Electronic Supplementary Information for:
DNA Origami Characterized via Solid-State Nanopore: Insight into Nanostructure Dimensions, Rigidity and Yield

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S1. M13mp18 Scaffold Preparation and Characterization

Scaffold preparation

The linearized M13 single-stranded DNA scaffolds were prepared as described in the Methods Section in the main text, using M13mp18 circular single-stranded DNA (New England Biolabs, N4040S). A primer strand was added in a mixture with 10 μg M13mp18 circular single-stranded DNA at a ratio of 10:1 in 1x NEB 3.1 buffer, the mixture was heated to 95 ^{o}C and slowly cooled down to room temperature in a thermal cycler. A mixture of prepared circular scaffold (with primer attached), and 10 units of HincII restriction enzyme (New England Biolabs, R0103S) in a total reaction volume of 50 μL in 1x NEB 3.1 buffer were incubated at 37 ^{o}C for 3 hours, then heat inactivated at 65 ^{o}C for 20 minutes in a thermal cycler.

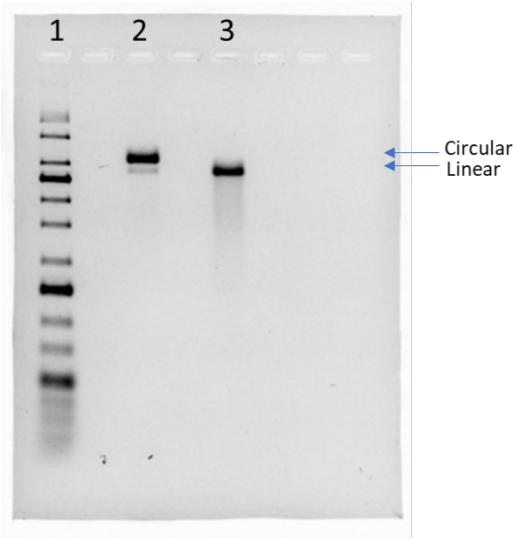

Figure S1. M13mp18 scaffold gel electrophoresis in denaturing gel. Lane 1: GeneRuler 1kbp plus DNA Ladder (ThermoFisher Scientific, SM1331). Lane 2: M13mp18 circular ssDNA scaffold (New England Biolabs, N4040S). Lane 3: M13mp18 linear ssDNA, digested by HincII (New England Biolabs, R0103S). The gel electrophoresis was done using 1% Agarose gel, prepared and performed in 1xTAE buffer at pH 10.5 (40 mM Tris, 20 mM acetic acid, and 2 mM EDTA, pH 10.6) on ice, with an applied voltage of 70 V.

The linearized products were characterized by denaturing agarose gel electrophoresis at 1% in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, and 2 mM EDTA, pH 10.5). Upon completion, the agarose gel was submerged in 1xTAE buffer at pH 8 and incubated on incubator at 60 RPM for an hour to bring gel pH back to 8. The gel was then submerged in 2x gel volume de-ionized

water with 1x GelRed (Biotium, #41003) for 45 minutes on incubator then store at 4 °C overnight to post stain. As shown in Figure S1, linear single-stranded M13 (lane 3) migrate faster than circular single-stranded M13 molecules (lane 2). The disappearance of circular band is an indication of complete cut.

Sequence of linearized M13mp18

The sequence of the M13mp18 scaffold is shown below, showing linearization by HincII (New England Biolabs, R0103S). The underlined sequence represents the region where the primer strand is attached, and the red sequences are the recognition site for HincII restriction enzyme.

GACCTGCAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTT
CGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACCAGAA
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CCATGATTACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTC

S2. 3HB Nanostructure Design and Sequence

Structure design

The caDNAno design of 3HB is shown in Figure S2. Due to its length and repetitive nature, only a partial design is shown here. The long blue strand represents the M13mp18 scaffold, and the short, coloured arrows are the staple strands, with the arrow side being the 3' end.

