Programmable self-assembly of three-dimensional nanostructures from 10,000 unique components

Contents

S1	Ma	terials an	d Methods	1
	S1.1	Design	and formation of Structures	1
	S1.2	Charact	terization of Structures	1
		S1.2.1	Agarose gel electrophoresis and purification	1
		S1.2.2	Electron microscopy imaging	2
		S1.2.3	3D DNA-PAINT super-resolution setup	2
		S1.2.4	Sequencing sample preparation and analysis	5
S2	Str	and desig	n schematic	6
S 3	Мо	tif charac	terization	7
	S3.1	Domain	n uniqueness analysis	8
	S3.2	Thermo	by namic analysis of interaction	8
	S3.3	Compa	rison of structures with a similar number of strands	10
		S3.3.1	Comparison of $6H \times 6H \times (8$ -domain) structure with domains of length 8-nt, 13-nt, and 18.5-nt	10
		\$3.3.2	Comparison of assembly efficiency under varying reaction times for 32-nt, 52-nt, and 74-nt DNA brick	
			structures	10
		S3.3.3	Strand diagram of the $6H \times 6H \times 64B$ structure composed of 32-nt DNA bricks	12
		S3.3.4	Strand diagram of the $6H \times 6H \times 104B$ structure composed of 52-nt DNA brick	13
		\$3.3.5	Strand diagram of the $6H \times 6H \times 148B$ structure composed of 74-nt DNA bricks	14
	S3.4	Compa	rison of the $6H \times 6H \times 104B$ structure composed of 32-nt versus 52-nt DNA bricks	15
		S3.4.1	Formation differences of the $6H \times 6H \times 104B$ structures	15
		\$3.4.2	Thermal stability of $6H \times 6H \times 104B$ structures assembled with 32-nt and 52-nt DNA bricks	16
		\$3.4.3	Annealing/Melting curves of 32-nt versus 52-nt DNA brick $6H \times 6H \times 104B$ structure	17
	625	53.4.4 Maniant	Strand diagram for the 6H×6H×104B structure composed of 32-nt DNA bricks	18
	33.3		S of the 52-fit DNA blick $OH \times OH \times 104B$ structure composed of variations of the 52 nt DNA briefs	19
		SS.J.1 S3 5 2	Strand diagram for the $6H \times 6H \times 104B$ structure composed of alternating 52 nt DNA bricks	20
		S3.5.2 S3.5.3	Strand diagram for the 52 nt $6H \times 6H \times 104B$ structure composed of alternating 52-iit DIVA blocks	20
		55.5.5	Strand diagram for the 52-nt off × 011× 10+D structure containing boundary strands	21
S4	An	nealing pi	rotocol optimization	22
	S4.1	Anneal	ing ramp optimization	22
	S4.2	Reactio	on time optimization	23
	S4.3	Format	ion temperature range	24
S 5	Gel	results a	nd TEM images of fully addressable cuboids	25
	S5.1	Gel res	ults and TEM images for the 4.3 MDa (8H×8H×104B) origami structure	25
		S5.1.1	Gel electrophoresis of 8H×8H×104B origami structure	25
		S5.1.2	Wide field view of $8H \times 8H \times 104B$ origami structure	26
		S5.1.3	Select particles of the $8H \times 8H \times 104B$ origami structure	27
	S5.2	TEM ir	nages for the 10.1 MDa ($10H \times 10H \times 156B$) structure $\dots \dots \dots$	29
		\$5.2.1	Wide field views of the $10H \times 10H \times 156B$ structure	29
		S5.2.2	Select particles of the $10H \times 10H \times 156B$ structure	30

	\$5.2	TEM in	pages for the 26.5 MDe $(14H)(14H)(200P)$ structure 2	2
	35.5		$\frac{1}{3} = \frac{1}{3} = \frac{1}$	2
		55.5.1	whether the set of the $14H \times 14H \times 208B$ structure	2
		\$5.3.2	Select particles of the $14H \times 14H \times 208B$ structure	3
	\$5.4	TEM in	nages for the 67.6 MDa $(20H \times 20H \times 260B)$ structure	5
		S5.4.1	Wide field views of the $20H \times 20H \times 260B$ structure	5
		\$5.4.2	Select particles of the $20H \times 20H \times 260B$ structure	6
	S5.5	TEM in	nages for the 152.1MDa ($30H \times 30H \times 260B$) structure $\ldots \ldots 3$	8
		S5.5.1	Wide field views of the $30H \times 30H \times 260B$ structure	8
		S5.5.2	Select particles of the 30H×30H×260B structure	9
	S5.6	TEM in	nages for the 262.8 MDa (36H×36H×312B) structure	1
		S5.6.1	Wide field views of the 36H×36H×312B structure	1
		\$5.6.2	TEM images of broken 36H×36H×312B structures	-2
		\$5.6.3	Select particles of the 36H×36H×312B structure	.3
		\$5.6.4	Particle size measurement distribution 4	.5
	S5 7	TEM in	pages for the 351 5 MDa $(40H \times 40H \times 338B)$ structure 4	.6
	55.7	S5 7 1	Wide field views of the $40H \times 40H \times 338B$ structure	.6
		S5 7 2	Salect particles of the $\Lambda 0H \times \Lambda 0H \times 338B$ structure	7
	\$5.8	Design	of the 536 4 MD ₂ ($A6H \times A6H \times 300$ B) structure	0
	55.0	TEM :-	of the 536.4 MDa $(4611 \times 300B)$ structure 5	2
	35.9		$\frac{1}{3} \frac{1}{3} \frac{1}$	0
		55.9.1	whether the state of the 40H × 40H × 390B structure	0
		\$5.9.2	Select particles of the 46H×46H×390B structure	1
56	Too	allations	5	3
30	S6 1	Upod to	band dimara	3
	30.1		Piedu uniters	2
		50.1.1	Diagram of head-to-head dimens	Э 1
		S0.1.2	Get electrophoresis of the head-to-nead dimers	4
	060	\$6.1.3	IEM images of head-to-head dimers	4
	\$6.2	Tail-to-	tail dimers	5
		\$6.2.1	Strand diagram of tail-to-tail dimers	5
		\$6.2.2	Gel electrophoresis of the tail-to-tail dimers	6
		\$6.2.3	TEM images of tail-to-tail dimers 5	6
	S6.3	Side-to-	-side dimers	7
		S6.3.1	Strand diagram of side-to-side dimers	7
		\$6.3.2	Gel electrophoresis of the side-to-side dimers	8
		S6.3.3	TEM images of side-to-side dimers	8
	S6.4	Rotatio	nal tetramers	9
		S6.4.1	Strand diagram of rotational tetramers	9
		S6.4.2	Gel electrophoresis of a tetramer comprised of 6H×6H×104B units	0
		S6.4.3	TEM images of a tetramer comprised of $6H \times 6H \times 104B$ units $\dots \dots \dots$	0
		S6.4.4	Strand diagrams of a tetramer composed of $36H \times 36H \times 312B$ units	1
		S6.4.5	TEM images of a tetramer comprised of 260 MDa units	4
		S6.4.6	Select particles of a tetramer composed of $36H \times 36H \times 312B$ units	5
				-
S7	3D	DNA-PAI	NT analysis of the 30H×30H×260B canvas 6	6
	S7.1	Design	of DNA-PAINT extensions	6
		\$7.1.1	3D DNA-PAINT imaging of the 30H×30H×260B structure	7
S8	The	Nanobrio	cks Software 6	8
	S8.1	Overvie	ew	8
	S8.2	Using N	Vanobricks	9
		S8.2.1	Interface	9
		S8.2.2	Help guides	9

		\$8.2.3	Design Flow	59 70
		S0.2.4		70
	a 0, 0	58.2.5		10
	\$8.3	Tools .		/1
		\$8.3.1	Pointer	/1
		S8.3.2	Select	12
		S8.3.3	Rectangle	12
		S8.3.4	Strand	12
		S8.3.5	Strand Eraser	12
		S8.3.6	Strand Extender	72
		S8.3.7	Cut Strands	72
		S8.3.8	Ligate Strands	72
		S8.3.9	Add Voxels	72
		S8.3.10	Power Edit	72
		S8.3.11	Voxels to Strands	74
		S8.3.12	Generate Sequences	74
		\$8.3.13	Change Lattice	74
	S8.4	Workin	g with files	75
	5011	S8 4 1	Importing 3D objects	75
		S8 4 2	Importing and exporting caDNAno files	75
		S8 4 3	Saving a Nanobricks file	75
		S8 / /	Dluging	76 76
	5 9 5	Archita	riugins	76
	30.5			70 76
		50.5.1	Overview	10
		50.J.Z	Modules	10
		58.5.5		11 77
		58.5.4		11
		58.5.5		11 77
		58.3.0		//
S9	Sha	pe design	1 7	78
	S9.1	Designs	s	78
	S9.2	Shape a	approximation	79
	S9.3	3D rend	lering import files	30
	S9 4	Mathen	natical scrinting	R 1
	57.1	S9 4 1	Möbius Strin	21 21
		S9.4.1	Hyperboloid	21
		S9.4.2	Helicoid	27
	\$0.5	Soloot 7	FEM images used for averaging	22 22
	39.5	SQ 5 1	General observations	30
		57.5.1		10
S1	0 Eleo	ctron tom	ography 9)1
	S10.1	l Electro	n tomography analysis of the parallel channels structure) 3
	S10.2	2 Electro	n tomography analysis of the teddy bear cavity structure) 5
	S10.3	3 Electro	n tomography analysis of the bunny cavity structure	99
	S10.4	4 Electro	n tomography analysis of the helicoid cavity structure	3
	S10.5	5 Electro	n tomography analysis of the 'GEB' cavity structure)7
S1	1 Seq	uencing a	analysis 11	12
	S11.1	l Sequen	cing read analysis	12
	S11.2	2 Strand	categorization	12
	S11.3	3 Strand	visualization	13

S11.4	Voxel visualization					 																				•	 •			113
S11.5	2D projections	· •	•		• •	 •		•	•	•	•	•	•	•	•	•	•			•	•	•		•	•	•	 •			113

List of Figures

1	Distance measurements of the 30H×30H×260B cuboid using 3D DNA-PAINT	4
2	Detailed schematic for the 52-nt DNA brick structure	6
3	Domain sequence match analysis for varying numbers of randomly assigned domains	8
4	Gel electrophoresis of a 6H×6H×(8-domain) structure with domains of length 8-nt, 13-nt, and 18.5-nt	10
5	Gel yields and rate of change at varying times during structure assembly	11
6	Strand diagram for the 6H×6H×64B structure composed of 32-nt DNA bricks	12
7	Strand diagram for the 6H×6H×104B structure composed of 52-nt DNA brick	13
8	Strand diagram for the 6H×6H×148B composed of 74-nt DNA bricks	14
9	Gel electrophoresis of a 6H×6H×104B structure composed of 32-nt versus 52-nt DNA bricks	15
10	Thermal stability of purified 6H×6H×104B structures assembled with 32-nt and 52-nt DNA bricks	16
11	Annealing/Melting curves of 32-nt versus 52-nt 6H×6H×104B structure	17
12	Strand diagram for the 6H×6H×104B structure composed of 32-nt DNA bricks	18
13	Gel electrophoresis of a $6H \times 6H \times 104B$ structure composed of variations of the 52-nt DNA bricks	19
14	Strand diagram for the $6H \times 6H \times 104B$ structure composed of alternating 52-nt DNA bricks	20
15	Strand diagram for the 52-nt $6H \times 6H \times 104B$ structure containing boundary strands	21
16	Annealing ramp optimization	22
17	Assembly efficiency of the $30H \times 30H \times 260B$ cuboid across different reaction times	23
18	Range of temperature formation	24
19	Gel electrophoresis of 8H×8H×104B origami structure	25
20	TEM image of the $8H \times 8H \times 104B$ origami structure	26
21	Select helical TEM images of the $8H \times 8H \times 104B$ origami structure.	27
22	Select lateral TEM images of the 8H×8H×104B origami structure	28
23	TEM images of the $10H \times 10H \times 156B$ structure.	29
24	Select helical TEM images of the 10H×10H×156B structure	30
25	Select lateral TEM images of the 10H×10H×156B structure	31
26	TEM images of the $14H \times 14H \times 208B$ structure	32
27	Select helical TEM images of the 14H×14H×208B structure	33
28	Select lateral TEM images of the 14H×14H×208B structure	34
29	TEM images of the $20H \times 20H \times 260B$ structure	35
30	Select helical TEM images of the 20H×20H×260B structure	36
31	Select lateral TEM images of the 20H×20H×260B structure	37
32	TEM images of the $30H \times 30H \times 260B$ structure	38
33	Select helical TEM images of the 30H×30H×260B structure	39
34	Select lateral TEM images of the 30H×30H×260B structure	40
35	TEM images of the $36H \times 36H \times 312B$ structure	41
36	TEM images of the broken 36H×36H×312B structures	42
37	Select helical TEM images of the 36H×36H×312B structure	43
38	Select lateral TEM images of the 36H×36H×312B structure	44
39	Histogram of measured edge lengths for the 36H×36H×312B particles	45
40	TEM images of the $40H \times 40H \times 338B$ structure	46
41	Select helical TEM images of the 40H×40H×338B structure	47
42	Select lateral TEM images of the 40H×40H×338B structure	48
43	<i>Nanobricks</i> design of the 46H×46H×390B structure	49
44	TEM images of the 46H×46H×390B structure	50
45	Select helical TEM images of the 46H×46H×390B structure	51
46	Select lateral TEM images of the 46H×46H×390B structure	52
47	Design schematic for the head-to-head dimerization	53
48	Gel electrophoresis of a head-to-head dimer composed of 6H×6H×104B units	54
49	TEM images of a head-to-head dimer comprised of $6H \times 6H \times 104B$ units	54
50	Design schematic for the tail-to-tail dimerization	55

51	Gel electrophoresis of a tail-to-tail dimer composed of 6H×6H×104B units	56
52	TEM images of a tail-to-tail dimer comprised of 6H×6H×104B units	56
53	Design schematic for the side-to-side dimerization	57
54	Gel electrophoresis of a side-to-side dimer composed of $6H \times 6H \times 104B$ units	58
55	TEM images of a side-to-side dimer comprised of $6H \times 6H \times 104B$ units	58
56	Design schematic for the rotational tetramers	59
57	Gel electrophoresis of a tetramer comprised of $6H \times 6H \times 104B$ units	60
58	TEM images of a tetramer comprised of $6H \times 6H \times 104B$ units	60
59	<i>Nanobricks</i> diagram of a tetramer comprised of $36H \times 36H \times 312B$ units	61
60	Cross-sectional helical diagram of a tetramer comprised of $36H \times 36H \times 312B$ units	62
61	Strand diagram of select helices in the $36H \times 36H \times 312B$ unit of a tetramer	63
62	TEM images of a tetramer comprised of $36H \times 36H \times 312B$ units	64
63	Select helical TEM images of a tetramer composed of $36H \times 36H \times 312B$ units	65
64	Select lateral TEM images of a tetramer composed of $36H \times 36H \times 312B$ units	65
65	Schematic of strand design of 30H×30H×260B for DNA-PAINT super-resolution imaging	66
66	DNA-PAINT characterization of the 30H×30H×260B structure	67
67	Nanobricks software	68
68		69
69	Design flow	70
70	Nanobricks tools	71
70	Add voxels tool	73
72	Power edit tool	73
72		75
73	Importing 3D structures	76
75	3D rendering of shape approximation	70
76	3D models used for structure designs	80
70	Selected particles used for class averages	8/
78	Selected particles used for class averages	85
70	Selected particles used for class averages	86
80	Selected particles used for class averages	87
80 81	Selected particles used for class averages	80
82	Selected particles used for class averages	80
82 83	TEM structural analysis of cavities	00
8J 84	Series of x z slices extracted from the perellel channels cuboid tomogram along the v direction	90
85	Series of $x - y$ slices extracted from the parallel channels cuboid tomogram along the y direction.	95
86	Wide field view of teddy hear cavity structures	05
80 87	Series of y z slices extracted from the teddy bear cavity tomogram along the x direction	95
88	Series of $x = y$ slices extracted from the teddy bear cavity tomogram along the z direction.	90
80	Series of x z slices extracted from the teddy bear cavity tomogram along the y direction.	97
09	Series of $x-z$ sinces extracted from the feddy bear cavity tomogram along the y direction	90
90	Series of x z slices extracted from the Bunny cavity tomogram along the y direction	100
02	Series of $x - z$ sinces extracted from the bunny cavity tomogram along the x direction.	100
92 03	Series of $y - z$ sinces extracted from the Bunny cavity tomogram along the z direction.	101
93	Wide field view of balicoid covity structures	102
94 05	Series of x y slices extracted from the baliceid equity tomogram along the z direction	103
95 06	Series of x-y sinces extracted from the helicoid cavity tomogram along the y direction.	104
90 07	Series of $y \neq z$ shows extracted from the helicoid cavity tomogram along the y direction.	105
71 08	Series of $y - \zeta$ shows extracted from the hencold cavity tomogram along the x direction	100
90 00	white-heith view of OED cavity shutchines	107
77 100	Solices of $y - \zeta$ shows extracted from the GED cavity tomogram along the χ direction.	100
100	Solution of the relation of the solution of the theory of the solution of the relation of the	109
101	Series of x - z sinces extracted from the OLD cavity tomogram along the y direction	110

102	Series of <i>x</i> - <i>y</i> slices extracted from the 'GEB' cavity tomogram along the <i>z</i> direction.	111
103	Categorization of canvas strands based on sequencing read numbers for the teddy bear structure	114
104	Diagram of every strand in the molecular canvas based on its classification in the teddy bear structure	115
105	Strand-to-voxel translation scheme and counts for the different types of neighboring strands.	116
106	Representation of the sequencing results from four different angles	117
107	Representation of the sequencing results from four different angles	118
108	Representation of the sequencing results from four different angles.	119
109	Representation of the sequencing results from four different angles.	120
110	Representation of the sequencing results from four different angles.	121
111	Representation of the sequencing results at different projections.	122
112	Representation of the sequencing results at different projections.	123
113	Comparison of sequencing data and TEM images.	124

S1 Materials and Methods

S1.1 Design and formation of Structures

Sample preparation

Chemically synthesized DNA oligomers were purchased from Integrated DNA Technologies, inc (www.idtdna.com). Strands were adjusted to concentrations of 2 - 100 nM per strand in $0.5 \times$ TE buffer (5 mM Tris, pH 8, 1 mM EDTA) containing 10 - 80 mM MgCl₂. For some of the larger structures, strand solutions were concentrated through evaporation by using the vacuum centrifuge (Thermo Scientific, Savant SPD131DDA Speedvac concentrator) before being added to the folding buffer.

