3	77.3 +1.1/ - 1.2	97.1 +35/ -5	-1.97 +0.3/ - 0.3	-1.13 +0.36/ - 0.45	11.7 +0.12/ - 0.12	358.4 +16/ -15	544.2
	35	57.4 + 2.4/ - 2.5	71.6 +1.2/ - 1.2	87.5 +38/ -6	-2.82 +0.4/ - 0.5	-1.48 +0.5/ - 0.4	11.7 +0.1/ -0.1	367.2 +16/ -15	517
	45	58.05 +1.8/ - 1.8	66 +1.2/ -1.3	119.4 +10/ - 35	-2.07 +0.2/ - 0.2	-1.7 +0.7/ - 0.5	11.2 +0.1/ -0.1	376.5 +15/ -14	469.7

While the betaine mixed-base +15 (iCy3)₂ dimer-labeled DNA construct and the AT-rich mixed-base DNA construct exhibit similar levels of conformational disorder (σ), the value of σ obtained under TEA conditions exceeds the values obtained for (iCy3)₂ dimer-labeled duplex DNA under physiological buffer salt conditions. This observation suggests a different mode of

action for the two iso-stabilizing salts: one in which a single ‘right-handed’ local DNA structure is stabilized in the presence of betaine (and energetically favored by the AT-rich DNA construct), and another mechanism in which a similar, but energetically and conformationally distinct ‘right-handed’ DNA structure is favored under TEA conditions. It should be noted that for the AT-rich DNA construct, TEA and betaine increase the amplitude of the CD signal at 565 nm, indicating a general stabilization of the ‘right-handed’ local DNA conformation within the ensemble of available structures, in comparison to the mixed-base and GC-rich DNA constructs.

Iso-stabilizing salts through the lens of (iCy3)₂ Dimers in DNA: TMA

To examine the dependence of base composition on the effects of different iso-stabilizing salts, we studied two (iCy3)₂ dimer-labeled duplex DNA constructs with homogeneous base composition (AT-rich and GC-rich) under iso-stabilizing salt (TMA) conditions (see Figure 3). The base sequences of these DNA constructs are shown in Table 1. When the melting profile of the GC-rich sequence was analyzed under isostabilizing amounts of TMA, the CD signal decreased linearly with increasing temperature, which was not significantly altered from the melting profile of the same DNA construct under normal physiological conditions. However, for the AT-rich duplex DNA construct under iso-stabilizing TMA conditions, the CD signal remained robust until much higher temperatures. Under normal physiological conditions, the bisignate CD signal, which indicates a dominant, coupled, chiral orientation of the DNA vanishes entirely at 55° C. However, the CD signal remains until 75° C in the presence of iso-stabilizing TMA conditions. While the GC-rich DNA construct under isostabilizing conditions shows a linear temperature dependent for CD₅₆₅, the AT-rich sequence exhibits an abrupt, temperature-dependent decrease in the CD signal, which we interpret as an indication of cooperative melting of the local DNA structure near the (iCy3)₂ dimer probe. It is important to note that under iso-stabilizing TMA conditions, the amplitude of the CD₅₆₅ is comparable between the base compositions, indicating a similar free energy landscape describing distribution of local DNA conformations at this position. These melting behaviors also deviate from predicted melting behaviors under an increase in simple positive cation concentration, such as Na⁺.

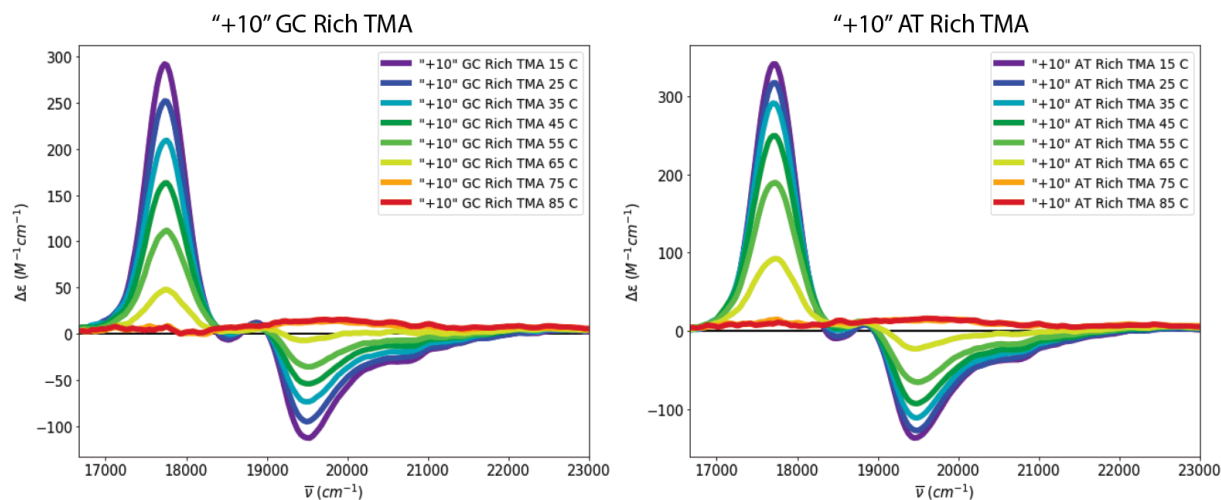


Figure 3. The temperature-dependent CD behavior of GC-rich and AT-rich +10 (iCy3)₂ dimer-labeled DNA constructs under iso-stabilizing salt conditions of tetramethylammonium chloride (TMA).

Base Composition-Dependence at the +1 DNA Fork Position

In previous chapters, we discussed the clear change in handedness that characterizes (iCy3)₂ dimer-labeled positions moving from the double-stranded to single-stranded DNA regions in the proximity of a DNA fork junction. To investigate the generalizability of these trends, we examined two additional constructs with altered neighboring base composition at the +1-labelling position, the sequences of which are shown in Table 1. The duplex and ss regions of these sequences are identical to the +1 labeled DNA found in Chapter III. However, these constructs have been altered so that the neighboring bases are comprised AT or GC base pairs.

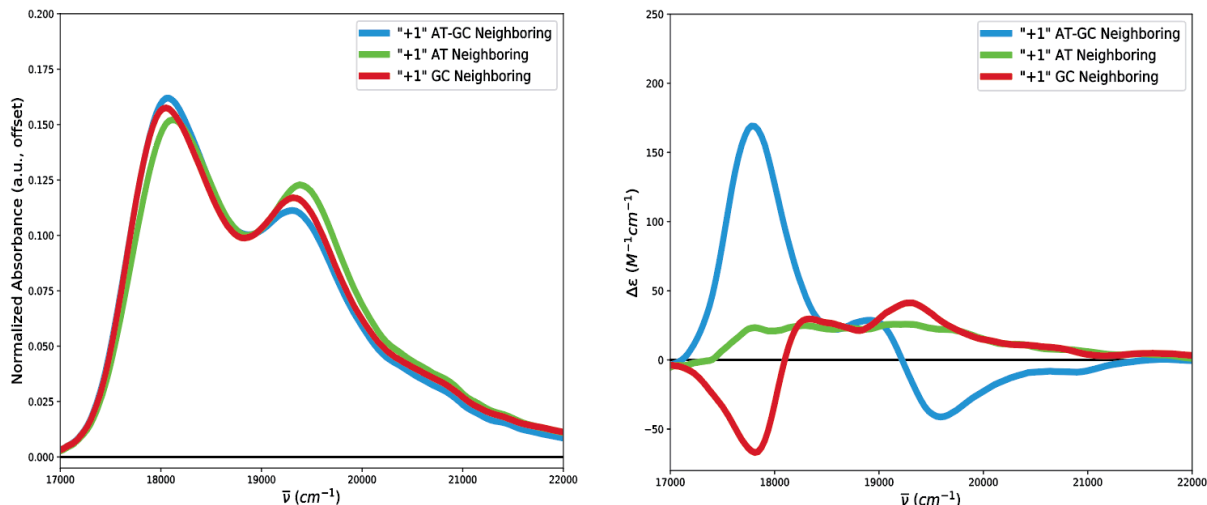


Figure 4. Local base sequence-dependence of room temperature absorbance and CD spectra for (iCy3)₂ dimer-labeled DNA fork constructs (labeled at the +1 position) with AT-rich, GC-rich and mixed-base sequences.

Interestingly, we find that there are significant structural changes that accompany alterations to the local base composition near the fork junction. It is well known that the inclusion of adenine and thymine directly at the ss-ds DNA junction results in significantly more fraying into the double-stranded DNA region (2), which is partly due to the same thermodynamic considerations that lead to the destabilization of AT-rich duplex DNA. This phenomenon is captured experimentally by the CD spectrum of the +1 AT-rich DNA fork construct (see Figure 4), which appears to lack a clear bisignate line shape. Nevertheless, the absorbance spectrum of the AT-rich DNA constructs exhibits the intensity borrowing indicative of the vibronically coupled (iCy3)₂ dimer. Satisfactory fitting of these spectra is not possible under our current methodology. These data suggest that the iCy3 probes occupy a heavily disordered region, with no singular stable average conformation of identifiable chiral orientation, and in general features a weak electrostatic coupling interaction. Our observations suggest the presence of a heterogeneous distribution of local conformations for the (iCy3)₂-dimer-labeled DNA construct, in which the structures within the ensemble contribute non-negligible values of J , and no singular conformation dominates the distribution when the barriers are lowered by the extensive fraying of AT terminal bases near the DNA fork junction. Conversely, the +1 GC rich spectrum adopts a weak ‘left-handed’ conformation that differs from the strong ‘right-handed’ conformation

previously observed for the mixed-base DNA fork construct. Our ability to correctly simulate this apparent left-handed conformation is also limited, in this instance by the similar amplitude between the positive and negative Cotton effects observed in the CD spectrum, which cannot be reliably captured under an assumed single average structural model. This could be due to an unexplained positive CD background, or from CD contributions attributed to the conformational distributions observed in CD signals that resemble that of the +1 AT-rich DNA fork junction.

The Effects of Iso-stabilizing TMA and Local Base-Sequence Composition on the Local Conformation of +1 (iCy3)₂ dimer-labeled DNA Fork Constructs

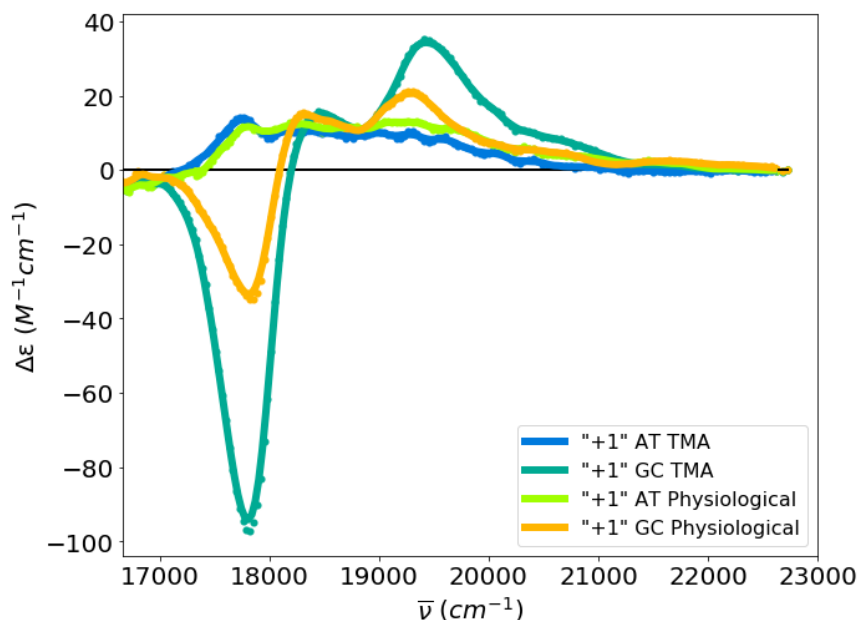


Figure 5. The effects of iso-stabilizing salt TMA at the +1 (iCy3)₂ dimer-labeled DNA fork junction composed of AT-rich and GC-rich regions.

From the analysis of CD data shown in Figure 5, even in the presence of large concentrations of iso-stabilizing salts, there is no structural stabilization of the +1 AT-rich ss-ds DNA junction. In this instance, the effects of fraying dominate the interactions near the ss-ds DNA junction and no single chiral conformation is favored. For the GC-rich +1 DNA fork construct, there appears to be an enhancement of the local stability due to the presence of TMA, which is evident from the increased magnitude of the CD signal. The presence of TMA also acts

to reduce the ‘right-handed’ CD signal of +1 mixed-base DNA fork construct, which has been omitted from the plot for simplicity. The measurements presented in Figure 5, in combination with the reduced “right-handed” CD signal observed for +1 mixed base DNA fork construct in the presence of TMA, indicates that TMA acts to stabilize a ‘left-handed’ local conformation within the available ensemble. While a left-handed local conformation indicates a deviation from the B-form-like structure at the ss-ds DNA junction, it might also indicate a ‘supercoiled’ geometry in which the twist angle is increased to a value $\phi_{AB} > 90^\circ$. Such a structure would likely exhibit an increased negative value of the coupling J , as seen above.

The Effects of Single-Stranded DNA Length on the Local Conformation Near (iCy3)₂ Dimer-Labeled ds-ss-DNA Junctions

Table 3. (iCy3)₂ dimer-labeled DNA fork constructs with short single-stranded DNA regions.

DNA construct	Nucleotide base sequence
All ds short dimer	3'–GAT TAT ACG CTC GCT <i>iCy3</i> AA TAT ACC ACG–5' 5'–CTA ATA TGC GAG CGA <i>iCy3</i> TT ATA TGG TGC–3'
+3 (iCy3) ₂ short ss dimer	3'–GAT TAT ACG CTC GCT <i>iCy3</i> AA TAT ACC ACG–5' 5'–CTA ATA TGC GAG CGA <i>iCy3</i> TT AGC ACA GTA–3'
+2 (iCy3) ₂ short ss dimer	3'–GAT TAT ACG CTC GCT <i>iCy3</i> AA TAT ACC ACG–5' 5'–CTA ATA TGC GAG CGA <i>iCy3</i> TT TGC ACA GTA–3'
+1 (iCy3) ₂ short ss dimer	3'–GAT TAT ACG CTC GCT <i>iCy3</i> AA TAT ACC ACG–5' 5'–CTA ATA TGC GAG CGA <i>iCy3</i> TA TGC ACA GTA–3'
-1 (iCy3) ₂ short ss dimer	3'–GAT TAT ACG CTC GCT <i>iCy3</i> AA TAT ACC ACG–5' 5'–CTA ATA TGC GAG CGA <i>iCy3</i> AA TGC ACA GTA–3'
-2 (iCy3) ₂ short ss dimer	3'–GAT TAT ACG CTC GCT <i>iCy3</i> AA TAT ACC ACG–5' 5'–CTA ATA TGC GAG CGT <i>iCy3</i> AA TGC ACA GTA–3'

To investigate how changes to the length of the single-stranded DNA region affect the local (iCy3)₂ dimer conformation, or distribution of conformations, at and near the ss-dsDNA fork junction we investigated ss-dsDNA fork constructs that only contain ~10 nucleotides in the ssDNA region. Examination of the (iCy3)₂ dimer-labeled ss-dsDNA fork constructs permits us to assess the effects that ssDNA length has on establishing structure at the junction, which may be due to the steric effects, coordinated motion of the long strands, or uncharacterized secondary structures adopted by the single-strand DNA region.

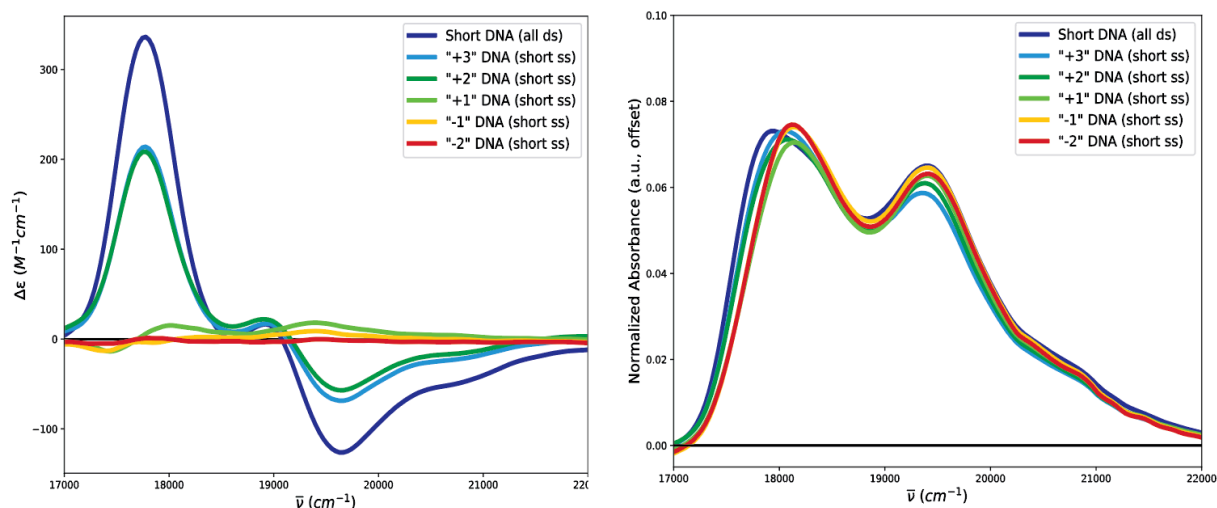


Figure 6. Circular dichroism and absorbance spectra taken for short ss DNA constructs under physiological buffer conditions.

Upon examination of the (iCy3)₂ dimer-labeled ss-ds DNA constructs with short ssDNA regions, we observed that the effects of fraying again reduce the stability of chiral conformers at the +1 position of the ss-dsDNA junction. This is comparable to the effect seen in the +1 dimer-labeled ss-dsDNA fork constructs with long ssDNA regions and AT-rich bases near the +1-labelling position, as both constructs are straddled by AT bases at the ds-ss DNA junction. While the DNA fork constructs with short ssDNA regions show reduced CD beginning at the +1 position, it appears that the reduced CD signal could be theoretically recaptured by using a linear combination of the characteristic ‘right-handed’ and ‘left-handed’ CD traces obtained from the +1 and -1 (iCy3)₂ dimer-labeled ss-dsDNA constructs with long ssDNA regions and mixed-base sequence composition. This suggests that the local conformation of the +1 (iCy3)₂ dimer-labeled ss-dsDNA constructs with short ssDNA regions (shown in Figure 6) occupies two stable iso-energetic conformational minima, one ‘right-handed’ and one ‘left-handed.’ As the CD signal regains its ‘duplex-like’ structure at the +2 position, it appears unlikely that the length of the ssDNA region has an effect on the local conformation that penetrates into the duplex DNA region. However, the length of the ssDNA region does appear to affect the distribution of structures within the ssDNA region at the -1 and -2 positions.

Structural Distributions and Conformational Dynamics of (iCy3)₂ Dimer-Labeled DNA Analyzed by Polarization-Sweep Single Molecule Fluorescence (PS-SMF) Microscopy

Background

To understand the distribution of structures and kinetics governing conformational exchange at ds-ss DNA fork junctions, we have implemented a novel technique, polarization-sweep single molecule (PS-SMF) microscopy, to study labeled (iCy3)₂-DNA at microsecond resolution and at the single molecule level. Using PS-SMF microscopy, we are able to monitor the conformational dynamics of the (iCy3)₂ dimer-labeled DNA constructs via time and phase resolved tracking of emission from the polarized excitons of the (iCy3)₂ dimer probe. Here we briefly outline the experimental methodology and preliminary results found from using this technique to study the conformational dynamics of the (iCy3)₂-DNA ss-ds junction constructs, which appear in Chapter III. A computational routine to complete analysis of higher order correlation functions and associated rate matrices is currently being prepared for publication as Maurer *et al.* The following descriptions serve to provide illustrative, if incomplete, preview of the dynamics of conformational exchange at ds-ss DNA junctions.

Experimental Techniques

Polarization-sweep single molecule fluorescence (PS-SMF) microscopy is a total internal reflection (TIRF) based microscopy which uses a narrow band, continuous wave, 532 nm laser to excite the polarized components (symmetric and antisymmetric) of the (iCy3)₂ dimer exciton. To prepare our samples, we use biotin labeled DNA and a linkage of biotin-neutravidin to tether the DNA to the surface of a quartz microscope slide and immobilize the molecule. The details of the sample chamber preparation can be found in Lee *et al.* The DNA constructs used in this study can be found in Table 3.1, however with an additional biotin added to the 5' end of the duplexed side of the DNA constructs.

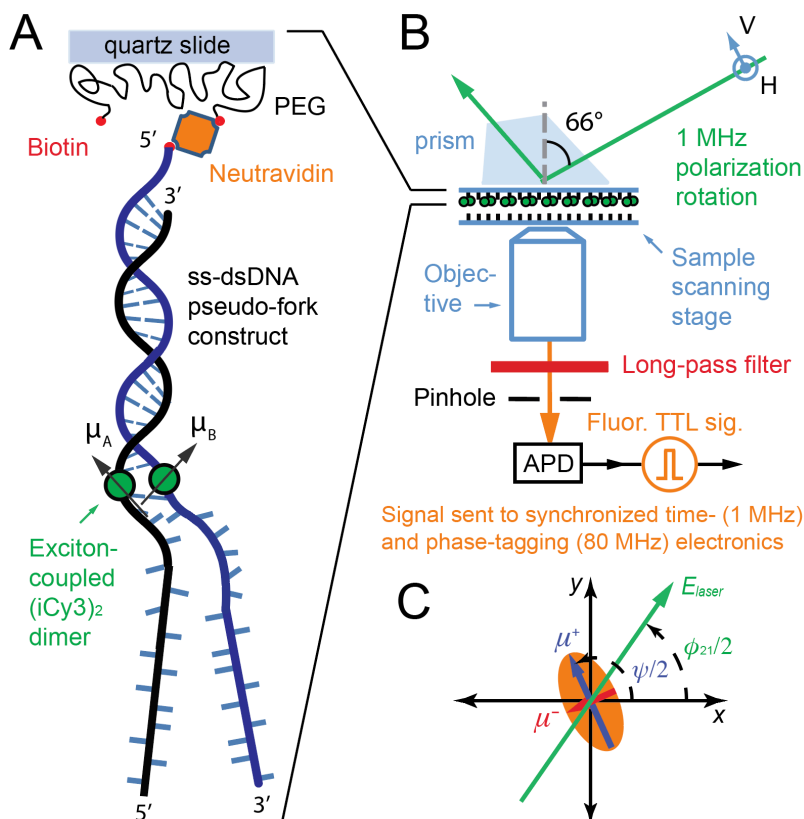


Figure 7. Single molecule polarization sweep microscopy experimental setup **(A)** The labeling scheme and attachment chemistry used to immobilize DNA molecules to the surface of microscope slides. **(B)** The optical setup for the single molecule polarization spectroscopy and its fluorescence collection geometry. **(C)** The polarized exciton components of the Cy3 dimer (μ_+ and μ_-) in the frame of the rotating, linearly polarized 532nm laser

PS-SMF microscopy is an adaptation of previously published Förster resonance energy transfer (FRET) experiments completed by Phelps et al (9). A 532 nm beam is passed through a half wave plate to rotate the polarization state, and through a polarizing beam splitter, which forms the entry port of a Mach-Zender interferometer. The relative phase of the H and V polarized beams is swept at 1 MHz using acoustic optical modulators in each arm of the interferometer. The arms are then recombined and directed into a Brewster's prism at the optimal angle for total internal reflection. Fluorescence from the (iCy3)₂ dimer is collected through a high numerical aperture objective and subsequently spectrally and spatially filtered through a long pass filter and pinhole. Two fundamental modifications in the experiment have been adopted since Phelps et al: 1) the use of a quarter wave plate in the path of the combined output

of the interferometer to prepare a purely linear polarization state rotating at 1 MHz and 2) the implementation of a custom-built field programmable gate array (FPGA) to bin and tag individual photons with the arrival times and phase of the interferometer. The details of this phase-tagging procedure are available in Tamimi et al (10). These experimental methods allow for the measurement of the ‘visibility’ of the polarized excitons of the (iCy3)₂ dimer chromophore probe, which can be conceived as the difference in absorption along the polarized symmetric and antisymmetric components of the dimer, resulting in an ‘ellipticity’ in the frame of the rotating linear polarization of the 532 nm laser (Figure 7C). The probability for differential absorption during a particular phase in the polarization cycle of the 1 MHz rotating field is related to the relative strength of the symmetric and anti-symmetric exciton components for a given (iCy3)₂ dimer conformation (see Figure 8 E).

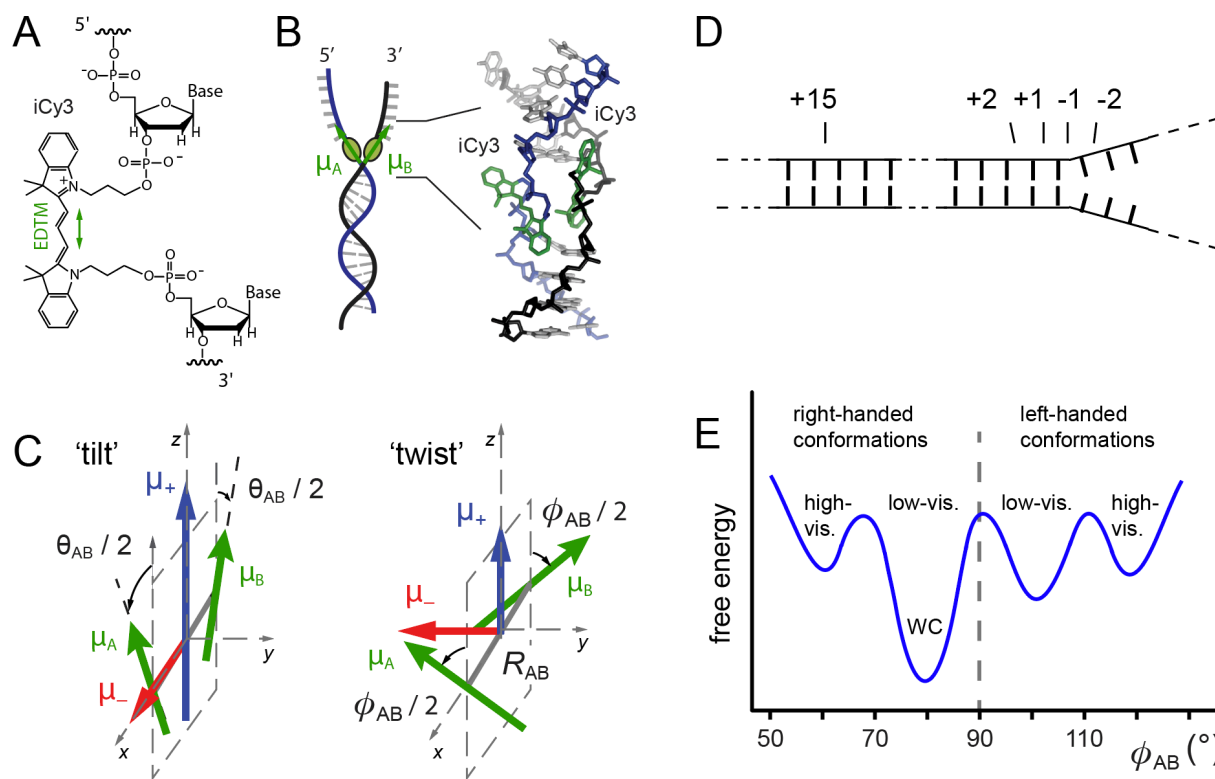


Figure 8. Panels **A**, **B**, **C**, **D** depict the previously discussed insertion chemistry of the Cy3 dyes in DNA, coordinate systems describing the relative orientation of the dyes, and labeling scheme. Panel **(E)** proposes a physical interpretation of the visibility of the single molecule polarization sweep measurement as a function of the crossing angle formed between chromophores.