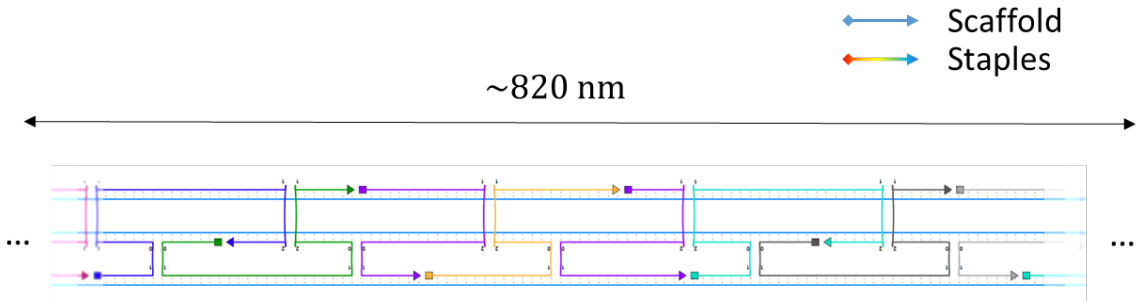

Figure S2. caDNAo design of 3HB on a honey-comb lattice, using M13mp18 scaffold (circular, New England Biolabs, N4040S), linearized at HincII (New England Biolabs, R0103S) site, using 191 staple strands with an average oligo length of 38 nt.

Nanostructure assembly

The 3HB molecules were assembled by mixing the linearized single stranded M13 scaffold with 191 staple strands at a molar ratio of 1:10 in assembly buffer (at final 40 mM Tris, 20 mM acetic acid, 2 mM EDTA, and 16 mM $MgCl_2$, pH 8). The mixture was heated to 95 °C for 5 minutes, cooled to 90 °C, ramped from 90 °C to 60 °C at a rate of 0.4 °C per minute, then from 60 °C to 26 °C at a rate of 0.03 °C per minute, and snap cooled to 4 °C using minicamp Plus Thermal Cycler (ThermoFisher Scientific, #A37835). The nanostructures were subsequently washed three times using 100 kDa Amicon Ultra-0.5 Centrifugal Filter Unit (Millipore Sigma, UFC500396) using the assembly buffer to remove the excess staple oligos.

Once assembled, the 3HB molecules were run on a 1% non-denaturing Agarose gel on ice, as shown in Figure S3, showing one clear band. The excess staple strands were removed by three washes using the assembly buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, and 16 mM

MgCl2, pH 8) through 100 kDa Amicon Ultra-0.5 Centrifugal Filter Unit (Millipore Sigma, UFC500396).

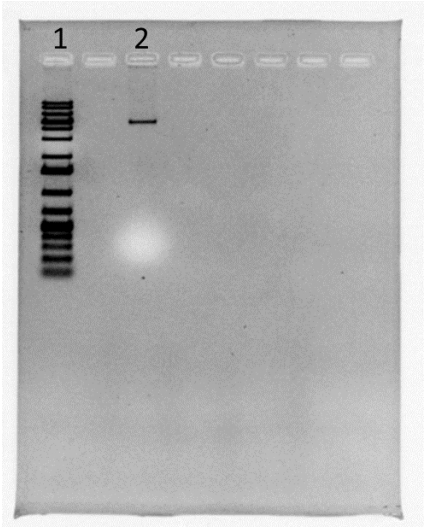

Figure S3. Gel electrophoresis characterization of 3HB assembly. Lane 1: Generuler 1 kb plus DNA Ladder (ThermoFisher Scientific, SM1331). Lane 2: 3HB. The gel electrophoresis was done using 1% Agarose gel, which was prepared and performed in 1xTAE buffer (40 mM Tris, 20 mM acetic acid, and 2 mM EDTA) at pH 8 on ice, with an applied voltage of 70 V.

Staple strand sequences

The sequences of the 3HB staple oligomers are shown in Table S1.

Oligo	Sequence	Length
1	CGACGGCGTTATACACCGGAATCATAATTACTAGAAAAAG	40
2	CCCAGTCATAAAGCAGGCGTTAAATAAG	28
3	GTTGGGTAGGGCTTATACCGACCGTGTG	28
4	AAAGGGGGATGTGCATATTTAACCTAAATTTAATG	35
5	CGGTGCGCGAGCCAAGAAAACTTTTTCAAATATATGGCAGAG	42
6	AGGCTGCGCAACTGTAAAGTAAATCGCAAGACAAA	35
7	GTGCCGGACAATAAAGGTTGGGTTATATAACTATACTGTCCA	42
8	TCCAGCCAGCTTTCTAATGCATACCTTTTTAACCT	35
9	GGACGACAACAAGAAGAGTCAATAGTGAATTTATCATAGATA	42
10	TCGTAACCGTGCATTCCTAATGCTTAGATTAAGAC	35
11	TAATGGGTCTTTCCATTAATTAATTTTCCCTTAGATCAATAA	42
12	TCCGTGGGAACAAAACGGGTATTGCTTCTGTAAAT	35
13	CATTAAAGCCGTTTAATGGAAACAGTACATAAATCTCGAGAA	42
14	TGGCCTTCCTGTAGGGAATCATTCATTTGAATTAC	35
15	AACCAATAGGCTTAGAAACAAACATCAAGAAAACAAAATCAG	42
16	AAATTTTTGTTAAAAACGCGAACCTGAGCAAAAGA	35
17	ATTGTAATTGAAGCCAAGTTACAAAATCGCGCAGAACTTGCG	42
18	CAGGAAGATTGTATAGTTGCTTCGCCTGATTGCTT	35

