Electronic Supplementary Information

Fabrication of Multi-layered DNA Nanostructures Using Single-strand and Double-crossover Tile Connectors†

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Materials and Methods

DNA lattice fabrication. Synthetic oligonucleotides purified via High-Performance Liquid Chromatography (HPLC) were purchased from Bioneer (Daejeon, Korea). The DNA nanostructures were formed by mixing a stoichiometric quantity of each strand in physiological 1× TAE/Mg^{2+} buffer [40 mM Tris, 20 mM Acetic acid, 1 mM EDTA (pH 8.0), and 12.5 mM magnesium acetate]. We adopted a SAG method with mica as a substrate to form the base layer. This method involved three steps: seeding, nucleation and finally lattice structure formation. The electrostatic interaction between the DNA tiles and the mica sheets created a constructive environment for lattice formation, and as depicted in Figure 1-(c), a number of unit tiles laid the foundation of the structure by randomly seeding on the mica substrate. Subsequently, the complementary sticky ends hierarchically assembled from the seeding tiles, resulting in lattice growth. A multi-step annealing method was employed to construct the double-layer and multi-layered structure. However, we used a free-solution annealing method to form individual tiles.

Free-solution annealing to form SAC, DAC, DGT, EL tiles (Tiles of single strand-based design) and DXA_1, DXB_1 (Tiles of DX-based design). 200 nM each of SAC, DAC, DGT, EL tiles and DXA_1, DXB_1 tiles were formed by mixing a stoichiometric quantity of each of their strands in 1× TAE/Mg^{2+} buffer. These tile strand mixtures were annealed in a test tube by slowly cooling from 95 down to 25 °C in a Styrofoam box**.**

Mica-assisted growth (MAG) to form the base layer. A 50 nM base layer was formed by mixing a stoichiometric quantity of each strand of two pair of tiles, SGT, EB and DXA_1, DXB_0 in case of single strand-based and DX tile-based design, respectively, with cleaved mica substrate (5 × 5 mm^2) in 1× TAE/Mg^{2+} in a test tube, as shown in Figure 1-(c). To facilitate selective hybridization, the mixture of the two tile strands with the mica substrate was annealed in a test tube by slowly cooling them from 95 down to 25°C for 24 hours in a Styrofoam box containing 2 L of boiled water. This resulted in the formation of base layer.

Multi-step annealing to form double-layer and multi-layered structures. The mica substrate of the pre-formed base layer was inserted in a new test tube containing 50 nM of pre-annealed layering and connector tiles at a volume of 200 µL. These samples were then cooled from 42 to 25 °C in a Styrofoam box for 24 hours to facilitate DNA hybridization for double- and multi-layer formation [Figure 1-(d)]. At the end of each annealing step, the sample was incubated overnight at 4°C to ensure structural stability.

AFM imaging. AFM imaging of the samples prepared via MAG involved taking the mica substrate from a test tube and placing it on a metal puck with instant glue. Then, 30 µL of 1× TAE/Mg^{2+} buffer were pipetted onto the substrate, and another 20 µL of 1× TAE/Mg^{2+} were dispensed onto a silicon nitride AFM tip (Veeco Inc. USA). Imaging was carried out for the samples prepared via free-solution annealing by dropping 5 µL of DNA sample on freshly cleaved mica surface. 30 µL of 1× TAE/Mg^{2+}

were then placed onto the mica, and another 20 μL of 1× TAE/Mg^{2+} were placed onto the silicon nitride AFM tip. AFM images were then obtained by using a Multimode Nanoscope (Veeco Inc., USA) in the fluid tapping mode.

Table S1. Schematics of different tiles with their respective strands involved in the assembly of DX multi-layer nanostructure using single-strand DNA as a connector. The name of each strand and the sticky-end sequences are indicated as well.