Polarization of (iCy3)₂ Dimer Conformation as Measured by the Signal Visibility

The visibility of the fluorescence can be explained theoretically by considering the expected intensity of the polarized exciton components, $I_+ = |E_{laser} \cdot \mu_+|^2$ and $I_- = |E_{laser} \cdot \mu_-|^2$. The time dependence of the signal visibility can be expressed as normalized difference in intensity between the two polarized exciton components, $v(t) = \frac{I_+(t) - I_-(t)}{I_+(t) + I_-(t)}$. The amplitude of this visibility, $2fv$, where $f = \frac{1}{2}(I_+ + I_-)$ is the mean flux, which is constructed experimentally from analysis of the probability distribution function of the time- and phase-tagged fluorescent photons at a specified temporal resolution. For the results outlined below, we correlated the fluctuations in the amplitude of the measured visibility at a resolution of 250 μ s. To calculate the experimental two-point time correlation functions (TCFs) in Figure 10, we use the formula:

$$C^2 = \langle A(0)A(\tau) \rangle$$

where the angle brackets denote a rolling time-average that has been performed over all possible starting times.

To estimate the anticipated visibility of our PS-SMF signal for a given (iCy3)₂-DNA conformation, we can estimate the spectral overlap of the symmetric and anti-symmetric components of the (iCy3)₂ dimer excitons with the 532 nm narrow band laser. An estimation of the visibility can be done by calculating the product of the homogenously broadened transitions of the dimer eigen-spectrum with the narrow band Gaussian laser.

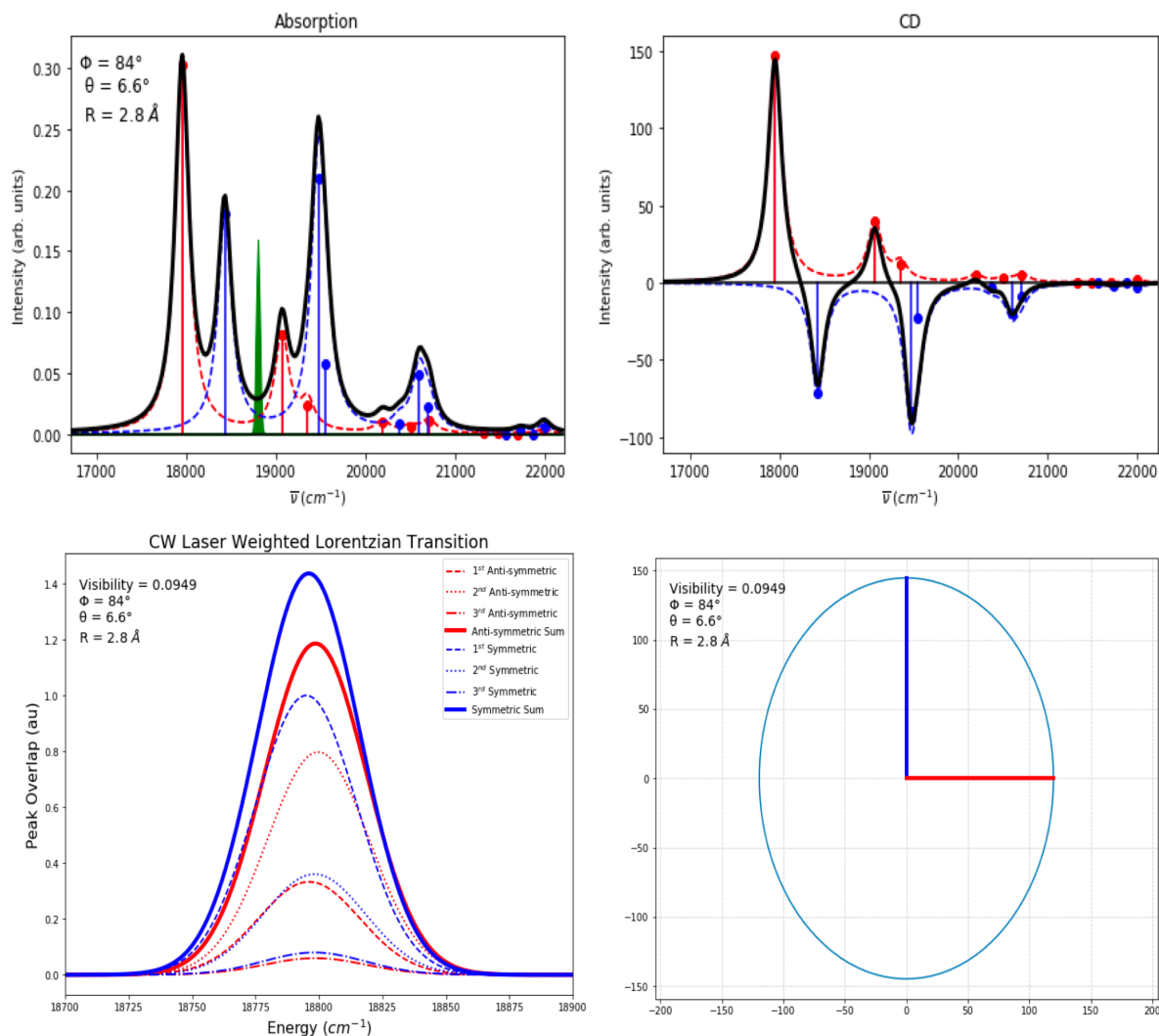


Figure 9. Calculated estimations of the visibility of the +2 labeled dimer using the conformation and spectral calculation routine outlined in Chapter III. *(top)* The homogeneously broadened absorbance and circular dichroism spectra overlaid with the gaussian 532 nm laser spectrum. *(bottom, left)* The sum of the product of the resonant anti-symmetric (solid, red) and symmetric transitions. *(bottom, right)* The predicted ‘elliptical’ character of the $(\text{Cy3})_2$ exciton for the calculated visibility.

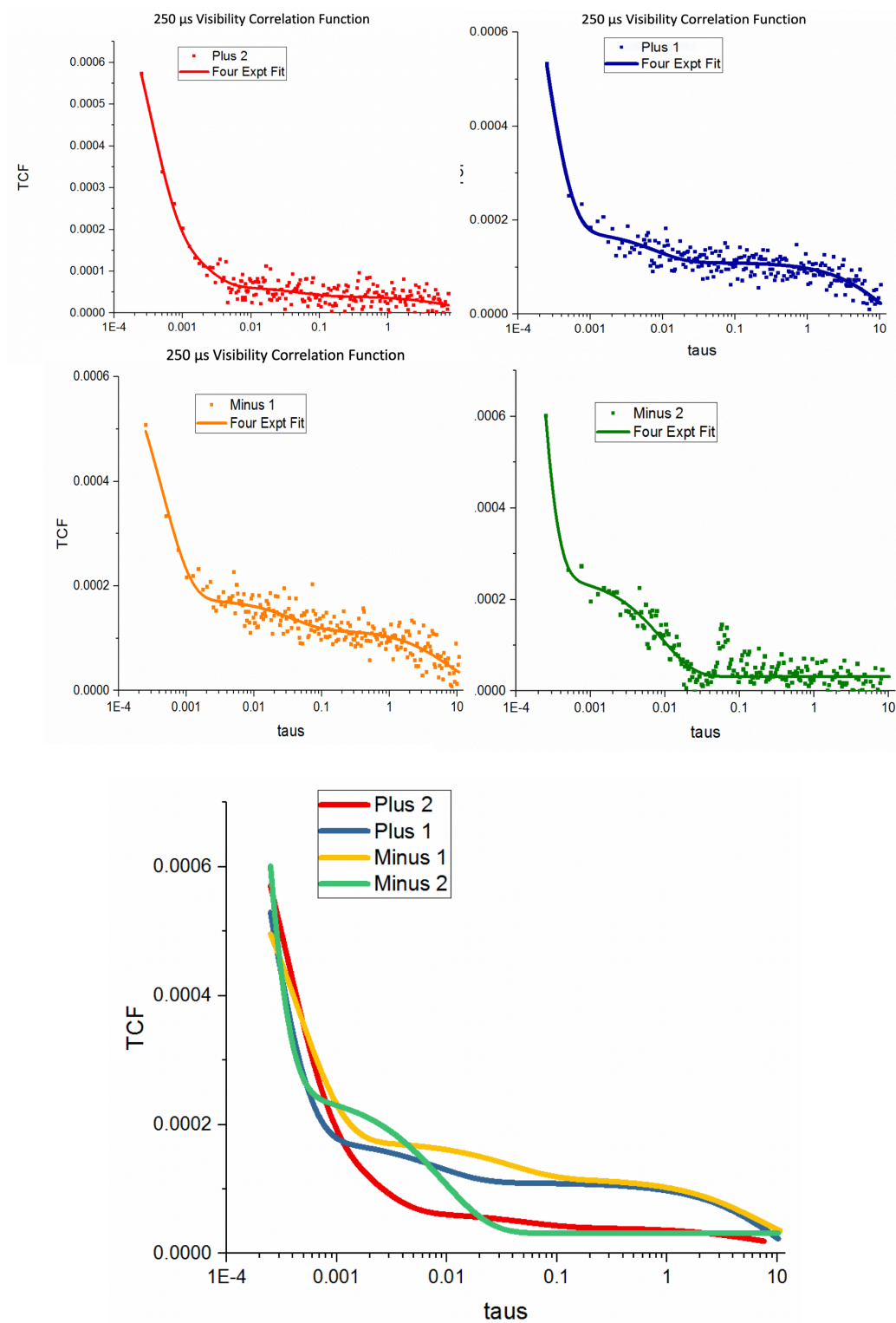


Figure 10. Two-point correlation functions describing the fluctuations of the amplitude of the visibility as a function of the waiting time, τ (in seconds) for ds-ss DNA junction labeled fork constructs. Provided fits were completed with an unbounded four component exponential decay function for illustrative purposes.

Discussion of Single Molecule Polarization Sweep Microscopy Results

The different labeled (iCy3)-DNA positions at and near the ss-ds DNA fork junction show markedly different time-correlated fluctuations (TCFs) in the amplitude of the visibility. The correlated fluctuations occur due to the presence of thermally driven fluctuations at room temperature that push the local DNA structures into different conformational states. In Chapter II, we methodically characterized the average conformation and the degree of conformational disorder at the ss-dsDNA junction for these same constructs. We found that the degree of structural disorder increases at the +1 position and remains relatively high for those positions labeled within the single-stranded DNA region. The results of our PS-SMF experiments reinforce our assignments of different degrees of conformational disorder at each location and reveal unique information about the structural basis of the measured disorder. Each of the TCFs shown in Figure 9 can be accurately fit using a three-exponential decay function, from which we infer an interconnected four-state system. An additional decay component was required to fit the experimental noise at long decay times, with the number of identifiable structural components being equal to the number of required exponential decays plus one.

While a three-exponential decay function fits our experimentally-determined TCFs well, the ‘right’ handed +2 position appears to be dominated by a single exponential decay component. This result indicates that conformational fluctuations at this position are characterized by fewer available structures at room temperature, and in tandem with our spectroscopic structural characterizations, we interpret this as evidence of a dominant ‘right-handed’ B-form conformation that has relatively higher barriers to structural exploration when compared to the other labeled sites. At the +1 and -1 labeled positions, we observe the emergence of correlated fluctuations in the visibility at 10 ms delay times, which are not present in the +2 data, and which indicate an emergence of conformational diversity through a lowering of the barriers to adopting other conformations at the junction (see Fig 10). The distribution function of visibilities evaluated at 10 ms resolution (shown in Figure S1) can be decomposed to predict the thermodynamic stability of conformational states of differing visibility, as seen in Figure 8E, per the methodology outlined in Israels et al. (4).

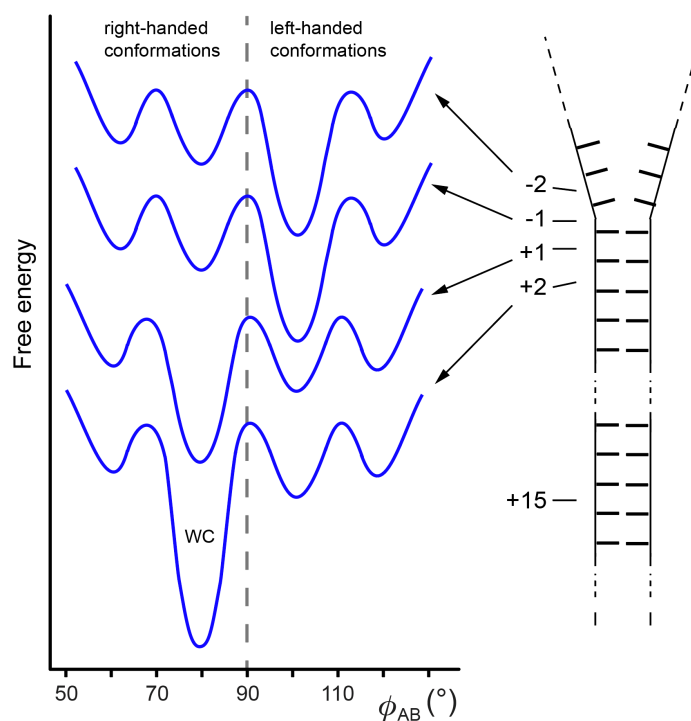


Figure 11. A proposed free energy landscape (FEL) of ss-dsDNA fork constructs labeled using (iCy3)₂ dimer probes at the designated positions relative to the ds-ss DNA fork junction. The hypothetical FELs are plotted as a function of the crossing angle formed between the iCy3 chromophores and are vertically displaced for clarity.

Conclusions

A comprehensive evaluation of the results presented in this chapter leads us to believe that the free energy landscape (FEL) near ss-dsDNA junctions can be altered by both the position relative to a ss-ds DNA junction, as outlined in previous chapters and recovered by our single molecule polarization sweep analysis, and by changes to the buffer salt conditions and the local base composition. By examining the local conformation and conformational disorder at positions relative to the ds-ss DNA junction, and with different base composition and varying ssDNA lengths, we have found examples of optical measurements that cannot be explained by invoking a structural model based on a single conformational species. Information about the average conformations of these constructs is blurred by the degree of conformational disorder. These constructs are better suited for study using single molecule fluorescence methods or 2DFS techniques, which are sensitive to conformational disorder. As the use of iso-stabilizing salts can

modify the average structure determined through bulk spectroscopy, the rates of fluctuations between distinct conformational states under these conditions may be determined using single-molecule methods.

The DNA fork constructs and conditions analyzed here illustrate the complexity of ss-ds DNA junctions under a variety of base-sequence composition and solvent-dependent conditions. DNA fork junctions can visit multiple conformational states, and experience observable, correlated fluctuations between states, the rates of which likely act to govern the kinetics of DNA-protein interactions at these ss-dsDNA junctions. These rates of interconversion are accessible through single molecule methods, which likely depend on base-sequence, solvent conditions, and the presence of replication proteins that can bind at these biologically relevant sites.

Bridge to Chapter VI:

As is alluded to in the previous chapter, there are likely thermally-induced fluctuations at and near ss-dsDNA fork junction between unique conformations that serve to regulate protein-DNA interactions and complex formation. Studies aimed at analyzing the assembly processes of these DNA-protein complexes using protein components of the T4 bacteriophage replisome are discussed in detail in Chapter VI.

Chapter VI

T4 Bacteriophage Replisome Protein-DNA Interactions at ss-ds DNA Junctions Studied Using (iCy3)₂ Dimer-Labeled DNA Fork Constructs

The writing, experiments and calculations found in this section were completed by Dylan Heussman, with some experiments done by Tom Steinberg and Lulu Enhkbataar under my direction. Patrick Herbert helped with the experimental design and preparation of the manuscript. Peter von Hippel aided in the experimental design and gave general feedback. Andrew Marcus was the principal investigator of this work.

Introduction

DNA replication, and the related processes of genome expression, require binding, assembly and function of protein complexes at and near single-stranded (ss) – double-stranded (ds) DNA junctions. These central protein-DNA interactions are likely influenced by thermally induced conformational fluctuations of the DNA scaffold across an unknown distribution of functionally relevant states to provide regulatory proteins access to properly conformed DNA binding sites. Understanding the nature of conformational fluctuations and the associated structural disorder at ss-dsDNA junctions is critical for interpreting the molecular mechanisms of these central biological processes, which may activity bound by leveraged by proteins during DNA binding interactions. Here we outline studies of the T4 bacteriophage replication proteins, the single-stranded DNA binding protein (gp32), and helicase loading protein (gp59), which have been evaluated based on their interaction and structural consequences at (iCy3)₂ dimer-labeled ss-dsDNA junctions.

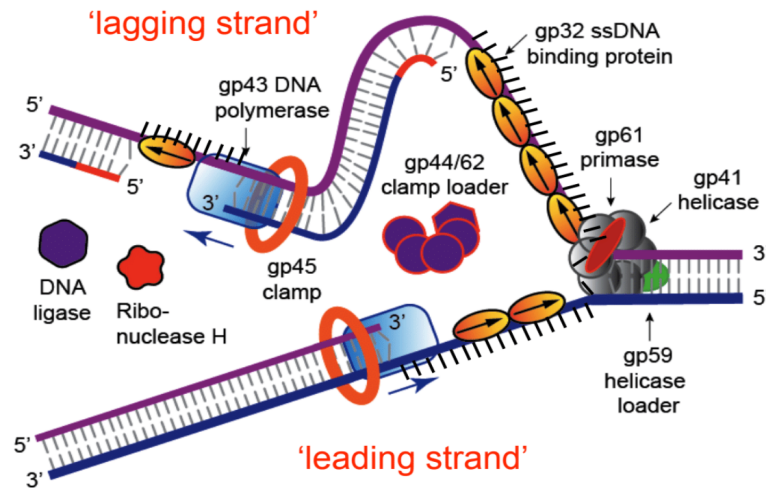


Figure 1. Cartoon depiction of the T4-bacteriophage replisome. *In vitro* reconstitution of a T4 replication system capable of leading- and lagging-strand synthesis on a model DNA replication fork framework requires a minimum of seven proteins: the DNA polymerase (gp43); the helicase (gp41); the ssDNA binding protein (gp32); the clamp loader (gp44/62); the clamp (gp45); and the primase (gp61). The helicase loader (gp59) speeds up the reconstitution of the replication machinery but is not essential once assembled.

The T4 bacteriophage is a virus that infects *E. coli* and uses the host bacteria to replicate and proliferate. The first direct evidence of the protein components that carry out T4 replication was detected by the Alberts group, who found small DNA fragments capable of carrying out the replication of plasmids *in vitro* (1,2). Over the years, the prerequisite protein elements required for the replication of the T4 bacteriophage have been elucidated and have been named according to their gene-products (hence the ‘gp’ identification convention) that result from the transcription of and translation of their genetic content. A cartoon illustration of the full replisome complex is presented in Figure 1. The replisome requires a minimum of seven protein components to form the fully functional complex (3). The two proteins investigated here, gp32 and gp59, are thought to be multifaceted in their consequences on the processivity and accuracy of replication. Understanding the functional mechanisms of the T4 bacteriophage proteins has historically been an excellent route to illuminating the roles played by the replication proteins of higher order organisms, which have evolved more complex levels of regulation involving additional protein-protein interactions.

The gp32 single-stranded DNA binding protein is thought to cooperatively assemble to form nucleoprotein filaments to protect ssDNA regions from nuclease activity and prevent the formation of unproductive ssDNA secondary structures that may interfere with proper replisome function (4). The mechanism for binding of gp32 to ssDNA involves a conformational change of the gp32 C-terminal domain that exposes a positively charged sequence of amino acid residues. These residues interact with the negatively charged ssDNA sugar-phosphate backbone (5). Given that the gp32 protein binding site size is seven nucleotide residues, and monomeric gp32 binds to ssDNA relatively weakly, cooperative assembly of at least two gp32 proteins is necessary for stable gp32-ssDNA binding (6). To assemble in a cooperative manner, the N-terminal domain of each gp32 protein must bind to the core domain of an adjacent gp32 protein. Previously, our group has utilized single-molecule FRET microscopy to study the interplay between conformational dynamics of the ssDNA region and gp32 assembly (7,8). As the binding of gp32 occurs up to and near the ds-ss DNA junction, cooperative interactions between gp32 and other protein complexes that assemble near the junction may play an important role. The gp32 proteins may also play a role in structural selection and stabilization of DNA structures at the junction, as have been outlined in this dissertation.

The gp59 helicase loading protein is a small protein (26 kDa) thought to be nearly indispensable for promoting the assembly of the gp41 helicase onto ssDNA molecules that are covered with saturating amounts of gp32 (9, 12). While not critical for replication to occur *in vitro*, gp59 acts to speed up the process significantly. gp41 and gp59 form a 1:1 hexameric complex with the lagging strand of the DNA replication fork, which eventually passes through the center of the ring-shaped helicase and accelerates the proper loading of helicase (3). Some experiments have indicated that gp59 facilitates the loading of gp41 onto gp32 by destabilizing the interaction between gp32 and ssDNA (10). Studies of gp32 carried out by the Nossal group indicate that gp59 preferentially binds to the ss-ds DNA fork junction with preference over ssDNA and dsDNA regions (12). Direct spectroscopic analysis of the (iCy3₂) dimer-labeled DNA fork junction may help to reveal the structural consequences for gp59 assembly at the junction, and local implications for gp32-gp59 interactions.

Experimental Protocols

Gel Electrophoresis

DNA gel shift analyses were carried out in Novex 6% DNA retardation gels ran at 100V for approximately 1 hr. Gel buffer solutions were made with 0.5x TBE and 6mM MgOAc. Gels were loaded with 8 μ L of protein:DNA solution in 100mM NaCl 6 mM MgCl₂ and 10mM TRIS with 2 μ L Native Gel loading buffer (40% sucrose 0.2% BPB).

Protein Titrations

CD and absorbance measurements were done with a JASCO model J-720 CD spectrophotometer. Temperature was not altered during our measurements from room temperature. For all absorbance and CD measurements, the samples were housed in a 1x 0.5 cm quartz cuvette. Proteins solutions were added to DNA containing solutions and allowed to incubate for 5 minutes prior to data collection. DNA construct sequences for the labeled junctions are listed in Table 1.3, unless otherwise specified.

Discussion of Results

Studies of gp32 Assembly on (iCy3)₂ dimer-labeled DNA Fork Construct

To investigate the structural changes of the DNA fork junction that accompany the loading of single-stranded DNA binding protein to ssDNA, we examined the effects of protein titrations on the absorbance and CD spectra of (iCy3)₂ dimer-labeled ss-dsDNA fork constructs. These studies aimed to determine: 1) whether (iCy3)₂ dimer-labeled DNA fork constructs can be used to characterize any structural changes associated with the loading/unloading state, and 2) which, if any, conformations are actively stabilized by specific protein binding events.

To rigorously establish whether the presence of (iCy3)₂ dimer probes at the ss-dsDNA junction are disruptive to the overall binding of single-stranded DNA binding protein gp32, we

carried out preliminary gel retardation assays to resolve protein-DNA complex formation at different stoichiometric ratios of protein to DNA. The band locations were resolved using the fluorescence from the (iCy3)₂ dimer chromophores as an indicator of the location of the labeled DNA in the gel. In Figure 2E, we see clear evidence that gp32 binds to the (iCy3)₂ dimer-labeled ds-ss DNA fork construct. In gel retardation assays, the DNA or DNA protein complex begins at the top of the well and migrates downward due to the differential voltage, which attracts negatively charged DNA and pulls it downward through the gel matrix. In Lane 5, which is the condition without gp32 present, there is a clear band visible in the middle of the gel indicating the presence of dsDNA. Lane 5 also contains two faintly visible bands at the bottom of the gel indicating the presence of some non-hybridized ssDNA, which travels farther due to its lower molecular weight. In Lane 4, which contains a stoichiometric ratio of 5:1 gp32:DNA, there is a clear shift in the position of the dsDNA band to positions higher up in the gel due to the increased molecular weight of the gp32:DNA complex. Some of the ssDNA remains unbound, indicating a small preference for our ds-ss DNA constructs. In Lane 2, a stoichiometric ratio of 20:1 gp32:DNA is present, in which case all of the detectable DNA in solution is bound with gp32. The spikes in the digital readout of Lane 2 indicate there is some variability in the number of gp32 proteins loaded onto the (iCy3)₂ dimer-labeled DNA fork construct, as the ssDNA regions of these ds-ss DNA constructs are long enough to accommodate 10 bound gp32 proteins per DNA-gp32 complex.

Figure 2. (next page) The CD-detected binding of gp32 to various (iCy3)₂ dimer-labeled DNA fork constructs in which the probe positions are varied across the ds-ss DNA junction. DNA titrated with increasing amounts of gp32 for ds-ss DNA containing (iCy3)₂ at the (**A**) +2 position, (**B**) +1 position, (**C**) -1 position, and (**D**) -2 position. The sequences for these constructs are listed in Table 2.1. (**E**) Gel retardation assay of gp32 binding -1 DNA with digital readout of lanes 2,4, and 5 showing the integrated fluorescence intensity at each vertical pixel location. Conditions for each gel lane can be found in Table 1.