To anneal the structures, samples were subjected to either a thermal ramp or an isothermal annealing protocol. For the 3-day thermal ramp, samples were cooled from 80 °C to 60 °C over 1 hour and from 60 °C to 25 °C over 3 days at a linear rate. For a linear annealing ramp, samples were cooled from 80 °C to 25 °C at the rate of 0.5 °C/hr.

To identify the optimal annealing temperature for isothermal folding, we used a fixed thermal gradient on our thermocycler (Eppendorf, Mastercycler Nexus GX2) to test multiple fixed annealing temperatures simultaneously. Note that only the high and low temperatures forming this gradient were set, and that the reaction temperatures we report were values indicated on the instrument (Supplementary Table 1). For the isothermal folding, samples were subjected to a denaturation step at 80 °C for 10 minutes before being held at the optimal temperature for 5 - 7 days. Cuboids and their optimal folding temperature, as reported by the thermocycler, at 5 nM per strand are listed in Supplementary Table 1. Alternatively, narrow annealing ramps spanning a two-degree range around the optimal folding temperature may also be used to form structures.

Structures are named by the number of helices $(H) \times H \times$ base-pairs (B) formed. Optimal temperatures at other strand concentrations can be obtained from the gels shown in Supplementary Fig. 18, Extended Data Fig. 1. Note that samples were analyzed on the gel immediately after annealing was complete to avoid aggregation of the structures upon cooling.

Structure	Temperature (°C)
10H×10H×156B	49.1
$14H \times 14H \times 208B$	50.3
20H×20H×260B	51.4
30H×30H×260B	51.4
36H×36H×312B	50.7
40H×40H×338B	50.7
46H×46H×390B	49.3

Supplementary Table 1. Optimal formation temperature as identified from a thermal gradient experiment for isothermal assembly of different cuboids at 5 nM per strand.

Shape design and structure formation

Structures were designed using our in-house *Nanobricks* software. Depicted 2D strand diagrams were generated from associating caDNAno files.¹ Oligomers were selected from a library of strands and concentrated to 5 nM per strand in $0.5 \times$ TE buffer (5 mM Tris, pH 8, 1 mM EDTA) containing 20 mM MgCl₂. Structures were annealed for 6 – 7 days in a narrow annealing ramp between 52.5 °C and 51 °C, or isothermally at a constant temperature.

S1.2 Characterization of Structures

S1.2.1 Agarose gel electrophoresis and purification

Samples were subjected to 0.3 - 2% agarose gel electrophoresis at 80 V for 2 hours in an ice water bath. Gels were prepared with $0.5 \times$ TBE buffer (1 × TBE: 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8) containing 10 mM MgCl₂ and with a 1:10,000 to 1:20,000 dilution of SYBR Safe loading dye.

Quantification of band intensities were obtained with the Typhoon FLA 9000 gel imager. Intensities were measured manually by using ImageJ² or by using the TotalLab Quant v12.2 (Cleaver Scientific, ltd) with the rubber band background subtraction method and a fixed edge width. Gel yields describe the target band intensity compared to that of the entire lane. Since this method considers roughly 70% of the total peak intensity, the reported percentages may be an underestimate of the target structure yields.

For purification, target bands were excised and crushed into fine pieces in Freeze 'N Squeeze tubes (Bio-Rad Laboratories, inc.) and centrifuged at 100 - 250 g for 5 - 10 minutes. Flow-through was collected and analyzed on the TEM.

S1.2.2 Electron microscopy imaging

TEM imaging

Formvar/carbon coated grids from Electron Microscopy Sciences were glow-discharged using PELCO easiGlow Glow Discharge system (Ted Pella Inc., USA). The current used was 15 mA and grids were glow discharged for 30 seconds. $2.5 - 7 \mu$ L of samples were incubated for 5 minutes on the glow-discharged formvar/carbon coated grids. After removing the excess solution, samples were then stained for 1 - 60 seconds with 2% uranyl formate solution containing 25 mM NaOH. Imaging was performed by using a JEOL JEM-1400 TEM operating at 80 kV.

Note: After 60 seconds of incubation, the heavy metal salts surround the different cuboids and penetrate into the structural cavities by diffusion through the channels. In addition, we observed that small heavy metal salts, such as uranyl formate or uranyl acetate, can also diffuse across the layers of DNA helices and accumulate within cavities. These processes result in images in which the different cuboids appear electron lucent against the dark electron dense cavities and background which correspond to stain accumulated regions.

Microscopy and image processing

5 μ L of cuboid solutions were deposited onto glow-discharged, carbon-coated 300 mesh copper grids for 3 minutes. The excess solution was blotted, and 5 μ L of 1% uranyl acetate solution were applied to the grids for 2 minutes. The grids were then dried with Whatman filter paper Number 4 and transferred into a JEOL 2200FS FEG transmission microscope using the JEOL high tilt holder. Series of tilted images were collected at a magnification of 50,000 fold by using a 4k × 4k slow-scan CCD camera (Gatan, inc.) with defocus values of -3 μ m and -5 μ m.

The acquisition was performed semi-automatically using a version of SerialEM (http://bio3d.colodaro.edu) adapted for operating the JEOL microscope, allowing recording of individual images in the zero-energy-loss mode with a slit width of 20 eV.

Tilt series consisting of about 60 images, were collected by tilting the specimen between -60° and 60° , imaging at 2° increments, with a total dose of about 700 electrons/Å².

Image alignment and three-dimensional reconstructions were performed by using the Etomo package (Boulder University, Colorado, USA). Montage of 2D slices through the 3D tomogram was performed by using ImageJ. For the 3D visualization of cuboid with Chimera software, a C4 symmetry along z axis and surface smoothing iterations were imposed.

In electron tomography, as a series of tilted images of the specimen was recorded, handedness was determined from two different angles. Magnifications of the electron microscope were calibrated by recording images of tobacco mosaic virus and handedness was validated by using DNA origami gold nanoparticle helices as the standard.³ After these calibrations, we determined the handedness of the helix cuboid structure by observing how the helix rotated as sections from the tomogram were inspected from the bottom to the top.

S1.2.3 3D DNA-PAINT super-resolution setup

Fluorescence imaging was carried out on an inverted Nikon Eclipse Ti-E microscope (Nikon Instruments, Melville, NY) with the Perfect Focus System, applying an objective-type TIRF configuration with an oil-immersion objective (CFI Apo TIRF $100 \times$, NA 1.49, Oil). For Cy3b excitation a 561 nm laser (300 mW nominal, Coherent Sapphire) was used. The laser beam was passed through a cleanup filter (ZET561/10, Chroma Technology, Bellows Falls, VT) and coupled into the microscope objective using a single-band beam splitter (ZT561rdc, Chroma Technology). Fluorescence light was spectrally filtered with an emission filter (ET600/50m, Chroma Technology) and imaged onto an sCMOS camera (Zyla 4.2, Andor Technologies, North Ireland). Imaging

was performed with a 2×2 pixel binning without additional magnification in the detection path, yielding a 130 nm pixel size. 3D images were acquired using a cylindrical lens (FL = 1 m) in the detection path.

Buffer solutions

Buffer A: 10 mM Tris (pH 8.0), 100 mM NaCl, 0.05% Tween20

Buffer B: 5 mM Tris (pH 8.0), 10 mM MgCl₂, 1 mM EDTA, 0.05% Tween20

PCA: 154 mg (Protocatechuic acid, Sigma-Aldrich CN: 37580-25G-F) was dissolved in 10 mL H₂O and the pH was adjusted to 9.0

PCD: 9.3 mg PCD (protocatechuate- 3,4- dioxygenase, Sigma CN: P8279) was dissolved in 13.3 mL of the following buffer: a 50% glycerol stock in 50 mM KCl, 1 mM EDTA and 100 mM Tris-HCl, pH 8.

Trolox: 100 mg Trolox ((+-)-6-Hydroxy-2,5,7,8-tetra-methylchromane-2-carboxzlic acid, Sigma-Aldrich: 238813-5G) was dissolved in 430 μ L of 100% methanol, 3.2ml H₂O, 345 μ L of 1 M NaOH.

Imaging buffer: $2 \mu L$ of Trolox, $2 \mu L$ of PCD and $5 \mu L$ of PCA were diluted in 191 μL of buffer B.

Sample preparation and imaging

For sample preparation, a piece of coverslip (No. 1.5, $18 \times 18 \text{ mm}^2$, 0.17 mm thick) and a glass slide ($3 \times 1 \text{ inch}^2$, 1 mm thick) were sandwiched together by two strips of double-sided tape to form a flow chamber with inner volume of approximately 20 μ L. First, 20 μ L of biotin-labeled bovine albumin (Sigma A8549, 1 mg/mL, dissolved in buffer A) was flown into the chamber and incubated for 2 minutes. The chamber was then washed using 40 μ L of buffer A. 20 μ L of streptavidin (Thermo S888, 0.5 mg/mL, dissolved in buffer A) was then flown through the chamber and was allowed to bind for 2 minutes.

After washing the chamber with 40 μ L of buffer A and subsequently with 40 μ L of buffer B, 20 μ L of biotin-labeled cube DNA structures suspended in buffer B was flown three times into the chamber and incubated for 10 minutes each. These structures were purified from a 5 nM reaction of strands with 75× excess of the DNA-PAINT handles, and the purified concentration was estimated to be approximately 300 pM of structures. The chamber was washed using 40 μ L of buffer B. The final imaging buffer solution contained 2 nM Cy3b-labeled imager strands in buffer B with Trolox, PCA and PCD. The chamber was sealed with epoxy before subsequent imaging. The sCMOS readout bandwidth was set to 200 MHz at 16 Bit. Imaging was performed using oblique illumination with an excitation intensity of ~160 W/cm² at 561 nm. Images were acquired for 15,000 frames (400 ms integration time, total imaging time 100 minutes).

Image processing and drift correction

Super-resolution DNA-PAINT images were reconstructed using spot-finding and 2D- Gaussian fitting algorithms programmed in LabVIEW.⁴

3D calibration was carried out according to the calibration function previously presented.⁵ A custom MATLAB script was used to determine the calibration curves. Based on this calibration, a z value was assigned to each localization of the sample. Drift correction was performed on the DNA structures, as previously described.⁶

Determination of localization precision

Localization precision in x and y were determined by calculating the average separation of single-molecule localizations in neighboring frames, which can be attributed to an imager strand binding to a single docking strand.⁷ As multiple docking strands are used in each corner of the cube (10 strands per corner), one cannot fit the distribution of binding events per corner, as this would result in an overestimation of the localization precision; The measured value per corner would represent a convolution of the actual localization precision with the spatial extent of the binding sites in this corner.

Image representation

Z-calibration was additionally corrected for refractive-index-mismatch by measuring a reference structure with given height, resulting in a correction factor of 1.3.⁸ For visualization of single-particle localizations in three dimensions, the software package ViSP was used.⁹ The following settings were used for all samples: Intensity offset -0.99, Detection Size 15 nm, min depth -60 nm, max depth 60 nm.

Individual localizations were exported with a custom MATLAB script, each localization was given an arbitrary intensity value of 10. After exporting from ViSP, images were contrast-adjusted and corresponding color bars were loaded in Fiji.¹⁰ Auto-brightness was applied on a single projection and propagated to all other projections.

140



Identify single structures (n=1016) on 2D Projection





Rotate, discard structures not standing upright



100

b

Select 200 structures and fit distances on localizations, discard outliers (<50, >150nm, 10.5%)

Supplementary Fig. 1. Distance measurements of the 30H×30H×260B cuboid using 3D DNA-PAINT. a, Individual structures were identified based on their localization count with a binary mask and extracted. A total of n = 1016 structures were detected in the super-resolution image. **b**, Each structure was rotated to be aligned in the x-y plane. Structures that were not standing upright (when the 4 lower and 4 upper spots were not overlapping) were discarded. c, A total of 200 structures were picked and used for analysis. The position of each corner and the length of each corner was calculated. Outliers (less then 50 nm or more than 150 nm length for an edge, $\sim 10.5\%$) were discarded. **d**, Histograms of measured lengths were fitted with a Gaussian Distribution.

Data analysis and length measurements

For 3D DNA-PAINT super-resolution length measurements, 100 structures were selected from the field of view and analyzed (Supplementary Fig. 1). Not all cube structures attached perpendicular to the surface. To increase measurement accuracy, only un-tilted structures were selected for evaluation. Due to the length of DNA-PAINT handles and structure geometry, a longer and a shorter side of the structure could be identified. Individual lengths were determined as the mean-to-mean distance of individual localizations in each corner. For structures with corners missing, respective distances were omitted. Lengths were binned and fitted with a Gaussian to estimate average lengths. As expected, measurements on the cube face parallel to and closest to the glass surface showed a smaller deviation compared to the cube face farther from the glass surface due to the different localization precisions in the different z-planes.

S1.2.4 Sequencing sample preparation and analysis

The sample was denatured at 95 °C for 5 minutes. Afterwards, it was purified and concentrated by using the Oligo Clean and Concentrator kit from Zymo Research, and eluted in 6 μ L MQ-H₂O.

Ligation

An adapter phosphorylated 5'-end ddC-protected 3'-end (5'-phossequence with а and а AGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCTCGTATGCCGTCTTCTGCTTG-ddC-3') was ligated to the 3'-ends of the brick strands by using T4 RNA ligase 1 (New England Biolabs). A 20 μ L ligation reaction contained 10 units of enzyme, 1× T4 RNA ligase buffer, 25% (w/v) PEG-8000, 10 pmol of the 3'-ddC adapter, 1 mM ATP and 10 pmol brick strands. The reaction mixture was incubated for 18 hours at room temperature (rt) and then heat-inactivated at 65 °C for 15 minutes.

Purification

The sample was purified by polyacrylamide gel electrophoresis. It was mixed 1:1 with $2 \times RNA$ loading dye (New England Biolabs), denatured for 10 minutes at 70 °C and loaded on a 10% TBE-urea gel. Electrophoresis was performed at 65 °C for 55 minutes at 180 V with $0.5 \times TBE$ as running buffer and the gel was post-stained with $1 \times SYBR$ Gold (Invitrogen) for 30 minutes in an orbital shaker. The ligation product band was excised by using Gel Cutting Tips (MidSci) on a Safe Imager 2.0 Blue-Light Transilluminator (Invitrogen).

