

# Designed Two-Dimensional DNA Holliday Junction Arrays Visualized by Atomic Force Microscopy

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**Abstract:** A two-dimensional DNA crystal has been designed and constructed from Holliday junction analogues that contain two helical domains twisted relative to each other. The Holliday junction is not an inherently rigid system, but it can be made less flexible if it is combined into a larger construct. We have fused four junctions into a rhombus-like molecule consisting of four six-turn helices, two on an upper layer and two on a lower layer; the branch points, which define vertices, are separated by four double helical turns each. Ligation of the rhombus-like motifs produces no cyclic species, when assayed by ligation-closure experiments. Self-assembly of the rhombuses in one dimension leads to a linear pattern. The rhombuses can be directed to self-assemble by hydrogen bonding into a two-dimensional periodic array, whose spacing is six turns in each direction. The expected spacing is seen when the array is observed by atomic force microscopy (AFM). Variation of the dimensions of the repeat unit from six turns  $\times$  six turns to six turns  $\times$  eight turns results in the expected increase in unit cell dimensions. Hence, it is possible to assemble periodic arrays with tunable cavities using these components. This system also provides the opportunity to measure directly the angles or torsion angles between the arms of branched junctions; here we measure the torsion angle between the helical domains of the Holliday junction analogue. We find by AFM that the torsion angle between helices is  $63.5^\circ$ , in good agreement with previous estimates.

The construction of periodic arrays from branched DNA molecules has been a goal of DNA nanotechnology<sup>1</sup> since the early 1980s.<sup>2</sup> The idea behind DNA nanotechnology is that the associations of branched DNA molecules can be directed by sticky-ended cohesion.<sup>2</sup> We have reported previously the construction of individual objects using this strategy, including a cube,<sup>3</sup> a truncated octahedron,<sup>4</sup> and Borromean rings.<sup>5</sup> In addition to the specificity of sticky ends, crystalline arrays also require rigid components.<sup>6</sup> Ligation-closure experiments on three-arm,<sup>7</sup> four-arm,<sup>8</sup> and bulged three-arm<sup>6,9</sup> branched junctions produce a variety of cyclic products, in addition to unclosed molecules. These experiments have been interpreted to mean that the branched junction is flexible, and that a more rigid motif should be sought. To this end, the antiparallel DNA double crossover (DX) molecule was shown to produce only unclosed species in the same assay, suggesting its lack of flexibility.<sup>10</sup> Attempts to ligate DX molecules into two-dimensional arrays have produced ambiguous results; nevertheless, we have been able to demonstrate the formation of two-dimensional crystals

from this motif when we do not ligate, but merely allow the components to self-assemble by hydrogen bonding.<sup>11</sup> We can form a series of arrays with well-defined topographic patterns that are readily visualized by the atomic force microscope. These surface patterns are produced by DNA hairpins protruding out of the plane of the array; predictable variation in surface features can be achieved by deliberate alteration of the sticky ends on the lattice components. Recently, we have been able to modify the surface features by restriction, ligation, or annealing, to produce a diversity of patterns from an initial starting set of DX molecules.<sup>12</sup>

This success with hydrogen-bonded arrays has led us to reexamine the earlier conclusions of the ligation-closure experiments. In particular, we decided to see whether a conventional four-arm immobile branched junction<sup>2</sup> could be used as the branching component of a one-dimensional array and a two-dimensional lattice held together only by hydrogen bonding. Immobile branched junction molecules with four arms have been characterized extensively,<sup>13,14</sup> because they are analogues of the Holliday intermediate in genetic recombination. In solution, the four arms of the structure stack on each other in pairs to form two helical domains.<sup>15</sup> These domains are oriented roughly antiparallel to each other, with a right-handed twist, producing a torsion angle estimated to be about  $60^\circ$ .<sup>16–18</sup> Two strands of each junction appear to retain their helical structures, and the