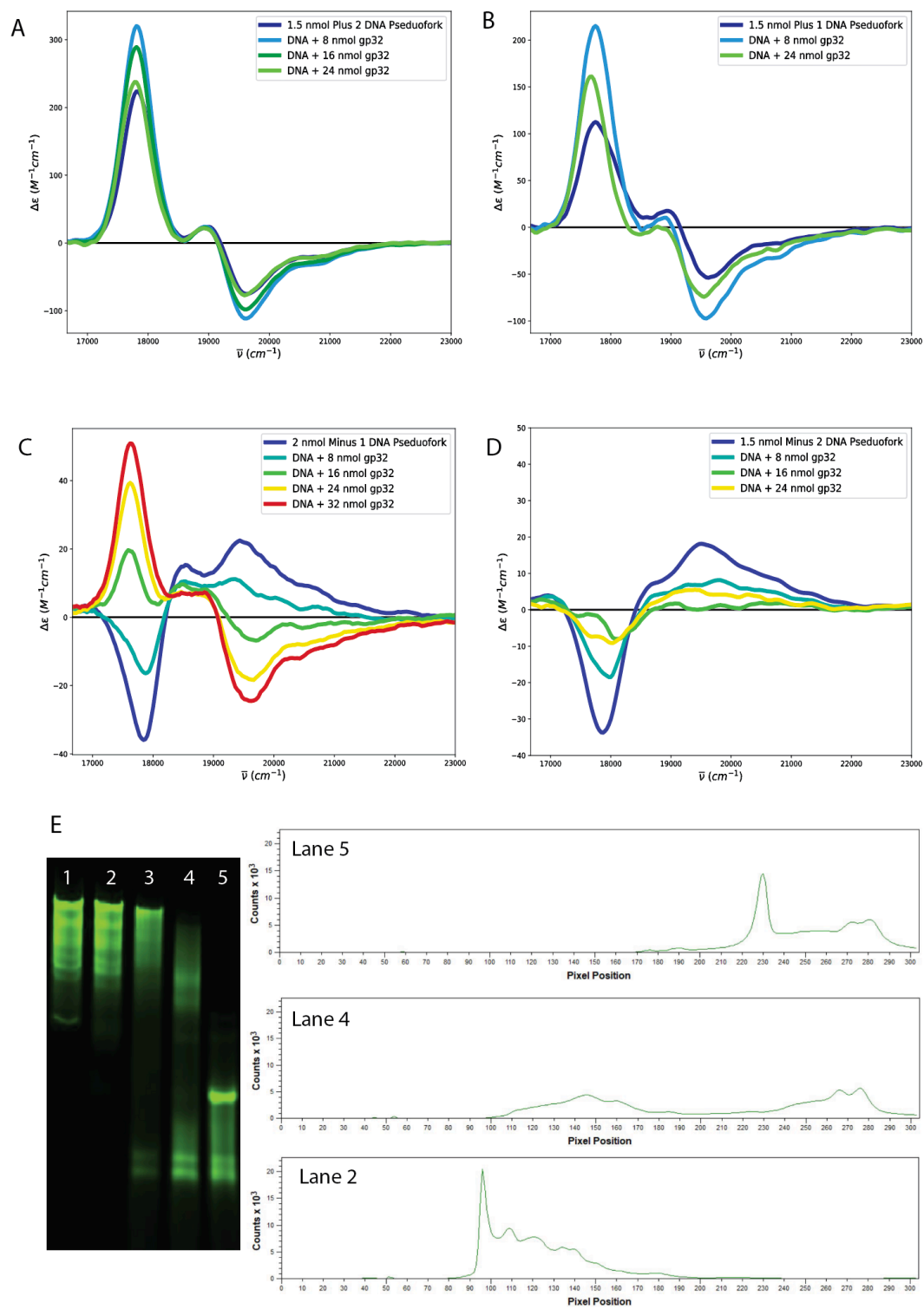


Table 1. “-1” (iCy3)- DNA, gp32 Retardation Gel Well Identification

Lane	Condition
5	1 μ M -1 (iCy3) ₂ -DNA
4	1 μ M -1 (iCy3) ₂ -DNA, 5 μ M gp32
3	1 μ M -1 (iCy3) ₂ -DNA, 10 μ M gp32
2	1 μ M -1 (iCy3) ₂ -DNA, 20 μ M gp32
1	1 μ M -1 (iCy3) ₂ -DNA, 40 μ M gp32

After establishing the viability of labeled (iCy3)₂ DNA junctions as protein binding targets for the single-stranded DNA binding protein gp32, we next investigated the local secondary structure of the DNA fork constructs at and near the ss-dsDNA junction as a function of protein concentration using optical methods. For this study, we determined whether there is any differential effect of protein binding at different probe positions relative to the ds-ss DNA junction. Interestingly, all the (iCy3)₂ dimer-labeled DNA fork constructs exhibited sensitivity to the presence of gp32. However, the effect varied depending on the labelling position. While the ‘duplex-like’ labeling positions (+2 and +1) maintained their right-handed character based on their CD spectra, they additionally exhibited small shifts in the CD peak positions and widths (Fig 2A and 2B). At higher concentrations of gp32, these titrations reveal a two-step loading sequence, as the CD signal increases dramatically and then subsequently decreases, which is consistent with the preferential loading of one ssDNA region before the other. For the (iCy3)₂ dimer-labeled DNA fork construct labeled at the -1 position, there is an inversion of the handedness of the CD signal, which is consistent with the spectra measured for the ‘duplex-like’ positions under saturating conditions of gp32. This result suggests gp32 has a direct role in stabilizing a right-handed, duplex-like average conformation directly at the ss-dsDNA fork junction. Finally, for the (iCy3)₂ dimer-labeled DNA fork construct labeled at the -2 position, the effect of gp32 binding can be characterized as a weakening of the electrostatic coupling of the (iCy3)₂ dimer chromophores, as the magnitude of the CD signal decreases at saturating concentrations of gp32. Since the (iCy3)₂ dimer probe occupies the -2 position, which is extended furthest into the ssDNA region of all the ss-dsDNA fork constructs, the loading of gp32 at the ss-ds DNA junction likely separates the iCy3 chromophores enough to disrupt their electrostatic interaction.

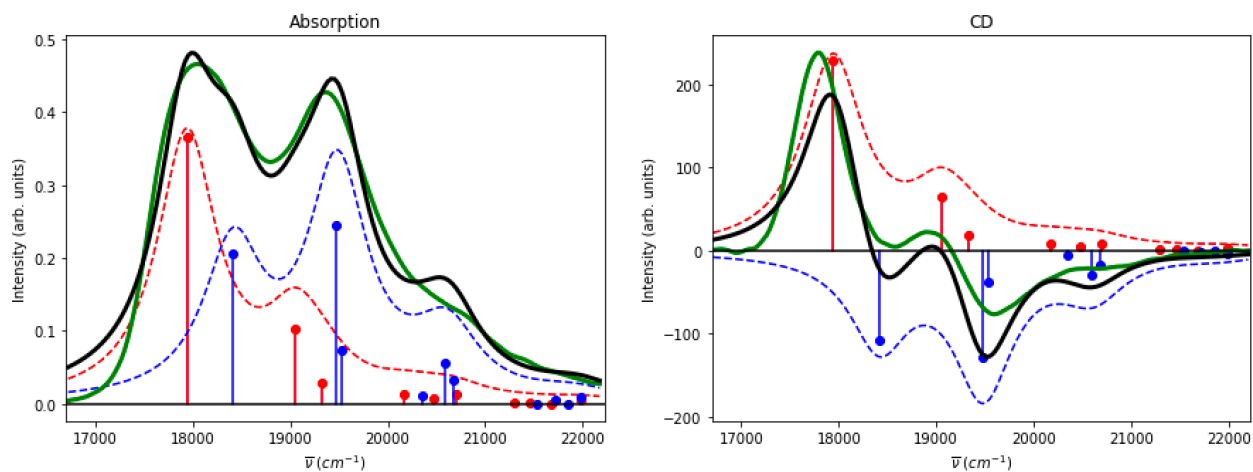


Figure 3. Experimental absorbance and circular dichroism spectra (green) and results of the optimized fitting routine (black) of gp32 protein of the +2 (1.5 nmol) (iCy3)₂ dimer-labeled ss-dsDNA fork construct saturated with gp32 protein (24 nmol). To carry out the optimizations of protein:DNA complex related spectra, we increased the weight of residuals for the CD signal in our χ^2 minimization procedure by 100-fold as compared to previous studies. The heterogeneity of these samples precluded us from simultaneously optimizing to both CD and absorbance spectra for all (iCy3)₂ dimer-labeled ss-dsDNA fork constructs under protein:DNA conditions.

To assign conformations to the protein-DNA complexes that we studied, we analyzed the CD and absorbance spectra following the procedures outlined in Chapters I and III. The results of these analyses are summarized in Figure 4. This analysis implemented an atomistic transition charge (TQ) model in which the relative roll angle (η_{AB}) was varied symmetrically. While the mean local conformation of the (iCy3)₂ dimer-labeled ss-dsDNA constructs change in the relative twist angle from $\phi_{AB} = 53.6^\circ$ to 98.3° (i.e., from right-handed to left-handed) as the dimer probe position is varied from the +1 to -1 position, the presence of gp32 bound to the DNA fork construct appears to maintain the right-handed B-form character at both positions such that there is only a small change in ϕ_{AB} from 66.8° to 55.5° . In the absence of gp32, the tilt angle at the +1 position is $\theta_{AB} = 74^\circ$, which drops off rapidly to $\theta_{AB} = 6.2^\circ$ at the -1. However, in the presence of gp32, a relatively large value of the tilt angle is maintained ($\theta_{AB} \approx 50^\circ$) as the dimer probe position is varied across the ss-dsDNA junction. While the degree of structural disorder is comparable between the bound and unbound conditions for most positions, the disorder does slightly decrease under gp32 saturation at the +1 position from $\sigma = 301$ to 280 cm^{-1} . Finally, the sign of the electrostatic coupling J does not change from positive to negative, as it does in the absence of protein, and actually increases under saturating conditions of gp32, as the value of J

ranges from 453 to 761 cm^{-1} as the (iCy3)₂ dimer probe position is varied across the ss-dsDNA junction in the presence of gp32, which is markedly higher than is observed for any other values of J reported in this dissertation.

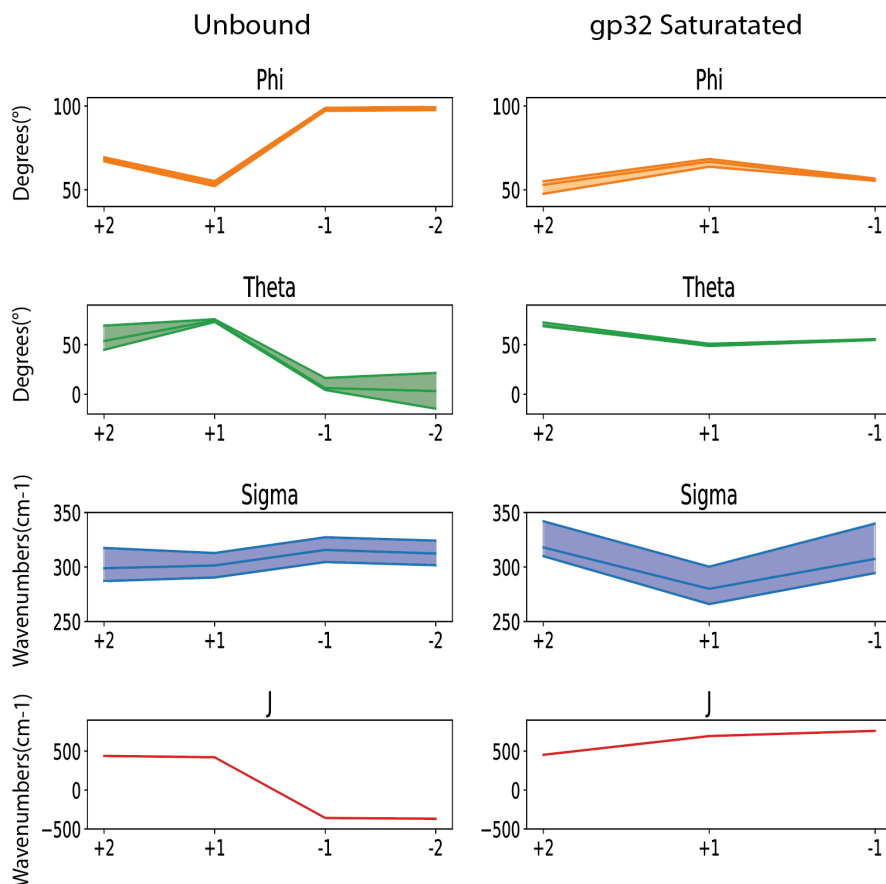


Figure 4. Changes to the mean local conformation of (iCy3)₂ dimer-labeled ds-ss DNA constructs under saturating concentrations of the single-stranded DNA binding protein gp32. For +2,+1 labeling sites, saturating conditions are 24 nmol gp32; for -1 labeled sites the saturating gp32 condition was found to be 32 nmol gp32.

Multistate Modeling of gp32 Assembly at (iCy3)₂ Dimer-Labeled ss-dsDNA Junctions

An important issue that has become apparent during the course of our analysis is the inability of our computational models to reliably simulate the local conformations of (iCy3)₂ dimer-labeled ss-dsDNA constructs at various concentrations of gp32. A likely source of this problem is the oversimplified interpretation of the (iCy3)₂ dimer-labeled ss-dsDNA conformation in terms of a single average structure with some structural disorder. This assumption leads to – in

some cases – an inability to simultaneously simulate CD and absorbance measurements using the same structural model. In such instances, there is likely a mixture of conformational species present in our samples, such that our measurements represent the weighted average CD spectra resulting from multiple different stable conformations, which depend on the fraction of the DNA that is bound by gp32. While the saturation of the -1 (iCy3)₂ dimer-labeled ss-dsDNA fork construct with gp32 results in a shift in the average chirality of the CD signal, this CD amplitude is significantly smaller than that found for ‘duplex’ (+2) labeled DNA. This observation suggests that the ss-dsDNA junction can adopt several energetically stable conformations, and that the free energy of the ‘right-handed’ (iCy3)₂ conformation in duplex DNA is much lower than that of the average ‘right-handed’ conformation found for (iCy3)₂ dimer-labeled ss-dsDNA labeled at the -1 position under saturating conditions of gp32. Preliminary polarization-sweep single molecule fluorescence (PS-SMF) experiments indicate that the correlated rates of local fluctuations at and near the ss-dsDNA junction, as probed by (iCy3)₂, are not significantly altered by the presence of gp32, and are currently in preparation.

In Figure 5, we illustrate the results of a different approach to analyzing (iCy3)₂ dimer-labeled ss-dsDNA:gp32 titrations, in which we fit the gp32 titrated CD spectra to linear combinations of two basis functions represented by the saturated (+32 nmol gp32) and unbound CD traces. From this analysis we can calculate the normalized fraction of bound sites and plot this against the relative concentration of gp32 in solution ([gp32]/[DNA]). The clearly visible isosbestic point in our data indicates the probable existence of multiple conformers in solution. It is interesting to note that the binding curve shows evidence of the known cooperative binding of the DNA, as demonstrated by the sigmoidal shape of the binding curve. It is also curious to note that the 50%-bound relative gp32 concentration falls off around 1:10 ratio of gp32, which is the number of total binding sites available on the ssDNA regions of the -1 (iCy3)₂ dimer-labeled ds-ssDNA fork construct.

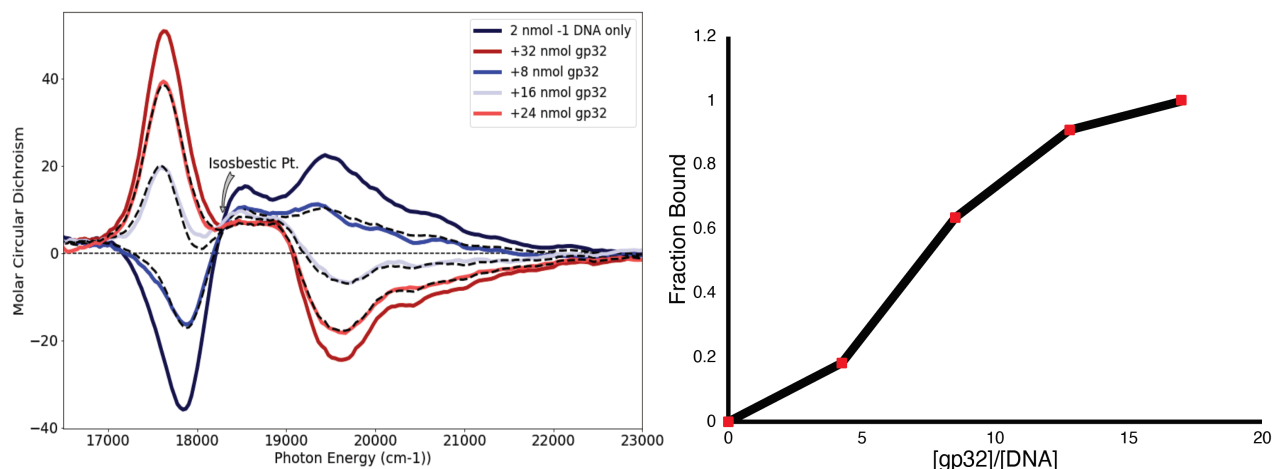


Figure 5. Fitting of the -1 mixed DNA fork junction protein titration to linear combinations of the unbound and bound spectra (*left*). The dotted lines indicate the reconstructed curves based on linear combinations of the 2 nmol -1 DNA only spectra and +32 nmol gp32 spectra. Plot depicting the fraction bound DNA vs. the $[gp32]/[DNA]$ (*right*). The fraction bound is retrieved from an optimized fit of each circular dichroism spectra linear combination of the 2 nmol -1 DNA only spectra and +32 nmol gp32 spectra.

Gp32 Removes Sequence-Dependent Structures at the ds-ss Junction

In Chapter IV we looked briefly at the effect that local base-sequence composition has on the conformations of (iCy3)₂ dimer-labeled ss-dsDNA fork constructs at positions at and near the ss-dsDNA junction. We found that changing the composition of neighboring bases at the +1-position resulted in significant variation of the CD spectra, which indicates that the conformational parameters that we assigned in Chapters I-III for mixed-base sequences must be re-evaluated in the context of specific-base sequences. While we were able to reliably simulate absorbance and CD spectra for the +1 mixed-base sequence, and found it adopted a ‘right-handed’ conformation with a slightly larger tilt angle than other labeled sites, we were not able to achieve equally good simulations for the corresponding GC-rich and AT-rich (iCy3)₂ dimer-labeled ss-dsDNA fork constructs (Table 5.1, Figure 5.3).

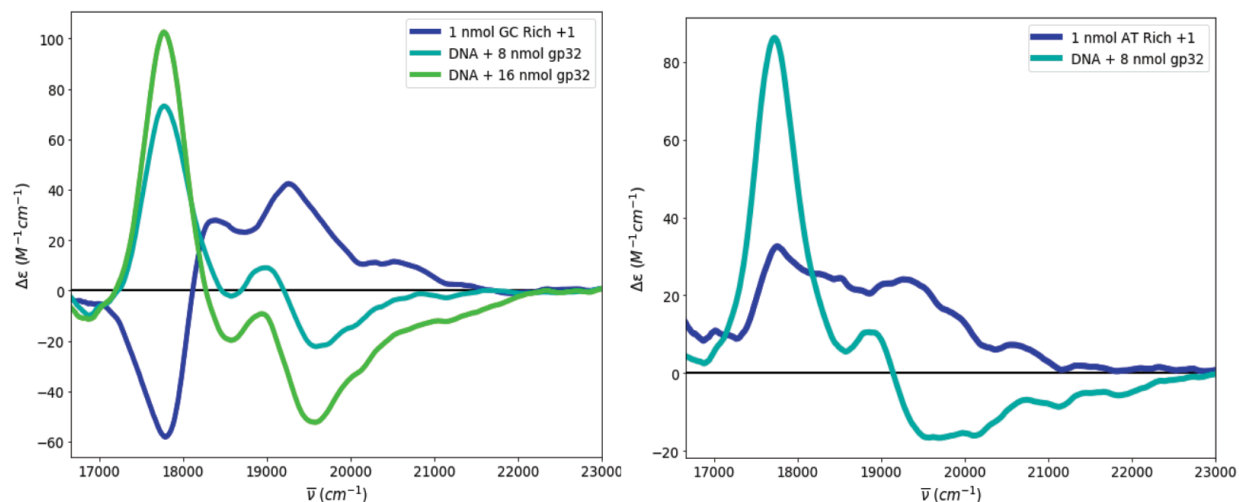


Figure 6. Gp32 homogenizes the probable structure of the ss-dsDNA junction at the +1 position, regardless of local base composition. The circular dichroism spectra found for “+1” labeled (iCy3)₂-DNA with neighboring GC bases under gp32 titration (*left*) and for the “+1” labeled (iCy3)₂-DNA with neighboring AT bases (*right*)

In Figure 6 we see the effects of gp32 titration on the CD spectra of the +1 (iCy3)₂ dimer-labeled ss-dsDNA fork constructs with different local base-sequence composition. The binding of gp32 enforces the adoption of a ‘right-handed’ conformation at the +1 position regardless of the average structure or distribution of structures in the unbound state. This result suggests a regulatory role at the junction for gp32 by homogenizing the dominant local conformation at positions near the ds-ss DNA fork junction. As proteins such as the gp59 helicase loading protein and gp41 helicase must assemble at the DNA replication fork junction, and unwind DNA regardless of DNA sequence, gp32 likely plays a role in establishing a ‘right-handed’ structure that can be easily bound by the helicase complex. The elimination of sequence-dependent local structure by stabilizing particular secondary structural motifs might help to regulate the rates at which these proteins can load and unwind dsDNA, and ensure that the proper loading and processivity rates are not impeded by sequence-dependent secondary structure.

Gp32 Exhibits Destabilizing Effects When Unable to Bind Cooperatively to ssDNA At and Near ss-dsDNA Junctions

In previous sections, we have established that the dominant role of gp32 at the ds-ss DNA junction is to provide a stabilizing effect for ‘right-handed’ structures within the conformational ensemble at the +1 and -1 positions. However, the binding of gp32 to the previously examined (iCy3)₂ dimer-labeled ss-dsDNA constructs was carried out cooperatively, with multiples proteins able to bind to the same ssDNA regions once it had been occupied by a single gp32. This cooperativity is illustrated in Figure 1E, which demonstrates the gp32:DNA complexes predominantly contain clusters of cooperatively-bound gp32 proteins. For DNA constructs containing a relatively long ssDNA region (accommodating 10 gp32 proteins total, 6 gp32 proteins on one ssDNA ‘arm’ of the ss-dsDNA fork construct and 4 gp32 proteins on the other ssDNA arm), the gp32:DNA complex was stabilized by the ability of multiple gp23 proteins to bind the ssDNA region and to form a stable ss-dsDNA-gp32 complex by forming preferential contacts with the N termini of adjacent proteins loaded onto the ssDNA regions of the ss-dsDNA fork constructs.

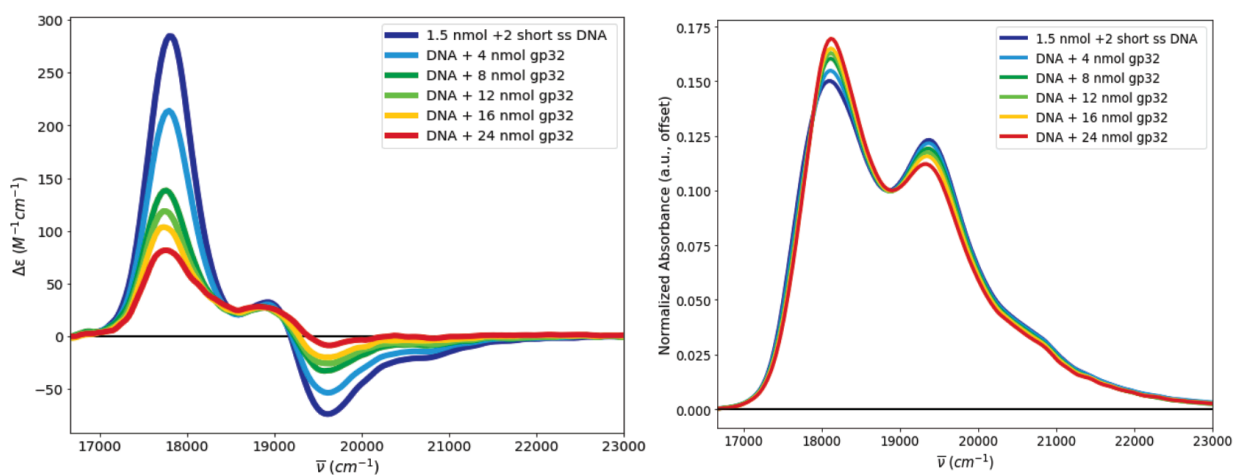


Figure 7. The effects of gp32 binding without cooperativity to the short single-strands of +2 (iCy3)₂ dimer-labeled ss-dsDNA fork constructs. When the ssDNA regions can only accommodate one gp32 binding register, the gp32:DNA complexes appear to disrupt the local structure at the junction.

Figure 7 demonstrates the opposing effects that gp32 has when it is unable to load cooperatively to the ssDNA regions of the ss-dsDNA fork construct. We see in Figure 2A that gp32 can perform its role in marginally stabilizing the ubiquitous ‘right-handed’ structure at the +2 position when it is able to cooperatively bind to form nucleoprotein filaments. However, the results shown in Figure 7 demonstrate that loading of gp32 in a 1:1 ratio with each short ssDNA arm destabilizes the right-handed conformation at the junction and is likely unable to create a stable ss-dsDNA-gp32 complex.