19	TGTACCCACCAACGACAGTAACAGTACCTTTTACATTATCCT	42
20	TAATCGTAAAACTATCCAGAGGGTTTAACGTCAGA	35
21	GAGAGTCATTTATCTTTGCACGTAAAACAGAAATATAAACAG	42
22	ATCTACAAAGGCTAAAACGATGAACCTACCATATC	35
23	AAATTAATTACAGATCCTGATTGTTTGGATTATACGAAAATA	42
24	AATATGATATTCAAAACAGGGGATGGCAATTCATC	35
25	GGGTGAGGAACAAACCAGAAGGAGCGGAATTATCATAACTGA	42
26	TGAGTAATGTGTAGGAGCGCTTTTTGCGGAACAAA	35
27	TTTTTAGGCCCAATGCCCGAACGTTATTAATTTTAAAGAATT	42
28	AGCCTTTATTTCAAACAATGAACAACTCGTATTAA	35
29	AAAACATGAAAAGTACATTTGAGGATTTAGAAGTAGAAGCCC	42
30	AGAGCATAAAGCTAACAAAGTTTAGAGCCGTCAAT	35
31	GGCAAAGGAATACCAGGTTATCTAAAATATCTTTAACGCAAT	42
32	ATTAACATCCAATATGATTAAACAGTTGAAAGGAA	35
33	GAAAAGGAAATACAAATATCAAACCCTCAATCAATTTAGCAA	42
34	AGCTATATTTTCATAACATATGCATCACCTTGCTG	35
35	TTAGATATTTGTCAACAGTGCCACGCTGAGAGCCACGGAATA	42
36	CTGCGAACGAGTAGTTCATATTCAGTATTAACACC	35
37	GAAGTTTAACCGATCCGAACGAACCACCAGCAGAAAAAGGGC	42
38	TAAATATGCAACTATAAATATAAAACATCGCCATT	35
39	AATTGCTGTCACCGTTTGAATGGCTATTAGTCTTTGGTGAAT	42
40	GGTCATTTTTGCGGGGAATTAATACGTGGCACAGA	35
41	TTAGAGAAGCAAGGTGGCCAACAGAGATAGAACCCGCACCAT	42
42	ACCGGAAGCAAACTAATGAAACCAGTAATAAAAGG	35
43	TATCGCGATCAAGTTGAAATGGATTATTTACATTGTCAGTAG	42
44	ATTAAGAGGAAGCCAGACTGTACATTTTGACGCTC	35
45	TTATAGTCTTATTACCGCCAGCCATTGCAACAGGATCGGTCA	42
46	ATCAAAAATCAGGTTTCATAAGTAATATCCAGAAC	35
47	GCTTTAAGCCTCCCTCACTTGCCTGAGTAGAAGAACCACCAC	42
48	ATAAATATTCATTGCTCAGAACTTCTTTGATTAGT	35
49	TTAGACTACCAGAAAAAAGAGTCTGTCCATCACGCCCCTCAG	42
50	TTTGCCAGAGGGGGGCCGCCATAATCAGTGAGGCC	35
51	AAACCAAGCCTTGATTAGACAGGAACGGTACGCCAAGGTCAG	42
52	ATAACCCTCGTTTATAAATCCAGGAGGCCGATTAA	35
53	AAGGAATTTTACCGTATAACGTGCTTTCCTCGTTAGCGCAGT	42
54	TTCAACTAATGCAGATACATGATGGTTGCTTTGAC	35
55	GAAAGATTTTAACGCCACACCCGCCGCGCTTAATGTACTGGT	42
56	AACTAACGGAACAAAGTAACACGGTCACGCTGCGC	35
57	GGACGTTTCGGAACAGAAAGCGAAAGGAGCGGGCGGCCCCCT	42
58	TTTAAGAACTGGCTCATGAAAACGTGGCGAGAAAG	35
59	TTCAACTAGGATTAAAGGGAGCCCCCGATTTAGAGTCAAGAG	42
60	TAGTAAATTGGGCTAGTACCAAAGCACTAAATCGG	35
61	ATAAGGCATAGCCCAACCATCACCCAAATCAAGTTGGGTTGA	42
62	CAACGTAACAAAGCACCGTACAGGGCGATGGCCCA	35
63	AATCTTGACCCTCAAGAACGTGGACTCCAACGTCACCCTCAG	42
64	CATAGGCTGGCTGAAGAGCCAGGAACAAGAGTCCA	35
65	GAAAGAGAACCCATCAAAAGAATAGCCCGAGATAGGCAAGCC	42
66	TCATAAGGGAACCGAGTTTCGTCGGCAAAATCCCT	35
67	ATGTTACCAGACAGTGCCCCAGCAGGCGAAAATCCCCTGTAG	42
68	AATTGTGTCGAAATGTAACGAAGCAAGCGGTCCAC	35
69	CAAAGTATGAATTTAACAGCTGATTGCCCTTCACCCAGACGT	42
70	ACCCCCAGCGATTATGCTAAACTTTTCACCAGTGA	35
71	AGAGGCAAGGAACACGGGGAGAGGCGGTTTGCGTAGAGTGAG	42