The gel slice was submerged in 50 mL $0.5 \times$ TBE in a dialysis tube (Slide-A-Lyzer MINI Dialysis Device, 2K MWCO, 0.1 mL). Electroelution was performed for 30 minutes at 90 V in $0.5 \times$ TBE and 1 more minute with reversed electric field. The electroeluate was concentrated by using Oligo Clean and Concentrator kit from Zymo Research and eluted in 6 μ L MQ-H₂O. The attained purified ligation product was phosphorylated at the 3'-end by using T4 polynucleotide kinase (New England Biolabs). A 10 μ L reaction with 10 units of enzyme, in 1 × T4 polynucleotide kinase buffer and 1 mM ATP was carried out. The mixture was incubated at 37 °C for 30 minutes and afterwards heat-inactivated at 65 °C for 20 minutes.

Amplification

A previously tested¹¹ adapter sequence with an integrated barcode was ligated to the 3'-end of the brick strands by using T4 RNA ligase 1 (New England Biolabs). Three 20 μ L reactions were set up. Each reaction contained 10 units of enzyme, 1× T4 RNA ligase buffer, 25% (w/v) PEG-8000, 1mM ATP, 10 pmol of the adapter species and 3 μ L of the phosphorylated product. The ligation reactions were incubated at room temperature for 18 hours and then heat-inactivated at 65 °C for 20 minutes. The three reactions belonging to the same sample were pooled and afterwards concentrated by using Oligo Clean and Concentrator kit from Zymo Research, and eluted in 10 μ L MQ-H₂O.

The sample was amplified using six cycles of PCR with a Q5 polymerase ($2 \times$ master mix from New England Biolabs). A 25μ L reaction contained $1 \times$ Q5 master mix, 300 nM previously tested Illumina qPCR primers¹² and $2 - 8 \mu$ L doubly ligated sample. Afterwards the library was quantified with quantitative PCR. For this purpose, Q5 polymerase, Illumina qPCR primers at a final concentration of 300 nM and SYTO13 (Molecular Probes/ Life Technologies) as a fluorescent indicator dye was used according to the manufacturer's instructions. DNA standard 1 - 6 from Kapa NGS library quantification kit were used as standards.

Sequencing

Multiple samples with different barcodes were pooled and sequenced with an Illumina MiSeq machine according to the manufacturer's instructions by using the MiSeq V2 paired end 50 kit (Illumina Inc., San Diego, CA). A modified library denaturation and loading protocol for lower concentration libraries was used.¹²

S2 Strand design schematic

Structures were assembled using single-stranded 52-nt oligomers with four 13-nt binding domains, and each DNA strand can be conceptualized as a DNA brick (Supplementary Fig. 2a). Each of these DNA bricks interacts with an adjacent brick through hybridization of one pair of complementary domains. A 13-bp duplex confers to roughly 1.25 turns of the helix and a 90° dihedral angle between two strands (Supplementary Fig. 2b). By implementing this connection pattern and using unique domain sequences, we can create a structure with our desired size and shape (Supplementary Fig. 2c).



Supplementary Fig. 2. Detailed schematic for the 52-nt DNA brick structure. a, The top panel shows a strand model for the basic component of the structure. Each brick can be divided into 13-nt domains. A LEGO-like model is shown in the bottom panel with corresponding colors to match the strand model. **b,** Two strands interact via hybridization of 13 base pairs, marked by complementary domains a and a*, to form a 90° dihedral angle. **c,** DNA brick model of a $6H \times 6H \times 104B$ cuboid is shown with a pair of interacting bricks highlighted.

S3 Motif characterization

In a square-lattice DNA structure, a DNA brick contains four single-stranded domains, and two neighboring DNA bricks form a 90° dihedral angle. In B-form DNA duplex, a full helical turn corresponds to 10.5 base pairs. Thus, a DNA brick domain should contain roughly $10.5 \times (0.5n + 0.25)$ nucleotides, where *n* is a non-negative integer.

Compared with previous canonical 32-nt DNA bricks,¹³ DNA bricks with longer domains could offer a number of potential advantages for assembling highly complex structures. First, the longer domain will substantially increases sequence design space, and thus reduce the number of domains with same or similar sequences (see Supplementary Fig. 3 for detailed analysis of sequence design space). For example, out of 1440 randomly assigned domains analyzed (the size of the largest previously published 8-nt domain structure),¹³ 8-nt domains would contain on average 30 repeated domains. In contrast, a structure comprised of 13-nt domains would contain on average no repeated domains. Structures assembled using the 13-nt approach can contain over 8000 domains before an average of one repeat is encountered, substantially expanding the sequence design space (Supplementary Fig. 3). Second, the additional 5 bp of hybridization increases the stability of the interaction by approximately 10 kcal/mol, which allows for more stable formation and interaction at lower component concentrations (Supplementary Table 2). Third, longer binding domains could help increase the nucleation rate for highly complex self-assembled systems, and reduced domain sequence similarity could also increase assembly kinetics by mitigating unwanted spurious domain-domain binding interactions.

Firstly, we tested 3D structures that contain the same number of binding domains. Cuboids were designed to have $6H \times 6H$ cross-section and measure eight x-bp in length, where $x = \{8, 13, 18.5\}$ is the length of the domain of different bricks to be tested. The cuboids were then assembled from 32-nt DNA bricks, 52-nt DNA bricks, or 74-nt DNA bricks in 10 - 80 mM MgCl₂ using a linear annealing ramp and compared for assembly efficiency (Supplementary Figs. 4 and 5, design details can be found in Supplementary Figs. 6 - 8). After annealing, yields of DNA structures were measured by comparing the intensity of the target band in the gel with that of the entire lane. The 52-nt brick structures had the highest gel yield of at least 32% across all salinities tested. The 74-nt brick design with longer domains (and hence stronger inter-domain interactions) did not produce increased gel yields, but instead showed more undesired products besides target bands.

Secondly, we compared formation rates between different designs by monitoring the gel yields of product bands during 72-hour isothermal annealing reactions (Supplementary Fig. 5). The assembly of 32-nt bricks, 52-nt bricks and 74-nt bricks all exhibited three distinct phases: (1) a lag period when no product is formed, (2) a rapid increase in target formation, and (3) a plateau in growth near equilibrium.

Cuboids composed of 32-nt bricks with boundary strands¹³ and 74-nt bricks assembled comparably, while 52-nt brick structures assembled the fastest kinetically and showed highest yields after 72 hours. Furthermore, direct comparison of 52-nt brick structures and 32-nt brick structures with similar overall dimensions reveal that 52-nt brick structures assembled with higher percentages and also showed higher thermal stability (Supplementary Figs. 9 – 12). Annealing curves and isothermal assembly studies further demonstrated that the 52-nt brick structures assembled around 50 – 53 °C at 5 – 100 nM strand concentration, while the 32-nt bricks were found to fold around 35 °C at 100 nM strand concentration (Supplementary Fig. 11). The formation temperatures fall between the melting temperatures of one and two hybridized domains (Supplementary Table 2) as expected.

Upon finding the optimal domain length, we also tested different versions of the 52-nt DNA bricks, including one with alternating crossover direction and another in which the 26-nt half strands are merged with the adjacent full length strands (Supplementary Fig. 13, design details in Supplementary Fig. 14, 15). The 52-nt brick structure with disjoint bricks oriented in the same direction gave the highest yields. As a result, the 52-nt unidirectional brick motif was selected for assembling large structures.

S3.1 Domain uniqueness analysis

We wanted to analyze the sequence uniqueness of structures formed using randomly assigned domains. For the 8-nt domain design, we have a total of $\frac{4^8-4^4}{2} + 4^4 = 32,896$ unique domains. The 4⁴ subtraction results from the presence of palindromic sequences. In contrast, 32-nt DNA brick designs have a total of $\frac{4^{13}}{2} = 33,554,432$ unique domains. To compare the sequence design space of the different motifs, we applied the birthday paradox theory to estimate the probability of encountering a single match when a certain number of domains are used (Supplementary Fig. 3a, Equation. 1). For this calculation, we assume that the frequency differences for the 8-nt domains are negligible. From our estimates, we find that the 8-nt domains reach a 50% probability of having a match when structures are sized at roughly 200 domains. This limit is at roughly 6,500 domains for 52-nt DNA brick structures.

$$p(N) = 1 - e^{\frac{-N^2}{2d}} \tag{1}$$

We additionally simulated random sequences and analyzed the number of matches for structures of different sizes. We tested 1000 simulations for structures of each size to obtain averages and standard deviations (Supplementary Fig. 3b). We found that 8 MDa 32-nt DNA brick structure would contain roughly 30 repeats and the 0.5 GDa 13-nt brick structure would contain 60 repeats. In both cases, the fraction of repeats of the total domains used comprise of less than 1% of the total number of domains in the structure. We can assign unique sequences to each domain to further reduce the number of unintended interactions.



Supplementary Fig. 3. Domain sequence match analysis for varying numbers of randomly assigned domains. a, Estimated probabilities of achieving at least a single matching domain given a specified number of random domains. Gray dotted lines highlight the number of domains necessary to reach a 50% probability of having a match. **b,** Simulated averages of total number of domains with repeated sequences, n = 1,000. Error bars represent standard deviation. Gray lines denote the largest fully addressable structures formed using 8-nt and 13-nt domains. The largest structure formed using 8-nt domains contained a total of 1,440 domains, which confers to roughly 30 reused sequences. The largest number of 13-nt domains used was 63,480 unique domains, which has roughly 60 matches.

S3.2 Thermodynamic analysis of interaction

We wanted to better understand the underlying thermodynamic and kinetics of the brick interactions. We used the nearest-neighbor models to study the energy of the binding interaction of two brick strands. By estimating an average base-stacking energy, we can calculate and estimate the melting temperature. From SantaLucia and Hicks, we find that the average $\Delta H_{bs} = -8.2$, $\Delta H_{init} = 0.2$, $\Delta H_{sym} = 2.2$, $\Delta H_{dangles} = -2.5$, $\Delta S_{bs} = -22$, $\Delta S_{init} = -5.7$, $\Delta S_{sym} = 6.9$, and $\Delta S_{dangles} = 6.9$.¹⁴ These average values of ΔH are in units of kcal/mol, while ΔS is in cal/mol. We can then use equation 2 to calculate the average ΔG for a hybridization, where *n* is the number of base-stacks present.

$$\Delta G = n\Delta H_{bs} + \Delta H_{init} + \Delta H_{sym} + 3\Delta H_{dangles} - T(n\Delta S_{bs} + \Delta S_{init} + \Delta S_{sym} + 3\Delta S_{dangles} + \Delta S_{salt})$$
(2)

where

$$\Delta S_{salt} = 0.368n \ln([Na^+] + 3.3\sqrt{[Mg^{2+}]}) \tag{3}$$

We can estimate the melting temperatures in Celcius of these structures by equation 4.

$$T_m = \frac{1000\Delta H}{\Delta S + R \ln([strands]/4)} - 273.15 \tag{4}$$

Various domain lengths and their energies are listed in Supplementary Table 2. Melting temperatures are estimated at 5 nM strand concentration. Generally, we observe that the melting temperatures of 8-bp of hybridization is far below that of 13-bp and 18.5-bp (the average of the 18/19-nt domain). Additionally, the melting temperature differences between two hybridized domains and one hybridized domain is roughly 10 $^{\circ}$ C larger for the 8-nt domains than for the 13-nt domains. As the domain lengths increase, the melting temperature differences between two versus one hybridized domains shrinks. Previous models of the DNA brick system have suggested that the nucleation barrier for assembly is composed of structures where each monomer contains two hybridized domains.

Hybridization Length	$MgCl_2 (mM)$	ΔH (kcal/mol)	ΔS (e.u.)	$T_m(^{\circ}\mathrm{C})$ @5 nM	T_m (°C) @ 100 nM
8	40	-62.5	-175.8	16.9	25.1
16	40	-128.1	-352.0	53.0	58.1
13	20	-103.5	-287.0	42.6	48.4
26	20	-210.1	- 576.7	67.1	70.4
13	40	-103.5	- 285.5	44.1	50
26	40	-210.1	- 573.5	68.9	72.2
18.5	40	-148.6	-407.4	58.5	62.9
37	40	-300.3	-817.2	76.9	76.9

Supplementary Table 2. Average melting temperatures at 5 nM strand concentration and energies. Values are derived from the SantaLucia and calculations are performed following equations 2 to 4.

S3.3 Comparison of structures with a similar number of strands

S3.3.1 Comparison of 6H×6H×(8-domain) structure with domains of length 8-nt, 13-nt, and 18.5-nt



Supplementary Fig. 4. Gel electrophoresis of a $6H \times 6H \times (8\text{-domain})$ structure with domains of length 8-nt, 13-nt, and 18.5-nt. Cylinder models of the structures are depicted in the top panel. The banding pattern distinguishes the different domains along the helical axis. Structures composed of 32-nt (a), 52-nt (b), and 74-nt (c) DNA bricks were annealed at 100 nM/strand using a linear annealing ramp in the presence of $10 - 80 \text{ mM MgCl}_2$. Samples were assayed on a 2% agarose gel. Lane M contains a 1 kb ladder. Blue arrows point to the different types of structures observed: 1 aggregates, 2 dimers, 3 target structure, 4 unreacted bricks. Numbers listed indicate the gel yields.

To do a more thorough characterization of our system, we formed structures of size $6H \times 6H \times (8-\text{domain})$, where each domain measures 8-nt, 13-nt, or alternates between 18- and 19-nt, to compare the assembly capabilities of each brick motif. These structures are respectively composed of 32-, 52-, and 74-nt DNA brick strands. Structures were assembled in a linear annealing ramp from 80 °C to 25 °C at the rate of 2 hours per degree in varying magnesium concentrations. From the gel electrophoresis results, we found that the 52-nt DNA structures produced the highest gel yields (Supplementary Fig. 4). Because the dye intercalation scales with the number of nucleic acids present, the larger 52-nt and 74-nt DNA brick structures showed darker staining. Although both 32-nt and 74-nt DNA brick designs performed more poorly than the structure composed of 52-nt DNA bricks in gel yields, 32-nt DNA brick structures contained more unreacted monomers. In contrast, 74-nt DNA brick structures showed slightly more aggregation and more intermediates, possibly resulting from strand incorporation errors for the 74-nt DNA brick structures due to the presence of truncated oligomers in the unpurified strand pools.

S3.3.2 Comparison of assembly efficiency under varying reaction times for 32-nt, 52-nt, and 74-nt DNA brick structures

To better understand how the different domain lengths influence structure formation, we assembled different cuboids using 32-nt, 52-nt, and 74-nt DNA brick strands at 100 nM strand concentration. The strand number was controlled by designing structures containing the same number of voxels ($6 \times 6 \times 8$). Additionally, a $6H \times 6H \times 104B$ cuboid was tested using 32-nt and 52-nt DNA bricks. The conditions used to assemble each of the structures is listed in Supplementary Fig. 5a. Temperature of formation was selected to be at or near the optimal annealing temperature. Agarose gel electrophoresis was used to analyze the yields for the different structures at different time points (Supplementary Fig. 5b). Cuboids composed of 52-nt DNA bricks (in red) gave the fastest growth and highest formation percentages. Structures begin to appear starting after 5 minutes of assembly. In contrast, 32-nt and 74-nt DNA brick structures formed comparably, with structures appearing after 10 – 70 minutes of reaction. The larger $6H \times 6H \times 104B$ 32-nt brick cuboids. In absence of boundary strands, 32-nt DNA brick structures formed rather slowly and inefficiently with structures appearing on the gel after 70 minutes. Although structures appeared after 70 minutes of reaction, minimal additional assembly was achieved over longer reaction times. Additional analysis to determine the rate of percentage change reveals that structure completion occurs the fastest at the onset of formation and slows down after the initial formation events (Supplementary



Supplementary Fig. 5. Cuboids were assembled isothermally at their optimal temperatures with 100 nM strand concentration and subjected to 2% agarose gel electrophoresis with 10 mM MgCl₂. **a**, Table listing the structure assembly conditions for each structure and whether boundary strands were used in the design. Boundary strands are half-strands that are connected to its neighboring full length strand to further stabilize its incorporation.¹³ These boundary strands were used in the 32-nt DNA brick structures previously designed. To understand how salt concentrations affect assembly kinetics, we included a condition where a lower MgCl₂ was used. **b**, Gel yields for each structure was plotted against reaction time on a linear scale. **c**, The rate of change in gel yields (i.e. Δ target band intensity percentage / Δ time) at given time points.

Fig. 5c). Overall, these studies support our hypothesis of delayed nucleation growth mechanism, with fast growth occurring initially.



S3.3.3 Strand diagram of the 6H×6H×64B structure composed of 32-nt DNA bricks

Supplementary Fig. 6. Strand diagram for the 6H×6H×64B cuboid composed of 32-nt DNA bricks. Zoom in to see details.