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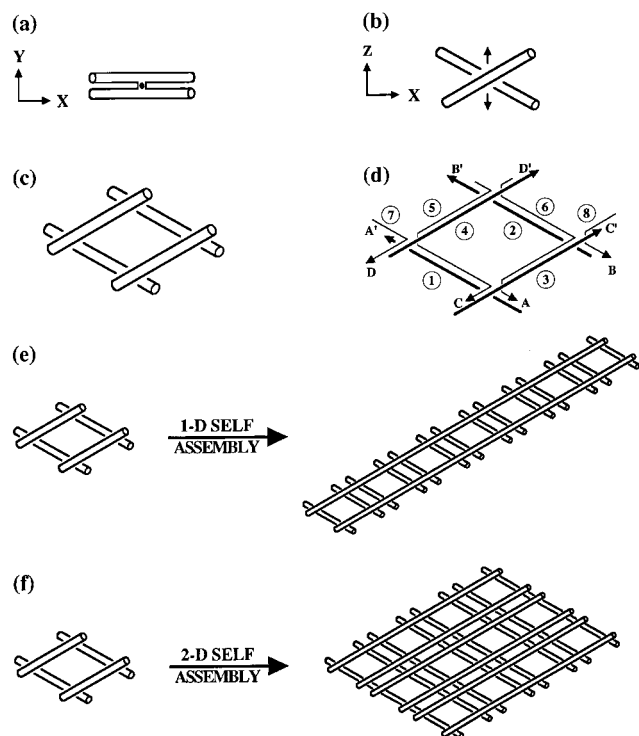
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**Figure 1.** Schematic representations of the molecular components and their assembly. (a) A view down the dyad axis of the Holliday junction. The dyad axis is indicated by the small lens-shaped figure. The upper helical domain is rotated  $30^\circ$  about the vertical so that its right end penetrates the page, and the lower helical domain is rotated  $30^\circ$  about the vertical so that its left end penetrates the page. The  $X$  and  $Y$  axes of a right-handed coordinate system are shown to help orient the reader. (b) A view with the dyad axis vertical. The molecule has been rotated  $90^\circ$  about the  $X$  axis, as indicated. The dyad axis is indicated by the double arrows. (c) The combination of four junctions into a rhombus-like motif. Four molecules, in the orientation of (b) are combined. There are six turns of DNA in each helix, and four turns between crossover points, leading to one-turn overhangs on the ends. (d) The strand structure of the rhombus-like motif. The strand structure of the molecule in (c) is shown. Strands that are continuous helices (numbered 1–4) are drawn with a dark line, and those involved in crossovers (numbered 5–8) are drawn with a thin line. Arrowheads indicate the 3' ends of the strands. Sticky ends are shown by the letters A, B, C, and D, and A', B', C', and D', respectively, represent their complements. The molecule is constructed from the strands synthesized as shown, rather than from the ligation of four junctions. (e) One-dimensional self-assembly of the motif into an array. One-dimensional self-assembly is shown to produce a railroad-track-like arrangement, with helices representing two "rails", extending for the length of the assembly, and "ties" separated by alternating distances of four turns and two turns. (f) Two-dimensional self-assembly produces a latticework of DNA. The array shows the two-dimensional self-assembly product of the motif; the long separations between helices contain four helical turns, and the short separations contain two helical turns. Note that the latticework array contains two separate layers, an upper layer oriented from lower left to upper right, and a lower layer oriented from lower right to upper left.

other two strands form the crossover between the helical domains. We have assumed this basic structure in the design of the crystal components constructed here.

The structure of the Holliday junction in solutions containing  $Mg^{2+}$  is shown at the top of Figure 1. Figure 1a is a view down

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its dyad axis,<sup>15</sup> and Figure 1b is a view rotated  $90^\circ$  about the horizontal. One helical domain is closer to the reader than the other in Figure 1b. The arrays we have built are designed to consist of two layers, one layer derived from each of the helical domains. As noted above, the two domains are twisted relative to each other about an axis perpendicular to the page in this view. Eis and Millar<sup>18</sup> have shown that there is normally a large dispersion about the mean torsion angle of  $60^\circ$  (or its supplement  $120^\circ$ ). Our design strategy has entailed preassembling four junctions into a rhombus-like molecule, as shown in Figure 1c; each edge contains four double helical turns (42 nucleotide pairs) of DNA (see Figure 1d for the strand structure). This is a three-dimensional motif, where the helix axes of two parallel edges are contained in one plane and the helix axes of the other edges are contained in a parallel plane, roughly 2 nm away; only the crossover points between helices are expected to be coplanar. The use of the terms "rhombus" and "parallelogram" below refers to the shapes of these structures in projection. Each of the helices of the four-junction unit contains six turns of double helical DNA, so there is a one-turn overhang beyond the vertices (crossover points) of the rhombus-like molecule in each direction. The angles of the rhombus-like molecules in projection are the interhelical torsion angles of the immobile branched junctions. The motivation for combining them in this fashion is the notion that coupling them might focus the angular dispersion to a central value.

The strand structure in Figure 1d shows sticky ends in both directions, which can lead to two-dimensional assembly. It is also possible to make blunt ends in one direction (i.e., eliminating sticky ends C and D and their complements), so that the molecules can cohere only in one dimension. Figure 1e shows that rhombuses assembled into a one-dimensional array are separated from each other by small parallelograms. The edges of these parallelograms contain two and four double helical turns of DNA. The two-turn edges result from the sum of the two single-turn overhangs that extend beyond the vertices of the rhombuses. The overall arrangement resembles a railroad track, where the long helices are analogous to the rails and the short helices correspond to the ties. Figure 1f shows that this pattern persists in two-dimensional arrays, and that a smaller rhombus, whose edges contain two turns of DNA, is also present. Thus, the crystallographic repeat unit in two dimensions is a rhombus, whose edges are six helical turns in length. Each branch point (vertex) is flanked locally with the base sequence of the well-characterized J1 junction,<sup>15</sup> so there is no ambiguity about which strands are involved in the crossover, and which strands assume a helical conformation. The unique sticky ends used to link the objects (Figure 1d) contain five nucleotides each. We are able to visualize the one-dimensional lattice of Figure 1e and the two-dimensional lattices of Figure 1f with the atomic force microscope. Visualization of the two-dimensional lattice results in a bonus: It provides a direct measurement of the average torsion angle between the helical domains of the Holliday junction.