72	CACTACGAAGGCACGAATAATATTAATGAATCGGC	35
73	GGAAGTTTCCAAAACACTGCCCGCTTTCCAGTCGGTCCAAAA	42
74	GCTTTGAGGACTAATATCGGTTCACATTAATTGCG	35
75	ACAGCATGCTTGATGCATAAAGTGTAAAGCCTGGGGAATTTC	42
76	GCGGGATCGTCACCCGACAATCACACAACATACGA	35
77	GCTGAGGCTTGCAGGGAGTTAAAGGCCCCACGCATTGTTA	40
78	CCTGTTTAGTATCATATGCCAGTGCCAAGCTTGCATGC	38
79	GCATTTTGGCCTCTTCGCTATTACGCCATAATTTATTTAGTT	42
80	GACGACGAAACCAGGCAAAGCGCCATTCAGTAATTTGTAAAT	42
81	AGTCCTGGACAGTATCGGCCTCAGGAAGATCAACAAAAATCA	42
82	TCGGCTGATAGGTCACGTTGGTGTAGATAAACCAAATCCTTG	42
83	CAAGCAATGTGAGCGAGTAACAACCCGTGCACTCAAATATAT	42
84	ATATAGAAGGAACGCCATCAAAAATAATAGCAAGCAAATTAA	42
85	GGAGGTTACGTTAATATTTTGTTAAAATCCTCCCGGGCGAAT	42
86	GAATCTTCGGTTGATAATCAGAAAAGCCTACAATTTCGGGAG	42
87	CCATATTTGGAGCAAACAAGAGAATCGATACAAAAAAGAAAT	42
88	GCAGCCTTGCCGGAGAGGGTAGCTATTTCAAAAATTTCTGAA	42
89	ACACCCTAAAGGCCGGAGACAGTCAAATGGAGAATTCATATT	42
90	GAGTTAAAACCCTCATATATTTTAAATGAACCCACAAAGTTT	42
91	TTTTTAATATGACCCTGTAATACTTTTGTCTTACCTTAGACT	42
92	AATAACGAATTAGCAAAATTAAGCAATACGAGGAAGGAGCAC	42
93	ACGTAGATGGCATCAATTCTACTAATAGCAGTATGATCTGGT	42
94	AGTTTATCATTTCGCAAATGGTCAATAAGACACCAGCAGCAA	42
95	GACATTCCATTCCATATAACAGTTGATTAAAGACAGATAAAA	42
96	TATCACCGAATATAATGCTGTAGCTCAACATTAAAAATGCGC	42
97	TACCATTGTACCTTTAATTGCTCCTTTTACCAGTATTCTGAC	42
98	CGACAGATTTTAATTCGAGCTTCAAAGCACCGTAAGCAGATT	42
99	TAGCCCCCAGAAGCAAAGCGGATTGCATGGCATTTAAAACGC	42
100	CGGAACCACAGTTCAGAAAACGAGAATGACCAGAGCTCAAAC	42
101	AGCCGCCGGATAGCGTCCAATACTGCGGGCCACCAAAATTAA	42
102	ACGATTGAATAGCGAGAGGCTTTTGCAATTGAGGCGAATCCT	42
103	CTCTGAATACGAGGCATAGTAAGAGCAAATGGAAAGAATCAG	42
104	AATAAGTTCATCAGTTGAGATTTAGGAAAGGAGTGCGCCGCT	42
105	GCCTATTGGGAAGAAAAATCTACGTTAAAGTTAATCTAGGGC	42
106	AAGGATTTTAATCATTGTGAATTACCTTAGACTCCCTTGACG	42
107	TATAAGTTTGCCCTGACGAGAAACACCAGTCGAGATTTTGGG	42
108	AACCGCCACAAGAACCGGATATTCATTAACCGCCAAAGGGCG	42
109	CAATAGGGACAGATGAACGGTGTACAGAAGGGATAGGTTGAG	42
110	CATTCCATTAGCCGGAACGAGGCGCAGATACAACGTGTTTGA	42
111	TAGTAAACAACGGAGATTTGTATCATCGGTCTTTCGCCTGGC	42
112	AATAGAAAAAGAATACACTAAAACACTCTTCAGCGTTGGGCG	42
113	AAAAGGCTCCATTAAACGGGTAAAATACGAAAATCGAAACCT	42
114	TTAAACACGGAACGAGGGTAGCAACGGCTCGAGGTGTGCCTA	42
115	AATAAACAAATTCTTACCAGTACGACGTTGTAAAA	35
116	ATAAATACAACGCTCAACAGTAACGCCAGGGTTTT	35
117	GTTTGAAAATTGAGAATCGCCTGCAAGGCGATTAA	35
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119	GAACGCGGTAATAAGAGAATATTGGGAAGGGCGAT	35
120	GCTGATGCAAATCCCCGACAAAAGGTAAGCCATTC	35
121	CCGGCTTACAACATGTTCAGCCGGCACCGCTTCTG	35
122	TAGGTCTGAGAGACGAACGCGCCTGTTTATCGCAC	35
123	GCTGAGAAAAATAATATCCCACTGCCAGTTTGAGG	35
124	AAAACATAGCGATATTACGAGCATGTAGGGGCGCA	35