35 34 33 32 31 12 13 14 15 16 17 11 10 9 8 7 6 1 2 3

S3.3.4 Strand diagram of the 6H×6H×104B structure composed of 52-nt DNA brick

Supplementary Fig. 7. Strand diagram for the 6H×6H×104B cuboid composed of 52-nt DNA bricks. Zoom in to see details.



S3.3.5 Strand diagram of the 6H×6H×148B structure composed of 74-nt DNA bricks

Supplementary Fig. 8. Strand diagram for the 6H×6H×148B composed of 74-nt DNA bricks. Zoom in to see details.

S3.4 Comparison of the 6H×6H×104B structure composed of 32-nt versus 52-nt DNA bricks

S3.4.1 Formation differences of the $6H \times 6H \times 104B$ structures



Supplementary Fig. 9. Gel electrophoresis of a $6H \times 6H \times 104B$ structure composed of 32-nt versus 52-nt DNA bricks. A $6H \times 6H \times 104B$ cuboid was designed containing 32-nt (a) and 52-nt (b) DNA bricks. The structures were annealed using a 3-day 2-stage annealing ramp in the presence of 10 - 80 mM MgCl₂. Samples were assayed on a 2% agarose gel. Lane M contains a 1 kb ladder. Numbers listed indicate the gel yields. Gray banding patterns indicate the different domains present.

We compared a $6H \times 6H \times 104B$ structure assembled using our original 32-nt DNA bricks with one using the new 52-nt DNA brick motif. Following 3-day annealing, the gel yields were compared. The 52-nt brick design showed a gel yield up to 40%, while the 32-nt bricks design showed a gel yield of only around 12.6% (Supplementary Fig. 9). This difference is consistent with the trends we observed in Supplementary Fig. 5, where the 52-nt DNA brick structures assemble much faster and with higher yields than the 32-nt DNA brick structures. Although the structures were designed with the same global morphology and molecular weight, 52-nt DNA brick structures migrated on the gel at a slower pace. This decrease could be attributed to the lower crossover density in these structures, resulting in expansion of the overall structure size.

S3.4.2 Thermal stability of 6H×6H×104B structures assembled with 32-nt and 52-nt DNA bricks



Supplementary Fig. 10. Thermal stability of purified $6H \times 6H \times 104B$ structures assembled with 32-nt and 52-nt DNA bricks. Cuboids formed from 32-nt (a) and 52-nt (b) bricks were annealed using a 3-day 2-stage annealing ramp in the presence of, respectively, 40 or 20 mM MgCl₂. Samples were assayed on a 2% agarose gel, and target bands were purified and adjusted to be 2.6 nM. Following, the structures were subjected to a higher temperature (30 - 48 °C) over 24 hours and assayed on a 2% agarose gel. Lane M contains a 1 kb ladder. Numbers indicate the percentage of the target gel yields.

Purified 32-nt and 52-nt $6H \times 6H \times 104B$ structures were compared for post-folding thermal stability at varying temperatures. The 52-nt structure was found to be stable up to 48 °C, while the 32-nt structure began to fall apart around 37 °C (Supplementary Fig. 10). Interestingly, the 52-nt structure shows a presence of increased higher molecular-weight products, possibly resulting from increased dimerization that occurs upon the loss of strands at the surface of the structures.



S3.4.3 Annealing/Melting curves of 32-nt versus 52-nt DNA brick 6H×6H×104B structure

Supplementary Fig. 11. Annealing/Melting curves of 32-nt versus 52-nt $6H \times 6H \times 104B$ structure. The $6H \times 6H \times 104B$ cuboid is folded using a 1-day linear annealing ramp and a 30 minute melting curve at 100 nM strand concentration in the presence of $0.3 \times$ SYBR Green I. The 32-nt structure (**a**) was folded in the presence of 40 mM MgCl₂, while 52-nt structure (**b**) was folded in the presence of 20 mM MgCl₂. Fluorescence signal was normalized to the those at 80 °C and 25 °C. The black vertical lines delineate the melting and annealing temperatures.

We also characterized the annealing curves for these structures and found that the optimal formation temperatures occurred around 35 °C for the 32-nt structure, in agreement with what was found previously for DNA crystal formation,¹⁵ and 53 °C for the 52-nt structure. Hysteresis is observed for the annealing and melting temperatures of both structures. The melting temperature for the 32-nt structure was around 48 °C, while the melting temperature for the 52-nt structure is around 62 °C.

S3.4.4 Strand diagram for the 6H×6H×104B structure composed of 32-nt DNA bricks



Supplementary Fig. 12. Strand diagram for the 6H×6H×104B structure composed of 32-nt DNA bricks. Zoom in to see details.

S3.5 Variants of the 52-nt DNA brick 6H×6H×104B structure

Variations of the 52-nt DNA brick motif was created to compare assembly effects on a formed structure. In the native design, the crossovers of each brick face the same direction (unidirectional). We also tested two additional designs: brick structures with crossover that alternate in each layer (alternating) and unidirectional brick structures that merge adjacent half strands to "boundary" strands (see ref. [13] for more details on boundary strands). For each design, 100 nM of each strand was annealed for three days in a thermal ramp in varying MgCl₂ concentration and analyzed for assembly yields using gel electrophoresis (Supplementary Fig. 13. We found that our uni-direction design with no boundary strands had the highest gel yields.

S3.5.1 Gel electrophoresis of a 6H×6H×104B structure composed of variations of the 52-nt DNA bricks



Supplementary Fig. 13. Gel electrophoresis of a $6H \times 6H \times 104B$ structure with varying connection patterns of 52-nt DNA bricks. The cuboid was folded using a 3-day 2-stage annealing ramp in varying concentrations with MgCl₂ (10 – 80 mM). Three different connection patterns were used: uni-directional 52-nt DNA bricks (**a**), alternating 52-nt DNA bricks (**b**), and uni-directional 52-nt DNA bricks with boundary strands (**c**). Percentages below the band indicate the target gel yields.



S3.5.2 Strand diagram for the 6H×6H×104B structure composed of alternating 52-nt DNA bricks

Supplementary Fig. 14. Strand diagram for the 6H×6H×104B structure composed of alternating 52-nt DNA bricks. Zoom in to see details.



S3.5.3 Strand diagram for the 52-nt 6H×6H×104B structure containing boundary strands

Supplementary Fig. 15. Strand diagram for the 52-nt 6H×6H×104B structure containing boundary strands. Zoom in to see details.

21

S4 Annealing protocol optimization

Annealing conditions are crucial for assembly of massive DNA brick structures. We tested a number of folding conditions with a $20H \times 20H \times 260B$ 67.6 MDa structure to obtain an optimal protocol. First, we annealed 5 nM strands at 10 – 80 mM magnesium in a two-stage linear folding ramp, and gel electrophoresis showed the sharpest band at 20 mM MgCl₂. Subsequently, we annealed the structures isothermally at 20 mM MgCl₂ and found that structures formed well in roughly a 2 °C window (Supplementary Fig. 16). A narrow annealing ramp using the similar strand concentrations and optimal salinity also showed roughly the same gel yield of 6% as that of the optimal formation temperature. Structure assembly times were varied, and reactions were found to near equilibrium in 5 – 7 days. With shorter reaction times, the structures showed lower yields (Supplementary Fig. 17).

To further characterize these large, complex structures, we compared the temperature window for assembly of each of the cuboids. We found that as the complexity of the structure increases, the range of temperatures at which the structures form becomes narrower (Supplementary Fig. 18). This result is in alignment with our hypothesis, as more complex structures have increased sequence diversity and larger component numbers that could limit efficient nucleation to a smaller window of reaction conditions.



S4.1 Annealing ramp optimization

Supplementary Fig. 16. Annealing ramp optimization. The $20H \times 20H \times 260B$ cuboid at 5 nM strand concentration was subjected to varying annealing conditions. **a**, Structures were annealed at varying MgCl₂ concentrations in a two-stage thermal annealing ramp with a fast cooling step from 80 °C to 60 °C over 40 minutes followed by a slow cooling step from 60 °C to 25 °C over 4.75 days. **b**, Cuboids were annealed at 20 mM MgCl₂ over 4.75 days isothermally. **c**, Cuboids were subjected to a slow, narrow annealing ramp between 52.5 and 50 °C over 4.75 days. Lane M contains a 1 kb plus DNA ladder. Numbers below the band indicate the gel yields.

S4.2 Reaction time optimization



Supplementary Fig. 17. Assembly efficiency of the $30H \times 30H \times 260B$ cuboid across different reaction times. $30H \times 30H \times 260B$ cuboids were assembled at 30 nM strand concentration isothermally at 53 °C in 10 mM MgCl₂ for varying amounts of times. Gel yields were analyzed using 2% agarose gel electrophoresis.

S4.3 Formation temperature range

To investigate how structure complexity affects the temperature of formation, we characterized the formation of seven 13-nt brick DNA structures with increasing size at varying temperatures. Structures were annealed isothermally for 5 days at temperatures from 45 - 55 °C using identical reactions conditions (5 nM strand concentrations and in the presence of 20 mM MgCl₂). The assembly efficiencies of the structures were assayed using agarose gel electrophoresis. The temperature range of structure formation narrowed with increasing structure size (Supplementary Fig. 18). For the smallest 10.2 MDa structure, structures were capable of forming across roughly a 5 °C range, while the largest 512 MDa structure formed only within a <1 °C window. This narrowing window observation fits with our proposed mechanism of delayed nucleation and growth. With increasing structure complexity, the higher sequence variation and larger component numbers can restrict nucleation to occur efficiently in a narrow range of temperatures. This limitation may restrict the size of structures that can be formed in a relevant time scale using this DNA brick method. Also note that while the absolute gel yields may vary between different gels, the relative yields and the narrowing temperature trend observed between the gels are still valuable for understanding underlying mechanisms driving structure formation.



Supplementary Fig. 18. Range of temperature formation. Agarose gel electrophoresis was used to analyze the formation of 13-nt brick structures assembled isothermally for 5 days at 5 nM strand concentration in the presence of $1 \times \text{TE}$ buffer with 20 mM MgCl₂. Structures tested include $10H \times 10H \times 156B$ (a), $14H \times 14H \times 208B$ (b), $20H \times 20H \times 260B$ (c), $30H \times 30H \times 312B$ (d), $36H \times 36H \times 312B$ (e), $40H \times 40H \times 338B$ (f), and $46H \times 46H \times 390B$ cuboids (g). a-d used a 0.5% agarose gels, while e-g used a 0.3% agarose gel. Lane M contains a 1 kb ladder. h, Plot of highest gel yields found in a-g with respect to number of strands present in the cuboid.

S5 Gel results and TEM images of fully addressable cuboids

S5.1 Gel results and TEM images for the 4.3 MDa (8H×8H×104B) origami structure

S5.1.1 Gel electrophoresis of 8H×8H×104B origami structure



Supplementary Fig. 19. Gel electrophoresis of $8H \times 8H \times 104B$ origami structure. a, Cylindrical model of the $8H \times 8H \times 104B$ origami cuboid. b, A 2% agarose gel was used to analyze the 4.3 MDa origami cuboid, which was annealed at 10 nM scaffold with 100 nM excess staples using a two-stage 3-day annealing ramp. MgCl₂ concentrations used are indicated above the lane. Lane 'scaf' contains scaffold only folded at 10 mM MgCl₂. Lane M contains a 1 kb ladder.

S5.1.2 Wide field view of 8H×8H×104B origami structure



Supplementary Fig. 20. TEM images of the 8H×8H×104B origami structure.

S5.1.3 Select particles of the 8H×8H×104B origami structure



Supplementary Fig. 21. Select helical TEM images of the $8H \times 8H \times 104B$ origami structure. a, Cylindrical model of the $8H \times 8H \times 104B$ origami cuboid. b, The helical projection of the cuboids. c, Select zoomed-in TEM images of these structures.



Supplementary Fig. 22. Select lateral TEM images of the $8H \times 8H \times 104B$ origami structure a, Cylindrical model of the $8H \times 8H \times 104B$ origami cuboid. b, The lateral projection of the cuboids. c, Select zoomed-in TEM images of these structures.

S5.2 TEM images for the 10.1 MDa ($10H \times 10H \times 156B$) structure

S5.2.1 Wide field views of the $10H \times 10H \times 156B$ structure



Supplementary Fig. 23. TEM images of the 10H×10H×156B structure.
S5.2.2 Select particles of the 10H×10H×156B structure



Supplementary Fig. 24. Select helical TEM images of the $10H \times 10H \times 156B$ structure. a, Cylindrical model of the $10H \times 10H \times 156B$ cuboid. b, The helical projection of the cuboids. c, Select zoomed-in TEM images of these structures.



Supplementary Fig. 25. Select lateral TEM images of the $10H \times 10H \times 156B$ structure. a, Cylindrical model of the $10H \times 10H \times 156B$ cuboid. b, The lateral projection of the cuboids. c, Select zoomed-in TEM images of these structures.

S5.3 TEM images for the 26.5 MDa (14H×14H×208B) structure

S5.3.1 Wide field views of the $14H{\times}14H{\times}208B$ structure



Supplementary Fig. 26. TEM images of the 14H×14H×208B structure.

S5.3.2 Select particles of the $14H \times 14H \times 208B$ structure



Supplementary Fig. 27. Select helical TEM images of the 14H×**14H**×**208B structure. a,** Cylindrical model of the 14H×14H×208B cuboid. **b,** The helical projection of the cuboids. **c,** Select zoomed-in TEM images of these structures.



Supplementary Fig. 28. Select lateral TEM images of the $14H \times 14H \times 208B$ structure. a, Cylindrical model of the $14H \times 14H \times 208B$ cuboid. b, The lateral projection of the cuboids. c, Select zoomed-in TEM images of these structures.

S5.4 TEM images for the 67.6 MDa (20H×20H×260B) structure

S5.4.1 Wide field views of the 20H×20H×260B structure



Supplementary Fig. 29. TEM images of the 20H×20H×260B structure.

S5.4.2 Select particles of the 20H×20H×260B structure



Supplementary Fig. 30. Select helical TEM images of the 20H×**20H**×**260B structure. a,** Cylindrical model of the 20H×20H×260B cuboid. **b,** The helical projection of the cuboids. **c,** Select zoomed-in TEM images of these structures.



Supplementary Fig. 31. Select lateral TEM images of the 20H×**20H**×**260B structure. a,** Cylindrical model of the 20H×20H×260B cuboid. **b,** The lateral projection of the cuboids. **c,** Select zoomed-in TEM images of these structures.

S5.5 TEM images for the 152.1MDa (30H×30H×260B) structure

S5.5.1 Wide field views of the 30H×30H×260B structure



Supplementary Fig. 32. TEM images of the 30H×30H×260B structure.

S5.5.2 Select particles of the 30H×30H×260B structure



Supplementary Fig. 33. Select helical TEM images of the $30H \times 30H \times 260B$ structure. a, Cylindrical model of the $30H \times 30H \times 260B$ cuboid. b, The helical projection of the cuboids. c, Select zoomed-in TEM images of these structures.



Supplementary Fig. 34. Select lateral TEM images of the $30H \times 30H \times 260B$ structure. a, Cylindrical model of the $30H \times 30H \times 260B$ cuboid. b, The lateral projection of the cuboids. c, Select zoomed-in TEM images of these structures.

S5.6 TEM images for the 262.8 MDa (36H×36H×312B) structure

S5.6.1 Wide field views of the 36H×36H×312B structure



Supplementary Fig. 35. TEM images of the 36H×36H×312B structure.

S5.6.2 TEM images of broken 36H×36H×312B structures



Supplementary Fig. 36. TEM images of the broken $36H \times 36H \times 312B$ structures.

S5.6.3 Select particles of the 36H×36H×312B structure



Supplementary Fig. 37. Select helical TEM images of the $36H \times 36H \times 312B$ structure. a, Cylindrical model of the $36H \times 36H \times 312B$ cuboid. b, The helical projection of the cuboids. c, Select zoomed-in TEM images of these structures.



Supplementary Fig. 38. Select lateral TEM images of the $36H \times 36H \times 312B$ structure. a, Cylindrical model of the $36H \times 36H \times 312B$ cuboid. b, The lateral projection of the cuboids. c, Select zoomed-in TEM images of these structures.