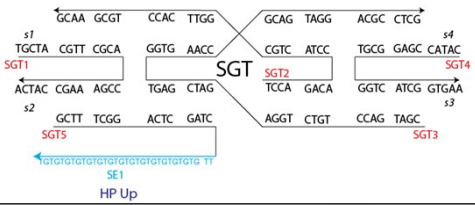
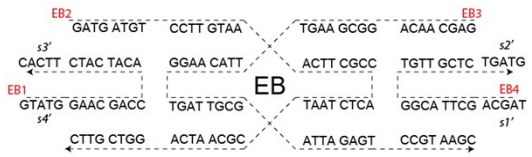
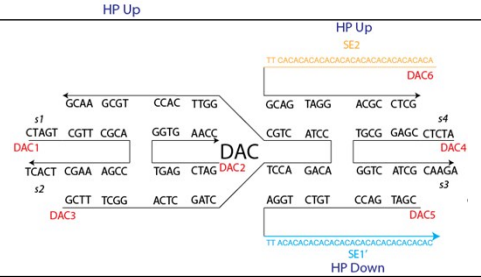
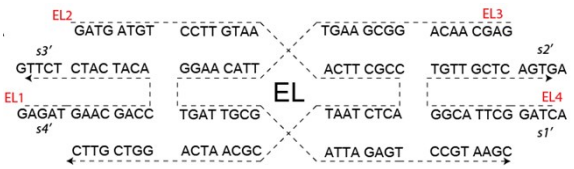
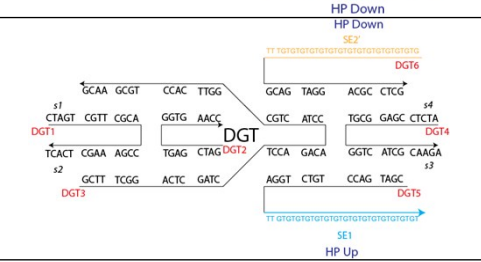
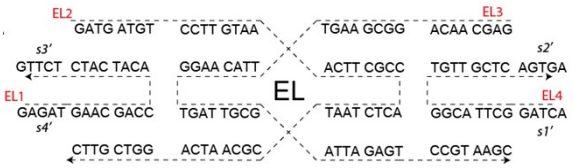
Layer	Tile Scheme	
Base-layer		
Double-layer		
Multi-layer		

Table S2. Details of the names of the strands, number of bases in each strand, and base sequence composition from the 5' to 3' direction required for multi-layer assembly.

Strand	Base	Sequence (5' to 3')
SGT		
SGT1	26	**TGCTA**CGTTCGCACCGAAAGC**CATCA**
SGT2	32	TCCAGACACCTACTGCGGTTCACCTGCGAACG
SGT3	48	CGATGACCTGTCTGGAGATCGAGTGGTGAACCGCAGTAGGACGCCTCG
SGT4	26	**CATCA**CGAGGCGTGGTCATCG**GTGAA**
SGT5	48	GCTTTCGGACTCGATCTTGTGTGTGTGTGTGTGTGTGTGTGTGTGT
EB		
EB1	26	**GTATG**GAACGACCACATCATC**TTCAC**
EB2	48	GATGATGTCCTTGTAAACTTCGCCACTCTAATCGCAATCAGGTCGTTC
EB3	48	GAGCAACAGGCGAAGTTTACAAGGTGATTGCGATTAGAGTCCGTAAGC
EB4	26	**TAGCA**GCTTACGG TGTTGCTC**TGATG**
EL		
EL1	26	**GAGAT**GAACGACCACATCATC**TCTTG**
EL2	48	GATGATGTCCTTGTAAACTTCGCCACTCTAATCGCAATCAGGTCGTTC
EL3	48	GAGCAACAGGCGAAGTTTACAAGGTGATTGCGATTAGAGTCCGTAAGC
EL4	26	**ACTAG**GCTTACGGTGTTGCTC**AGTGA**
DAC		
DAC1	26	**CTAGT**CGTTCGCACCGAAAGC**TCACT**
DAC2	16	GATCGAGTGGTGAACC
DAC3	48	GCTTTCGGACTCGATCTCCAGACACCTACTGCGGTTCACCTGCGAACG
DAC4	26	**ATCTC**CGAGGCGTGGTCATCG**CAAGA**
DAC5	48	CGATGACCTGTCTGGATTACACACACACACACACACACACACACAC
DAC6	48	ACACACACACACACACACACACACACACACACTTGCAGTAGGACGCCTCG
DGT		
DGT1	26	**CTAGT**CGTTCGCACCGAAAG**CTCACT**
DGT2	16	GATCGAGTGGTGAACC
DGT3	48	GCTT TCGGACTCGATCTCCAGACACCTACTGCGGTTCACCTGCGAACG
DGT4	26	**ATCTC**CGAGGCGTGGTCATCG**CAAGA**
DGT5	48	CGATGACCTGTCTGGATTGTGTGTGTGTGTGTGTGTGTGTGTGTGT
DGT6	48	GTGTGTGTGTGTGTGTGTGTGTGTGTGTGTTTGCAGTAGGACGCCTCG

Table S3. Schematic representation of two tiles (A-tile and B-tile) and their respective strands required to construct 3D-T based nanostructures in which a DX tile is used as a connector. (a) DXA_1 tile, (b) DXB_0 tile (connector tile), and (c) DXB_1 tile. Insets in (a) and (c) show the 3D view of A-tile and B-tile, respectively. The sticky ends and their sequences are represented in green and in red, respectively.