125	CGTCGCTTTATCATTCCAAGACGGCGGATTGACCG	35
126	GTGAGTGAATAACCTTAAACCAAGTACCCGGATTC	35
127	CTTTTTTTTATTTTCATCGTACCAGCTTTCATCAA	35
128	TTACATTTAACAATTTACCGCGCCCAATTCGCGTC	35
129	AGATGATTCCGGTATTCTAAGTCAGCTCATTTTTT	35
130	TATTCATTTCAATTGGCGTTTTAGCGAATCGCATT	35
131	TGAATACCTTAAATCAAGATTAAGCAAATATTTAA	35
132	AAACAATAACGGATATTTTGCACCCAGCCCAAAAA	35
133	TGAATATCTAACGAGCGTCTTGCATGTCAATCATA	35
134	TGCGTAGATTTTCACCTAATTTGCCAGTTGAACGG	35
135	AAAATTACCAATCCAAATAAGTCAGGTCATTGCCT	35
136	TAATGGAAGGGTTATTTTTGTTTAACGTTTGAGAG	35
137	AATATAAGAGAATAACATAAACCGTTCTAGCTGAT	35
138	CCTGATTATCAGATAAGCGCATTAGACGCACCATC	35
139	GAAACCAGTCAGAGGGTAATTGTAAAGATTCAAAA	35
140	GAGTAACATTATCAAATATCAGAGAGATCAATGCC	35
141	ATCCTTTAATAAGAGCAAGAACGCAAGGATAAAAA	35
142	TTACAAACAATTCGAATAGCAATAGCTACGGGAGA	35
143	AGATAATAAGCAGATAGCCGAAATCGGTTGTACCA	35
144	TAACAACTAATAGATACCAGAAGGAAACAAGCCTC	35
145	TTGAGGACAAAAGAACTGGCAAATCATACAGGCAA	35
146	CAGTTGGCAAATCAGACTCCTTATTACGTAGTAGC	35
147	AACCTCATACATAAAGGTGGCTTGGGGCGCGAGCT	35
148	ATGAAAAATCTAAAAAAAGAAACGCAAACCTGTTT	35
149	GCCTGCACAATCAATAGAAAAATTTAGTTTGACCA	35
150	CAGAGGTGAGGCGGGGTTTACCAGCGCCCCCAATT	35
151	AAAAATATGAGGGAGGGAAGGAAGTACGGTGTCTG	35
152	GAACTGATAGCCCTTGACGGAAATTATTCATGTTT	35
153	CAATATTACTTGAGCCATTTGATGGCTTAGAGCTT	35
154	CTGAAAGCGTAAGAGAGCCAGCAAAATCGATAAGA	35
155	GACATTCCCGGAAACGTCACCCCAACAGGTCAGGA	35
156	CACCAGTCACACGACCATCGATAGCAGCGAACCAG	35
157	AATCGTCTTGCCTTTAGCGTCCGAAAGACTTCAAA	35
158	TCATGGAAATACCTAGCGCGTTTTCATCCAAAAAG	35
159	AATATTAGCGTTTGCCATCTTCTTTACCCTGACTA	35
160	TATCGGCCTTGCTGTCAAAATCACCGGAACCATAA	35
161	AATAACATCAGAGCCGCCACCAATCCCCCTCAAAT	35
162	CCGTTGTAGCAATACCGCCACCCTCAGAAATCGTC	35
163	ACCGAGTCCACCACCAGAGCCTAATAGTAAAATGT	35
164	GAGAAGTGTTTTTAGCATTGACAGGAGGAAGAAGT	35
165	AGGGATTTATTCACAAACAAACCAGACGACGATAA	35
166	AGCGGGAGCTAAACTCATTAAAGCCAGACACTATC	35
167	GAGCACGTTCCAGTAAGCGTCATACATAACGCCAA	35
168	ACAGGGCGCGTACTGCTTTTGATGATACTACCACA	35
169	GTAACCAGGGTCAGTGCCTTGCATTATTACAGGTA	35
170	GCTGGCAAGTGTAGGTGCCCGTATAAACTAAAACG	35
171	GAAGGGACTATTATTCTGAAACATTATACCAGTCA	35
172	GGGAAAGCCGGCGAGTATTAAGAGGCTGATGCGAT	35
173	AACCCTAGCGGGGTTTTGCTCTGAGATGGTTTAAT	35
174	GTCGAGGTGCCGTAGGCGGATAAGTGCCGAACGAG	35
175	CTACGTGGGAATAGGTGTATCTGCTCATTCAGTGA	35
176	AAAAACCGTCTATCTCAGGAGGTTTAGTCCCAAAT	35
177	CTATTAAGAACCGCCACCCTCCCTTCATCAAGAGT	35