S5.6.4 Particle size measurement distribution

The edge lengths for the $36H \times 36H \times 312B$ cuboid were measured in two different TEM projection views: helical (denoted by *x*-*y* projection) and lateral (denoted by *x*-*z* or *y*-*z* projection). Because this cuboid is symmetrical, the *x*- and *y*-axes are indistinguishable. The measured lengths are plotted as a histogram in Supplementary Fig. 39. We expect the distance between bases to be approximately 0.34 nm and between helices to be 2.5 nm, which would respectively give lengths of 106 nm parallel to the helices and 90 nm perpendicular to the helices. The measured length along the helices peaked around 104 nm, which is close to our anticipated length. In contrast, the edge length perpendicular to the helices was measured to be 102 or 99 nm depending on whether the particles lay laterally or helically on the grid surface. This 10 nm discrepancy between the measured and expected lengths may result from electrostatic repulsion between the backbone that makes the lengths between helices to be larger. The distance between helices was 2.8 nm, slightly larger than the 2.6 nm measured for the 32-nt structures.¹³ This larger inter-helix distance may be due to the helical direction, especially when the structures are lying on the grid laterally. This larger variation may result from different compressive responses of the structure to adhesive forces or from surface forces that result during TEM sample deposition, since the structures are more flexible perpendicular to the helices compared to along the helices.



Supplementary Fig. 39. Histogram of measured edge lengths for the $36H \times 36H \times 312B$ particles. a, Lateral projection view of the cuboid, with the measured edge in (b) indicated by the blue arrow (n = 100). Average length measures 104.5 nm ± 1.8 nm s.d. c, Lateral projection view of the cuboid, with the measured edge in (d) indicated by the blue arrow (n = 100). Average length measures 102.1 nm ± 3.4 nm s.d. Note that the *x*- and *y*- axis are equivalent in these diagrams. e, Helical projection view of the cuboid, with the measured edges in (f) indicated by the blue arrow (n = 200). Average length measures 99.2 nm ± 2.9 nm s.d. All histograms display in the *x*-axis the length rounded to the nearest nm and the number of structures measured in the *y*-axis. Red curve shows the Gaussian fit of these histograms.

S5.7 TEM images for the 351.5 MDa (40H×40H×338B) structure

S5.7.1 Wide field views of the $40H \times 40H \times 338B$ structure



Supplementary Fig. 40. TEM images of the $40H{\times}40H{\times}338B$ structure.

S5.7.2 Select particles of the 40H×40H×338B structure



Supplementary Fig. 41. Select helical TEM images of the 40H×40**H**×338B structure. **a**, Cylindrical model of the 40H×40H×338B cuboid. **b**, The helical projection of the cuboids. **c**, Select zoomed-in TEM images of these structures.



Supplementary Fig. 42. Select lateral TEM images of the 40H×**40H**×**338B structure. a,** Cylindrical model of the 40H×40H×338B cuboid. **b,** The lateral projection of the cuboids. **c,** Select zoomed-in TEM images of these structures.

S5.8 Design of the 536.4 MDa (46H×46H×390B) structure



Supplementary Fig. 43. Nanobricks design of the 46H×46H×390B structure. Red shows zoomed-in views of the strand diagram.

S5.9 TEM images for the 536.4 MDa (46H×46H×390B) structure

S5.9.1 Wide field views of the $46H \times 46H \times 390B$ structure



Supplementary Fig. 44. TEM images of the 46H×46H×390B structure.

S5.9.2 Select particles of the 46H×46H×390B structure



Supplementary Fig. 45. Select helical TEM images of the 46H×**46H**×**390B structure. a,** Cylindrical model of the 46H×46H×390B cuboid. **b,** The helical projection of the cuboids. **c,** Select zoomed-in TEM images of these structures.

51



Supplementary Fig. 46. Select lateral TEM images of the $46H \times 46H \times 390B$ structure. a, Cylindrical model of the $46H \times 46H \times 390B$ cuboid. b, The lateral projection of the cuboids. c, Select zoomed-in TEM images of these structures.

S6 Tessellations

One can create larger structures by designing multimers containing a defined number of units. We first used a small $6H \times 6H \times 104B$ cuboid to test the different one-pot homo-multimeric designs. The design of these structures is analogous to the previously assembled DNA crystals¹⁵ in that complementarities are specified across different planes of helices. In contrast to the periodic structures, these multimeric structures are designed to grow to a defined and prescribed structure size based upon their starting unit and connection pattern. We explored the formation of dimers and tetramers using a number of different approaches. We then applied these approaches to a larger cuboid to create gigadalton-sized structures.

S6.1 Head-to-head dimers

Head-to-head dimers were created by removing the protecting head strands and connecting the remaining quartered strands in a symmetric manner across a plane parallel to the helical axis (Supplementary Fig. 47).

S6.1.1 Diagram of head-to-head dimers



Supplementary Fig. 47. Detailed design schematic for the head-to-head dimerized structure. a, The helical cross section of the repeating unit. **b,** A brick model for the repeating unit with the matching colored bricks indicating connected bricks. **c,** A brick model of the expected final design. **d,** A detailed strand diagram for the designed structure. Red color indicates the connecting strands. Zoom-in to see the details.

S6.1.2 Gel electrophoresis of the head-to-head dimers



Supplementary Fig. 48. Gel electrophoresis of a head-to-head dimer comprised of $6H \times 6H \times 104B$ units. A 2% agarose gel was used to analyze the tetramer which was formed at 20 nM/unit strand and 10 nM/connector strand concentration in the presence of 20 mM MgCl₂ using a 3-day two-stage annealing ramp. Blue arrow points to the expected location of a single $6H \times 6H \times 104B$ cuboid structure. Lane M contains a 1 kb DNA ladder. Numbers listed indicate the target gel yields.

S6.1.3 TEM images of head-to-head dimers



Supplementary Fig. 49. TEM images of the head-to-head dimer comprised of 6H×6H×104B units.

S6.2 Tail-to-tail dimers

Tail-to-tail dimers were created by removing the protecting tail strands and connecting the remaining half strands in a symmetric manner across a plane parallel to the helical axis (Supplementary Fig. 50).





Supplementary Fig. 50. Detailed design schematic for the tail-to-tail dimerized structure. a, The helical cross section of the repeating unit. **b**, A brick model for the repeating unit with the matching colored bricks indicating connected bricks. **c**, A brick model of the expected final design. **d**, A detailed strand diagram for the designed structure. Strand colored other than blue or gray indicate the connecting strands. Zoom in to see the details.

S6.2.2 Gel electrophoresis of the tail-to-tail dimers



Supplementary Fig. 51. Gel electrophoresis of a tail-to-tail dimer comprised of $6H \times 6H \times 104B$ units. A 2% agarose gel was used to analyze the tetramer which was formed at 20 nM/unit strand and 10 nM/connector strand concentration in the presence of 20 mM MgCl₂ using a 3-day two-stage annealing ramp. Blue arrow points to the expected location of a single $6H \times 6H \times 104B$ cuboid structure. Lane M contains a 1 kb DNA ladder. Numbers listed indicate the target gel yields.

S6.2.3 TEM images of tail-to-tail dimers



Supplementary Fig. 52. TEM images of the tail-to-tail dimer comprised of 6H×6H×104B units.

S6.3 Side-to-side dimers

Side-to-side dimers were created by connecting half strands within the same face of a structure such that the dimerized product results in two stacked cuboids that are shifted out of phase in the helical direction (Supplementary Fig. 53).





Supplementary Fig. 53. Detailed design schematic for the side-to-side dimerized structure. a, The helical cross section of the repeating unit. **b**, A brick model for the repeating unit with the matching colored bricks indicating connected bricks. **c**, A brick model of the expected final design. **d**, A detailed strand diagram for the designed structure. Red color indicates the connecting strands. Zoom-in to see the details.

S6.3.2 Gel electrophoresis of the side-to-side dimers



Supplementary Fig. 54. Gel electrophoresis of a side-to-side dimer comprised of $6H \times 6H \times 104B$ units. A 2% agarose gel was used to analyze the dimer which was formed at 200 nM/core strand and 100 nM/connecting strand concentration in the presence of 20 mM MgCl₂ using a 3-day two-stage annealing ramp. Blue arrow points to the expected location of a single $6H \times 6H \times 104B$ cuboid structure. Lane M contains a 1 kb DNA ladder. Numbers listed indicate the target gel yields.

S6.3.3 TEM images of side-to-side dimers



Supplementary Fig. 55. TEM images of the side-to-side dimer comprised of 6H×6H×104B units.

S6.4 Rotational tetramers

To create rotationally symmetric structures, we connected half-strands on two adjacent faces together. The resulting expected product would be a strained tetramer where each strand is connected to another in a rotational manner (Supplementary Fig. 56c). A potential byproduct can result by forming structures with no strain where two adjacent cuboids are not connected (indicated by the arrow in Supplementary Fig. 56d).

S6.4.1 Strand diagram of rotational tetramers



Supplementary Fig. 56. Detailed design schematic for the rotational tetramers. a, The helical cross section of the repeating unit. **b**, A brick model for the repeating unit with the matching colored bricks indicating connected bricks. **c**, A brick model of the expected final design. Note that formation of this structures would result in a strained structure **d**, A brick model of a defectively formed structure. **e**, A detailed strand diagram for the designed structure. Red color indicates the connecting strands. Zoom in to see the details.

S6.4.2 Gel electrophoresis of a tetramer comprised of 6H×6H×104B units



Supplementary Fig. 57. Gel electrophoresis of a tetramer comprised of $6H \times 6H \times 104B$ units. A 2% agarose gel was used to analyze the tetramer which was formed at 400 nM/strand concentration in the presence of 20 mM MgCl₂ using a 3-day two-stage annealing ramp. Blue arrow points to the expected location of a single $6H \times 6H \times 104B$ cuboid structure. Lane M contains a 1 kb DNA ladder. Numbers listed indicate the target gel yields.

S6.4.3 TEM images of a tetramer comprised of $6H \times 6H \times 104B$ units



Supplementary Fig. 58. TEM images of the tetramer comprised of 6H×6H×104B units.



S6.4.4 Strand diagrams of a tetramer composed of 36H×36H×312B units

Supplementary Fig. 59. *Nanobricks* diagram of the tetramer comprised of $36H \times 36H \times 312B$ units. **a**, Schematic depicting a single $36H \times 36H \times 312B$ unit with its connecting strands. **b**, Schematic depicting only the connecting strands in context of a $36H \times 36H \times 312B$ unit. Red outlines shows zoomed-in views of the corner helices. **c**, Schematic depicting the full $72H \times 72H \times 312B$ tetramer. **d**, Schematic depicting only the connecting strands in the context of the full $72H \times 72H \times 312B$ tetramer. Note that these diagrams do not show the *z*-axis distortion that results from the strained connections.



Supplementary Fig. 60. Cross-sectional helical diagram of the $36H \times 36H \times 312B$ unit forming a tetramer. Schematic of select helices in the $36H \times 36H \times 312B$ unit with associating strand diagram is depicted in Supplementary Fig. 61.



Supplementary Fig. 61. Strand diagram of select helices in the 36H×36H×312B unit of a tetramer. Red color indicates the connecting strands. Zoom-in to see the details. Background color matches those in the associating helical cross-sectional view depicted in Supplementary Fig. 60.

S6.4.5 TEM images of a tetramer comprised of 260 MDa units



Supplementary Fig. 62. TEM images of the tetramer comprised of $36H\times36H\times312B$ units.



S6.4.6 Select particles of a tetramer composed of 36H×36H×312B units

Supplementary Fig. 63. Select helical TEM images of a tetramer composed of $36H \times 36H \times 312B$ units. a, Cylindrical model of the 1 GDa structures. b, The helical projection of the cuboids. c, Select zoomed-in TEM images of these structures. Note that some of the structures shown in the bottom row are not fully connected.



Supplementary Fig. 64. Select lateral TEM images of the a tetramer composed of $36H \times 36H \times 312B$ units. a, Cylindrical model of the 1 GDa structure. b, The lateral projection of the cuboids. c, Select zoomed-in TEM images of these structures.
S7 3D DNA-PAINT analysis of the 30H×30H×260B canvas

S7.1 Design of DNA-PAINT extensions

DNA-PAINT super resolution imaging was applied to our $30H \times 30H \times 260B$ canvas. To create a structure with addressable PAINT extensions, we removed some of the crossovers on the surface of a helical face and added new crossovers on the opposite side of the strand (Supplementary Fig. 65). Such redesign was applied only to the face of the structure with poly-T loops. Each corner of the structure along the helical axis was designed to contain PAINT handles on five 5' ends and five 3' ends. Twelve 26-nt strands were modified with biotin extensions to enable docking of the structure on a surface. To fold the structure, these new PAINT strands were added in 75 times excess of the original cuboid structure.



Supplementary Fig. 65. Schematic of strand design of $30H \times 30H \times 260B$ for DNA-PAINT super-resolution imaging. a, Model of the $30H \times 30H \times 260B$ cuboid with the PAINT extensions depicted in black and how the structure is expected to be docked on a glass slide. Views of the different faces are shown in (c)-(e) b, Legend of the strand diagrams. c, Bottom layer of the structure with the *y* strands depicted. Strands with extensions for the biotin-labeled handles are shown in orange. d, The "head" of the structure where poly-T crossover protecting strands are included. Changes to the structure architecture are shown in red. Extensions for PAINT handles are indicated in black and blue to represent a 5' and 3' extension, respectively. e, The "tail" of the structure where poly-T protector strands are replaced by the PAINT handles.

S7.1.1 3D DNA-PAINT imaging of the 30H×30H×260B structure

In order to further characterize our $30H \times 260B$ canvas, we applied DNA-PAINT super-resolution imaging by labeling 10 docking sites at each corner (Fig. 2f, Supplementary Fig. 65 and 66a). By modifying one surface of the cube with a biotin labeled DNA strand, the cube was immobilized via biotin-streptavidin interaction and imaged with a TIRF microscope using optical astigmatism for 3D super-resolution fluorescence microscopy.^{5,8} The overview shows that most particles are well formed (Fig. 2f). Zoomed-in views of a representative particle is shown in the panels below. Measurement of dimensions across 100 individual structures showed that structures in solution matched our expected design and was in good agreement with our TEM analysis and theoretical calculations (see Supplementary Fig. 65 for additional calculations). Due to the optical astigmatism, localizations closer to the surface were observed with higher precision compared to localizations further away. (Supplementary Fig. 66b and c). Single-particle analysis reveals that most structures in the field of view (33.28 μ m × 32.28 μ m, in total ~ 1000 structures) contain all eight corners, suggesting well formed structure edges.



Supplementary Fig. 66. DNA-PAINT characterization of the 30H \times 30H \times 260B structure. a, Labeling scheme of $30H \times 30H \times 260B$ with DNA-PAINT-handles. Each corner of the cube is modified with ten DNA-PAINT handles to allow for identification of the longer and shorter sides of the cube structure. The bottom surface of the structure is tethered to the glass-slide via biotin-streptavidin interactions. b, Representative 3D-DNA-PAINT super-resolution images of the cuboid. Upper panel depicts representative fluorescent image of four cuboids on the surface. Color indicates height as displayed in the color bar. The lower panel depicts zoomed-in views of a single cube structure. Boxes show different projections of the respective structure: 3D-View, *x-y, y-z* projection (left to right). Scale bars measure 100 nm. **c,** Length measurements of cuboids with 3D-DNA-PAINT. Statistical analysis was performed on 100 structures. Histograms in the upper right and lower panel show distance distributions. Colors of bars match those indicated in the schematic in **a**.

S8 The Nanobricks Software

S8.1 Overview



Supplementary Fig. 67. Nanobricks software. We developed a software package for automating the process of converting a shape into a set of voxels, compiling these voxels into a set of strands, and generating sequences for these strands. The software runs on the web and has an intuitive interface for adding and manipulating voxelized structures.

To facilitate the design of sequences for our large structures, we created a comprehensive software package, *Nanobricks* (Supplementary Fig. 67). This software automates the process of converting a 3D shape into a set of strands and of mapping sequences onto those strands. A suite of tools are available to users for modifying shapes, strands, and sequences, either through the interface or programmatically through scripts. These capabilities and tools are discussed below.

S8.2 Using Nanobricks

S8.2.1 Interface

The *Nanobricks* interface has been designed to be intuitive to new users, by employing the DNA brick abstraction directly into a dynamic 3D canvas. Users can click on points in space to fill in voxels and build up their desired structures. With a single button press, these structures can be converted into a set of strands routed through the structure according to the desired DNA brick motif.¹³ With another button press, sequences can be automatically generated and mapped onto these structures. These sequences can then be outputed in several formats and the oligos ordered directly.