## Methods

**Molecular Design and Synthesis.** The sequences of the strands have been designed by the program SEQUIN,<sup>19</sup> and they are listed in Table 1. All DNA molecules in this study have been synthesized on an Applied Biosystems 380B automatic DNA synthesizer, removed from the support, and deprotected, using routine phosphoramidite procedures.<sup>20</sup> DNA strands have been purified by denaturing gel electrophoresis; bands were cut out of 12–20% denaturing gels and eluted in

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**Table 1.** Sequences of the Molecules Used in This Work<sup>a</sup>

<b>Strand 1:</b>
5'-GTATGCTGATAGACAATGAGTAGCTATTGGTATCAACGTTAAGTACCAGTGGACGAATCG-3' (63).
<b>Strand 2:</b>
5'-CAGTATGGACGTAGATACTGTGCTAACGATATTCGAACTAGCGTCATCGGACGATCAGAGACG-3' (63).
<b>Strand 3:</b>
5'-CATTGGTAGTGCCTGTAATAATGTTGACTGCGGTTACCGTACTAATTGCTGTACCTGAGTGG-3' (63).
<b>Strand 4:</b>
5'-TGACAGCCTGTCGAGTAGATCGTATGAATAGATGGCATCGCTGTAAATCCTGTGCACCTCAC-3' (63).
<b>Strand 5:</b>
5'-GTGACACACCGATGACGCTAGTTCGAATATCGTTAGCACAGTATCTACGTGGTACAGCAATTAGTACGGTAACCGCAGTCAACATTATTACACCTACAG-3' (100).
<b>Strand 6:</b>
5'-CTGATCGTGGATTTACAGCGATGCCATCTATTTCATACGATCTACTCGACACCACTGGTATCTTAACGTTGATCACC AATAGCTACTCATTGTGGCACTAC-3' (100).
<b>Strand 7:</b>
5'-CAATGCTCACTACCATACTGCGTCT-3' (26).
<b>Strand 8:</b>
5'-CATACCGATTGCTGGCTGTCAGTGAG-3' (26).
<b>Strand 1a:</b>
5'-CTGATAGGACAATGAGTAGCTATTGGTATCAACGTTAAGATACCA GTGGACGAATCGTTTTTCGATTGCTGGCTGTCAGTGAG-3' (83).
<b>Strand 2a:</b>
5'-CAATGCTCACTACCATAGAAATTCGTTTTTACGAATTCATGGACGTAGATACTGTGCTAACGATATTCGAACTAGCGTCATCGGACGATCAG-3' (93).
<b>Strand 3b:</b>
5'-CATTGGTAGTGCCTGTAATAATGTTGACTATGTTGTTGTGAACAGC ACTGCGGTTACCGTACTAATTGCTGTACCTGAGTGAG-3' (84).
<b>Strand 4b:</b>
5'-TGACAGCCTGTCGAGTAGATCTTCAGATTAGTTATCTGCTTGTATG AATAGATGGCATCGCTGTAATCCTGTGTCACCTCAC-3' (84).
<b>Strand 5b:</b>
5'-CTGACACACCGATGACGCTAGTTCGAATATCGTTAGCACAGTATCTACGTGGTACAGCAATTAGTACGGTAACCGCAGTGGTTCACAACCATAGTCAAACATTATTACACCTATCAG-3' (121).
<b>Strand 6b:</b>
5'-CTGATCGTGGATTTACAGCGATGCCATCTATTTCATACAAGCAGATAA CTGAATCTGAAGATCTACTCGACACCACTGGTATCTTAACGTTGATCA CCAATAGCTACTCATTGTGGCACTAC-3' (121).

<sup>a</sup> Strand numbers correspond to those shown in Figure 1d. The strand length is given in parentheses after the sequence. The components of the rhombus-shaped array  $(4 + 2) \times (4 + 2)$  consist of strands 1, 2, 3, 4, 5, 6, 7, and 8. The components of the one-dimensional array consist of strands 1a, 2a, 3, 4, 5, and 6. The components of the parallelogram-shaped array consist of strands 1, 2, 3b, 4b, 5b, 6b, 7, and 8.

a solution containing 500 mM ammonium acetate, 10 mM magnesium acetate, and 1 mM EDTA.

**Formation of Hydrogen-Bonded Complexes.** Complexes were formed by mixing a stoichiometric quantity of each strand (50 nM), as estimated by OD<sub>260</sub>, in 5 mM Hepes (pH 7.0), 2 mM MgCl<sub>2</sub>, and 0.5 mM EDTA. This mixture was cooled slowly from 90 °C to room temperature in a 1 L water bath.

**Denaturing Polyacrylamide Gel Electrophoresis.** Gels contained 3.5–20% acrylamide (19:1 acrylamide/bisacrylamide) and 8.3 M urea; they were run at 55 °C. The running buffer consisted of 89 mM Tris·HCl, pH 8.0, 89 mM boric acid, and 2 mM EDTA (TBE). The sample buffer consisted of 10 mM NaOH and 1 mM EDTA, containing 0.1% xylene cyanol FF tracking dye. Gels were run on a Hoefer SE 600 electrophoresis unit at 60 °C (31 V/cm, constant voltage). They were then dried onto Whatman 3MM paper and exposed to X-ray film for up to 15 h. Autoradiograms were analyzed on a BioRad GS-525 molecular imager.