178	TGTTGTTCCAGTTTCCACCCTCATTTTCCCAGGCG	35
179	TATAAATGTACCGTAACACTGAACTGACCAACTTT	35
180	TGGTGGTTCCGAAATCACCAGTACAAACCGGTCAA	35
181	GCTGGTTCCCTCATAGTTAGCCCGCGACCTGCTCC	35
182	CCTGAGAGAGTTGCTCTAAAGTTTTGTCCCTGATA	35
183	GACGGGCTCTGTATGGGATTTTACCAAGCGCGAAA	35
184	CCAGGGTGGTTTTTCAACTTTCAACAGTATCTTTG	35
185	CAACGCGACTAAAGGAATTGCCAACCTAAAACGAA	35
186	GTCGTGCCAGCTGCAATTTTTTCACGTTGTAATGC	35
187	TTGCGCTGGAGCCTTTAATTGAGACTTTTTCATGA	35
188	ATGAGTGAGCTAACTTATCAGCTTGCTTTACAGAG	35
189	GCCGGAAACCGATAGTTGCGCCTCAGCAGCGAAAG	35
190	TCCGCTCACAATTCGACAACAACCATCGCGCTTTT	35
191	TAGCTGTTTCCTGTGTGAAATAACCGATATATTCGGTC	38
HincII Primer	CTGCAGGTCGACTCTAGAGG	20

Table S1. Staple strand sequences for 3HB, and primer sequence for HincII restriction enzyme.