S8.2.2 Help guides

🗞 NanoBricks Help	Getting Started		
Help Topics	Welcome to NanoBricks; there are 3 easy steps to start building nanostructures:		
Getting Started	Draw voxels Build strands Generate sequences		
Lattices and Translation Schemes			
Sequences	1. Draw voxels		
Plugins	When you begin, you'll already be using the Pointer tool, which you can use to place voxels; click to place, shift-click to delete, or hold alt and move the mouse to paint. Drag the mouse to look around (orbit). Instructors for using the tool show up in a blue box to the top left, ri under the toolbar.		
Scripting	If you don't want to draw, you can also import files from another 3D modeling program, using the import button in the main toolbar. Follow		
CoffeeScript	the instructions in the top left.		
API Documentation	2. Build strands		
Base	Click the "Voxels > Strands" button in the toolbar to generate strands from your voxel design; you should see a bunch of blue and black lines appearthose are your strands. You can toggle the Voxels view off from the "views" pane on the right in order to see your strands better.		
Strand	3. Generate sequences Click the "Sequences" button in the toolbar to open the sequences window. Choose "Random" from the dropdown. Click "Generate & Thread", you should see a bunch of sequences.		
Voxel			
Strand Controller	Next store		
Voxel Controller	Next steps		
Sequence Controller	You're done! Now you can • Export your sequences to a CSV file and order them from a DNA synthesis company		
1.000	 Export a picture of your structure to an SVG file (for editing in Inkscape or Adobe Illustrator) Export a model of your structure in STL format (for 3D modeling or OBJ (for editing in another 3D modeling process). 		

Supplementary Fig. 68. Help menu. New *Nanobricks* users can find several help guides for using the software by accessing the menu at the top of the screen.

Several guides are available to help users learn the *Nanobricks* software. These can be accessed from the menu at the top right section of the screen (Supplementary Fig. 68).

S8.2.3 Design Flow

The overall *Nanobricks* design flow occurs in three basic steps (Supplementary Fig. 69). First, users design their voxelized structures, either by manipulating voxels on the 3D canvas or by directly importing a 3D object. Next, strands are routed onto the voxelized structure following a translation scheme, such as the 8nt-per-voxel DNA brick abstraction.¹³ Finally, sequences can be mapped onto the strands in a couple of ways. The sequences may be randomly generated by the software, or an existing set of sequences can be uploaded for use.



Supplementary Fig. 69. Design flow. With the *Nanobricks* interface, users can easily convert their voxelized shapes into a set of strands. Sequences can be loaded into the software or randomly generated and then mapped onto the strands.

S8.2.4 Navigating the 3D canvas

Structures are displayed on a 3D canvas that can be rotated by clicking and dragging outside the gridded area. Scrolling zooms the camera in and out. A set of axes is included to aid visualization, with the x, y, and z axes denoted by red, green, and blue, respectively. The z axis corresponds to the helical axis for strand routing.

The *Views* menu on the right-hand side of the screen provides additional options for viewing the canvas. Three different viewing modes are available - orthographic, perspective, and anaglyph. The camera can also be snapped to one of five preset viewing angles from this menu - top, left, right, bottom, and front.

In the same right-hand menu, different slices of the canvas can be disabled to allow for easier access to and modification of the internal voxels of a structure. These slices are adjusted using sliding bars and can be reset to show all voxels once internal editing is complete.

The last options in this menu denote the different elements that can be displayed on the canvas. When the *Voxels* option is checked, filled voxels are shown on the canvas. When the *Strands* option is selected, any strands that have been routed onto the structure will also be displayed. Selecting the *Sequences* option displays the identity of the individual base pairs that have been mapped onto all voxels (including unfilled ones) on the canvas. If no sequences have been generated and mapped yet, base pairs are shown in gray.

S8.2.5 Current version

The current version of the software can be found at http://nanobricks.software. The Chrome browser (https://www.google.com/chrome/browser/desktop/) is recommended for use with *Nanobricks*.

S8.3 Tools



Supplementary Fig. 70. *Nanobricks* tools. **a**, The *pointer* tool allows users to add and remove individual voxels. **b**, The *select* tool allows selected (yellow) sets of voxels to be manipulated. **c**, The *rectangle* tool allows large areas of the canvas to be voxelized at once. **d**, The *strand* tool allows users to add crossovers between strands in a structure. **e**, Tools are selected from the toolbar found at the top of the screen.

Several tools are available to users for modifying their structures and strands (Supplementary Fig. 70). Typically, these tools are accessed as different modes, so that only one tool is active at a time. While using these tools, it is good to remember that there are undo and redo options available, which can be accessed at the top of the screen.

S8.3.1 Pointer

By default, users are set to use the *pointer* tool (Supplementary Fig. 70a). The pointer is the simplest mode to add individual voxels. Using a point-and-click method, the user can add voxels at any canvas position and shift-click to remove voxels at any position. Holding alt (or option key on Macs) enables the paint mode, whereby all voxels traversed by a mouse path become filled. Similarly, mouse movement while holding shift-alt activates an eraser tool. Pressing the arrow keys while in pointer mode will shift the cursor by a single voxel position.

The pointer mode also includes an X-ray option, which can be toggled on/off by pressing the Enter key. If active, voxels around the pointer position become semi-transparent to better show the interior details of a structure.

S8.3.2 Select

The *select* tool allows the user to select and manipulate a set of voxels by dragging and dropping (Supplementary Fig. 70b). Multiple voxels can be selected by shift-clicking them, and they are removed from selection by a subsequent shift-click.

S8.3.3 Rectangle

The *rectangle* tool allows for rapid prototyping by providing the user with the ability to paint voxels in rectangular regions in the canvas by clicking corners of the rectangle (Supplementary Fig. 70c). If one of the corners is above the base level of the canvas, then the rectangular area will be filled starting from the highest point of the corner to the base level.

S8.3.4 Strand

The *strand* tool allows the user to edit strands on the canvas by clicking to choose crossover positions (Supplementary Fig. 70d). To add a crossover, a user clicks the 3' end of an existing strand and then the 5' end of the joining strand. Longer crossover strands can also be created *de novo*, by clicking to place the 5' end and specifying the crossover path of the strand before double clicking to terminate the strand.

S8.3.5 Strand Eraser

The *strand eraser* tool can be used to delete strands by directly clicking on them on the canvas.

S8.3.6 Strand Extender

The *strand extender* tool allows the user to extend strands on the canvas by clicking on the end of a strand and dragging. This tool is used for creating off-lattice strand extensions. These extensions will be visibly different from the other on-lattice since they extend at a 45° angle from the canvas.

S8.3.7 Cut Strands

Strands can be cut at specific base pair locations with the *cut strands* tool. The affected strand will be cut into two adjacent strands.

S8.3.8 Ligate Strands

The *ligate* tool can be used to concatenate two strands together, by connecting the 3' end of the first strand selected with the 5' end of the second strand.

S8.3.9 Add Voxels

The *add voxels* tool vastly extends the capability of users to manipulate their voxelized structures by providing them with the opportunity to programmatically define which voxels should be filled with a script. Coffeescript code (http://coffeescript.org/) can be input into the *Add/Remove voxels* window and applied directly to the canvas. A couple basic examples of code and the results are shown in Supplementary Fig. 71. In addition to calling a library of functions already available to them, users can also write their own functions and execute them for filling in voxels.

S8.3.10 Power Edit

The *power edit* tool is another script-based editing tool that allows users to programmatically select and edit voxels and strands on the canvas. Although the features required to create simple structures are available through the user interface, the power edit functionality gives users much more control over the details of the shapes and sequences. Properties of voxels (such as color and position) and strands (such as length and sequence) can be easily modified with Coffescript code executed directly in the program. An example of this programmability is shown in Supplementary Fig. 72. More examples of possible uses for the power edit tool are given in the *Nanobricks* software.



Supplementary Fig. 71. Add voxels tool. **a**, Using the Coffeescript cuboid command, a cube with specified side lengths can be added to the canvas, centered at a particular point in space. **b**, Specific coordinates can be voxelized by setting the conditions for which coordinates are valid.



Supplementary Fig. 72. Power edit tool. Users can directly select and manipulate voxels and strands with Coffescript by using the *power edit* tool. The example above shows how to select a set of voxels and collectively change their color on the canvas.

S8.3.11 Voxels to Strands

DNA strands can be routed onto any voxelized structure with using the *Voxels to Strands* command. By default, the routing strategy matches the brick translation scheme described in ref. [13], where helical domains are 8-nt and crossovers are non-alternating. However, other routing options can be accessed through the dropdown menu on the command.

If some strands already exist in the structure, this tool will by default create strands on the canvas at any voxel positions which do not already have strands in place. If old strands should be deleted instead, this functionality is accessible by changing from the *Merge to Overwrite* option in the dropdown menu.

S8.3.12 Generate Sequences

The *Generate Sequences* tool assigns sequences to strand in two steps. First, sequences are generated for each voxel in the lattice. Then, those sequences are threaded onto the strands in the structure, based on which voxels the strand passes through. Strands that pass through voxels in opposite directions get complementary sequences.

Three sequence generation modes are available. In the *Random* mode, sequences are generated with a pseudo-random number generator. In *Linear* mode, the sequence block is loaded from a file or a string, and then sequences are threaded onto the strand. In *Excel* mode, existing strands are loaded from an Excel spreadsheet, then threaded into the sequence block (that is, sequences for voxels in the block are assigned based on the existing strands). Sequences can then be assigned to new strands by threading from the sequence block.

S8.3.13 Change Lattice

In addition to the default square lattice, *Nanobricks* supports other lattices for structure and strand routing. These lattices, such as flat, honeycomb, and hexagonal, can be accessed through the *Change lattice shape or size* menu in the toolbar. The user can also configure the dimensions (width, height, and depth) of the canvas for the given lattice type. Screenshots of example lattices can be seen in Supplementary Fig. 73.



Supplementary Fig. 73. Lattice types. The lattice type can be selected by the user. **a**, The cubic lattice uses 8-nt domains. **b**, The flat lattice uses 21-nt domains. **c**, The hexagonal lattice uses 9-nt domains. **d**, The honeycomb lattice uses 9-nt domains. For more information about lattices and domain lengths, see ref. [13, 16, 17].

S8.4 Working with files

S8.4.1 Importing 3D objects

3D object files in the .obj or .stl formats can be imported directly into the *Nanobricks* software (Supplementary Fig. 74). Several commands are available during import for placing, resizing, and rotating the object before it gets voxelized on the 3D canvas.

S8.4.2 Importing and exporting caDNAno files

Structures built using the caDNAno and caDNAnoSQ programs (http://cadnano.org/legacy) can be imported directly into the *Nanobricks* software. Similarly, structures built in *Nanobricks* can be exported to the caDNAno file formats.

S8.4.3 Saving a *Nanobricks* file

Nanobricks has a specialized file format with the .nbks extension for preserving the state of a *Nanobricks* program, including the voxels, strands, and sequences. This allows the user to re-open their programs across sessions and computers, enabling easy and unlimited access to their constructions. The name of the file can be designated at the top of the screen, by replacing the "Untitled" text in the appropriate box. Data are saved in a JSON-based serialization format.



Supplementary Fig. 74. Importing 3D structures. 3D object models can be imported directly into the software, and many options are available for changing their size and orientation on the canvas. Once positioned, users can voxelize the structures and continue through the *Nanobricks* design process.

S8.4.4 Plugins

The software has been designed to allow for simple and customized reconfiguration through the use of plugins. These plugins are compiled into javascript and can be linked to the software through the *Plugins* menu. A plugin must be hosted at a valid URL in an accessible directory and can be deleted by the user at any time. Examples of plugins include scripts for new types of lattices and translation schemes.

S8.5 Architecture

S8.5.1 Overview

The *Nanobricks* software was designed to be modular and scalable. Modules and libraries were combined in such a way that the software can easily be extended to new types of nanostructures, including ones with different lattice types, translation schemes for routing strands through structures, and new sequence generation methods. The software lays the foundation for a general tool for structural nanotechnology.

The *Nanobricks* software is written in several largely independent modules, with a particular focus on creating software that is reusable, modular, and extensible. The CommonJS module standard (http://wiki.commonjs.org/wiki/Modules/1. 1) was used to organize code, with most requisite dependencies loaded as this type of modules.

S8.5.2 Modules

The software has three core modules: *vox*, *C3D*, and *UI3D*. The *vox* module contains the core routines for manipulating voxels, lattices, and strands. It does not define any user interface. This code handles three general categories of voxel manipulation: lattice object manipulation and functions for mapping voxel coordinates to 3D space, translation schemes for defining how a set of voxels gets compiled into a set of strands, and utility functions for generating and manipulating base, domain, and strand objects.

The *C3D* module defines and handles user interactions with the 3D canvas interface. It is responsible for loading and managing the central data models. *C3D* is built largely around a Model, View, and Controller (MVC) paradigm, although it also handles the different modes associated with the tools described above. The C3D camera uses a projection matrix to show the mapping of the 3D space onto 2D. An inverse technique, known as unprojection, multiplies the inverse of the projection matrix to map a 2D point

back onto the 3D space. This strategy is combined with raycasting to determine what point in 3D space a user's mouse is hovering over.

Finally, the *UI3D* module handles buttons, toolbars, windows, and other elements that form the user interface as marked in chrome on top of the 3D canvas. Typically, each of these interface elements use Jade (http://jade-lang.com/) templates.

S8.5.3 Dependency management and builds

To build the software, two engines - browserify (http://browserify.org/) and usemin (https://github.com/ yeoman/grunt-usemin) - are used. The browserify engine parses *require* statements in our code, to ensure the relevant module dependencies are included in the build. As a few of the libraries the software uses are not available as CommonJS modules, they must be separately included and parsed by the usemin engine at build time to be included. Grunt (http://gruntjs.com/) is used to manage building the application, documentation, and other requirements.

S8.5.4 Unit tests

The Mocha (http://visionmedia.github.io/mocha/) framework was used for implementing unit tests for the software.

S8.5.5 Libraries

Nanobricks utilizes several available libraries, including but not limited to jQuery (http://jquery.com/), Backbone (http://backbonejs.org/), three.js (http://threejs.org/docs/), ndarray (https://github.com/mikolalysenko/ndarray), Rivets (http://rivetsjs.com/), Bootstrap (http://getbootstrap.com/), and CodeMirror (http://codemirror.net/).

S8.5.6 Coffeescript

Most of the *Nanobricks* code is written in Coffeescript (http://coffeescript.org/), and this is also the language used by the *Add Voxels* and *Power Edit* tools described above. Learning the Coffeescript syntax will be especially helpful for users who want to take full advantage of the powerful capabilities of the Nanobricks software.

S9 Shape design

S9.1 Designs

Because we used a 152 MDa cuboid as our canvas without ordering additional modified strands with polyT replacing unused voxels, each structure would be missing several strands. As a result, features shown from our original design that are 4 voxels thick would actually contain strands that cover 3 voxels. Thus, our designs were made to be a bit more conservative to account for these differences. Generally, the structures can be sorted into four categories based upon the design process utilized: feature resolution testing, mathematical scripting, 3D model imported, hand-designed. For each of the categories, we describe the process and shapes that fall under this category. Through these designs, we demonstrate an unprecedented level of 3D feature complexity on the nanoscale. Our previous studies that used a $10 \times 10 \times 10$ voxel canvas was limited in feature patterning. By using our new 52-nt DNA brick to assemble a more massive structure containing just 18 times more voxels, we are already capable of patterning far more intricate features. Further, the larger canvas sizes can help stabilize fragile features by providing increased surrounding structural support for our intricate cavities.

Feature resolution testing

We gradually introduced finer and finer features to the top surface of a cuboid canvas containing a $26H \times 26H \times 52B$ cavity (Extended Data Fig. 2). When features were less than 3 helices, structures proved to be difficult to form due to the fragility of the structure and the increased number of missing strands. With an odd number of helices in the features, designs have an increased ratio of half strands which are thereby missing from the reaction stocks.

For sufficient structural stability, we selected to follow design principles we found from our previous studies.¹³ This ensured that there were at least four voxels in a helical feature, thereby allowing for at least one crossover to occur between each helix.

Other structures also demonstrate the limitations of features. The interconnected loop cavities show that TEM contrast will be visible if features are least 3 voxels thick along the helical direction and at least 2 helices thick (Fig. 3j).

3D model import

We used different 3D models to design a few complex cavities (Supplementary Fig. 76). These structures demonstrate the type of complex features that can be patterned only with larger scales. Previously, our attempts in creating varying defined cavities and features were limited due to the small canvas size. While we had the possibility of creating geometric patterns such as triangles and rectangles, it was difficult to create fine features. With the increase in voxel number, we have been able to accommodate cavities including a bunny, teddy bear, and even words to spell out 'LOVE' (Fig. 3b,e,f). The previous 3D DNA brick structures could only hold one letter or character at a time. Now the increased size allows not only for words to be created but also for control over lettering thickness.

Mathematical scripting

We were also able to rapidly design a number of mathematically inspired cavities, including a twisted Möbius strip, a helicoid cavity, and a hyperboloid (Fig. 3c,g,h), by using the scripting function of Nanobricks (Supplementary Section S9.4).