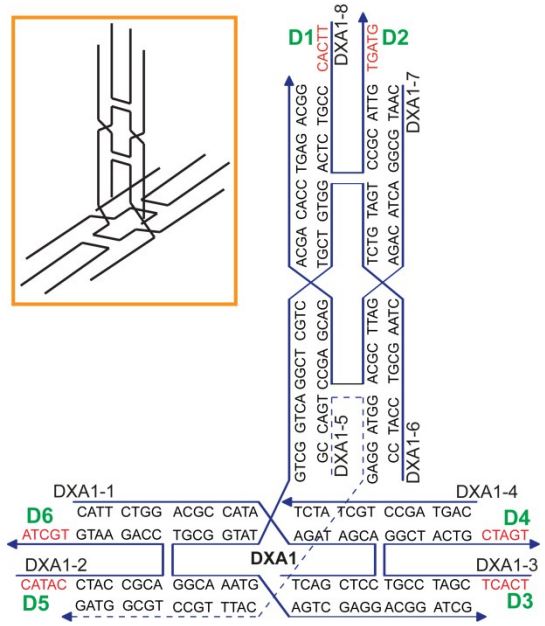
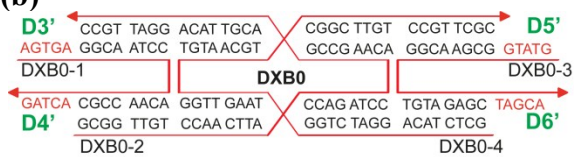
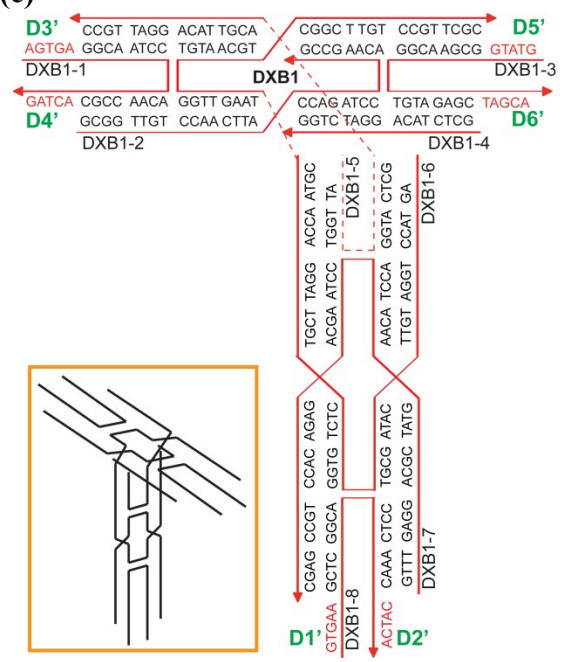
A-Tile	B-Tile
(a) 	**(b)** 
	(c) 

Table S4. Details of the names of the strands, number of bases, and sequence composition from 5' to 3' direction required for multi-layer assembly.