S3. Translocation Times *versus* Voltage and Salt-Concentration

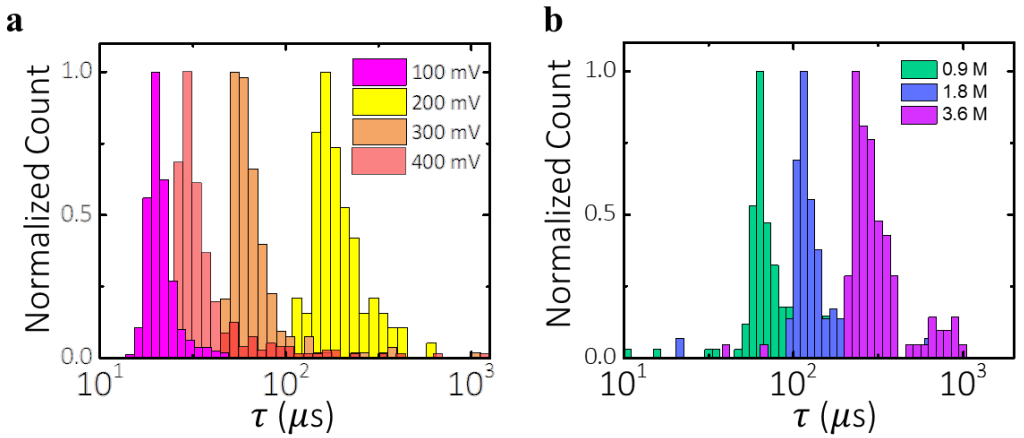

Figure S4. a) Distribution of single-file translocation times recorded under different voltages in a 13.3 nm nanopore, in 0.9 M LiCl. b) Distribution of single-file translocation times recorded under different salt concentrations for a 11.2 nm pore under a 200mV voltage.

S4. Estimates of Nanostructure Volume – ECD Comparisons

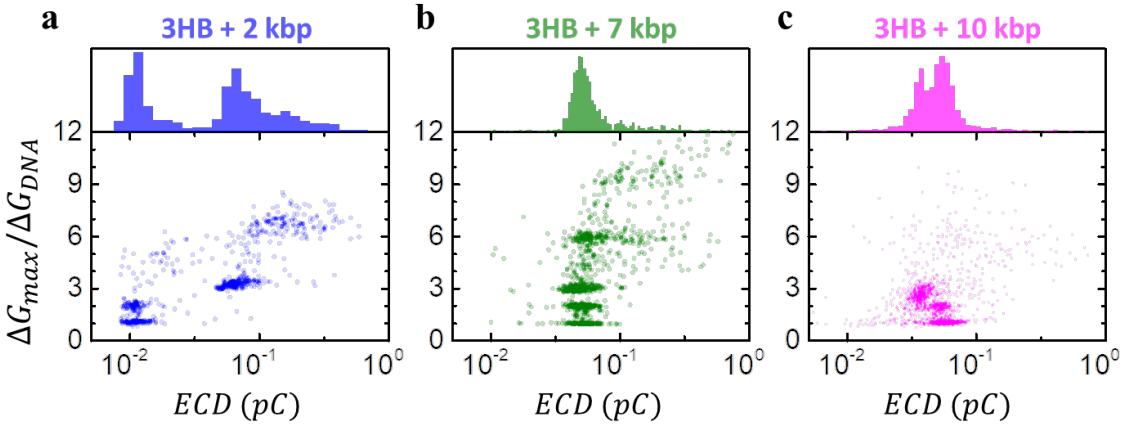

Figure S5. a) 3HB with 2kbp dsDNA through a 13.3 nm pore in 0.9 M LiCl under a 200 mV bias, N = 630. b) 3HB with 7kbp dsDNA through a 11.2 nm pore in 0.9 M LiCl under a 200 mV bias, N = 1189. C) 3HB with 10kbp dsDNA through a 25 nm pore in 0.72 M LiCl under a 200 mV bias, N = 1757.