Hand-designed shapes

Remaining shapes were made by combination of Nanobricks scripting to easily control voxel deposition and hand placement of voxels. Two particularly complex structures include a interconnected loop cavities and a cavity that penetrates into itself (Fig. 3j,k). Further, we were able to demonstrate a structure displaying projections of three different letters along each of the faces (Fig. 3d). Such structures can be particularly difficult to self-assemble because the features are tightly packed and separated by cavities.

Overall, these larger structures demonstrate unique features and designs that have previously been unachievable in a 3D structure in a single-pot reaction. Generally, this involves a diversity of fine features spread across a large volume. One would expect growth of such cavity structures to be difficult because different parts of the structure could potentially nucleate and grow separately, making it difficult to form one contiguous structure. Further, the increased voxel number allows for formation of structures stable enough to provide 3 different projections that show 3 different letters. Before with previous 32-nt brick designs, only 2 letters were possible and these designs were very fragile and formed with low yields.¹³ Additionally, forming words with detailed lettering thickness is possible, along with introducing numerous parallel non-intersecting channels.

S9.2 Shape approximation

Strands from the original cuboid canvas were reused to form the different shapes, and no new strands were ordered. As a result, the structures are missing a number of strand derivatives and half-strands typically used to protect the free edges within the cavity (See the Supplementary Information of reference [13] for more details about structure design and strand derivatives). Thus, final structures assembled may deviate slightly from the original intended design. To visualize these discrepancies, we generated a second model that depicts each voxel as either transparent, semi-transparent, or blue to indicate the presence of double strands, single strands, or no strands, respectively. We can observe from these models that fine cavity features may appear larger due to missing half-strands in the cavity. As an example, the voxels at the end of the helices are always single-stranded in the simplest shape with a missing cuboid center (Supplementary Fig. 75n).



Supplementary Fig. 75. 3D rendering of shape approximations. For all subfigures, left shows the original cavity design where blue voxels represent no strands present. In contrast, the right panel shows the actual strands present, with semi-transparent gray voxels representing single strands.

S9.3 3D rendering import files

The original 3D rendered files and their original source are shown in the following sections alongside the link to the original references (Supplementary Fig. 76).



Supplementary Fig. 76. 3D models used for structure designs a, Low poly Teddy bear by nonon from http://www.thingiverse.com/thing:653095 **b,** LOVE Sculpture by phineasjw from http://www.thingiverse.com/thing:22952/ **c,** Stanford bunny from http://graphics.stanford.edu/data/3Dscanrep/ http://www.thingiverse.com/thing:653095

S9.4 Mathematical scripting

Scripts used to generate the mathematical shapes are included in the following subsections.

S9.4.1 Möbius Strip

```
add = 12
bdd = 15
cdd = 10
r = 8
m = []
for v in [-100..100]
  m.push v/20
n = []
for u in [0..100]
  n.push u*Math.PI/50
a = []
b = []
c = []
d = []
for u in n
   for v in m
      a= Math.round(((r + v*Math.cos(u/2))*Math.cos(u)))
      b= Math.round(((r + v*Math.cos(u/2))*Math.sin(u)))
      c= Math.round(v*Math.sin(u/2))
      d.push([a+add,b+bdd,c+cdd], [a+add+1, b+bdd, c+cdd], [a+add-1, b+bdd, c+cdd], [a+add,
          b+bdd, c+cdd-1], [a+add, b+bdd, c+cdd+1])
(x, y, z) ->
   for [x2, y2, z2] in d
      if x == x^2 and y == y^2 and z == z^2
         return true
```

S9.4.2 Hyperboloid

 $(x, y, z) \rightarrow (x-15) * *2 + (y-15) * *2 - (z-11) * *2 >= 9$

S9.4.3 Helicoid

```
add = 15
bdd = 15
cdd = 10
m = []
for v in [-100..100]
  m.push v/12
n = []
for u in [-100..100]
  n.push u*Math.PI/100
a = []
b = []
c = []
d = []
r = 5
p=1
for u in n
   for v in m
      a= Math.round(v*Math.cos(p*u))
      b= Math.round(v*Math.sin(p*u))
      c= Math.round(r*u)
      d.push([a+add,b+bdd,c+cdd], [a+add+1, b+bdd, c+cdd], [a+add-1, b+bdd, c+cdd], [a+add,
          b+bdd-1, c+cdd], [a+add, b+bdd+1, c+cdd])
(x, y, z) ->
   for [x2, y2, z2] in d
      if x == x^2 and y == y^2 and z == z^2
         return true
```

S9.5 Select TEM images used for averaging

At least six particles were selected for averaging for each given projection. Particles that were used for averaging are depicted in Supplementary Figs. 77 - 82. Supplementary Table 3 lists the number of particles that were averaged for each figure. Note that the particle shape is referenced by a naming scheme matching those in Fig. 3 and Extended Data Fig. 2. Structures were averaged by hand alignment or using the EMAN2 software. For a few of the shape designs, the lateral projections are difficult to distinguish from one another. In this case, a single lateral projection is used, and the expected average projection is shown in Fig. 3.

Shape	Helical	Lateral	
	xy	XZ	yz
1	6	6	
2	6	6	
3	6	6	
4	6	6	
5	6	6	
6	6	6	
7	6	6	
b	22	12	9
с	18	21	10
d	7	14	9
e	6	16	20
f	17	8	8
g	16	14	11
h	6	6	
i	9	8	
j	6	6	6
k	6	6	6
1	6	6	
m	6	6	
n	6	6	

Supplementary Table 3. Number of particles used for class averages across the different projections. Note that shapes with symmetrical lateral projections are listed with only one value under the 'Lateral' column.



100 nm

Supplementary Fig. 77. Particles used for class averages. TEM images of the particles used for the different class averages. The averaged image is depicted on the left.



100 nm

Supplementary Fig. 78. Particles used for class averages. TEM images of the particles used for the different class averages. The averaged image is depicted on the left.



Supplementary Fig. 79. Particles used for class averages. TEM images of the particles used for the different class averages. The averaged image is depicted on the left.



Supplementary Fig. 80. Particles used for class averages. TEM images of the particles used for the different class averages. The averaged image is depicted on the left.



Supplementary Fig. 81. Particles used for class averages. TEM images of the particles used for the different class averages. The averaged image is depicted on the left.



Supplementary Fig. 82. Particles used for class averages. TEM images of the particles used for the different class averages. The averaged image is depicted on the left.

S9.5.1 General observations

Wide field images of both unpurified and purified samples showed that some particles are destroyed. To analyze the structures, we performed counting experiments to determine the structural integrity of the formed shapes. The cavity structures showed generally lower structural integrity when compared with a the solid cuboid $30H \times 30H \times 260B$ canvas (Supplementary Fig. 83), as expected since the rigidity of the structures are reduced with the introduction of cavities. Overall, structures have TEM yields of approximately 65 - 80%.



Supplementary Fig. 83. TEM structural analysis of cavities. Example wide-field TEM images obtained for the **a**, bear, **b**, helicoid, and **c**, 'GEB' structures are shown. Blue circled structures match the expected design. Red circled structures have observable defects. Respective counts for each structure are listed in a table (**d**).

S10 Electron tomography

In conventional transmission electron microscopy, images of DNA brick cuboids result from the projection of the complete thickness of the DNA nanostructure, limiting the visualization of internal features of the objects. To circumvent these limitations, we performed structural analysis by electron tomography on several DNA brick nanostructures. We first investigated a cuboid $(30H \times 30H \times 260B)$ that features sixteen-parallel crossing channels $(2H \times 2H \times 260B)$ along the helical axis (Fig. 4a). Imaging of the sample revealed monodisperse particles in multiple orientations with the expected dimensions and shape (Supplementary Figs. 86 – 90). Several tilted series of purified structures in different projections were recorded from -60° to $+60^{\circ}$ with 2° angular increment. Image alignment and 3D reconstruction of tomograms were performed using IMOD¹⁸ (Fig 4a, b). Series of 2D slices through the reconstructed cuboid are shown in Fig. 4a, Supplementary Figs. 84 - 93 and the Supplementary Videos. The cuboid reconstructions from electron tomography showed typical shape artifacts at the extreme top and bottom of the particles in the direction of the electron beam due to the missing wedge.¹⁹ Nevertheless, the reconstruction unambiguously verified the morphology of the network of channels in the cuboid.

The sequences of images extracted from the tomogram (*x*-*y* plane) along the helical (*z*) direction show sixteen-parallel cavities belonging to the $2H \times 2H$ channels design (Fig. 4a, Supplementary Figs. 84, 85). In the perpendicular orientation (*x*-*z* plane), 2D slices display continuous crossing channels. The 3D visualization in mesh surface representation reveals the 3D channels network with both external and internal features of the cuboid. The top and side view of the filtered 3D volume of the cuboid clearly show continuous crossing, independent channels (Fig 4b). Therefore, the gallery of slices and the filtered 3D volume of the cuboid confirm that the global topology of the reconstructed density is in agreement with the expected architecture of the object. In our TEM experiments, the tomogram of cube containing parallel channels displays apparent diameters between 4 - 6 nm, suggesting a resolution of 2 to 3 nm, which is in agreement with the literature.²⁰ It is established that in negative stain, resolution of reconstructions is limited to 1.5 nm both in electron tomography and in single particle analysis. This is related to the origin of image contrast and more specifically to the balance between amplitude and phase contrasts. So in our TEM experiments, the limiting factor for the obtained resolution is directly related to the sample preparation, *i.e.* the negative stain.

We then performed electron tomography on four distinct cuboids exhibiting sophisticated interior cavities and thin features: the teddy bear, the bunny, the helicoid cavity, and the 'GEB' cavity. Several tilt-series were collected for the three different projection views of each cuboid. The sequences of slices extracted from these tomograms are provided in Fig. 4c-e and Supplementary Figs. 86 - 102. For most cuboid particles, reconstructed tomograms revealed that they did fold into expected designs.

Sophisticated three-dimensional structures containing features as thin as 2 nm have been observed in the teddy bear and the bunny design, such as the bear's snout and limbs or the bunny's ears. Figure 4c summarizes the series of slices through the cuboid featuring the teddy bear along the three orthogonal axes (see also Supplementary Figs. 87 - 89 and Supplementary Movies). The diffuse gray structure visible in the center of the cube section along the *z* axis (slice 34 in plane *x-y*) corresponds to the bear's snout, indicated by the red arrow (Fig. 4c). It can also be visualized from side view (slice 57 in the *x-z* plane), indicated by the red arrow (Fig. 4c). In addition, from the face view, we can recognize two thin oval shapes on each side of the the bear's head, corresponding to the ears (slice 51 along the *z* direction, Fig. 4c). The bear's ears are also recognizable from the top view (in slice 64 along the *y* axis, indicated by the red arrows, Fig. 4C). The teddy bear's hind paws are clearly distinguished from the three orthogonal projections. In slice 26 along the *z* axis, we can see the O-shaped paws near the bottom of the body (Fig. 4c). The hind paws are visible from the top view in slice 41 along the *y* axis and from the side view in slices 37 along the *x* axis. Similarly, fine structural features are seen on the reconstructed tomograms of the bunny. The series of *x-y* slices along the *z* axis shows the bunny's ears in slices 70 - 80, the bunny's head in slices 60 - 70, and the body in slices 30 - 70 (Supplementary Fig. 93).

Tomography was also used to confirm directionality of our structures. For example, moving along the stack in the *z* direction from the bottom of the helicoid cavity shape clearly shows a continuous counter-clockwise spiral rotation of the right-handed helix through the entire tomogram (Fig. 4d, Supplementary Fig. 95). On this reconstructed tomogram we can observe one complete helix turn. This feature is cross-validated by the two other projections (*x*-*y* and *y*-*z*), where we can follow the helical path along the edge of the helix through the tomogram (Fig. 4d, Supplementary Figs. 96 – 97). It is worth noting that magnifications of the electron microscope were calibrated by recording images of tobacco mosaic virus and handedness was validated by using DNA origami gold nanoparticle helices as the standard.³

In the slices of the reconstructed tomogram of the cuboid featuring the projections of 'G', 'E' and 'B', each capital letter is visible at the expected depth in the cuboid. We can clearly see the letter 'B' in slices 40 to 60 along the *z* axis, which is in accordance with prediction (Fig. 4e, Supplementary Fig. 102). In images of the letter 'G', visualized by conventional TEM, we could not accurately resolve the correct shape of the letter. However, reconstructed tomograms of the letter 'G' present better resolution of the letter contour, which is in agreement with the design (slices 34 to 44 along the *y* axis, Supplementary Fig. 101). The letter 'E' is recognizable at three levels of depth, corresponding to the design (slices 21 - 31, slices 44 - 54, and slices 75 - 85 along the *x* axis, Supplementary Fig. 99).



S10.1 Electron tomography analysis of the parallel channels structure

Supplementary Fig. 84. Series of x-z slices extracted from the channels cuboid tomogram along the y direction. Gray level representation of x-z slices through the channels cuboid volume along the y direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.



Supplementary Fig. 85. Series of x-y slices extracted from the channels cuboid tomogram along the z direction. Gray level representation of x-y slices through the channels cuboid volume along the z direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.



S10.2 Electron tomography analysis of the teddy bear cavity structure

Supplementary Fig. 86. Wide-field view of teddy bear cavity structures. Typical field-of-view negative-stain TEM micrograph obtained from teddy bear cavity objects before tomogram acquisition, inset: zoom-in image.



Supplementary Fig. 87. Series of *y*-*z* **slices extracted from the teddy bear cavity tomogram along the** *x* **direction.** Gray level representation of *y*-*z* slices through the teddy bear cavity volume along the *x* direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.



Supplementary Fig. 88. Series of x-y slices extracted from the teddy bear cavity tomogram along the z direction. Gray level representation of x-y slices through the teddy bear cavity volume along the z direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.



Supplementary Fig. 89. Series of *x*-*z* **slices extracted from the teddy bear cavity tomogram along the** *y* **direction.** Gray level representation of *x*-*z* slices through the teddy bear cavity volume along the *y* direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.

200nm

E 50nm 0 29 0

S10.3 Electron tomography analysis of the bunny cavity structure

Supplementary Fig. 90. Wide-field view of bunny cavity structures. Typical field-of-view negative-stain TEM micrograph obtained from bunny cavity objects before tomogram acquisition, inset: zoom-in image.



Supplementary Fig. 91. Series of x-z slices extracted from the bunny cavity tomogram along the y direction. Gray level representation of x-z slices through the bunny cavity volume along the y direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.



Supplementary Fig. 92. Series of *y*-*z* slices extracted from the bunny cavity tomogram along the *x* direction. Gray level representation of *y*-*z* slices through the bunny cavity volume along the *x* direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.


Supplementary Fig. 93. Series of x-y slices extracted from the bunny cavity tomogram along the z direction. Gray level representation of x-y slices through the bunny cavity volume along the z direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.



S10.4 Electron tomography analysis of the helicoid cavity structure

Supplementary Fig. 94. Wide-field view of helicoid cavity structures. Typical field-of-view negative-stain TEM micrograph obtained from helicoid cavity objects before tomogram acquisition, inset: zoom-in image.



Supplementary Fig. 95. Series of x-y slices extracted from the helicoid cavity tomogram along the z direction Gray level representation of x-y slices through the helicoid cavity volume along the z direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.

Ň



Supplementary Fig. 96. Series of x-z slices extracted from the helicoid cavity tomogram along the y direction. Gray level representation of x-z slices through the helicoid cavity volume along the y direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.

z y

y-z



Supplementary Fig. 97. Series of y-z slices extracted from the helicoid cavity tomogram along the x direction. Gray level representation of y-z slices through the helicoid cavity volume along the x direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.

S10.5 Electron tomography analysis of the 'GEB' cavity structure



Supplementary Fig. 98. Wide-field view of GEB cavity structures. Typical field-of-view negative-stain TEM micrograph obtained from 'GEB' cavity objects before tomogram acquisition, inset: zoom-in image.



Supplementary Fig. 99. Series of y-z slices extracted from the 'GEB' cavity tomogram along the x direction. Gray level representation of y-z slices through the 'GEB' cavity volume along the x direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.



Supplementary Fig. 100. 3D rendering of the 'GEB' with three y-z slices extracted from the 'GEB' cavity tomogram along the x direction. a, 3D rendering of the 'GEB' cavity cuboid with different orientations of the 'E' projection. b, 3D rendering of the 'GEB' with the position of three extracted slices of the tomograms.



Supplementary Fig. 101. Series of x-z slices extracted from the 'GEB' cavity tomogram along the y direction. Gray level representation of x-z slices through the 'GEB' cavity volume along the y direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.