**Enzymatic Reactions. A. Radioactive and Nonradioactive Phosphorylation.** A 1 pmol sample of an individual strand of DNA was dissolved in 20 μL of a solution containing 66 mM Tris·HCl, pH 7.6, 6.6 mM MgCl<sub>2</sub>, and 10 mM dithiothreitol (DTT), and mixed with 0.5 μL of 2.2 μM [ $\gamma$ -<sup>32</sup>P]ATP (10 mCi/mL) and 3 units of polynucleotide kinase (Amersham) for 80 min at 37 °C. Radioactive labeling was followed by the addition of 1 μL of unlabeled 10 mM ATP, and incubation proceeded for another 5 min. The reaction was stopped by heating the solution to 90 °C for 10 min, followed by gel purification.

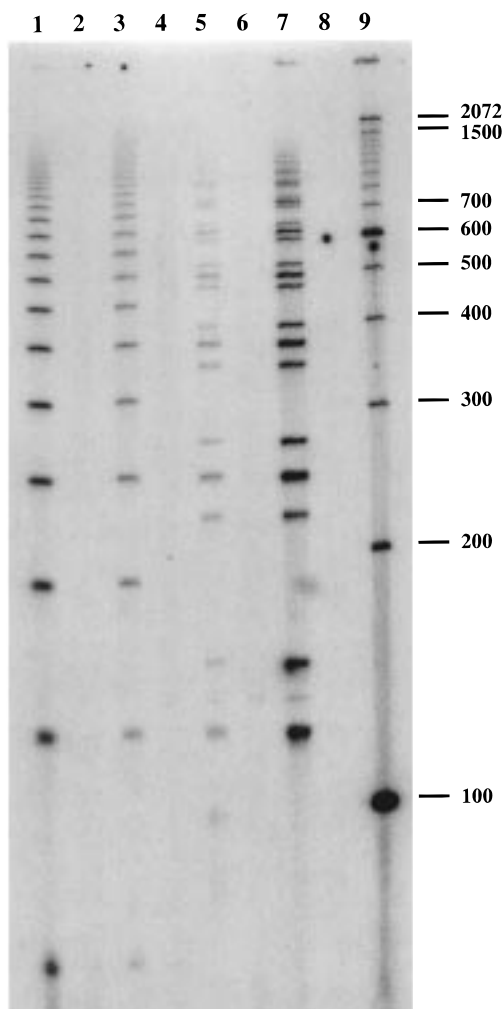
**B. Ligation.** Ligation reactions were performed in the phosphorylation buffer, which had been brought to 1 mM in ATP. All strands were mixed stoichiometrically (50 nM), and the solution was then heated to 90 °C for 7 min and cooled slowly to room temperature. Thirty units of T4 polynucleotide ligase (Amersham) were added, and the reaction was allowed to proceed at 16 °C for 16 h. The reaction was stopped by phenol/chloroform extraction. Samples were then ethanol precipitated.

**C. Exonuclease Treatment.** One hundred units of *E. coli* exonuclease III (exo III) (Amersham) were added directly to the ligation mixture, and the reaction was allowed to proceed for 0.5 h at 37 °C. The solution was heated to 90 °C for 5 min and cooled on ice for 2 min to generate single-stranded DNA. Ten units of *E. coli* exonuclease I (exo I) (Amersham) were added, and the digestion was continued for 0.5 h at 37 °C. The reaction was stopped by phenol extraction.

**Atomic Force Microscopy Imaging.** A 5 μL sample drop was spotted on freshly cleaved mica (Ted Pella, Inc.) and left to adsorb to the surface for 2 min. To remove buffer salts, 5–10 drops of doubly distilled water were placed on the mica, the drops were shaken off, and the sample was dried with compressed air. Imaging was performed under 2-propanol in a fluid cell on a NanoScope II, using commercial 200 μm cantilevers with Si<sub>3</sub>N<sub>4</sub> tips (Digital Instruments). The feedback setpoint was adjusted frequently to minimize the contact force to be approximately 1–5 nN.

## Results

**Ligation-Closure Experiments.** The ligation-closure experiment<sup>6–10</sup> is the way that we have previously determined whether a particular branched motif behaved qualitatively in a rigid or flexible fashion. Two sticky ends of the molecule are ligated, thereby oligomerizing the structure. The way in which these experiments are interpreted is by the inclusion of labeled reporter strands<sup>9</sup> within the motif; these are strands whose fates reflect the fate of the system. If reporter strands are found to be linear, the system has ligated linearly; if they are circular, the system has cyclized; furthermore, the sizes of reporter strands indicate the extent of ligation for both types of products. This system, too, contains reporter strands, those represented in Figure 1d as individual dark strands, as well as combinations of pairs of light strands. However, we have performed the ligation-closure experiment differently here. Formerly, we prepared a motif so that the only ligations that could occur would be those that included either the 5' or 3' end of the reporter strand. Other arms of the motif lack both sticky ends and phosphates. Such experiments report on the flexibility of the angles between those double helical arms containing the reporter strand. In the experiments performed here, all strands contain their sticky ends and phosphates, but only the reporter strand being examined is