Strand	Base	Sequence (5' to 3')
DXA1		
DXA1-1	32	CATTCTGGACGCCATAAGATAGCACCTCGACT
DXA1-2	26	**CATAC**CTACCGCACCAGAATG**TGCTA**
DXA1-3	26	**TCACT**CGATCCGTGGCTACTG**CTAGT**
DXA1-4	16	CAGTAGCCTGCTATCT
DXA1-5	30	GCCAGTGGTAGGAGCATTTGCCTGCGGTAG
DXA1-6	78	CCTACCTGCGAATCTCTGTAGTGGTGTCGTCTGCTCGGACTGG CTGTATGGCGTGGCAAATGAGTCGAGGACGGATCG
DXA1-7	48	CAATGCGGACTACAGAGATTCGCACCGAGCAGACGACACCTG AGACGG
DXA1-8	26	**TTCAC**CCGTCTCACCGCATTG**TGATG**
DXB0		
DXB0-1 (=DXB1-1)	26	**AGTGA**GGCAATCCACAACCGC**ACTAG**
DXB0-2	48	GCGGTTGTCCAACTTACCAGATCCACAAGCCACGTTACAGGAT TGCC
DXB0-3 (=DXB1-3)	26	**GTATG**GCGAACGGTGTAGAGC**TAGCA**
DXB0-4	48	GCTCTACAGGATCTGGTAAGTTGGTGTAACGCGGCTTGTCCGT TCGC
DXB1		
DXB1-2	32	GCGGTTGTCCAACTTACCAGATCCACAAGCCG
DXB1-4	16	GCTCTACAGGATCTGG
DXB1-5	30	ATTGGTGGTACTCGACGTTACAGGATTGCC
DXB1-6	78	AGTACCTGGATGTTCATAGCGTGGTGTCTCTCTTAGGACCA ATGCTAAGTTGGTGTAACGTGGCTTGTCCGTTCGC
DXB1-7	48	GTTTGAGGACGCTATGAACATCCACCTAAGCGAGACACC TGCCGAGC
DXB1-8	26	**GTGAA**GCTCGGCACCTCAAAC**CATCA**

Figure S1. A 2% agarose gel image (ethidium bromide stained) showing association complexes of equimolar combination (200 nM) of various DNA cross-tiles in order to check the purities of DNA tiles involved in the assembly of multi-layered DNA nanostructures. DNA tiles were loaded in the gel as; EB (148 nt), EL (148), SGT (180), SAC (180), DAC (212), DGT (212), DXB0 (148), DXA1 (284), and DXB1 (284) in the lanes 2, 3, 4, 5, 6, 7, 8, 9, and 10, respectively. 25 bp DNA Ladders were shown in lanes 1 and 11.

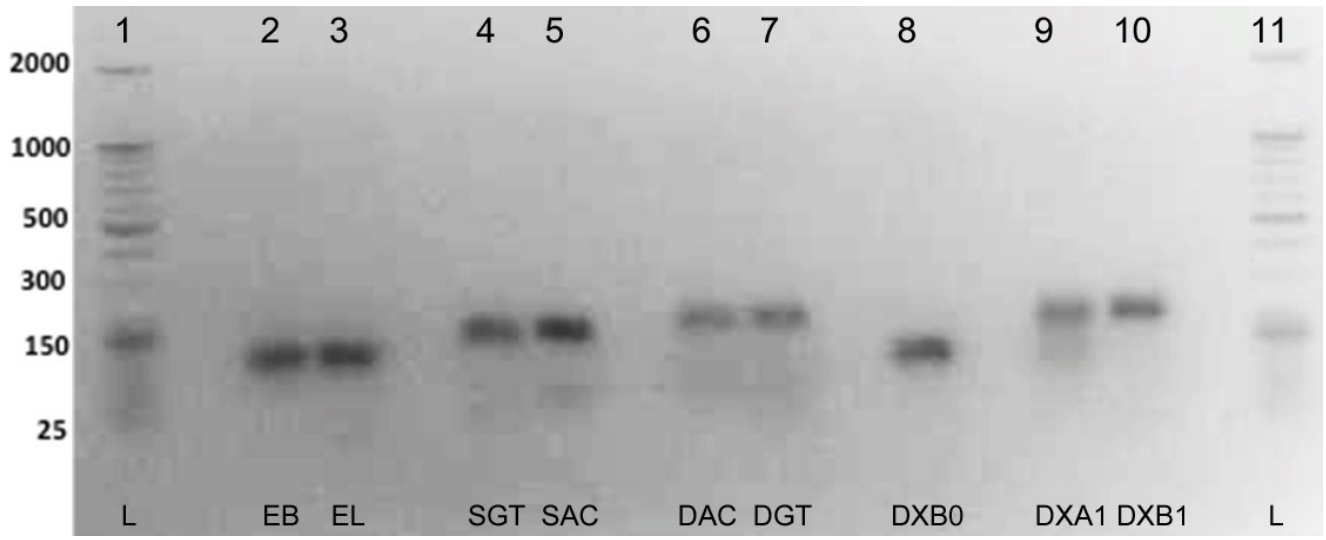


Figure S2. AFM image and height profile of the multi-layer assembly comprised of layering DXA_1 and DXB_1 tiles obtained via scratch method (contact mode AFM operation). Scale bar in an image is 1 μm.