Supplementary Fig. 102. Series of x-y slices extracted from the 'GEB' cavity tomogram along the z direction. Gray level representation of x-y slices through the 'GEB' cavity volume along the z direction. The order of the sections is indicated by the numbers. Scale bar measures 50 nm.

S11 Sequencing analysis

To analyze the relative strand incorporation into the designed structure, we adapted a previously developed sequencing analysis¹¹ on both the purified product band strand mix and the unreacted folding strand mix used for the structure assembly. Because of the introduction of distinct barcodes to each strand mixture, we could simultaneously sequence both of these samples and later distinguish the reads during analysis. Only full-length 52-nt strands were analyzed.

S11.1 Sequencing read analysis

We first established a method of classifying the different strands based on read numbers and whether the sequence is expected to be present in the designed structure (Supplementary Fig. 103). We plotted the decadic logarithm of the number of reads for the folding strand mix and the product band strand mix in a histogram (Supplementary Fig. 103a). Bars representing strands that were designed to be present in the structure were colored in blue (designed), while those that were designed to not be present because they constitute the cavity (not-designed) were colored red. The presence of not-designed strands is anticipated because different structures derived from the same canvas were processed on the same gels. Correspondingly, both the folding strand mix and the product band strand mix showed some not designed species with a non-zero number of reads. Such contamination while applying sequencing analysis on structures derived from the same canvas and processed in parallel was previously observed.¹¹

To distinguish which strands were actually present in the desired strand mix and which were contaminants, we set a threshold at the lowest number of reads at which a higher number of designed compared with not-designed strands can be observed (Supplementary Fig. 103a). In the ideal case, this threshold would separate the contamination from the designed strands. In reality, some poorly incorporated strands or strands with low ligation, amplification, or sequencing efficiency would fall under this threshold.

S11.2 Strand categorization

We established a pipeline to assess the relative incorporation of all strands of the molecular canvas for a given shape design (Supplementary Fig. 103b).

To achieve this categorization, we examined three different criteria for each of the strands:

- 1. whether the strand is a part of the shape design (i.e. not located in the cavity)
- 2. whether the number of reads in the unreacted folding strand mix exceeds the set threshold
- 3. whether the number of reads in the purified product band exceeds the set threshold

Based on the above information, we can categorize the relative abundance of given strand in a particular structure into one of four levels:

- intentionally high abundance in the structure (part of the structural design, gray)
- unintentionally low abundance in the structure (part of the structural design, red)
- intentionally low incorporation in the structure (strand is part of the cavity, black)
- difficult to categorize due to systematic errors (blue).

Thus, by sorting whether the strand is included in the design, has read numbers for the unreacted folding mix above the threshold, and has read numbers for the purified product band strand mix above the threshold, we can determine the potential cause of low abundance for a given strand species in a design – i.e low incorporation versus experimental errors arising from low ligation, amplification or sequencing efficiency, or potential pipetting errors. This pipeline was applied to all species in the $30H \times 30H \times 260B$ canvas since all of the strands could be potentially present in the sequencing mix – by design or contamination.

First, we distinguished between designed (6833 full-length strands) and not-designed strands (1432 full-length strands).

Second, for the designed strands, we examined whether strands pass the folding mix threshold. Only 62 out of the 6833 designed full-length strands fall beneath the folding mix threshold. While the incorporation state of these strands cannot be

determined based on sequencing data, some potential causes of low abundance of the designed strands include pipetting errors (i.e. unintentionally omitting some strands while preparing the folding strand mix) or low ligation, amplification or sequencing efficiency. Third, the 6771 strands which passed the folding strand mix threshold can now be evaluated for structural incorporation. If the number of reads is greater than the product band strand mix threshold, the strand is sufficiently incorporated (i.e. relatively high incorporation). While 6697 strands show intentionally high abundance, only 74 strands have read numbers below the product band strand mix threshold and are categorized as unintentionally low abundant.

The not-designed strands were examined only in comparison to the folding mix threshold. A large fraction of the not-designed strands fall below the folding mix threshold (1401 out of 1432 full-length strands), which is expected because these strands constitute the teddy bear shaped cavity. Possible causes of a few of the cavity strands exceeding the threshold include contamination combined with high ligation, amplification or sequencing efficiency, or pipetting errors arising from inadvertent adding of not-designed strands to the folding strand mix.

S11.3 Strand visualization

Based on the above analysis, one can subdivide and color-code the strands according to the different categories (Supplementary Fig. 103b) to visualize the sequencing results in a layer-by-layer manner through the structure (Supplementary Fig. 104). While the strands without sufficient information about the incorporation state seem to be distributed throughout the whole structure, the unintentionally low abundant strands, although present on almost all slices, seem to be more densely accumulated near the back of the bear shape. We assume that this phenomena arises from having only few crossovers between the structure's external surface and the cavity. Thus, limited structural stability can be expected at this location in the bear cavity structure.

S11.4 Voxel visualization

In order to visualize our structures in a more intuitive manner, as opposed to sliced strand-level visualization of the sequencing results, we applied voxel level visualization of our structures. Two strands are required to form one voxel. To account for the number of strands present in a voxel and the type of strands present, we displayed the voxels with different opacities and colors. The strand-to-voxel translation scheme is shown in Supplementary Fig. 105. Voxels with two intentionally high abundant strands are rendered completely transparent. A voxel composed of one intentionally high abundant strand and one strand belonging to a different category was rendered translucent with a color according to the latter strand. Voxels composed of two strands in categories besides the intentionally high abundant strands are rendered as completely opaque blocks with a color corresponding to the two strands forming this voxel. The numbers of corresponding voxels formed by two particular strands are shown in the right column in Supplementary Fig. 105.

By applying this visualization scheme to our renders, we can observe different voxel-level depictions, as in Supplementary Figures 106 - 110 with focuses on the different strand types. The voxels consisting of the low abundant cavity strands show a clear resemblance to the designed cavity. In addition, the "hot-spot" previously observed in the strand-level representation has a higher density of designed low abundant strands (in red) at the back of the teddy bear structure. Outside this particular region, the unintentionally low abundant strands seem to be sparsely distributed throughout the structure, suggesting an overall high integrity of the structure assembly.

S11.5 2D projections

To provide a means for comparing the sequencing data with the expected and observed TEM projections, we plotted 2D projections of the fraction of the different kinds of strands along the three axes (Supplementary Fig. 111). The depicted pixel intensity corresponds to the respective fraction of strands present in the projection. The low abundant cavity strands (gray) show good resemblance of the schematic views representing the designed shape. When we enhance the contrast by normalizing all strand fractions against the corresponding maximal strand fraction for all three projections, we can more easily visualize the strand types that occur infrequently (red, blue) (Supplementary Fig. 112). In general, strands without sufficient information about the incorporation state (blue) and low abundant designed strands (red) are both broadly distributed throughout the structure, suggesting overall high structural integrity. Further, in the x-z and y-z projections, larger fractions of the low abundant designed strands are

observed in the previously mentioned "hot-spot" at the back of the cavity shape. By comparing the normalized sequencing data with TEM data of some broken particles (Supplementary Fig. 103), we can verify a spatial correlation for this tenuous spot.



Supplementary Fig. 103. Categorization of canvas strands based on sequencing read numbers for the teddy bear structure. a, Histograms of the decadic logarithm of the number of reads for the folding strand mix (left) and the product band strand mix (right). Designed (blue) and not designed strands (red) are color-coded correspondingly. The applied threshold with the corresponding number of reads per species are shown in more detail in the inset. b, Categorization of the different strands of the canvas and the number of strands that follow a particular criteria. Final categories are color-coded.



Supplementary Fig. 104. Diagram of every strand in the molecular canvas based on its classification in the teddy bear structure. Schematic of the analyzed structure with corresponding slice number and position (top). Sliced strand-level rendering of the attained sequencing results (bottom). The strands are represented in a color scheme corresponding to Supplementary Fig. 103. Well incorporated designed strands are gray, relatively low incorporated designed strands are red, not designed and not observed or observed in very low quantities strands are black and strands without sufficient information about their incorporation state are blue.

	voxel-level representation	composition of a voxel				number of
strand-level representation		number of designed, incorporated strands	number of not designed, not incorporated strands	number of designed, low incorporated strands	number of strands without sufficient information about incorporation	corresponding voxels in the canvas
and and a second		2	0	0	0	14128
		1	1	0	0	981
- Aller		1	0	1	0	241
and the second s		1	0	0	1	250
		0	2	0	0	2240
		0	0	2	0	5
		0	0	0	2	1
		0	1	1	0	34
1		0	1	0	1	109
		0	0	1	1	11
					total:	18000

Supplementary Fig. 105. Strand-to-voxel translation scheme and counts for the different types of neighboring strands. Left columns depict the strand and voxel level schematic. Right columns show corresponding composition and statistics based on the whole canvas, consisting of 18,000 voxels. Well incorporated designed strands are not depicted in the voxel representation.



Supplementary Fig. 106. Representation of the sequencing results from four different angles. a, Schematics of the analyzed structure. b, Voxel-level representation of the not designed low abundant strands. c, Voxel-level representation of the designed strands with relative low incorporation. d, Voxel-level representation of the strands without sufficient information about the incorporation state.



Supplementary Fig. 107. Representation of the sequencing results from four different angles. a, Schematics of the analyzed structure. **b**, Voxel-level representation of the not designed (gray) and designed (red) strands observed in low quantities according to sequencing data. **c**, Voxel-level representation of the voxels with exactly one designed low incorporated strand and one not designed strand low abundant strand (dark red). **d**, Voxel-level representation of the voxels with one designed low incorporated strand and one not designed strand with low abundance (dark red). Voxel-level representation of not designed strands observed in low quantities according to sequencing data is added for guidance (gray).



Supplementary Fig. 108. Representation of the sequencing results from four different angles. a, Schematics of the analyzed structure. **b**, Voxel-level representation of the not designed strands (gray) and strands without sufficient information about the incorporation state (blue). **c**, Voxel-level representation of the voxels with one not designed low abundant strand and one strand without sufficient information about the incorporation state (dark blue). **d**, Voxel-level representation of the voxels with exactly one designed low incorporated strand and one strand without sufficient information about the incorporation state (dark blue). Voxel-level representation of not designed strands observed in low quantities according to sequencing data is added for guidance (gray)



Supplementary Fig. 109. Representation of the sequencing results from four different angles. a, Schematics of the analyzed structure. **b**, Voxel-level representation of designed low incorporated strands (red) and strands without sufficient information about the incorporation state (blue). **c**, Voxel-level representation of the voxels with exactly one designed low incorporated strand and one strand without sufficient information about the incorporated strand one not designed low abundant strand (purple). **d**, Voxel-level representation of not designed strands observed in low quantities according to sequencing data is added for guidance (gray).



Supplementary Fig. 110. Representation of the sequencing results from four different angles. a, Schematics of the analyzed structure. **b**, Voxel-level representation of designed low incorporated strands (red), not designed low abundant strands (gray) and strands without sufficient information about the incorporation state (blue).



Supplementary Fig. 111. 3D (left) and 2D (right) representations of the sequencing results. **a**, Schematic 3D representation and 2D projections of the analyzed cavity structure. **b**, Voxel-level representation of the intentionally low abundant strands and the respective 2D plots of the sequencing results along the three axis. **c**, Voxel-level representation of the strands without sufficient information about the incorporation state and the respective 2D plots of the sequencing results of the sequencing results along the sequencing results along the three axis. **d**, Voxel-level representation of the strands without sufficient information about the incorporation state and the respective 2D plots of the sequencing results along the three axis. **e**, Voxel-level representation of the respective 2D plots of the sequencing results along the three axis. **e**, Voxel-level representation of the strands without sufficient information about the incorporation state and the respective 2D plots of the sequencing results along the three axis. **e**, Voxel-level representation of the not designed (gray) and designed (red) strands observed in low quantities according to sequencing data and the respective 2D plots of the sequencing results along the three axis. For the 2D projections the opacity represents the number of corresponding strands along the axis.



Supplementary Fig. 112. Contrast-adjusted 3D (left) and 2D (right) representations of the sequencing results. **a**, Schematic 3D representation and 2D projections of the analyzed cavity structure. **b**, Voxel-level representation of the intentionally low abundant strands and the respective 2D plots of the sequencing results along the three axis. **c**, Voxel-level representation of the strands without sufficient information about the incorporation state and the respective 2D plots of the sequencing conductive 2D plots of the sequencing results along the sequencing results along the three axis. **c**, Voxel-level representation of the strands without sufficient information about the incorporation state and the respective 2D plots of the sequencing results along the sequencing results along the three axis. **e**, Voxel-level representation of the respective 2D plots of the sequencing results along the sequencing results along the sequencing results along the three axis. **e**, Voxel-level representation of the respective 2D plots of the sequencing results along the three axis. **e**, Voxel-level representation of the sequencing results along the three axis. **e**, Voxel-level representation of the sequencing results along the three axis. For the 2D projections the opacity represents the number of corresponding strands along the axis.



Supplementary Fig. 113. Comparison of sequencing data and TEM images. Correlation between sequencing data (left) and TEM obtained images of broken particles (right) for the structurally weak portions of the teddy bear structure. The structural weak part is circled for both represented projections. Color-adjusted 2D plots of the sequencing results of the analyzed structure plotted along two axis (left). The opacity represents the number of corresponding strands along the axis. Red and gray depict relatively low abundant designed and not designed species. **a**, *xz*-projection. **b**, *yz*-projection.

References

- 1. Douglas, Shawn M et al. Rapid prototyping of 3D DNA-origami shapes with caDNAno. Nucleic Acids Research 37, 5001–5006 (2009).
- 2. Schneider, C.A., Rasband, W.S. & Eliceiri, K.W. NIH image to ImageJ: 25 years of image analysis. Nature Methods 9, 671–675 (2012).
- 3. Briegel, A., Pilhofer, M., Maastronarde, D.N. & Jensen, G.J. The challenge of determining handedness in electron tomography and the use of DNA origami gold nanoparticle helices as molecular standards. *Journal of Structural Biology* **183**, 95–98 (2013).
- 4. Jungmann, R. et al. Multiplexed 3D cellular super-resolution imaging with DNA-PAINT and Exchange-PAINT. Nature Methods 11, 313–318 (2014).
- 5. Huang, B., Wang, W., Bates, M. & Zhuang, X. Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy. *Science* **319**, 810–813 (2008).
- 6. Lin, C. et al. Sub-micrometer geometrically encoded fluorescent barcodes self-assembled from DNA. Nature Chemistry 4, 832-839 (2012).
- Endesfelder, U., Malkusch, S., Fricke, F. & Heilemann, M. A simple method to estimate the average localization precision of a single-molecule localization microscopy experiment. *Histochem Cell Biol* 141, 629–638 (2014).
- 8. Iinuma, R. et al. Polyhedra self-assembled from DNA tripods and characterized with 3D DNA-PAINT. Science 344, 65-69 (2014).
- 9. El Beheiry, M. & Dahan, M. ViSP: representing single-particle localizations in three dimensions. Nature Methods 10, 689-690 (2013).
- 10. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nature Methods 9, 676–682 (2012).
- 11. Myhrvold, C. et al. Barcode extension for analysis and reconstruction of structures (BEARS). Nature Communications 8, 14698 (2017).
- 12. Quail, M.A. et al. A large genome center's improvements to the Illumina sequencing system. Nature Methods 5, 1005–1010 (2008).
- 13. Ke, Y., Ong, L. L., Shih, W. M. & Yin, P. Three-dimensional structures self-assembled from DNA bricks. Science 338, 1177–1183 (2012).
- 14. SantaLucia, J. & Hicks, D. The thermodynamics of DNA structural motifs. Annual Reviews Biophysics Biomolecular Structure 33, 415-440 (2004).
- 15. Ke, Y. et al. DNA bricks crystals with prescribed depths. Nature Chemistry 6, 994–1002 (2014).
- 16. Wei, B., Dai, M. & Yin, P. Complex shapes self-assembled from single-stranded DNA tiles. Nature 485, 623–626 (2012).
- 17. Myhrvold, C., Dai, M., Silver, P.A. & Yin, P. Isothermal self-assembly of complex DNA structures under diverse and biocompatible conditions. *Nano Letters* 13, 4242–4248 (2013).
- Kremer, J.R., Mastronarde, D.N. & McIntosh, J.R. Computer visualization of three-dimensional image data using IMOD. Journal of Structural Biology 116, 71–76 (1996).
- 19. Weyland, M. & Midgley, P.A. 3D electron microscopy in the physical sciences: the development of Z-contrast and EFTEM tomography. *Ultramicroscopy* **96**, 413–431 (2003).
- 20. Penczek, P.A. & Frank, J. Resolution in electron microscopy (Springer, 2006).