**Figure 2.** Ligation-closure experiments performed on the rhombus-like molecule. This is an autoradiogram of a 3.5% denaturing polyacrylamide gel. Lane 9 contains a ladder at 100 base intervals. Lane 1 contains the ligated reporter strand 2 (Figure 1d), and lane 3 contains the ligated reporter strand 4. Strands 2 and 4 both contain 63 nucleotides, so the bands are simple multiples of 63 nucleotides. Lane 5 contains the strand pairs 6 and 7, and lane 7 contains the ligated strand pairs 5 and 8. Strands 5 and 6 each contain 100 nucleotides, but strands 7 and 8 contain 26 nucleotides; consequently the bands seen are triples, where the central band contains a multiple of 126 nucleotides, and it is flanked by two other bands containing either 26 more or 26 fewer nucleotides. The products of adding *exo I* and *exo III* to these lanes are shown in the even-numbered lanes adjacent to them (2–8, respectively). No cyclic material is evident in any of the even-numbered lanes.

labeled radioactively. This type of experiment will yield less information about the flexibility of the joints between the arms containing the reporter strand, but may be more appropriate for providing information about the behavior of the motif when it self-assembles into a periodic array.

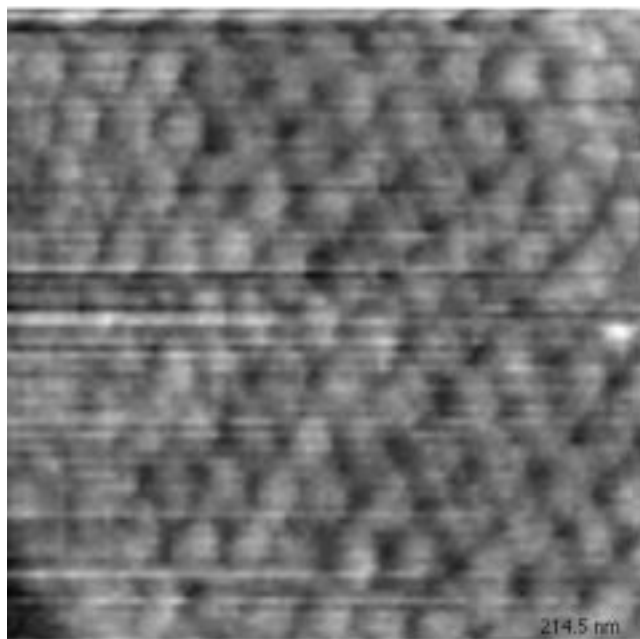
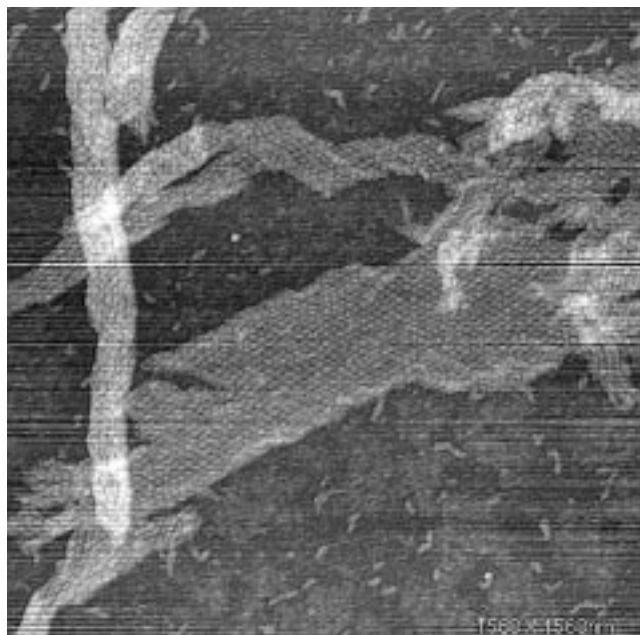
Figure 2 illustrates the results of the experiment. Lanes 1 and 3 contain the products of ligating the motif with labeled strands 2 and 4 (see Figure 1d). The ligations are quite extensive, producing products at least 20 units in length. Lanes 2 and 4 contain the results of digesting this material with *exo I* and *exo III*. No material is visible in those lanes, indicating that none of it is cyclic. Lanes 5 and 7 contain the results of ligating in the zigzag diagonal direction involving strands 6 + 7 and 5 + 8. An extensive pattern of multiple long strands plus one or two short strands is visible. Lanes 6 and 8 indicate that there is

no cyclic material produced by ligation in this direction either. Thus, the experiments suggest that the self-assembly of the rhombus-like motif will not lead to simple cyclic arrangements of matter parallel to the edges (the 10 or 01 direction) or the principal diagonal (the 11 direction). As with the double crossover molecule,<sup>11</sup> these experiments are suggestive of a successful motif for producing two-dimensional arrays, but not all arrangements can be excluded by these data. For example, a cylindrically curved sheet containing helical reporter strands would produce this same pattern. Direct observation of the array is necessary to confirm the nature of the association.