Gp59 Helicase Loading Protein Weakly Binds and Disrupts the Electrostatic Coupling of (iCy3)₂ Dimer-Labeled ss-dsDNA Junctions

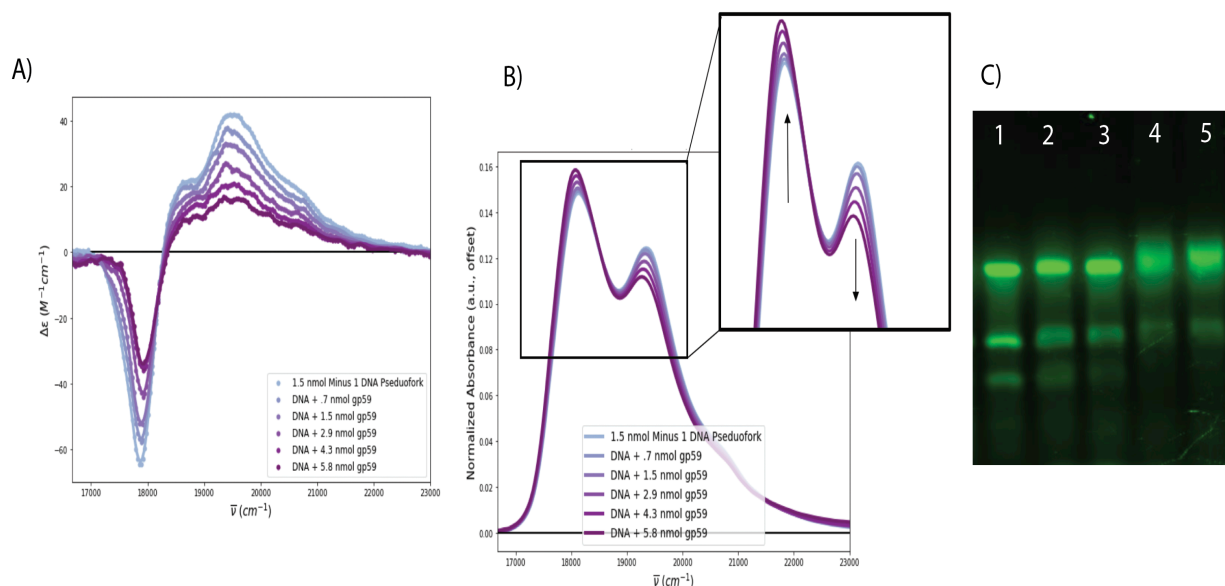


Figure 8. CD (*A*) and absorbance (*B*) measurements for gp59 ss-dsDNA fork titration of the long ssDNA -1 (iCy3)₂ dimer-labeled ss-dsDNA fork construct. Gel shift analysis of this construct under *1*) physiological buffer *2*) 1:1, *3*) 1:2, *4*) 1:4, *5*) 1:5 stoichiometric ratios of gp59.

An additional T4 replisome protein we investigated is the gp59 helicase loading protein. Upon comparing Figure 2E to Figure 8C, it is apparent that gp59 is a much smaller protein than gp32, and that gp59 binds weakly to (iCy3)₂ dimer-labeled ss-dsDNA junctions, as it does not show a prominent gel shift. While gp32 gel shifts display a distinct series of bands that result from the highly cooperative binding affinity of gp32, gp59 exhibits only a subtle gel shift that can be seen in the smearing of the dsDNA band. However, the affinity of the gp59 protein for ds-

ss DNA junctions does not seem to be disrupted by the presence of the (iCy3)₂ dimer probe at the ss-dsDNA fork junction, as similar results are found for gel shift controls performed with the dimer probes inserted at the +15 position within the duplex region of the ss-dsDNA fork construct.

The spectroscopic evidence shown in Figure 8 indicates that the gp59:DNA complex exhibits similar titration behavior to that is seen for non-cooperatively assembled gp32:DNA complexes. However, in this experiment, the ‘left-handed’ structure that dominates the mixed-base -1 (iCy3)₂ dimer-labeled ss-dsDNA fork construct with ‘long’ ssDNA regions is disrupted, as indicated by the reduction in the amplitude of the CD spectrum and the concomitant loss of intensity borrowing in the absorbance spectrum. The gp59:DNA complex is intrinsically unstable. However, it is interesting to note that the presence of gp59 loaded at the fork leads to a discernable reduction of the electrostatic coupling of the (iCy3)₂ dimer probe, either through a physical separation of the dyes in space or through a disruption of the local dimer conformation. The presence of gp59 exhibits a similar effect for -2 (iCy3)₂ dimer-labeled ss-dsDNA. Conversely, the effects of gp59 binding at the +2 and +1 positions are negligible, indicating that the conformational changes induced by gp59 binding to the ds-ss DNA fork junction do not extend deeply into the DNA duplex region. When gp59 is added to the -1 (iCy3)₂ dimer-labeled ss-dsDNA fork construct with long ssDNA regions in the presence of saturating amounts of gp32, there is no observable change to the spectroscopic signals. However, when the same -1 (iCy3)₂ dimer-labeled ss-dsDNA fork construct is first combined with saturating amounts gp59 and then followed by titration with gp32, the right-handed structure is stabilized. Therefore, the order of assembly for ss-dsDNA-gp59-gp32 complexes appears to initially involve a weakly bound ss-dsDNA-gp59 intermediate followed by the cooperative assembly of the full complex (see Figure 9).

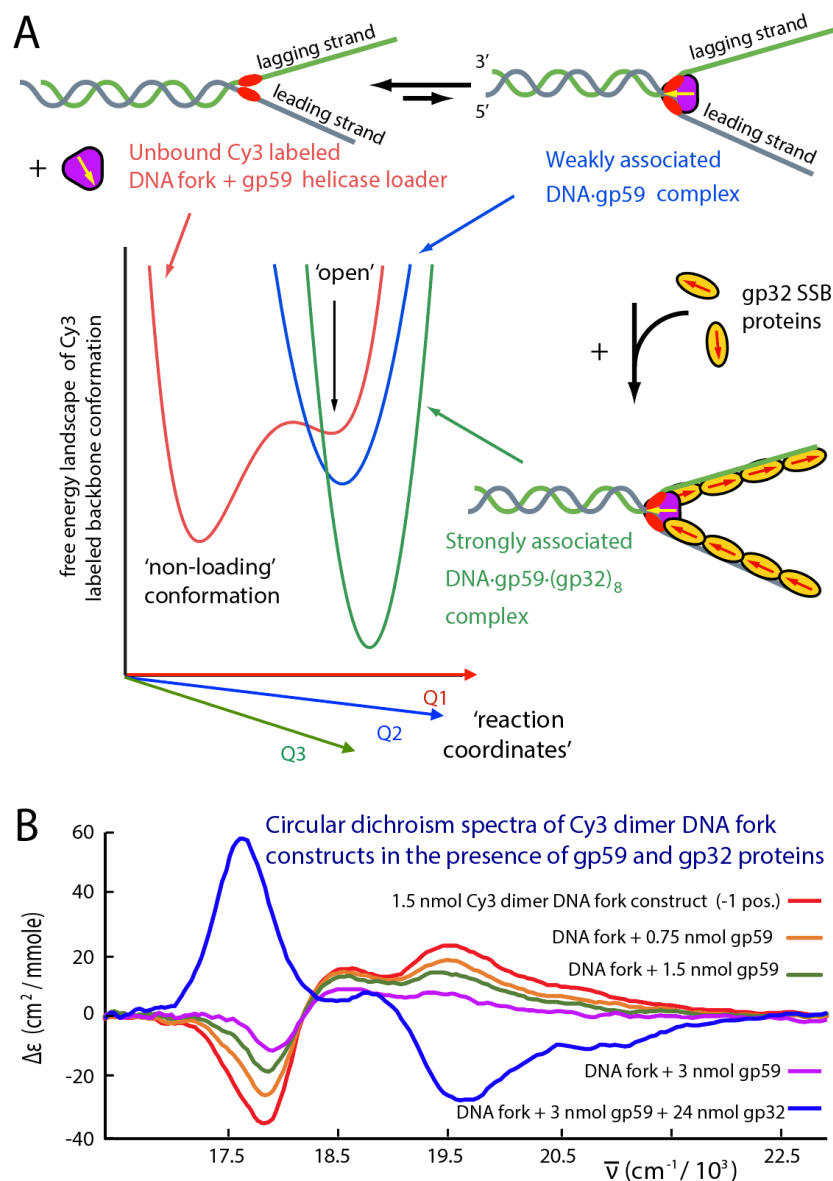


Figure 9. gp59, gp32 titration at the -1 (iCy3)-DNA Junction. **(A)** A proposed free energy landscape describing the loading of gp59 and gp32 onto the fork junk as a function of a generalized reaction coordinate, Q **(B)** Experimental measurement of (iCy3)₂-DNA circular dichroism as a function of protein concentration.

Conclusions

In this Chapter, we have outlined a novel application of our ‘essential-state’ Holstein-Frenkel Hamiltonian model to analyze the conformations and structural disorder associated with

T4 replisome gp32:DNA and gp59:DNA complex formation at the ds-ss DNA fork junction through simulation and modal parameter analyses of CD and absorbance spectra. We have characterized the effects of the assembly of single-stranded DNA binding protein gp32 and the helicase loading protein gp59 at model ds-ss DNA fork junctions that are site-selectively labeled using the exciton coupled (iCy3)₂ dimer located at varying positions relative to the ds-ss DNA junction. We find that the gp32 protein plays a unique role in organizing the local structure at the ss-dsDNA junction due to its ability to form cooperatively-bound nucleoprotein filaments near the ds-ss DNA junction. This process occurs in the presence of cooperatively interacting gp32 proteins that select for a specific ‘right-handed’ conformation at the DNA fork junction, which is stabilized regardless of local base sequence. The interactions between gp32 nucleoprotein filaments and the ss-dsDNA junction likely plays a role in regulating the kinetics of assembly other T4 replication proteins that form stable sub-assemblies at the DNA replication fork junction such as the gp41 helicase and the gp61 primase.

We find that multiple structures of relatively equal stability exist simultaneously during the course of gp32:DNA titrations. These structures can be quantified to assess the cooperative binding of gp32 as a function of concentration through a simple two-state decomposition of the CD spectra. The local DNA conformational selection thus determined for gp32 binding appears to be disrupted when the ssDNA regions are not long enough to accommodate cooperative binding of the gp32 protein. While gp59 binds weakly to our (iCy3)₂ dimer-labeled ss-dsDNA fork constructs, its presence acts to disrupt the electrostatic coupling of the (iCy3)₂ dimer probe, which may have meaningful consequences for local conformational selection when interacting in concert with other T4 replication proteins.

Chapter VII

Concluding Remarks

Chapter II outlines an experimental technique to examine the local conformation of (iCy3)₂ dimer-labeled ss-dsDNA fork constructs by implementing a site-specific labeling strategy. The structures of the sugar-phosphate backbones at these ss-dsDNA junctions and within the duplex regions of ss-dsDNA fork constructs were modeled using a single effective mode Holstein Hamiltonian to calculate and simulate experimental results from CD and absorbance measurements under different estimations of the electrostatic coupling interaction iCy3 chromophores. These studies establish that the local conformations of (iCy3)₂ dimer-labeled ss-dsDNA constructs are sensitive to both the temperature and labeling position of the iCy3 chromophores, as might be expected for DNA in the absence of a non-intrinsic chemical probe. The (iCy3)₂ dimer-labeled DNA constructs with the probe position deep within the duplex region exhibit sensitivity to increasing temperature, with a loss of local order associated with the ‘right-handed’ B-form DNA double-helix. At the ds-ss DNA fork junction a number of local conformations of near equal stability can be adopted. These can be characterized in terms of an average local conformation, which is ‘left-handed’ and splayed out at positions very close to the ss-dsDNA fork junction. Perhaps surprisingly, the local conformations at the DNA fork junction exhibit relatively little structural heterogeneity in comparison to regions deep in the duplex.

The nature of the conformational disorder, and the incremental changes to the average structure at and near the ds-ss DNA junction were outlined in Chapter III. Using 2DFS, a quantitative characterization of the conformational disorder was carried out as a function of temperature and site-labeling position. The ds-ss DNA junction was found to undergo a continuous change in the average structure, from a ‘right-handed’ to ‘left-handed’ average structure as the dimer probe position was varied across the ds-ss DNA junction. The ds-ss DNA junction appears to support a larger range of conformational distributions beginning at locations one base pair removed from the ds-ss DNA junction, which may serve functionally relevant roles in protein assembly and function. Conformational disorder in the duplex region was found to increase as a function of temperature, both with increasing temperature and decreasing temperature consistent with simple thermodynamic models of macromolecular stability. The

results of these studies indicate an increase in conformational fluidity that develops at temperatures far below the melting point transition and likely reflects the nucleation of ‘bubble’-like instabilities in the pre-melting regime.

The local conformations reported for (iCy3)₂ dimer-labeled ss-dsDNA constructs were re-examined in Chapter IV under a more precise depiction of the electronic transition charge density. Adaptation of these methods, alongside improvements to the optimization algorithm, confirmed our structural assignment for (iCy3)₂ dimer-labeled ss-dsDNA constructs at different labeling positions. These studies suggested that the DNA constructs with the dimer probes inserted deep within the duplex region adopt a ‘face-to-face’ stacking arrangement and helped to eliminate the assignment of structures that violated steric overlap constraints. These optimizations helped to provide plausible three-dimensional models of the (iCy3)₂ dimer-labeled ss-dsDNA fork constructs, which can be directly compared to MD simulations.

The generalizability of structural trends at site-labelling positions near the ds-ss DNA junction and within the duplex region were evaluated in Chapter V for DNA sequences of varied nucleobase composition and under iso-stabilizing salt conditions. The dynamics of conformational exchange were assessed at sites near the ss-dsDNA junction using polarization-sweep single-molecule fluorescence (PS-SMF) microscopy. While the proximity of the (iCy3)₂ dimer probe to the DNA junction appears to affect the average structure, the relative stability of conformations can be augmented by changes to the neighboring base sequence and through the addition of iso-stabilizing salts. Evidence for the presence of multiple conformations of near equal stability at the ds-ss DNA junction was confirmed through the direct observation of conformational fluctuations, which depend sensitively on the labeling site position relative to the ds-ss DNA junction.

Finally, the ability of the T4 bacteriophage single-stranded DNA binding proteins to leverage the diverse topology of ds-ss DNA junctions by selecting for local structures at the ds-ss DNA junction was assessed in Chapter VI. The viability of using (iCy3)₂ dimer-labeled ss-dsDNA constructs to study T4 replisome protein-DNA interactions was established by analyzing spectroscopic experiments using the T4 single-stranded DNA binding protein (gp32) and helicase loading protein (gp59). Saturation of the single-stranded DNA region of (iCy3)₂ dimer-labeled ss-dsDNA constructs appears to shift the relative stability of the available conformations

such that an average ‘right-handed’ conformation is stabilized. The binding of gp32 appears to similarly shift the average conformation near the ss-dsDNA fork junction to a stable right-handed structure regardless of the local DNA base-sequence composition. However, when the single-stranded DNA binding proteins are prevented from binding near the DNA fork junction in their cooperative modality, they are unable to stabilize the right-handed local conformation.

The work described in this thesis indicates that (iCy3)₂ dimer-labeled ss-dsDNA constructs can be used as an effective tool for studying the detailed assembly of non-base-sequence specific protein-DNA complexes through the lens of changes to the local DNA structure. The combination of absorbance spectroscopy, CD, 2DFS, and polarization-sweep single-molecule fluorescence (PS-SMF) spectroscopy, and molecular biology techniques permits a comprehensive assessment of local DNA structure and disorder, which may be implemented to study the functional roles of specific DNA conformations in facilitating productive base-sequence specific and nonspecific binding interactions with proteins. It would be informative to interrogate the correlation between simultaneous structural changes to the DNA and interacting proteins through the use multiplexed experiments, or through the simultaneous tracking of global biomolecular motions. While the (iCy3)₂ dimer-labeled DNA sugar-phosphate backbones studied in this work show the presence of fairly ordered chiral conformations, the fluctuations of the nucleobases are likely more strongly correlated to the kinetics governing the assembly of base-sequence specific DNA binding proteins and analogous routes to tracking nucleobase structures through fluorescent base analogs should be pursued.

Appendix A

Supplementary Figures by Chapter

From Chapter 2

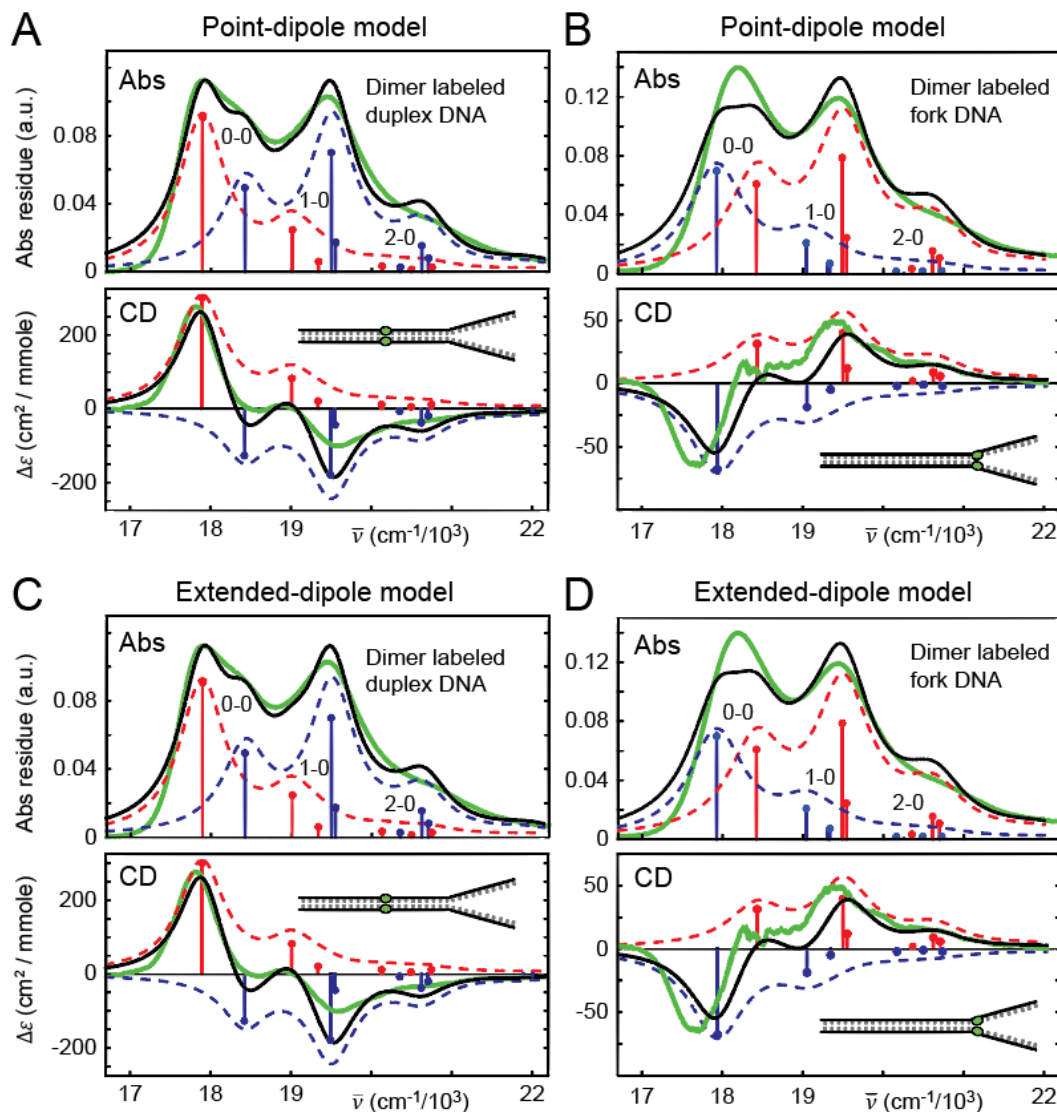


Figure S1. Comparison between 15 °C experimental and simulated absorbance and CD spectra of (Cy3)₂ dimer labeled duplex (**A & C**) and fork (**B & D**) DNA constructs. The simulated spectra are alternately based on the point-dipole model (**A & B**) and the extended-dipole model (**C & D**). Experimental spectra are shown in solid green, and the model total line shapes (inhomogeneous-plus-homogeneous) in solid black. Symmetric and anti-symmetric transitions determined from the model are shown as blue and red sticks, respectively. Symmetric and anti-symmetric contributions to the inhomogeneous line shapes are shown as dashed blue and red curves, respectively. The corresponding optimized values are presented in Table II for the duplex DNA construct, and in Table III for the fork DNA construct.

From Chapter 3

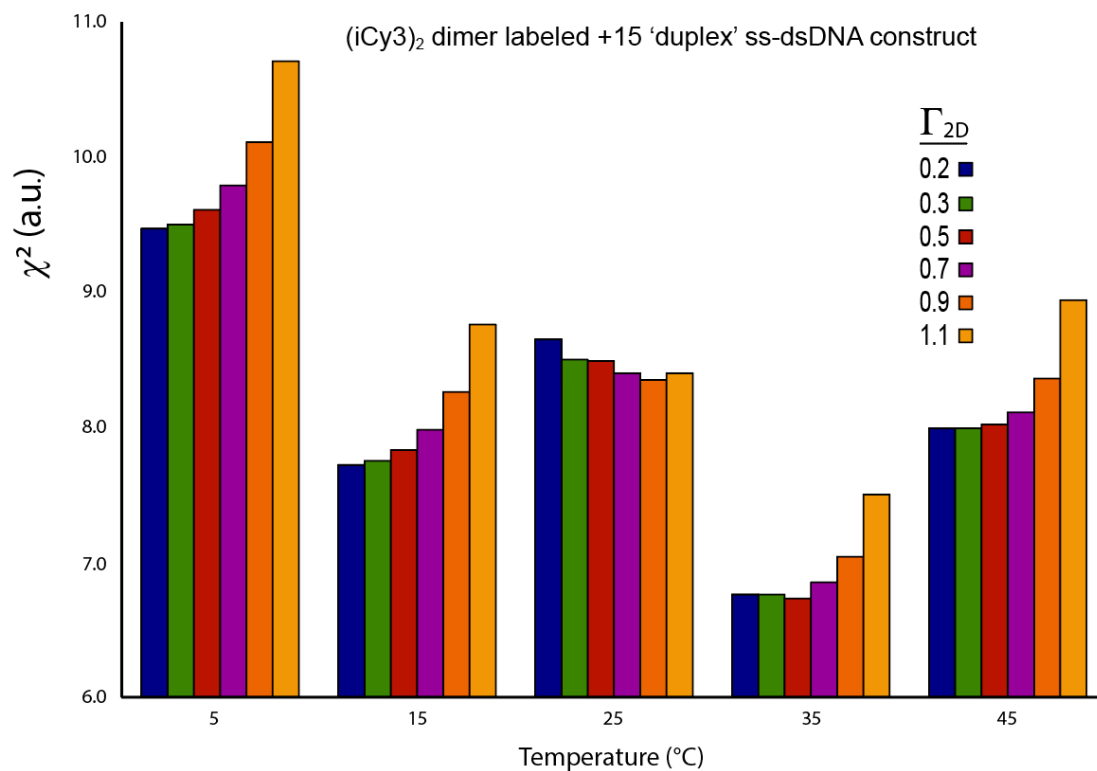


Figure S1. Temperature-dependent χ^2 parameter resulting from optimizations of the relative fluorescence quantum yield parameter Γ_{2D} , which is included in the dimer Hamiltonian given by Eq. (4) of the main text, to experimental 2DFS data of the (iCy3)₂ +15 'duplex' labeled ss-dsDNA construct.

Experimental 2DFS iCy3 Monomer +15 'duplex' ss-dsDNA Construct

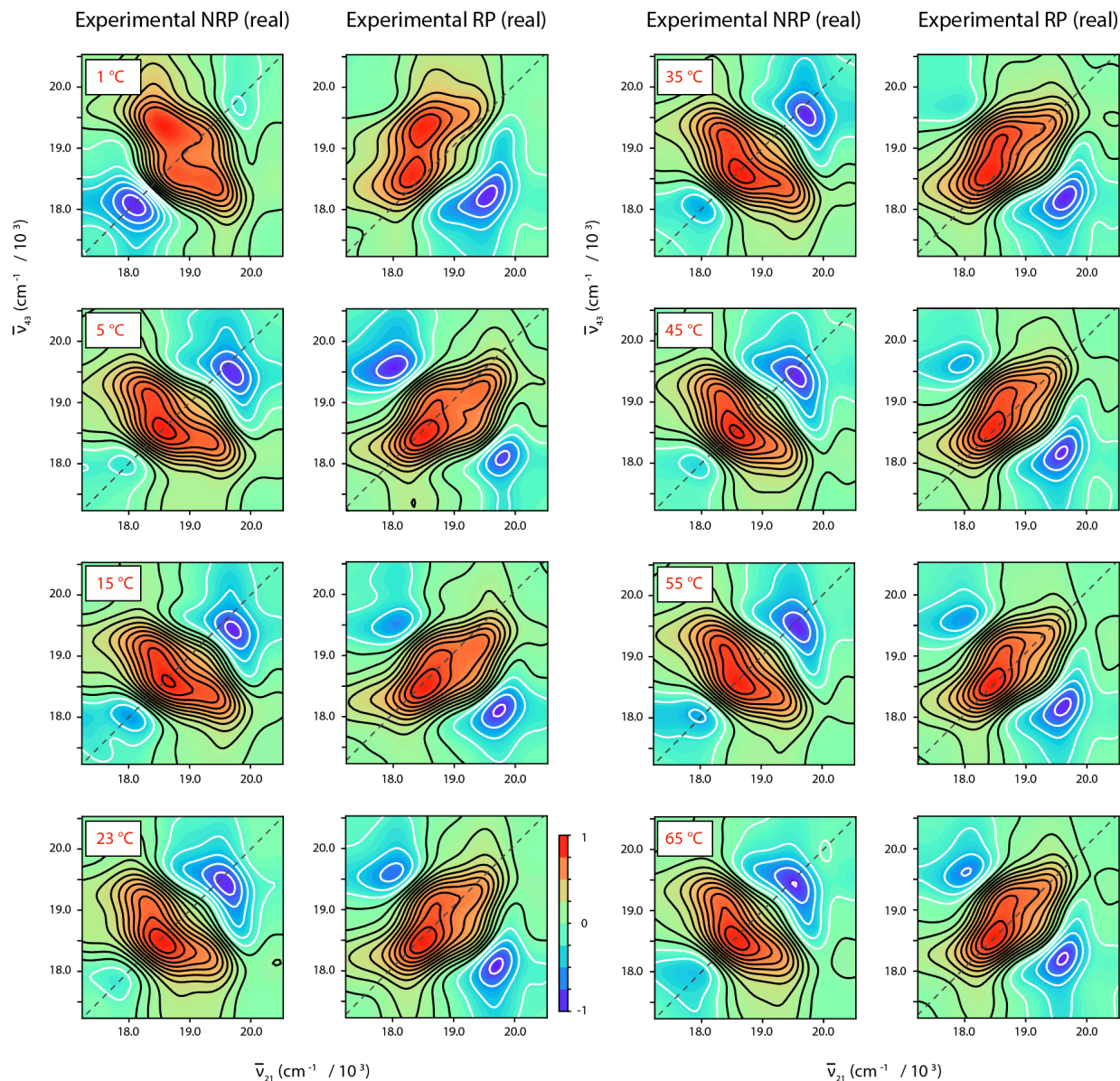


Figure S2. Temperature-dependent experimental NRP and RP 2DFS data (real part) for the iCy3 monomer labeled +15 'duplex' ss-dsDNA construct. The laser spectrum used for these measurements is shown in Fig. 4 of the main text. The melting temperature of the duplex region of the construct is ~ 65 °C.