**Two-Dimensional Arrays.** We have assembled the rhombus-like molecule with all four sticky ends into a two-dimensional hydrogen-bonded array that we have examined by atomic force microscopy (AFM). Figure 3a shows a well-ordered lattice whose repeat distances ( $20.5 \pm 0.5$  and  $21.1 \pm 0.2$  nm) are in good agreement with the expected six helical turn repeat ( $63 \times 3.4 \text{ \AA} = 21.42$  nm) for this system. After the sample is deposited on the mica surface, both layers of DNA seem to adhere to it electrostatically, and become one layer. However, at the branch point, the two layers do not compress, so the image is higher (thicker) than at other positions. This image does not resolve the two double helices separated by two turns of DNA (7.1 nm), so the features seen are the merged helices; their observed widths of 8.7 and 8.8 nm are in agreement with this interpretation, 7.1 nm ( $21 \times 3.4 \text{ \AA}$ ) separation + two 1 nm overhangs (half a helical thickness) beyond the branch points. Figure 3b shows a zoom of a different array, one that is less extensive, but in which the two helices separated by the 7.1 nm distance are resolved.

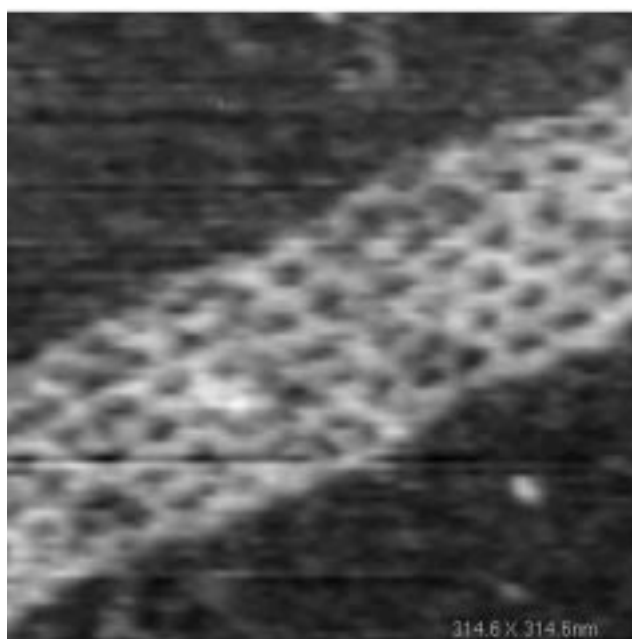
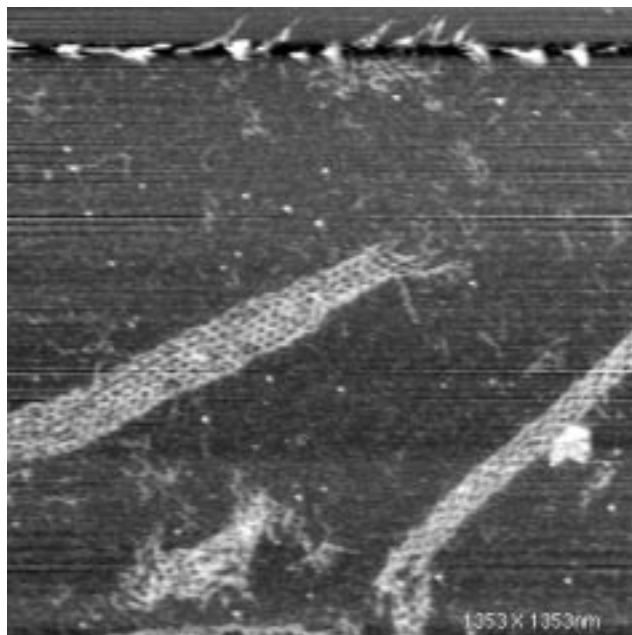
It is important to ensure that the image seen indeed derives from the design of the molecules we have assembled. A useful control that we have used previously<sup>11</sup> is to change the expected dimensions of visible features, and then to monitor the changes in the image. Here, we have altered one edge of the rhombus from four helical turns to six turns, thereby converting it to a six turn  $\times$  four turn parallelogram, and the repeat unit to eight turns  $\times$  six turns. This altered lattice is seen in Figure 4a. Its repeat units are now  $27.7 \pm 0.8$  and  $20.9 \pm 0.8$  nm. The longer dimension now corresponds to a repeat of eight helical turns ( $84 \times 3.4 \text{ \AA} = 28.56$  nm), in good agreement with our observation. This repeat unit seems to produce only narrow arrays. Figure 4b shows a higher magnification view of this same crystal.

**One-Dimensional Arrays.** As noted above, the rhombus-like molecule can be altered so as to contain only sticky ends A and A', and B and B' (see Figure 1d,e). When this modification is performed, hydrogen-bonded self-assembly can occur only in one dimension, to produce the railroad-track arrangement shown in Figure 1e. Figure 5a shows an AFM image containing the products of this one-dimensional assembly. Figure 5b shows a zoomed view of the boxed molecule in Figure 5a. The individual units are evident as regions of increased thickness, which appear whiter than their surroundings. These are likely to correspond to the "railroad ties". The array contains about 20 individual units, and is composed of two straight sections, with a kink in the middle. Inspection of the molecules visible in Figure 5a shows that all of them contain kink-like features. The kinks could result from the deposition procedure, or they could reflect a systematic feature of the one-dimensional arrangement. We are unable to distinguish between these two alternatives. We have observed no cyclic species in Figure 5 or in any other visualization of the one-dimensional arrays, suggesting that the rhombus-like molecules are also stiff.