Simulated 2DFS iCy3 Monomer +15 ‘duplex’ ss-dsDNA Construct

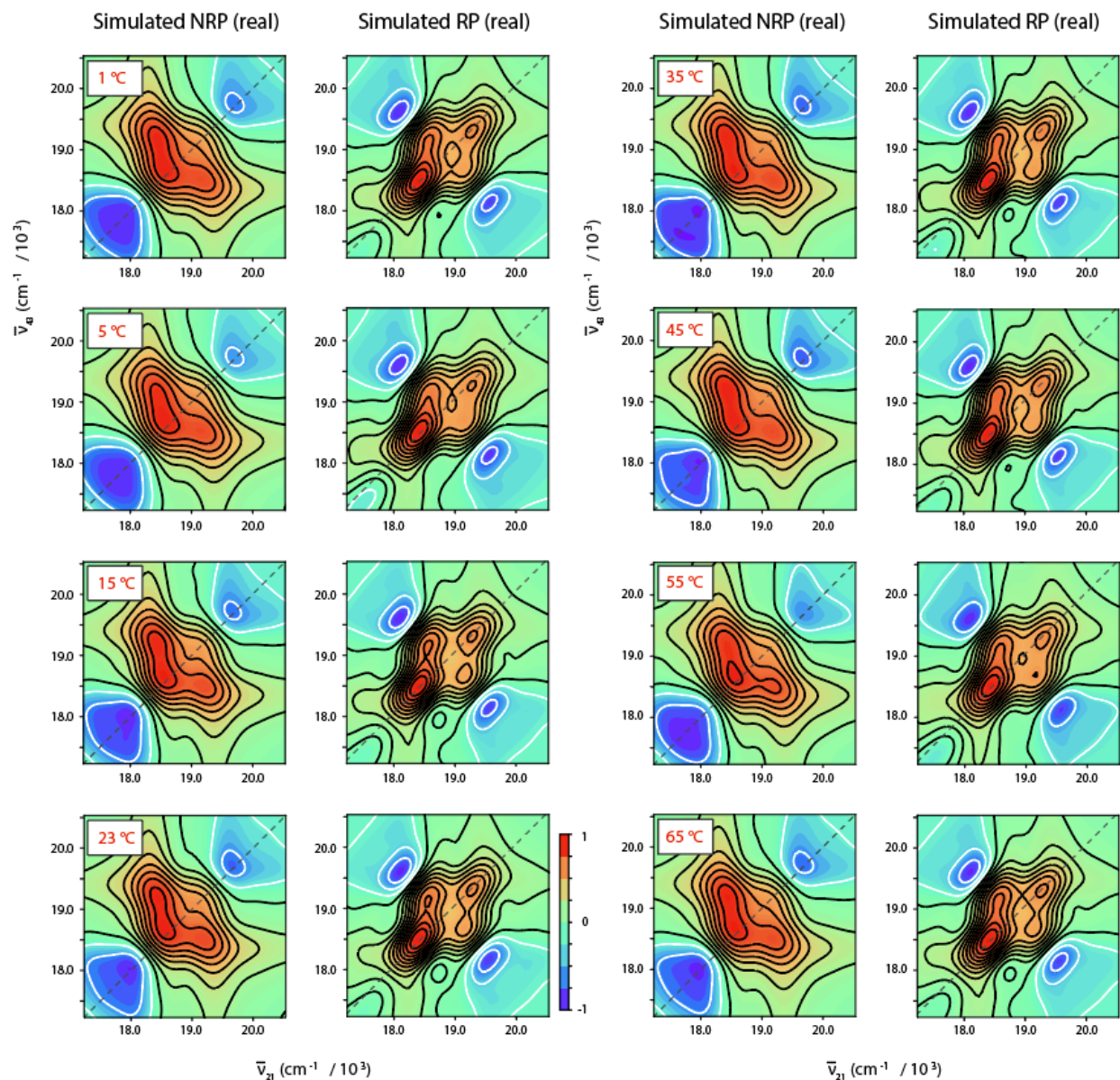


Figure S3. Temperature-dependent simulated NRP and RP 2DFS data (real part) for the iCy3 monomer labeled +15 ‘duplex’ ss-dsDNA construct. Simulations were performed using the monomer Hamiltonian given by Eq. (1) of the main text. The monomer Hamiltonian parameters used as input, in addition to the optimized homogeneous and inhomogeneous spectral line widths determined from our analyses, are listed in [Table S1](#).

Experimental 2DFS iCy3 Monomer +15 ‘duplex’ ss-dsDNA Construct

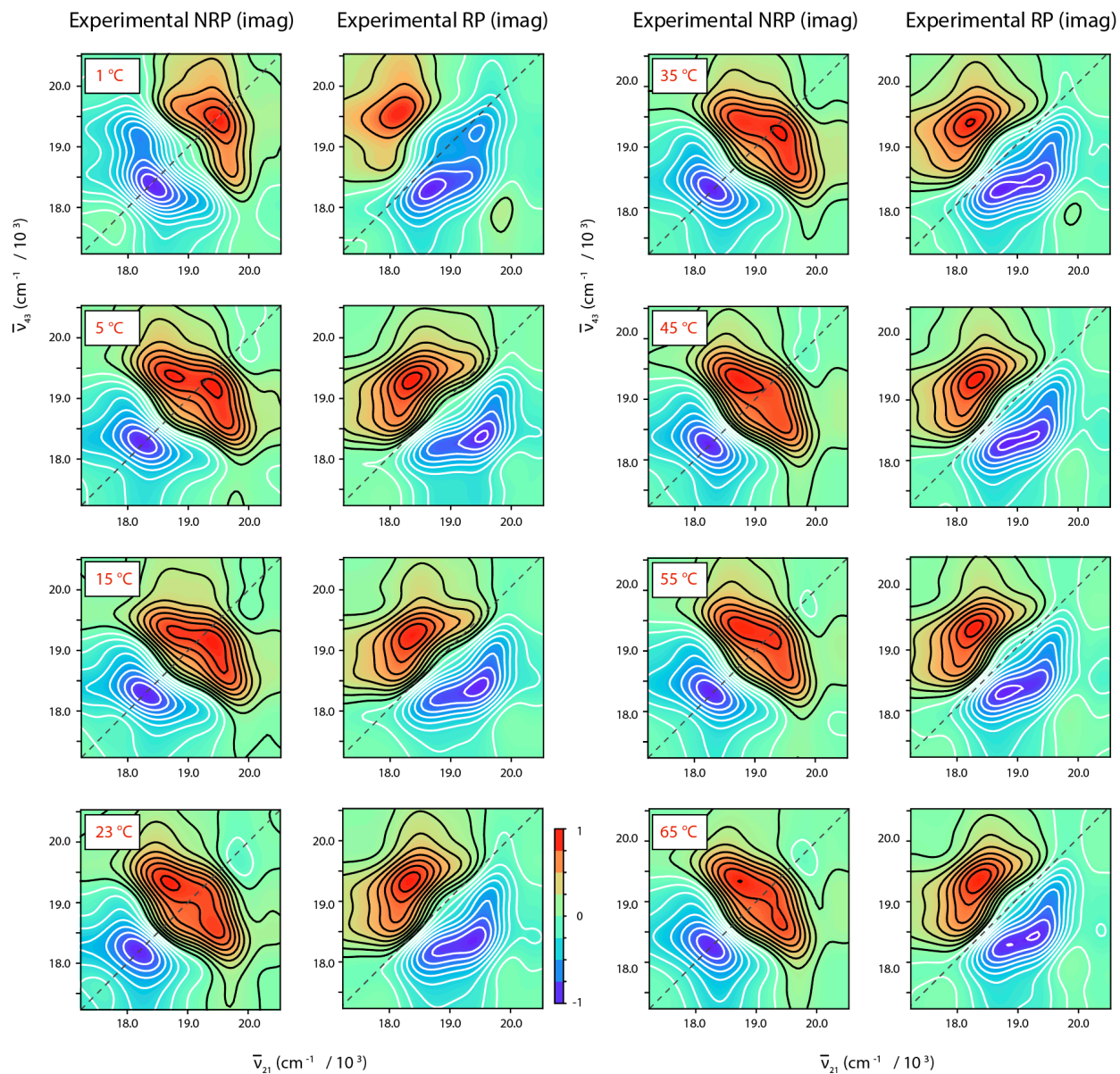


Figure S4. Temperature-dependent experimental NRP and RP 2DFS data (imaginary part) for the iCy3 monomer labeled +15 ‘duplex’ ss-dsDNA construct. The laser spectrum used for these measurements is shown in Fig. 4 of the main text.

Simulated 2DFS iCy3 Monomer +15 ‘duplex’ ss-dsDNA Construct

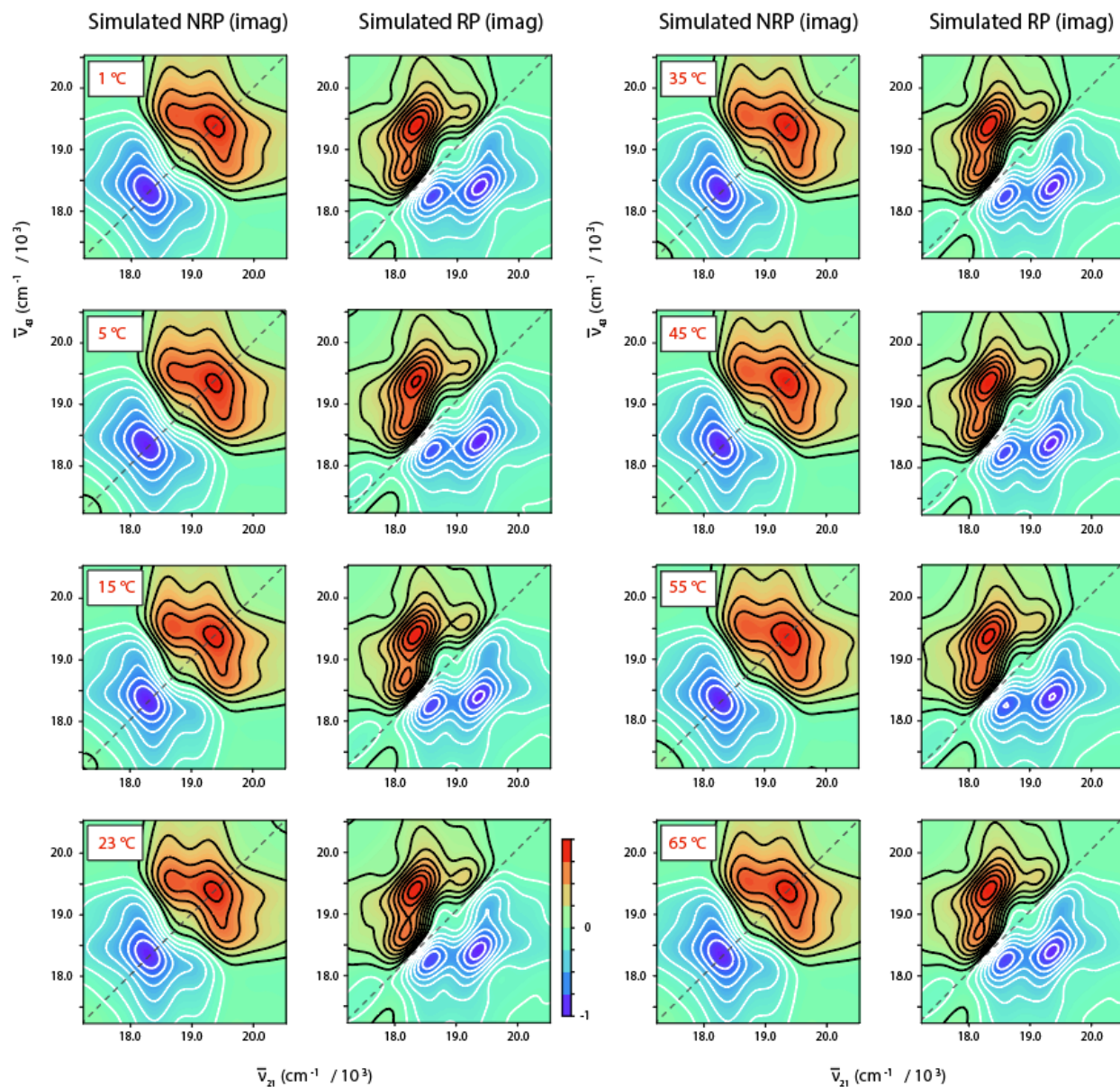


Figure S5. Temperature-dependent simulated NRP and RP 2DFS data (imaginary part) for the iCy3 monomer labeled +15 ‘duplex’ ss-dsDNA construct. Simulations were performed using the monomer Hamiltonian given by Eq. (1) of the main text. The monomer Hamiltonian parameters used as input, in addition to the optimized homogeneous and inhomogeneous spectral line widths determined from our analyses, are listed in [Table S1](#).

Experimental 2DFS (iCy3)₂ Dimer +15 ‘duplex’ ss-dsDNA Construct

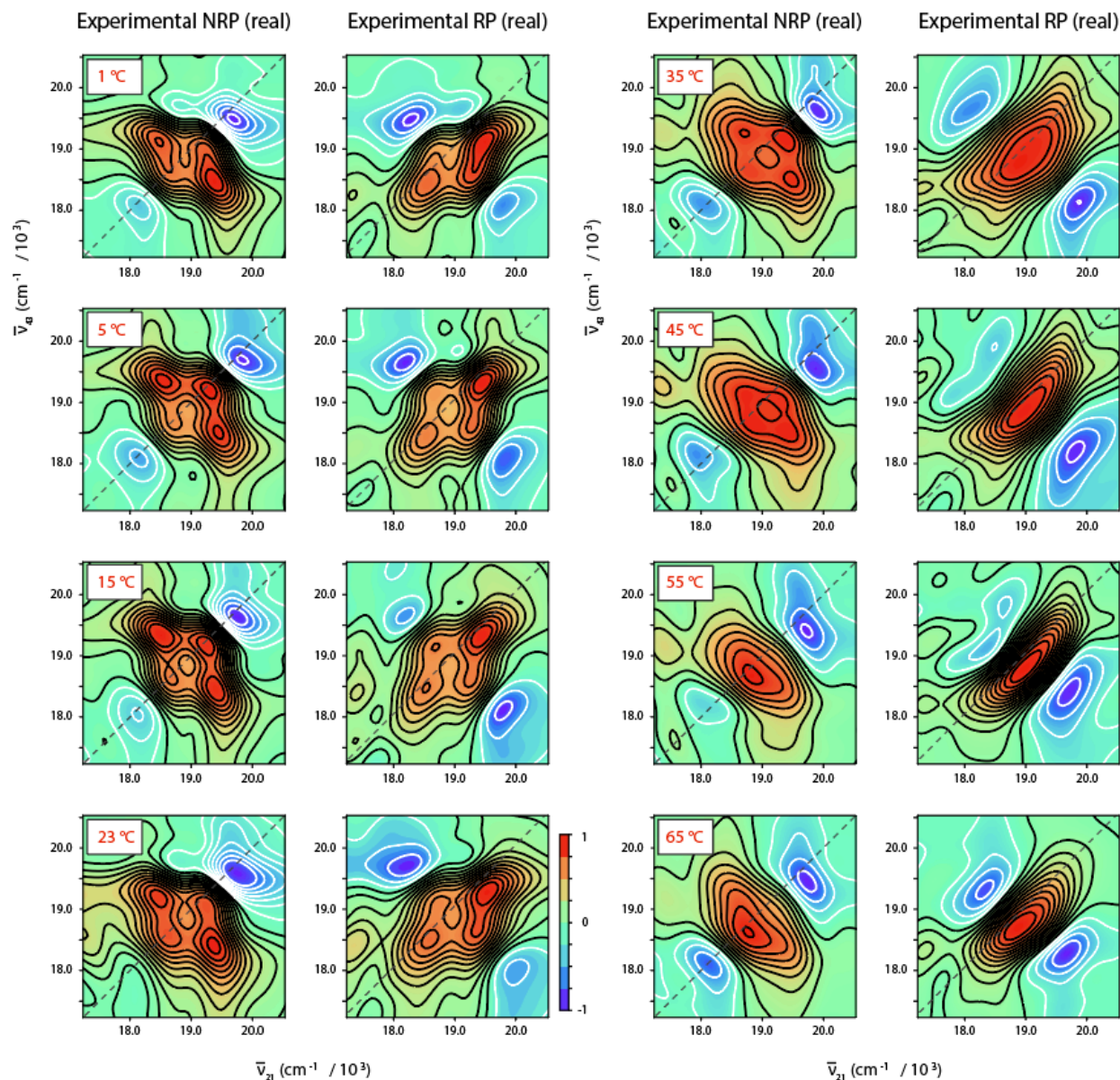


Figure S6. Temperature-dependent experimental NRP and RP 2DFS data (real part) for the (iCy3)₂ dimer labeled +15 ‘duplex’ ss-dsDNA construct. The laser spectrum used for these measurements is shown in Fig. 4 of the main text. The melting temperature of the duplex region of the construct is ~65 °C.

Simulated 2DFS (iCy3)₂ Dimer +15 ‘duplex’ ss-dsDNA Construct

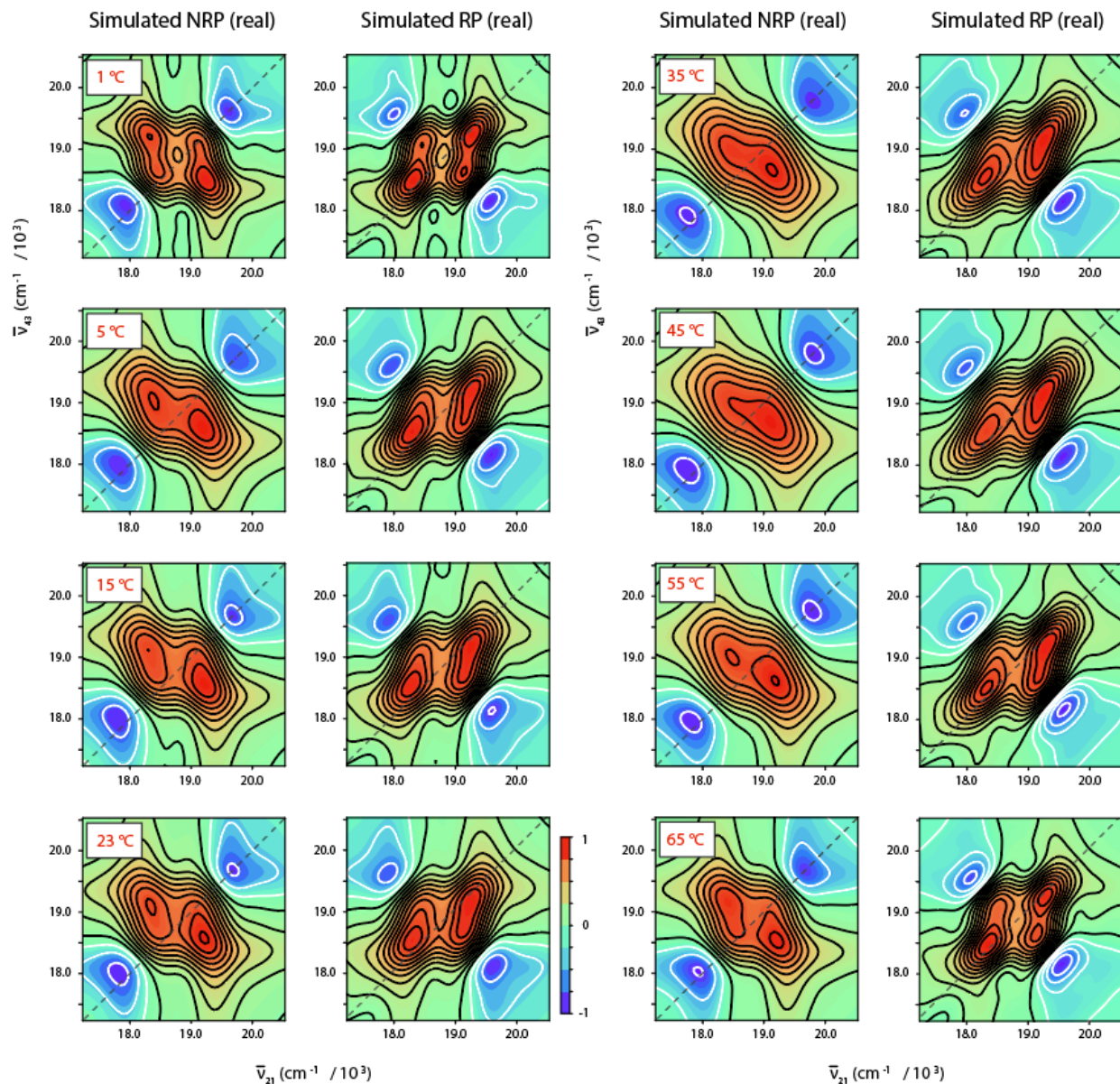


Figure S7. Temperature-dependent simulated NRP and RP 2DFS data (real part) for the (iCy3)₂ dimer labeled +15 ‘duplex’ ss-dsDNA construct. Simulations were performed using the dimer Hamiltonian given by Eq. (4) of the main text. The dimer Hamiltonian parameters used as input, in addition to the optimized homogeneous and inhomogeneous spectral line widths determined from our analyses, are listed in Table 2 of the main text.

Experimental 2DFS (iCy3)₂ Dimer +15 ‘duplex’ ss-dsDNA Construct

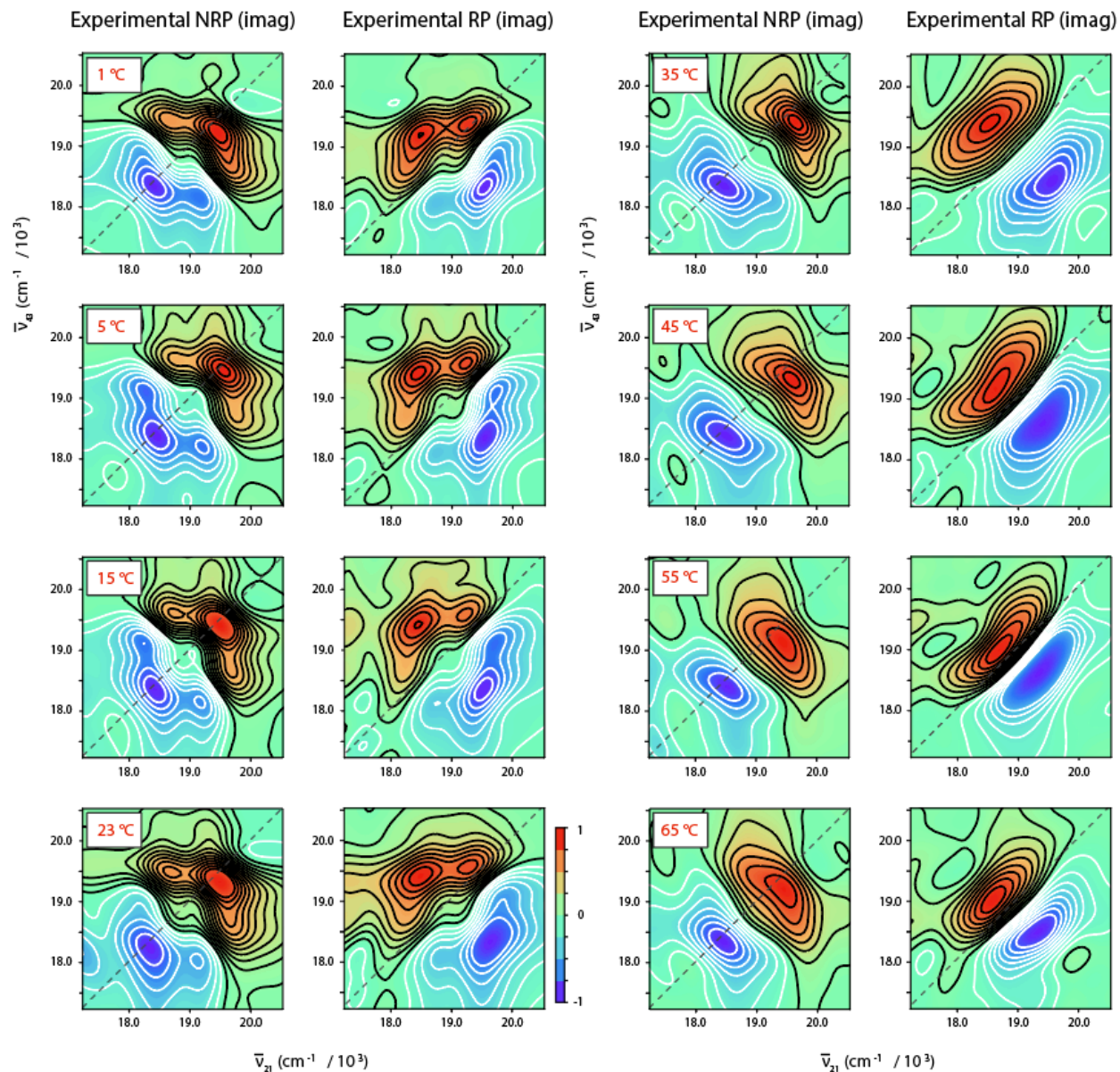


Figure S8. Temperature-dependent experimental NRP and RP 2DFS data (imaginary part) for the (iCy3)₂ dimer labeled +15 ‘duplex’ ss-dsDNA construct. The laser spectrum used for these measurements is shown in Fig. 4 of the main text.