**Figure 3.** AFM images of the assembled rhombus-shaped component (4 + 2 helices per repeat in each direction). (a, top) An image of dimensions  $1568 \times 1568$  nm. The unit cell is  $20.5 \pm 0.2$  and  $21.1 \pm 0.5$  nm, in good agreement with the expected dimensions. The features seen are fused helices, separated by two turns of double helix. (b, bottom) A zoomed view of a better-resolved but less extensive rhombus-shaped array. The narrow (two-turn) spacing between helices is evident in this view.

**The Torsion Angle between Helices.** The presence of an array of Holliday junction analogues provides a means of measuring the torsion angle between helical domains. Figure 6 shows the autocorrelation function of the array shown in Figure 3d. The averaging done to generate this image produces direct observation of the average angle, which we find to be  $63.5^\circ \pm 2.4^\circ$ . This estimate is in good agreement with the indirect estimates of the angle obtained by fluorescence measurements,<sup>16,18</sup> from calculations,<sup>17</sup> and from crystal structures of related systems.<sup>21</sup> We have shown in a related system (R. Sha,



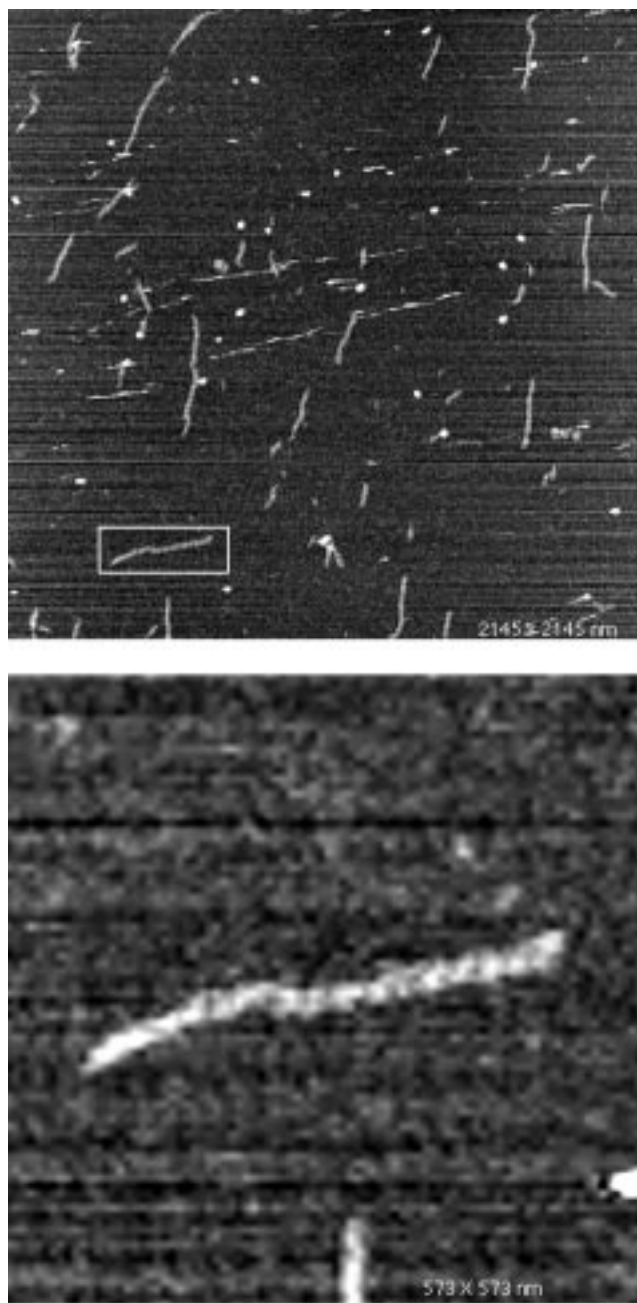
**Figure 4.** AFM images of the assembled parallelogram-shaped component (4 + 2 helices per repeat by 6 + 2 helices per repeat) (a, top) An image of dimensions  $1353 \times 1353$  nm. (b, bottom) A zoom to  $315 \times 315$  nm. The observed unit cell is  $27.7 \pm 0.8$  and  $20.9 \pm 0.8$  nm, in good agreement with the model.

F. Liu, and N. C. Seeman, manuscript in preparation) that the interaction of the DNA with the mica surface is not a serious perturbation on the interhelical angle, because the same values are obtained when the sample is deposited on glow discharge-treated graphite. It is important to point out that we know the sign of the angle, because we can tell which helix domain is above the other, but we cannot distinguish whether the domains are closer to parallel or to antiparallel.

## Discussion

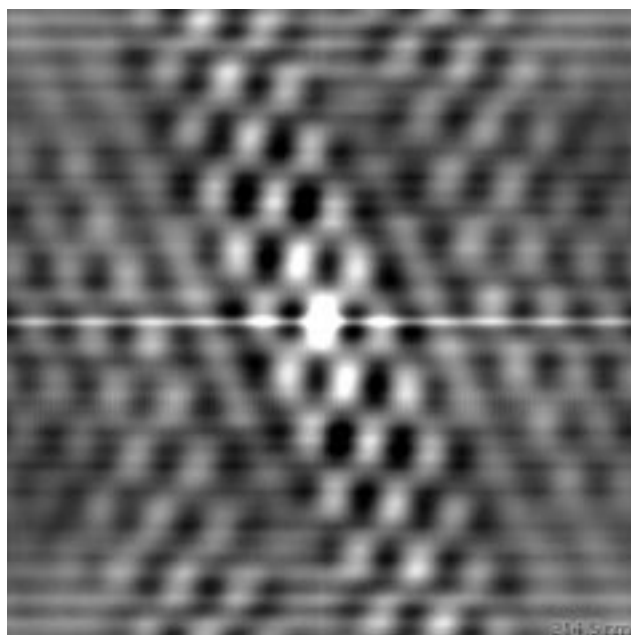
**Array Formation.** The results presented here demonstrate that coupled, but unconstrained, Holliday junction analogues can be condensed into periodic arrays via hydrogen-bonded interactions. This study differs from previous work with