Simulated 2DFS (iCy3)₂ Dimer +15 ‘duplex’ ss-dsDNA Construct

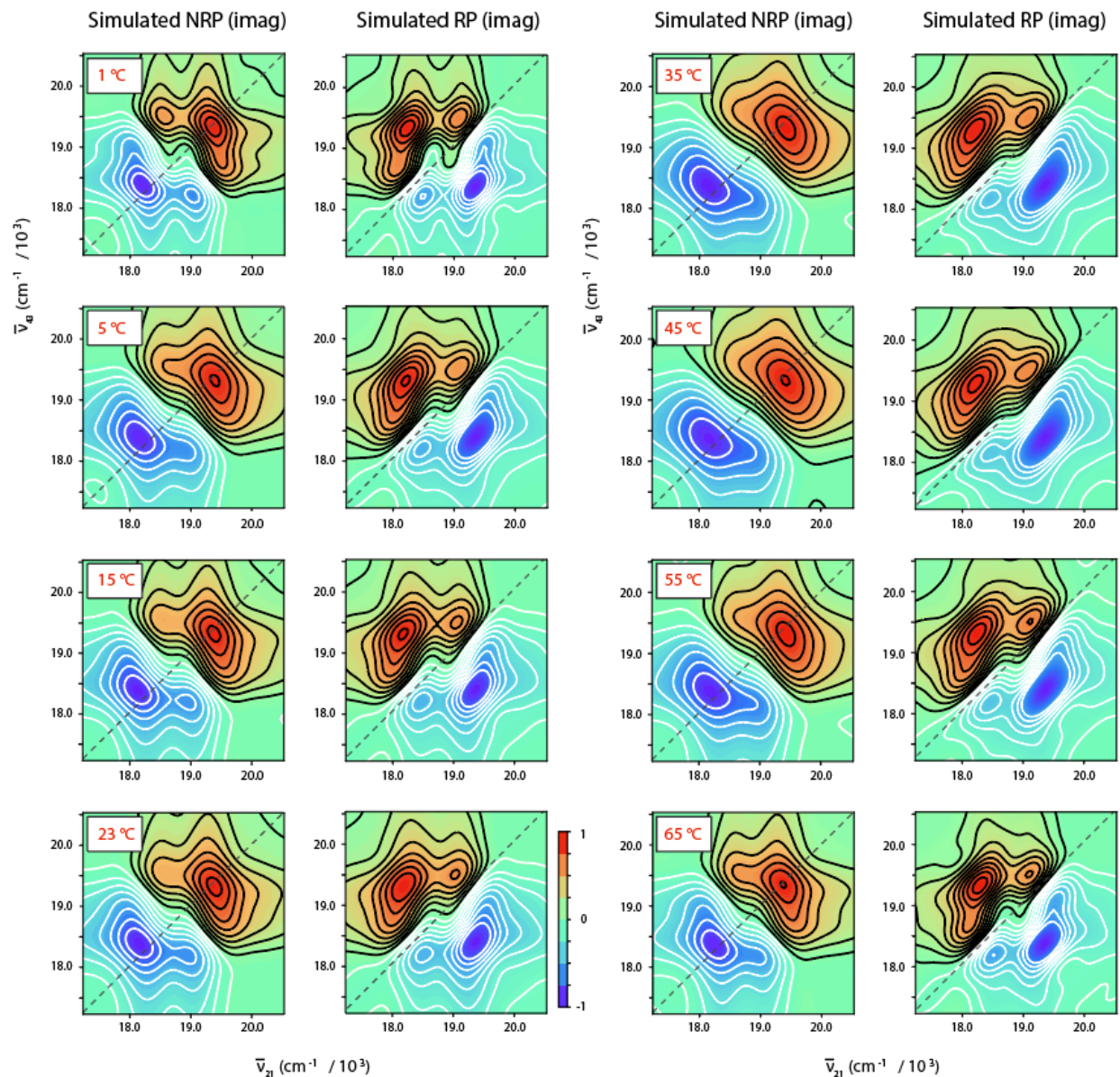


Figure S9. Temperature-dependent simulated NRP and RP 2DFS data (imaginary part) for the (iCy3)₂ dimer labeled +15 ‘duplex’ ss-dsDNA construct. Simulations were performed using the dimer Hamiltonian given by Eq. (4) of the main text. The dimer Hamiltonian parameters used as input, in addition to the optimized homogeneous and inhomogeneous spectral line widths determined from our analyses, are listed in [Table 2](#) of the main text.

Experimental 2DFS iCy3 Monomer -1 ‘fork’ ss-dsDNA Construct

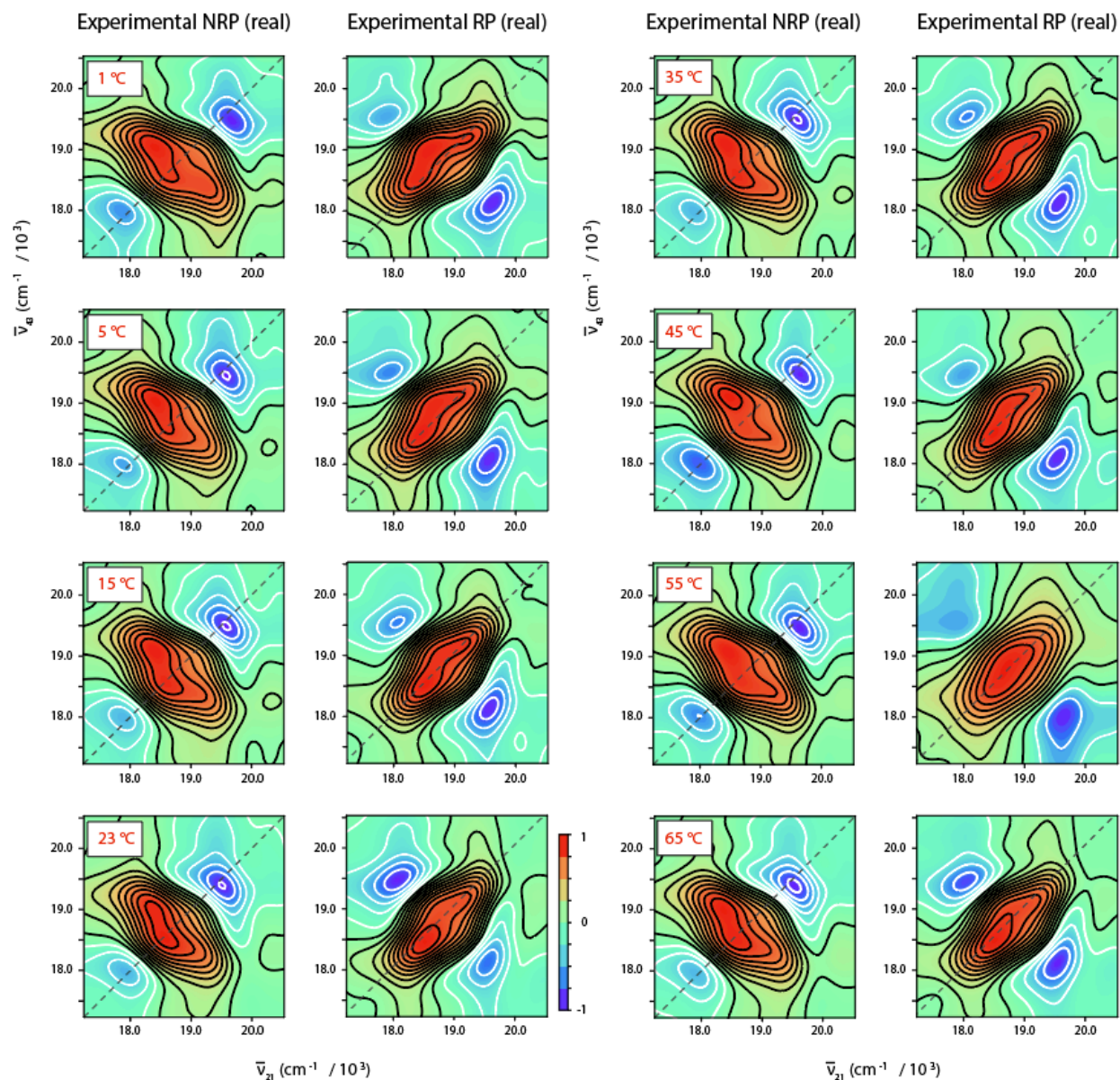


Figure S10. Temperature-dependent experimental NRP and RP 2DFS data (real part) for the iCy3 monomer labeled -1 ‘fork’ ss-dsDNA construct. The laser spectrum used for these measurements is shown in Fig. 4 of the main text. The melting temperature of the duplex region of the construct is ~ 65 °C.

Simulated 2DFS iCy3 Monomer -1 ‘fork’ ss-dsDNA Construct

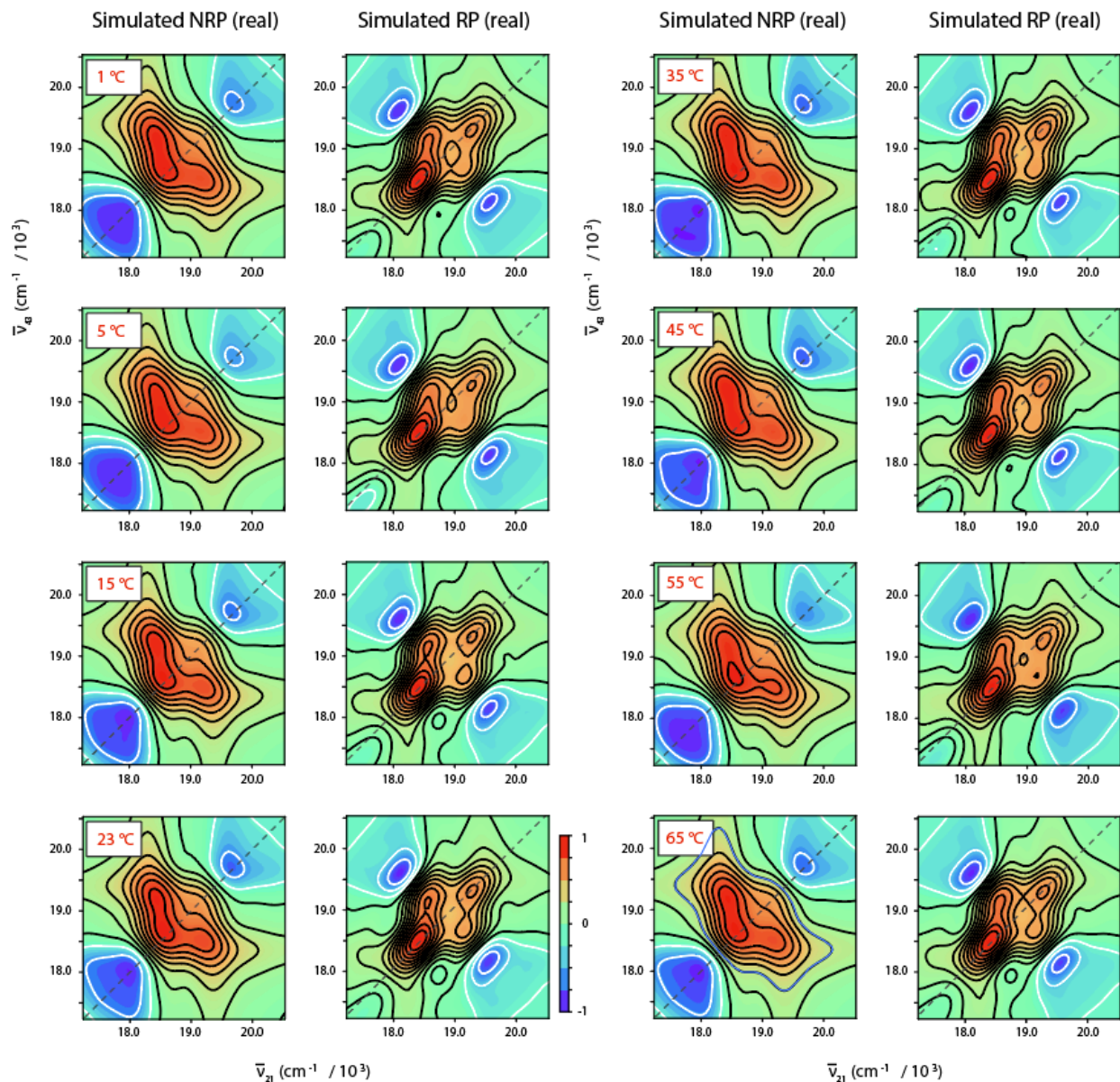


Figure S11. Temperature-dependent simulated NRP and RP 2DFS data (real part) for the iCy3 monomer labeled -1 ‘fork’ ss-dsDNA construct. Simulations were performed using the monomer Hamiltonian given by Eq. (1) of the main text. The monomer Hamiltonian parameters used as input, in addition to the optimized homogeneous and inhomogeneous spectral line widths determined from our analyses, are listed in [Table S2](#).

Experimental 2DFS iCy3 Monomer -1 ‘fork’ ss-dsDNA Construct

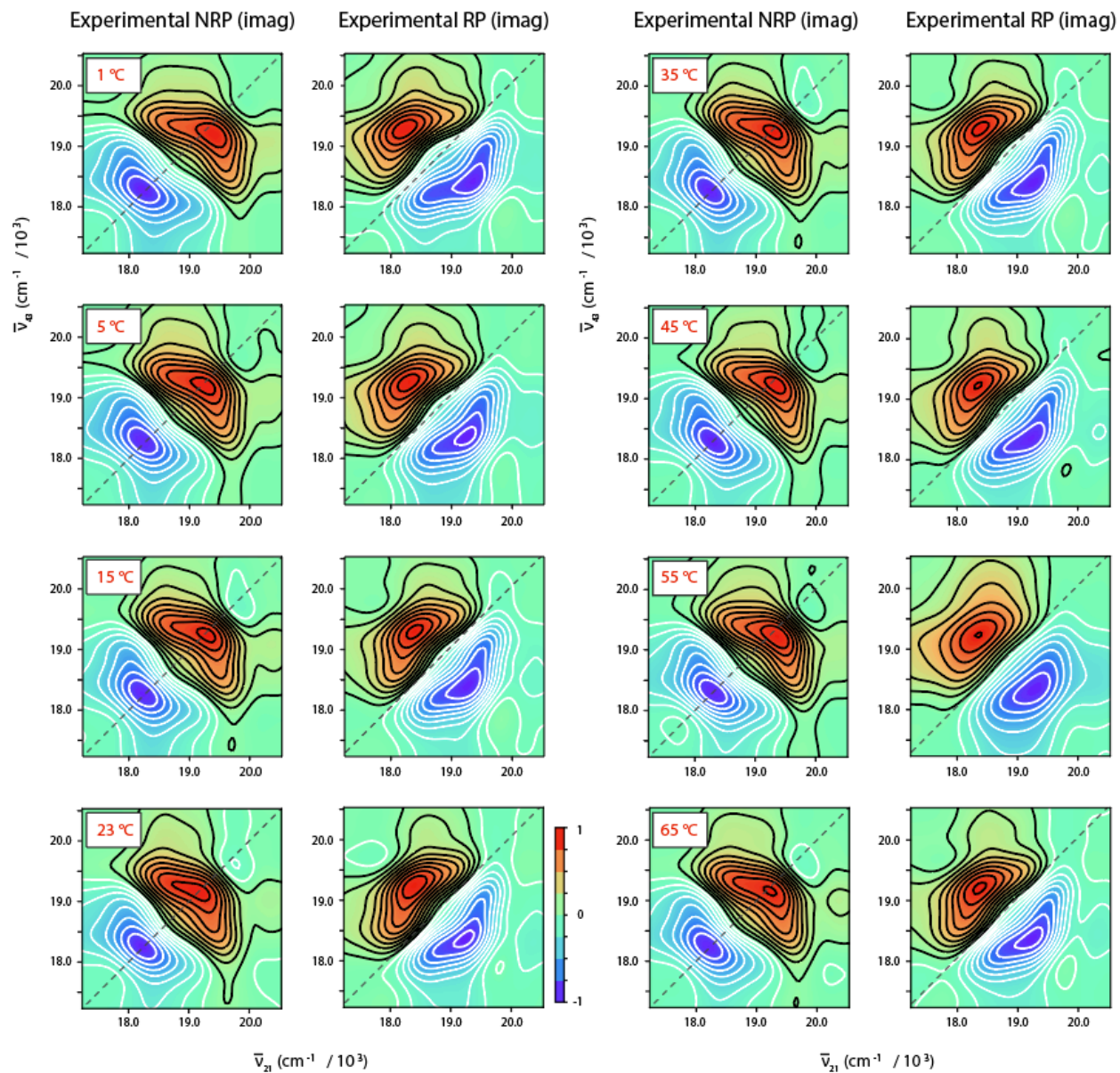


Figure S12. Temperature-dependent experimental NRP and RP 2DFS data (imaginary part) for the iCy3 monomer labeled -1 ‘fork’ ss-dsDNA construct. The laser spectrum used for these measurements is shown in Fig. 4 of the main text.

Simulated 2DFS iCy3 Monomer -1 ‘fork’ ss-dsDNA Construct

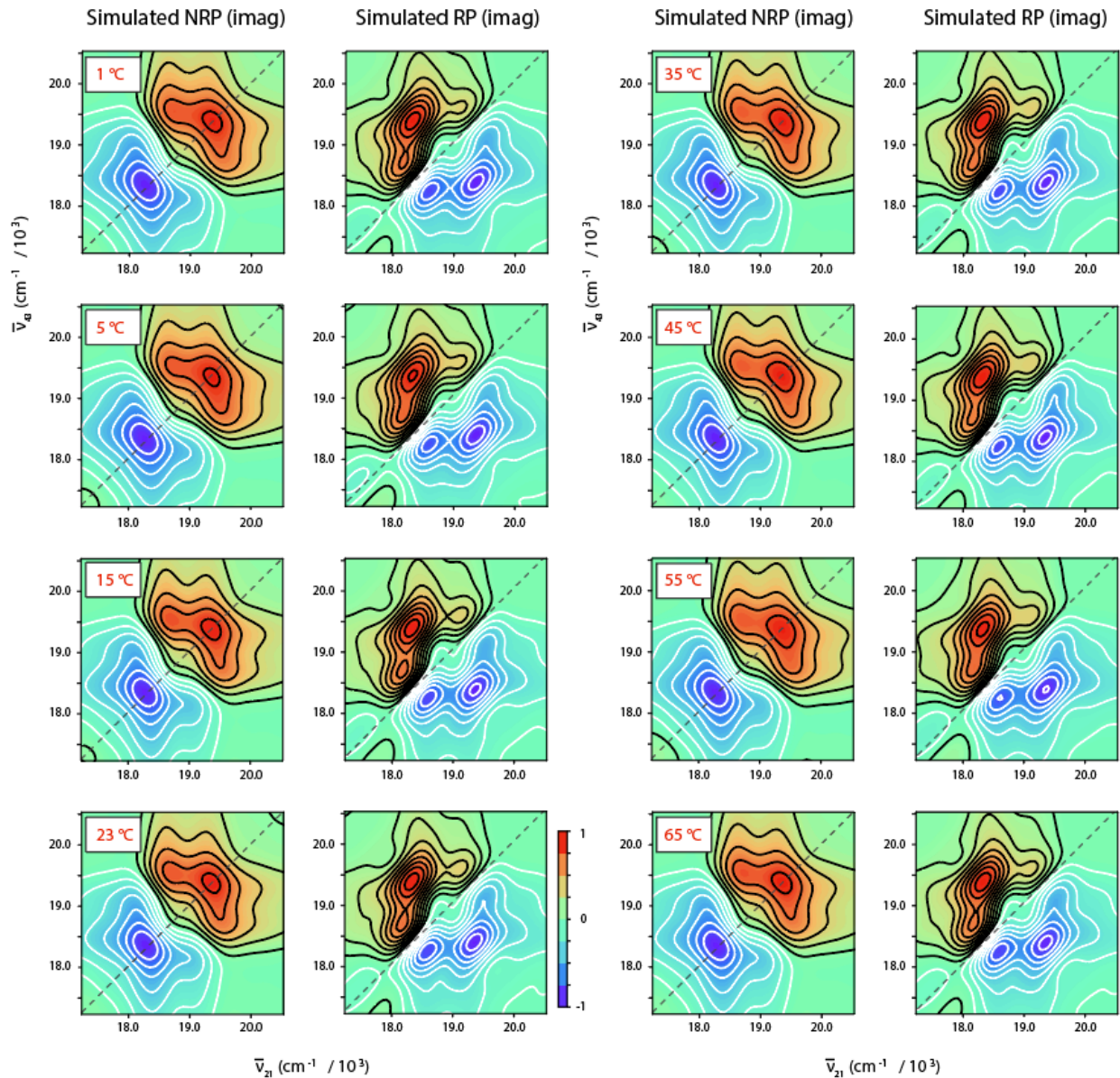


Figure S13. Temperature-dependent simulated NRP and RP 2DFS data (imaginary part) for the iCy3 monomer labeled -1 ‘fork’ ss-dsDNA construct. Simulations were performed using the monomer Hamiltonian given by Eq. (1) of the main text. The monomer Hamiltonian parameters used as input, in addition to the optimized homogeneous and inhomogeneous spectral line widths determined from our analyses, are listed in [Table S2](#).

Experimental 2DFS (iCy3)₂ Dimer -1 ‘fork’ ss-dsDNA Construct

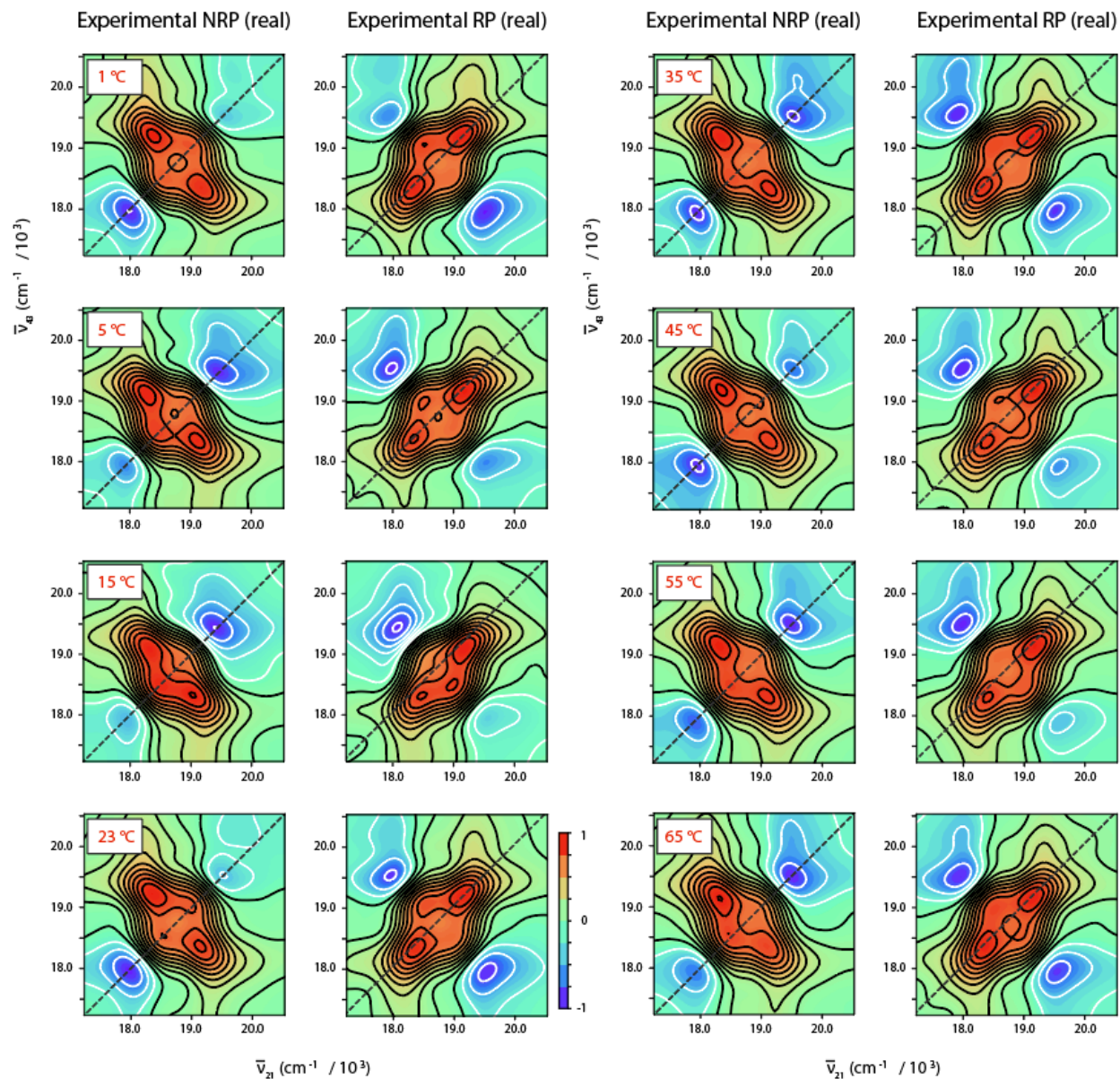


Figure S14. Temperature-dependent experimental NRP and RP 2DFS data (real part) for the (iCy3)₂ dimer labeled -1 ‘fork’ ss-dsDNA construct. The laser spectrum used for these measurements is shown in Fig. 4 of the main text. The melting temperature of the duplex region of the construct is ~65 °C.

Simulated 2DFS (iCy3)₂ Dimer -1 ‘fork’ ss-dsDNA Construct

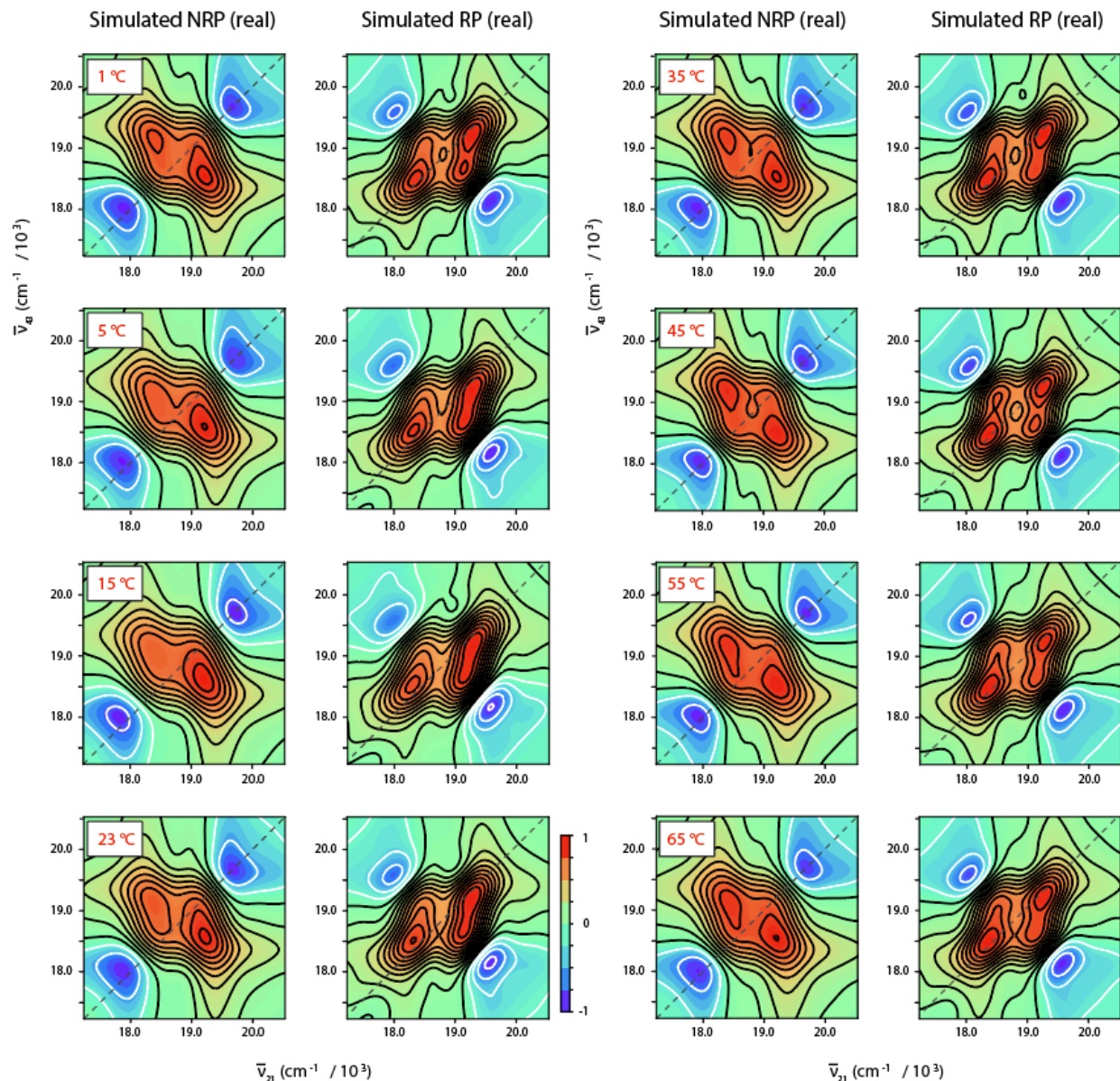


Figure S15. Temperature-dependent simulated NRP and RP 2DFS data (real part) for the (iCy3)₂ dimer labeled -1 ‘fork’ ss-dsDNA construct. Simulations were performed using the dimer Hamiltonian given by Eq. (4) of the main text. The dimer Hamiltonian parameters used as input, in addition to the optimized homogeneous and inhomogeneous spectral line widths determined from our analyses, are listed in Table 3 of the main text.

Experimental 2DFS (iCy3)₂ Dimer -1 ‘fork’ ss-dsDNA Construct

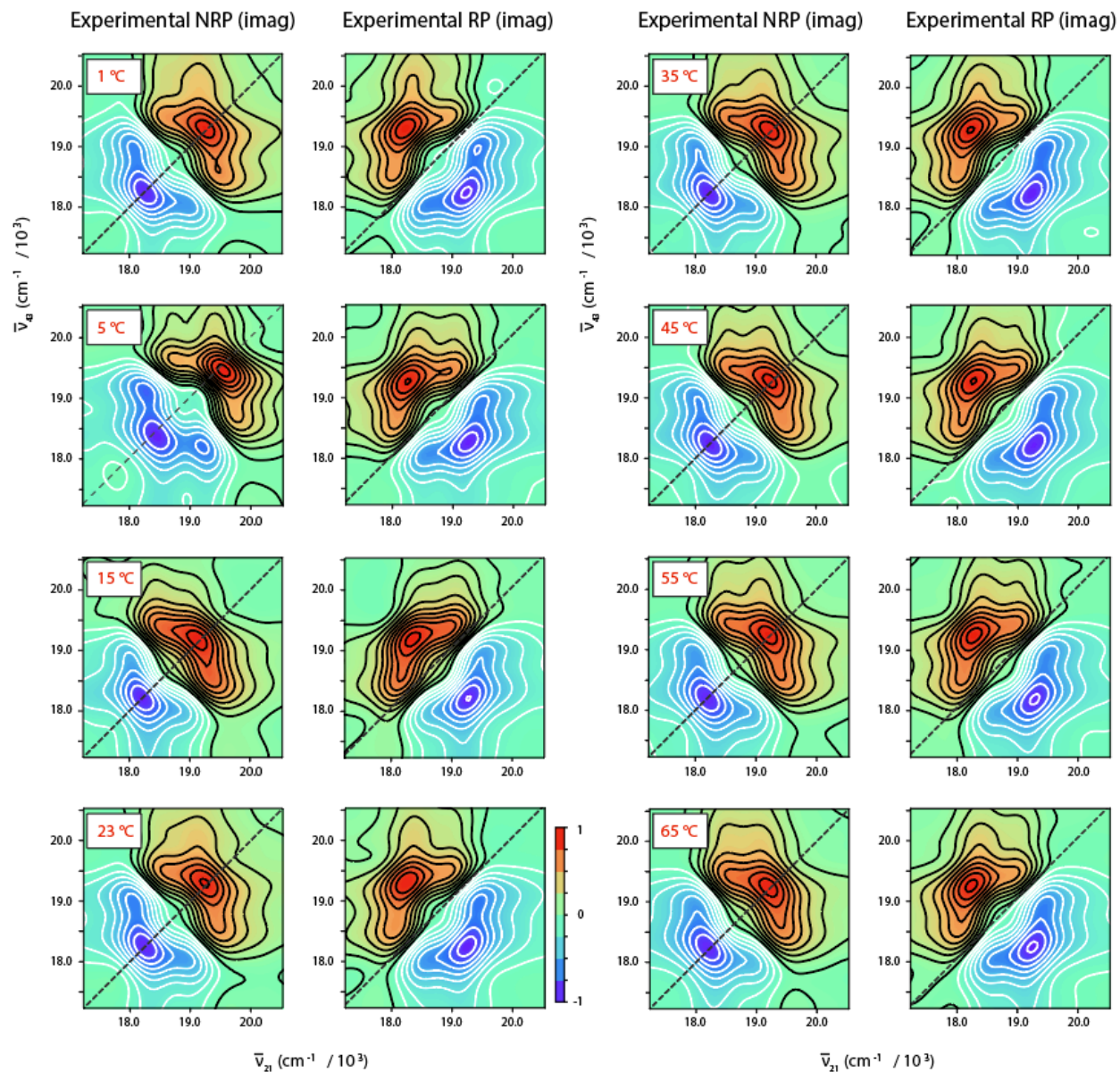


Figure S16. Temperature-dependent experimental NRP and RP 2DFS data (imaginary part) for the (iCy3)₂ dimer labeled -1 ‘fork’ ss-dsDNA construct. The laser spectrum used for these measurements is shown in Fig. 4 of the main text.

Simulated 2DFS (iCy3)₂ Dimer -1 ‘fork’ ss-dsDNA Construct

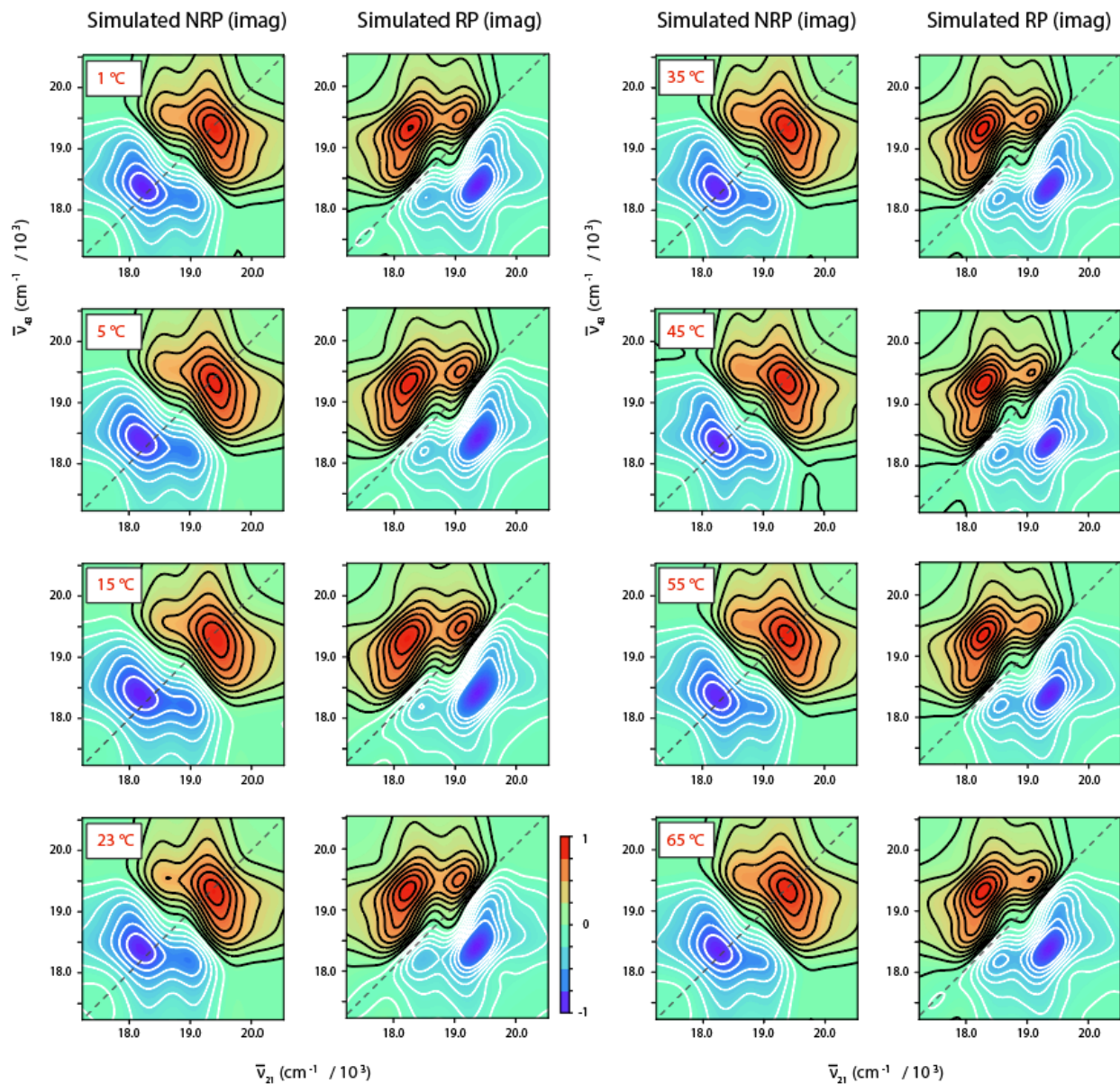


Figure S17. Temperature-dependent simulated NRP and RP 2DFS data (imaginary part) for the (iCy3)₂ dimer labeled -1 ‘fork’ ss-dsDNA construct. Simulations were performed using the dimer Hamiltonian given by Eq. (4) of the main text. The dimer Hamiltonian parameters used as input, in addition to the optimized homogeneous and inhomogeneous spectral line widths determined from our analyses, are listed in Table 3 of the main text.

Experimental and Simulated 2DFS (iCy3)₂ Dimer +2 ‘fork’ ss-dsDNA Construct

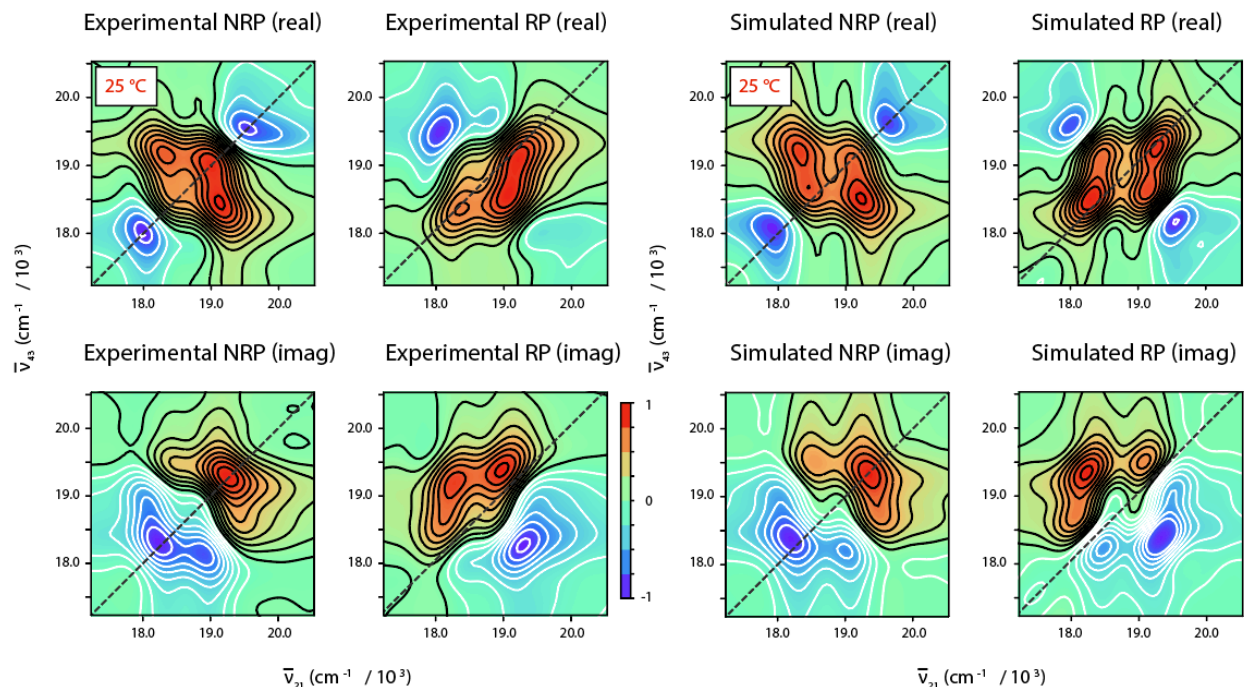


Figure S18. Room temperature (25 °C) experimental and simulated 2DFS data (RP, NRP, real, imaginary) for the (iCy3)₂ dimer labeled +2 ‘fork’ ss-dsDNA construct. The laser spectrum used for the measurements is shown in Fig. 4 of the main text. Simulations were performed using the dimer Hamiltonian given by Eq. (4) of the main text. The dimer Hamiltonian parameters used as input, in addition to the optimized homogeneous and inhomogeneous spectral line widths determined from our analyses, are listed in Table 4 of the main text.

Experimental and Simulated 2DFS (iCy3)₂ Dimer +1 ‘fork’ ss-dsDNA Construct

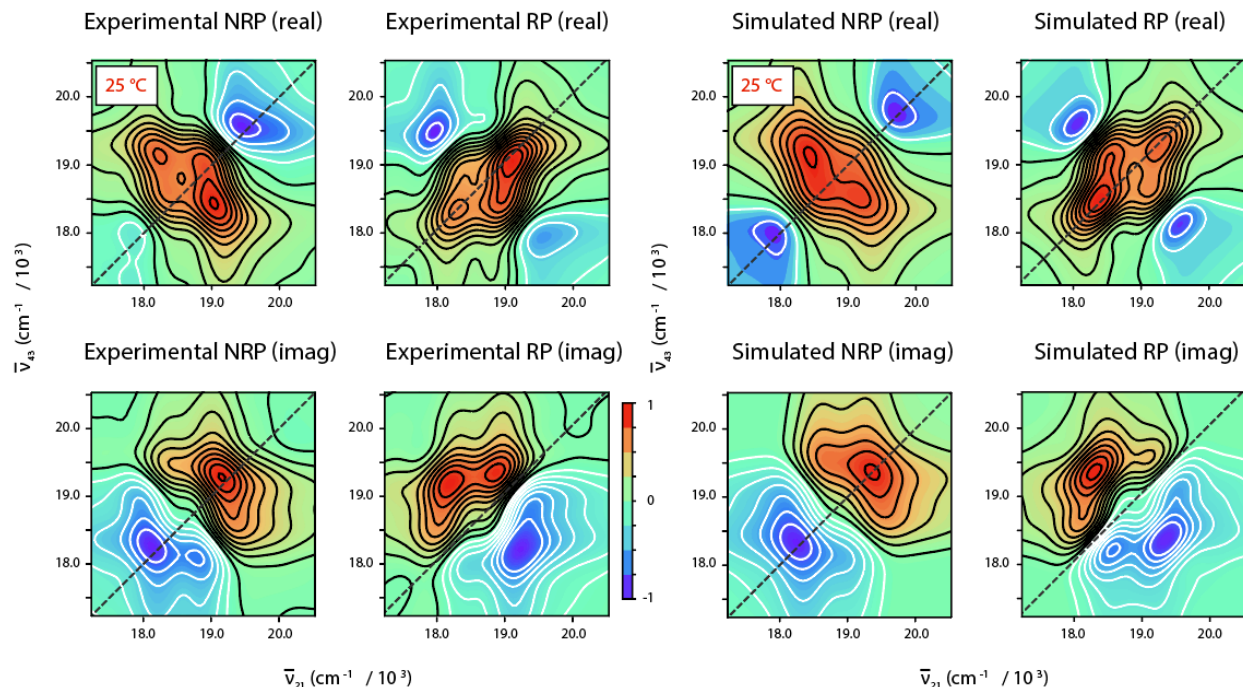


Figure S19. Room temperature (25 °C) experimental and simulated 2DFS data (RP, NRP, real, imaginary) for the (iCy3)₂ dimer labeled +1 ‘fork’ ss-dsDNA construct. The laser spectrum used for the measurements is shown in Fig. 4 of the main text. Simulations were performed using the dimer Hamiltonian given by Eq. (4) of the main text. The dimer Hamiltonian parameters used as input, in addition to the optimized homogeneous and inhomogeneous spectral line widths determined from our analyses, are listed in Table 4 of the main text.

Experimental and Simulated 2DFS (iCy3)₂ Dimer -1 ‘fork’ ss-dsDNA Construct

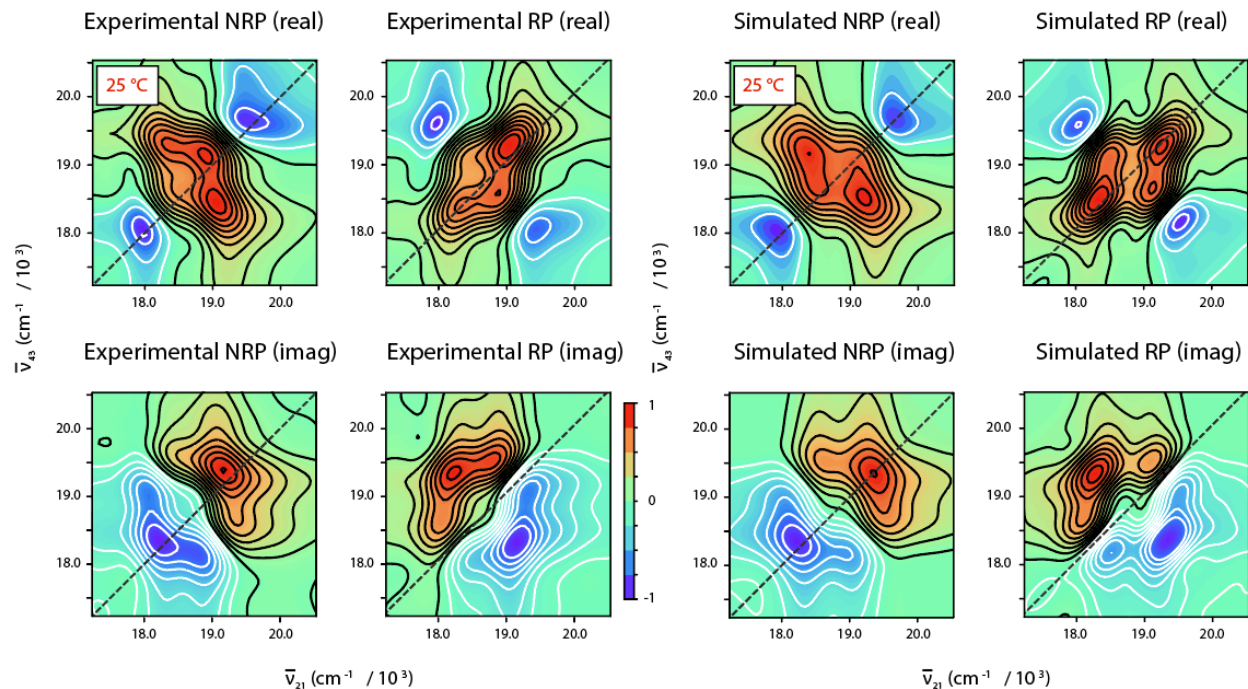


Figure S20. Room temperature (25 °C) experimental and simulated 2DFS data (RP, NRP, real, imaginary) for the (iCy3)₂ dimer labeled -1 ‘fork’ ss-dsDNA construct. The laser spectrum used for the measurements is shown in Fig. 4 of the main text. Simulations were performed using the dimer Hamiltonian given by Eq. (4) of the main text. The dimer Hamiltonian parameters used as input, in addition to the optimized homogeneous and inhomogeneous spectral line widths determined from our analyses, are listed in Table 4 of the main text.

Experimental and Simulated 2DFS (iCy3)₂ Dimer -2 ‘fork’ ss-dsDNA Construct

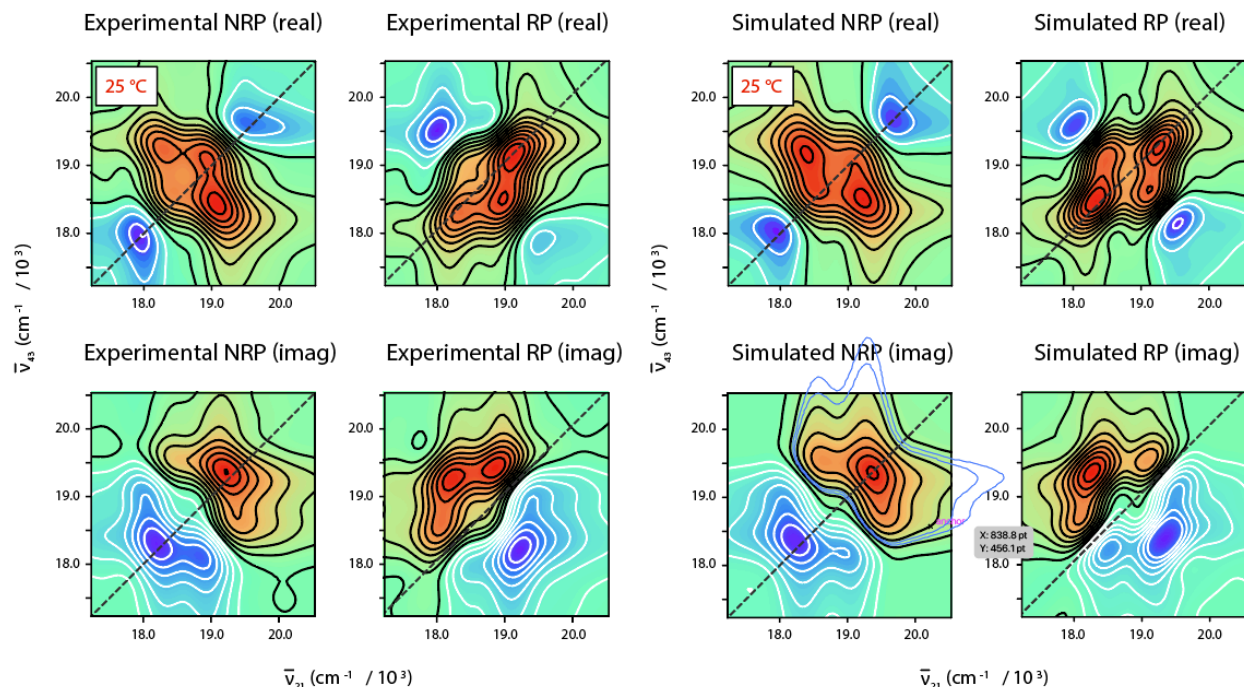


Figure S21. Room temperature (25 °C) experimental and simulated 2DFS data (RP, NRP, real, imaginary) for the (iCy3)₂ dimer labeled -2 ‘fork’ ss-dsDNA construct. The laser spectrum used for the measurements is shown in Fig. 4 of the main text. Simulations were performed using the dimer Hamiltonian given by Eq. (4) of the main text. The dimer Hamiltonian parameters used as input, in addition to the optimized homogeneous and inhomogeneous spectral line widths determined from our analyses, are listed in Table 4 of the main text

Experimental and Simulated 2DFS (iCy3)₂ Dimer -2 ‘fork’ ss-dsDNA Construct (70 °C)

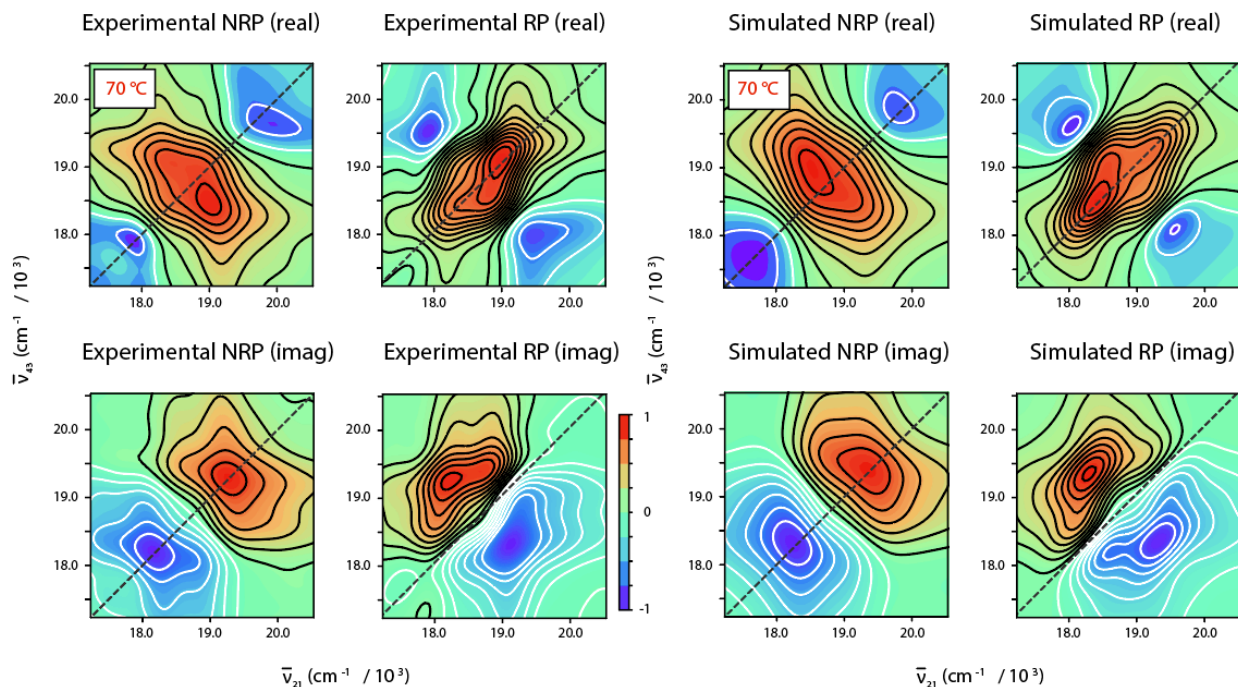


Figure S22. High temperature (70 °C) experimental and simulated 2DFS data (RP, NRP, real, imaginary) for the (iCy3)₂ dimer labeled -2 ‘fork’ ss-dsDNA construct. The laser spectrum used for the measurements is shown in [Fig. 4](#) of the main text. Simulations were performed using the monomer Hamiltonian given by Eq. (1) of the main text. The monomer Hamiltonian parameters used as input, in addition to the optimized homogeneous and inhomogeneous spectral line widths determined from our analyses, are listed in [Table S1](#).