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**Figure 5.** AFM images of the one-dimensional assembly of the rhombus-like molecule. (a, top) A large field of molecules. Numerous linear arrays are evident as white streaks, roughly 200–400 nm in length. They are primarily linear molecules, but all appear to contain at least one kink. (b) A zoomed view of the boxed molecule in (a). About 20 individual units are visible; there is a prominent kink near the middle of the molecule.

immobile branched junctions in that ligation has been avoided, and only hydrogen-bonded interactions have been utilized to stabilize the lattice. A reason for the success of the hydrogen-bonding approach may be that the array is not constrained tightly to respond to deviations from planarity that would be enforced by twist. Singly ligated arrays (one bond at each sticky end) also can be visualized successfully (data not shown), but doubly ligated arrays appear to curl. The uniform heights of comparable features in all two-dimensional arrays visualized suggest that they are planar in the context of deposition on mica; we cannot comment on the possible existence of deviations from planarity in solution.



**Figure 6.** Two-dimensional autocorrelation function of the rhombus-based lattice. This function has been calculated from the image shown in Figure 3d. The observed angle between helical domains is  $63.5 \pm 2.4^\circ$ , in good agreement with previous indirect estimates.

These results demonstrate the power of the DNA self-organization. By specific design, the units used here can self-assemble into well-defined structures with tunable parameters. Possible applications of the simplicity and universality offered by this system range from molecular electronics<sup>22</sup> to the determination of the structures of biological macromolecules<sup>2,23</sup> to DNA-based computing.<sup>24,25</sup> Unlike the system we have reported previously,<sup>11</sup> these arrays contain large unoccupied cavities with controlled dimensions; these empty spaces could be used to host covalently or noncovalently attached macromolecular guests of linear dimension 10–20 nm. It may be possible to extend the strategy used here to three-dimensional crystal design.<sup>2</sup>

**Ligation-Closure Experiments.** The revised protocol used for the ligation-closure experiments performed here produced results markedly different from those of the original ligation-closure experiments performed on four-arm branched junctions.<sup>8</sup> In those experiments, only the two arms involving the reporter strand contained phosphates or even sticky ends. Ligation experiments generated small cyclic molecules, such as trimers and tetramers as prominent products of the experiment, regardless of whether the reporter strand was a helical strand or a crossover strand. We have repeated those experiments, and get the same results (data not shown). The experiments reported here differ from those experiments in two ways: (1) All arms contain both sticky ends and phosphates, and (2) the repeat unit is the rhombus-like molecule, rather than a single four-arm branched junction. To remove one of these two differences, we have repeated the original ligation-closure experiments on a single J1-derived four-arm branched junction in which all arms contain sticky ends and phosphates. In this case, few cyclic products are produced by the helical strands, but they are produced readily by the crossover strands (data not shown). This

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finding is in line with expectations based on the well-defined crossover isomer<sup>15</sup> and strong crossover bias,<sup>26</sup> known for J1. We conclude that the approach to ligation-closure experiments used here is more appropriate for assessing the flexibility of branched junctions in assembly processes.

**Torsion Angle Measurement.** We have measured the average torsion angle between double helical domains in a Holliday junction analogue. This is a direct structural observation, one that does not rely on inferences from less direct techniques, such as fluorescence resonance energy transfer.<sup>16,18</sup> This is a new method for measuring well-defined torsion angles between the arms of branched molecules. It is worth emphasizing that there are no apparent forces in this system to distort the torsion angle, as long as the edge lengths are compatible with an integral number of full turns. For example, we have also measured the angle in the parallelogram system, and find it to be  $62.6 \pm 1.6^\circ$ , the same value within experimental error. It may also be possible to use it for measuring interhelix angles in planar molecules, such as the four-arm branched junction in low salt conditions.<sup>27</sup> In work to be reported elsewhere, we have measured the torsion angle between the helical domains of the

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bow tie<sup>28</sup> junction (R. Sha, F. Liu, and N. C. Seeman, manuscript in preparation). The key requirements for successful application of the method are (1) that the angles be well-defined, (2) that the angles or torsion angles about the point of interest sum to  $360^\circ$ , and (3) that the helix axes of opposite arms flanking the angle of interest are roughly collinear. The need for the first requirement is self-evident for a meaningful measurement; the second requirement derives from the necessity of having a planar (or pseudoplanar, as we had here) system so that measurements do not represent a distortion owing to projection. The third requirement enables one to tile the plane in a fashion similar to that used here, so that the angle between arms is the unit cell angle, leading to useful averaging by Fourier methods.

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