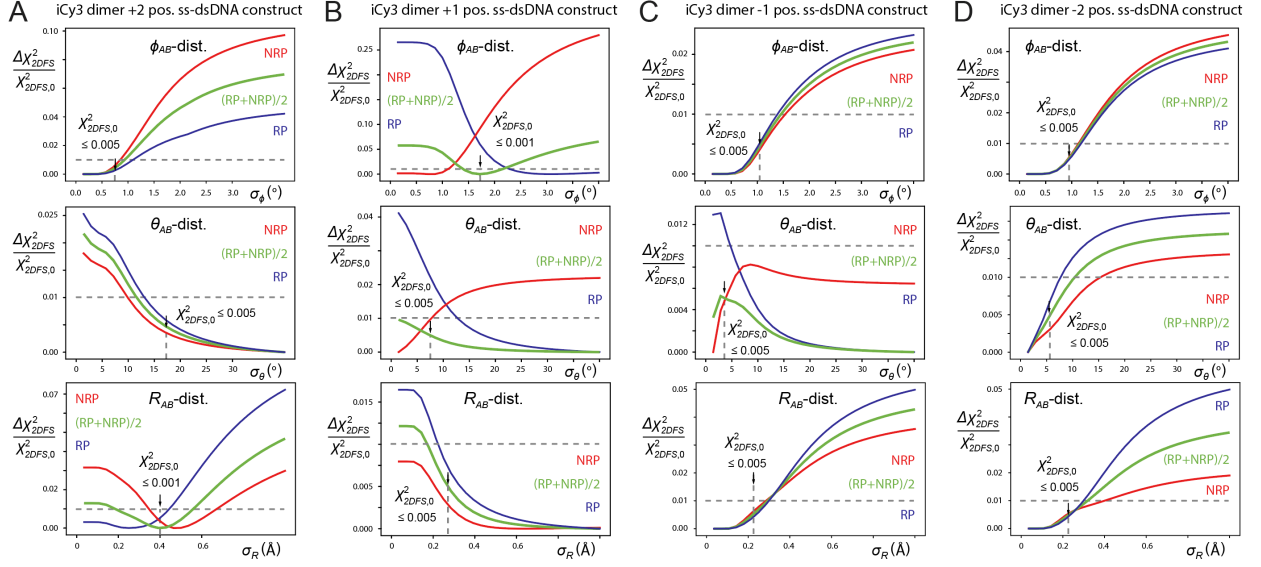


Figure S23. Cross-sections of the relative deviation of the linear least squares error functions, $\chi^2_{2DFS}(\sigma_R, \sigma_\theta, \sigma_\phi) / \chi^2_{2DFS,0} = [\chi^2_{RP}(\sigma_R, \sigma_\theta, \sigma_\phi) + \chi^2_{NRP}(\sigma_R, \sigma_\theta, \sigma_\phi)] / 2\chi^2_{2DFS,0}$, versus the standard deviations of the conformational coordinates σ_ϕ (top), σ_θ (middle) and σ_R (bottom) for the (iCy3)₂ dimer ss-dsDNA constructs at probe labeling sites (A) +2, (B) +1, (C) -1 and (D) -2. The error functions cross-sections are plotted relative to their ‘optimized’ values, $\chi^2_{2DFS,0}$ (indicated by vertical arrows). Optimized values of the standard deviation are defined as the 0.5% threshold for cases in which the error function approached its minimum asymptotically (as do the σ_ϕ and σ_θ cross-sections), and the 0.1% threshold for cases in which the error function exhibited a distinct minimum (as shown for the σ_R cross-section). ‘Confidence intervals’ are defined as a 1% deviation of the error function from its optimized value (indicated by horizontal dashed lines).

From Chapter 5

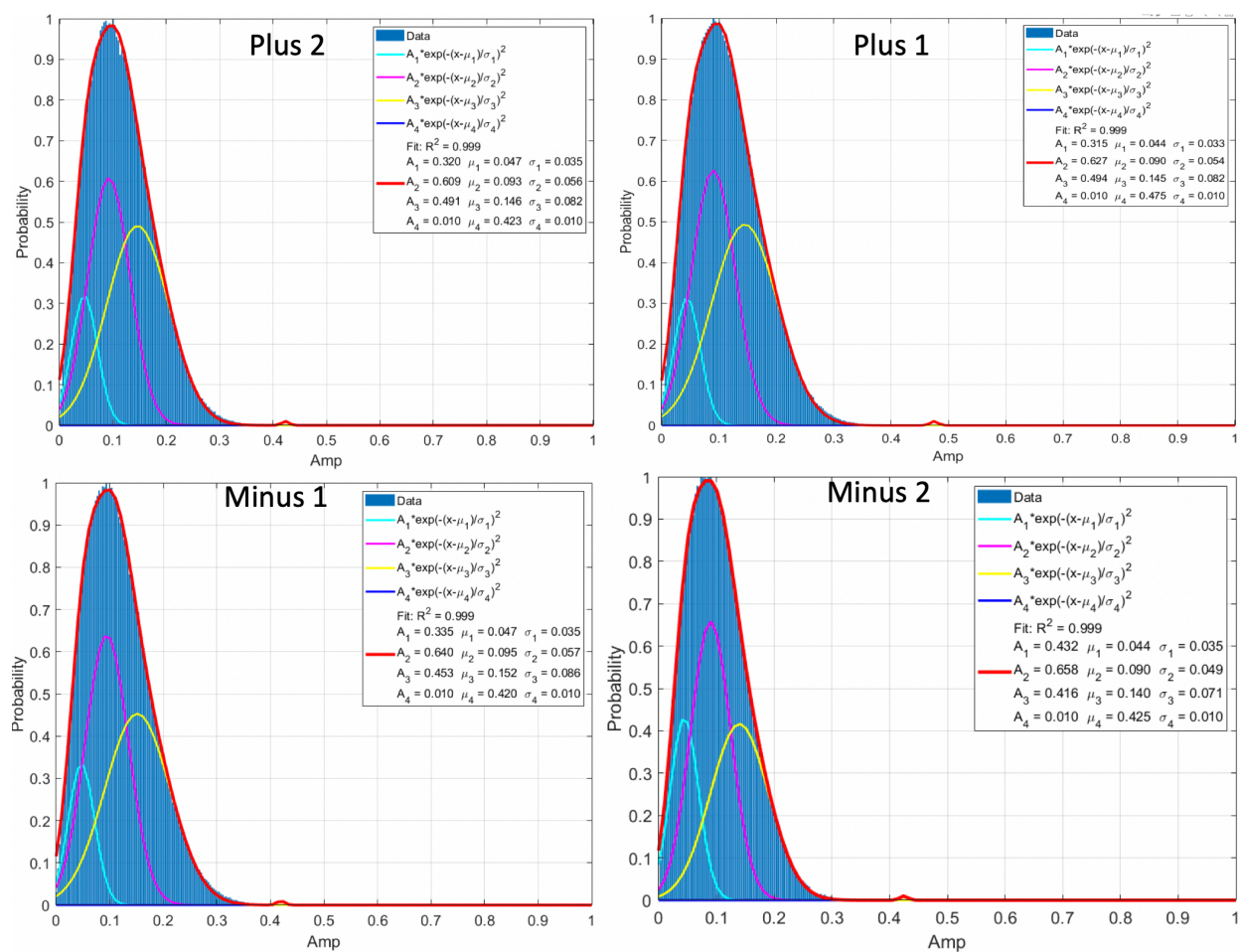


Figure S1. Histograms of the visibility of the labeled DNA fork junctions calculated at 10ms resolution and fit with an unbounded 4 component gaussian decomposition.

Appendix B

Supplementary Tables by Chapter

From Chapter 2

Table S1. Hamiltonian parameters of the Cy3 monomer duplex DNA construct at various temperatures, obtained from model fits of Eq. (1) to the absorbance spectra (17). The calculations used the electric transition dipole moment (EDTM) $\mu_{eg} = 12.8$ D and the homogeneous line width $\Gamma_H = 186$ cm⁻¹. The values listed correspond to the electronic transition energy ε_{eg} , the vibrational mode frequency ω_0 , the Huang-Rhys parameter λ^2 , and the standard deviation of the Gaussian disorder function $\sigma_{I,mon}$.

Cy3 Monomer Duplex DNA Construct				
T (°C)	ε_{eg} (cm ⁻¹)	ω_0 (cm ⁻¹)	λ^2	$\sigma_{I,mon}$ (cm ⁻¹)
15	18,285 +40/-39	1,116 +99/-103	0.54 +0.07/-0.06	333 +16/-15
25	18,277 +38/-37	1,109 +88/-90	0.56 +0.06/-0.06	347 +15/-14
35	18,266 +36/-35	1,119 +82/-84	0.56 +0.06/-0.06	353 +14/-13
45	18,262 +36/-35	1,113 +82/-82	0.56 +0.06/-0.05	380 -14/+13
55	18,280 +39/-38	1,124 +93/-96	0.55 +0.06/-0.06	380 +15/-14
65	18,301 +45/-45	1,103 +103/-107	0.54 +0.07/-0.07	367 +18/-16
75	18,323 +49/-48	1,072 +120/-120	0.54 +0.08/-0.07	376 +19/-17

Table S2. Hamiltonian parameters of the Cy3 monomer fork DNA construct at various temperatures, obtained from model fits of Eq. (1) to the absorbance spectra (17). The calculations used the same input parameters as those described in Table SI.

Cy3 Monomer Fork DNA Construct				
T (°C)	ε_{eg} (cm ⁻¹)	ω_0 (cm ⁻¹)	λ^2	$\sigma_{I,mon}$ (cm ⁻¹)
15	18,223 +37/-36	1,112 +83/-85	0.58 +0.06/-0.06	354 +15/-14
25	18,240 +37/-36	1,116 +81/-82	0.59 +0.06/-0.06	370 +15/-14
35	18,252 +36/-35	1,105 +80/-80	0.58 +0.06/-0.06	372 +14/-13
45	18,249 +37/-35	1,088 +79/-79	0.59 +0.06/-0.06	374 +14/-13
55	18,257 +39/-38	1,086 +83/-83	0.58 +0.06/-0.06	387 +15/-14
65	18,263 +38/-38	1,089 +83/-85	0.58 +0.06/-0.05	398 +15/-14
75	18,285 +38/-37	1,074 +83/-83	0.58 +0.06/-0.05	405 +14/-13

From Chapter 3

Table S1. Temperature-dependent Hamiltonian parameters and 2DFS line widths determined for the iCy3 monomer labeled +15 ‘duplex’ ss-dsDNA construct. The parameters determined from absorbance and CD are the electronic transition energy, ε_{eg} , the vibrational frequency, ω_0 , and the Huang-Rhys electronic-vibrational coupling parameter, λ^2 . The parameters determined from 2DFS data are the inhomogeneous and homogeneous line widths, σ_I and Γ_H . Error bars were calculated based on a 1% deviation of the χ^2 function from its minimum (optimized) value.

Optimized Parameters for iCy3 Monomer +15 ‘Duplex’ ss-dsDNA Constructs					
From Absorbance and CD Optimization				From 2DFS Optimization	
T (°C)	ε_{eg} (cm ⁻¹)	ω_0 (cm ⁻¹)	λ^2	σ_I (cm ⁻¹)	Γ_H (cm ⁻¹)
1	18,285 +40/-39	1,116 +99/-103	0.54 +0.07/-0.06	157.0 +40.2/-37.94	189.2 +29.3/-27.3
5	18,285 +40/-39	1,116 +99/-103	0.54 +0.07/-0.06	202.5 +26.3/-21.31	149.4 +17.2/-12.7
15	18,285 +40/-39	1,116 +99/-103	0.54 +0.07/-0.06	197.5 +20.8/-22.3	131.6 +12.7/-13.8
25	18,277 +38/-37	1,109 +88/-90	0.56 +0.06/-0.06	217.7 +24.6/-29.1	144.9 +20.4/-16.2
35	18,266 +36/-35	1,119 +82/-84	0.56 +0.06/-0.06	192.4 +24.2/-17.3	140.5 +14.6/-12
45	18,262 +36/-35	1,113 +82/-82	0.56 +0.06/-0.05	202.5 +29.8/-25.4	131.6 +18.3/-15
55	18,280 +39/-38	1,124 +93/-96	0.55 +0.06/-0.06	192.4 +22.3/-19.9	105.1 +15/-10.4
65	18,301 +45/-45	1,103 +103/-107	0.54 +0.07/-0.07	202.5 +24.8/-25.1	113.9 +15.1/-13.5

Table S2. Temperature-dependent Hamiltonian parameters and 2DFS line widths determined for the iCy3 monomer labeled -1 ‘fork’ ss-dsDNA construct. The parameters listed are the same as those described in [Table S1](#). Error bars were calculated based on a 1% deviation of the χ^2 function from its minimum (optimized) value.

Optimized Parameters for iCy3 Monomer -1 ‘Fork’ ss-dsDNA Constructs					
From Absorbance and CD Optimization				From 2DFS Optimization	
T (°C)	ε_{eg} (cm ⁻¹)	ω_0 (cm ⁻¹)	λ^2	σ_I (cm ⁻¹)	Γ_H (cm ⁻¹)
1	18,223 +37/-36	1,112 +83/-85	0.58 +0.06/-0.06	202.5 +22/-16.5	127.2 +11.8/-10.9
5	18,223 +37/-36	1,112 +83/-85	0.58 +0.06/-0.06	202.5 +22.5/-12.3	131.6 +12/-12.2
15	18,223 +37/-36	1,112 +83/-85	0.58 +0.06/-0.06	192.4 +20.8/-19.2	118.4 +11.4/-12.7
25	18,240 +37/-35	1,116 +81/-82	0.59 +0.06/-0.06	197.5 +26.5/-23.2	105.1 +15.4/-12.8
35	18,252 +36/-35	1,105 +80/-80	0.58 +0.06/-0.06	192.4 +21/-17	109.5 +13.7/-9.3
45	18,249 +37/-35	1,088 +79/-79	0.59 +0.06/-0.06	192.4 +18.3/-18.3	122.8 +10.6/-12
55	18,257 +39/-38	1,086 +83/-83	0.58 +0.06/-0.06	187.3 +17.9/-17.9	140.5 +12.1/-11.4
65	18,263 +38/-37	1,074 +83/-83	0.58 +0.06/-0.05	202.5 +26.1/-24.1	113.9 +14.2/-14.6

From Chapter 4

Table S1. DNA fork junction conformational parameters found under various models of electrostatic coupling and an asymmetric roll.

Position	Model (Asymmetric)	Phi (°)	Theta (°)	Eta (°)	Shift (Å)	Shear (Å)	R (Å)	Sigma	J
+2	TQ	61.5 +0.8/- 0.8	61.4 +1.2/- -1.3	103.6 +2/-5.7	1.3 +0.1/ -0.1	0.84 +0.22/- 0.18	8.36 +0.16/- 0.14	298.7 +11.9/- 11.4	439.1
	Extended	84.7 +0.9/- 0.9	30.8 +2.6/- 2.9	87.1 +70.3/- 266.6	-1.9 +0.7/ -0.6	2.86 +0.19/- 0.18	8.8 +0.1/- 0.1	298.7 +11.9/- 11.4	439.1
	Point	55.73 +2.55/ -2.6	66.62 +1.6/- 1.6	103.6 +49.7/- 61.2	-3 +1.2/- 1.7	-1.1 +2.7/ -0.5	12.28+ 0.12/- 0.12	298.7 +11.9/- 11.5	439.1
+1	TQ	71.95 +1.46/ -1.45	57.2 +1.0/- 1.1	97.9 +6.7/- 11.9	-2.24 +0.2/-0.2	0.16 +0.72/- 1.58	10.6 +0.12/- 0.12	300.2 +12.5/- 10.0	421.8
	Extended	71.2 +1.8/- 1.8	58.2 +1.3/- 1.3	103.5 +66.7/- 259.1	2.38 +0.3/ -0.4	1.0 +0.25/ -0.23	12.4 +0.12/- 0.11	301.4 +11.4/- 11.0	421.3
	Point	69.37 +1.27/ -1.26	60.55+1. 09/-1.12	82.6 +87.3/- 23.3	0.3 +0.1/ -0.1	2.68 +0.11/- 0.11	10.07+ 0.12/- 0.12	301.4 +11.4/- 11.0	421.3
-1	TQ	96.9 +1.3/- 1.3	19 +8.3/ -7.4	132.1 +2.4/- 2.5	1.88 +0.34/- 0.47	4.33 +3.66/- 0.66	7.4 +0. 12/- 0.11	315.8 +11.6/- 11.2	-358.8
	Extended	97.54 +0.74/ -0.76	13.92+6. 85/-4.83	129.43 +39.1/- 76.0	1.44 +0.4/ -1.4	- 1.01+0.06/ -0.06	7.85 +0.13/- 0.12	316.16+ 11.3/- 11.5	-358.6
	Point	93.44 +0.73/ -0.74	35.05+1. 67/-1.78	105.27 +28.63/ -47.15	1.04 +0.13/- 0.12	3.6 +0.13/- 0.13	7.65 +0.07/- 0.06	316.33+ 11.13/- 11.62	-358.5
-2	TQ	96.6 +1.3/- 1.3	23.6 +8.2/- 10.2	133.3 +2.25/- 2.38	1.5 +0.5/ -1.4	4.24 +3.6/ -0.6	7.5 +0. 1/-0.1	312.8 +11.4/- 11.0	-362.2
	Extended	98	15	110.8	-0.77	2.81	10.28	312.9	-362.2
	Point	96.06 +1.1/- 1.1	27.1 +4.5/- 8.1	64.4 +84.1/- 24.6	1.5 +1.05/- 0.52	4.6 +0.43/ -0.34	8.47 +0.09/- 0.09	313.1 +11.2/- 11.2	-362.1

From Chapter 5

Table S1. Temperature Dependent Conformational Parameters for AT Rich DNA Sequences

Temp	Model (Sym)	Phi (°)	Theta (°)	Eta (°)	Shift (Å)	Shear (Å)	R (Å)	Sigma	J
15	TQ	62.3 +1.2/-1.2	74.1 +0.9/-0.9	99 +5/-4	-0.58 +0.1/-0.1	-1.5 +0.3/-0.26	10.27 +0.1/-0.1	291.9 +11.6/-11.1	508.6
	Extend	81.1 +1.3/-1.3	52 +1/-1	82 +71/-25	-2.7 +0.2/-0.2	1.16 +0.2/-0.2	11 +0.08/-0.08	291.9 +11.6/-11.1	508.6
	Point	63.2 +1.9/-1.9	73.4 +1.2/-1.2	103 +61/-30	-0.4 +0.2/-0.2	1 +0.3/-0.5	11.8 +0.1/-0.1	292 +11.6/-11.1	508.6
25	TQ	72.75 +1.4/-1.3	59.8 +0.9/-0.9	104.45 +18/-11	-3.16 +0.16/-0.16	-0.884 +1.7/-0.6	10.27 +0.1/-0.1	307.86 +12/-11	500.75
	Extend	84.3 +1.2/-1.2	39.26 +1.1/-1.2	92.3 +85/-46	-3.25 +0.24/-0.26	2.03 +0.3/-0.25	10.5 +0.1/-0.1	307.8 +12/-11	500.75
	Point	56.2 + 2.2/-2.3	75.4 +1.2/-1.2	109 +49/-50	-0.86 +0.3/-0.3	-1.96 +0.2/-0.2	12.06 +0.1/-0.1	307.9 + 12/-11	500.74
35	TQ	61.2 +1.7/-1.7	66.1 +1/-1	126 +7/-13	-2.9 +0.2/-0.2	0.084 +0.7/-1.9	11.6 +0.1/-0.1	322.5 +12.3/-11.7	476.8
	Extend	70.8 +1.7/-1.7	55.4 +1.5/-1.6	110.8 +240/-74	-2.7 +0.5/-0.6	2.74 +3.6/-0.5	12.4 +0.1/-0.1	322.5 +12.3/-11.8	476.7
	Point	69.8 + 1.6/-1.6	56.8 +1.3/-1.3	91.5 +70.4/-41.7	-1.8 +0.3/-0.3	0.53 +0.5/-1.6	11.6 +0.1/-0.1	322.5 +12.3/-11.8	476.8
45	TQ	59.7 +1.4/-1.4	61.5 1.2/-1.2	102.5 +9/-6	-1.0 +0.2/-0.2	0.48 +0.6/-2	10.6 +0.14/-0.14	341.1 + 12.7/-12.1	432.1

	Extend	83.45 + 1/ -1	60.4 +1.7/ -1.8	103.36 +75/ -67	-3.02 +0.4/ -0.4	2 +0.16/ - 0.15	8.82 +0.1/ - 0.1	341.2 +12.6/ - 12.2	432.1
	Point	61.2 +2.1/ - 2.1	60 +1.6/ - 1.6	120.7 +38/ -90	-2.66 +0.6/ 3	-2.37 +0.25/ - 0.23	12 +0.1/ -0.1	341.2 +12.6/ - 12.2	432.1

Table S2. Temperature dependent conformational parameters for the GC Rich Structures

Temp	Model (Sym)	Phi (°)	Theta (°)	Eta (°)	Shift (Å)	Shear (Å)	R (Å)	Sigma	J
15	TQ	66.3 +1.3 /- 1.3	65.8 +0.8/ - 0.9	117.6 + 13/ -14	-2.45 +0.14/ - 0.14	1.1 +0.28/ - 0.33	10.5 +0.1/ - 0.1	294 +12/ - 11	506.9
	Extend	66.8 +1.8 /- 1.8	65.3 +0.9/ - 0.9	91.1 +57/ - 23	-2.64 +0.2/ - 0.3	-0.67 +0.1/ - 0.1	11.5 +0.1/ - 0.1	294 +12/ - 11	506.9
	Point	51.1 +2.6 /- 2.7	77.6 +1.3/ - 1.3	99.1 +68/ - 25	-1.27 +0.4/ - 0.6	0.85 +0.44/ - 2.1	12.6 +0.1/ - 0.09	294 +12/ - 11	506.9
25	TQ	58 +2/ -2	69.8 +1/ -1	97.3 +26/ -8	-3.3 +0.3/ - 0.4	0.08 +0.9/ - 1.2	11.9 +0.1/ - 0.1	309.9 +12/ -12	503
	Extend	73.6 +0.9 /- 0.9	52.8 +2/ -2	98.1 +99/ - 31	1.67 +0.12/ - 0.12	2.8 +0.2/ -0.2	9.2 +0.1/ - 0.1	309.8 +12.2/ - 11.7	503
	Point	51.8 6 +2/ -2.1	74.2 +1.2/ - 1.3	129.4 +41/ - 60	0.07 +0.2/ - 0.24	-0.3 +1.3/ - 0.7	12.2 +0.1/ - 0.09	309.9 +12/ -12	503

35	TQ	57.9 +1.5 /- 1.5	65 +1/- 1	85.1 +8/ -7	-1.44 +0.2/- 0.2	-2.2 +0.2/- 0.2	10.52 +0.1/- 0.1	323.65 +12.7/- 12.1	492.1
	Extend	79.7 +0.8 /- 0.8	31.7 +2/ -2	98.5 +91/- 45	-0.35 +0.18/- 0.18	2.6 +0.2/ -0.2	9.28 +0.1/- 0.1	323.7 +12.7/- 12.2	492.1
	Point	53 2.3/ -2.4	68.8 +1.3/- 1.4	91.2 +63/- 43	-2.06 +0.6/- 2.5	-2.0 +0.25/- 0.23	12.16 +0.1/- 0.1	323.65 +12.7/- 12.1	492.15
45	TQ	60.0 6 +1.7 /- 1.7	57.7 +1/ -1	109 +13/- 23	-3.75 +0.3/- 0.4	0.6 +0.5/ -2.4	11 +0.1/ -0.1	339.8 +13/ -13	484.7
	Extend	74.3 +1.2 /- 1.1	36 +1.5/ -1.5	127.5 +62/- 93	-2.4 +0.3/- 0.3	1.85 +0.24/- 0.22	10.68 +0.1/- 0.1	340 +13/- 13	484.6
	Point	51.1 +1.5 /- 1.5	65.6 +1.3/- 1.4	81.7 +93/- 21	1.25 +0.15/- 0.15	-1.86 +0.2/- 0.2	10.94 +0.1/- 0.1	339.8 +13/ -12.5	484.7
55	TQ	53 +1.5 /- 1.5	61.2 +1/ -1	98 +38/ -8	-1.72 +0.24/- 0.25	0.02 +0.9/- 1.2	11 +0.1/ -0.1	354.2 +13/ -12	480.35
	Extend	76.1 +0.8 /- 0.8	24.16 +2/ -2.4	101.2 +70/- 48	-0.38 +0.2/- 0.2	1.79 +0.1/-0.1	8.96 +0.1/- 0.1	354.2 +13/ -12.4	480.3
	Point	71 +0.9 /- 0.9	37.8 +1.2/- 1.2	77.2 +76/- 28	-0.74 +0.14/- 0.14	0.41 +0.4/- 1.2	9.94 +0.08/- 0.08	354.2 +13/ -12.4	480.35
65	TQ	58.7 +1.6 /- 1.6	55.7 +1.2/- 1.2	107.7 +13/- 25	-2.77 +0.3/- 0.3	-1.82 +0.5/- 0.4	10.8 +0.3/- 0.1	367.5 +13/ -13	445.3

	Extend	61.2	52.9	80	3.3	3.46	10.2	367.5	445.3
	Point	65.5 +1.5 /- 1.5	47.2 +1.5/- 1.5	103.7 +63/- 76	-2.17 +0.4/- 0.5	-0.67 +1.8/- 0.5	11.56 +0.1/- 0.1	367.5 +13.4/- 12.8	445.3

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