S5. Strong Correlation of Metastable State and Total Duration

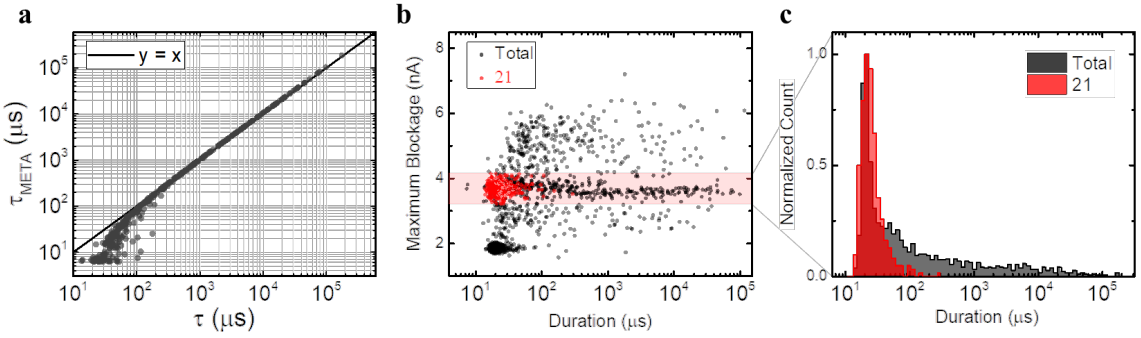

Figure S6. a) Metastable durations *versus* total translocation time for 3HB molecules on a log-log plot. A very strong correlation is observed for longer translocations. b) Scatter plot of Maximum blockage *versus* τ for all types of translocation events and *versus* τ_{21} for folded events contained withing the horizontal red band, N (total) = 1333, and N(21) = 321. c) Distributions of τ and τ_{21} for folded events of b). In calculating τ_{21}, the contribution of the metastable state to the total translocation times is omitted. The distribution of τ_{21} is well-described by a log-normal distribution, and the spread of τ_{21} is significantly smaller than that of τ_{total}, and comparable to that of single-file translocation times. This further supports the interpretation that events with $\Delta G_{max} = 2 \times \Delta G_{3HB}$ are indeed folded translocation events preceeded by a metastable state. The data for a-c were acquired using a 13.3 nm pore in 0.9 M LiCl, under an applied bias of 400 mV.

S6. Voltage Dependence of Metastable State Duration Distributions

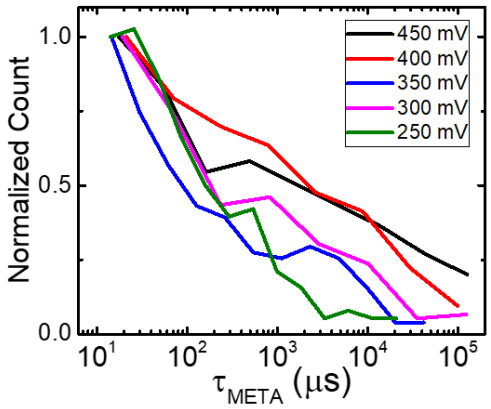

Figure S7. Distribution of metastable state durations τ_{META} measured at different voltages, in a 13.3 nm nanopore, in a 0.9 M LiCl solution. Distributions are normalized such that the count at the smallest τ_{META} bin is 1.

S7. Metastable State Power Spectra

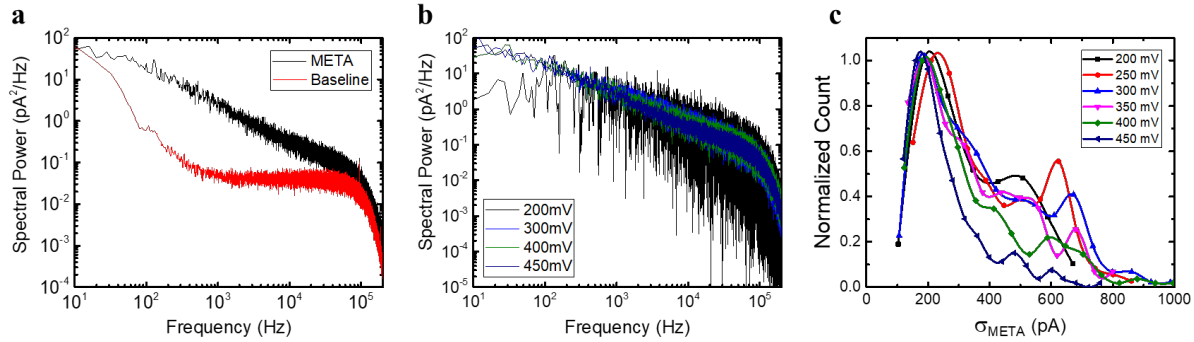

Figure S8. Spectral Power vs Frequency comparison of a) the metastable state and the baseline ionic current, and b) the metastable state current measured under different voltages. c) Distribution of the standard deviation of individual metastable state current traces. Results were obtained from a 13.3 nm pore, in 0.9 M LiCl.

Figure S8a plots and compares the Power Spectral Densities (PSD) of the ionic current traces of the open-pore baseline and of the metastable states: $1/f$ noise dominates the metastable state PSD for the entire bandwidth used, unlike the baseline current. Note that the PSD traces for the metastable state were obtained by concatenating the zeroed metastable

traces, after which the spectral power was calculated over the entire concatenated trace. Interestingly, Figure S8b shows that amplitude of the metastable state PSD traces is independent of the applied voltage. Given that the current's root mean square (RMS) is equivalent to the integral of PSDs, Figure S8b therefore suggests that the RMS of metastable states is voltage independent. To confirm this, Figure S8c plots the normalized histograms of the metastable state current standard deviation $\sigma_{META} = \langle [I_{META} - \langle I_{META} \rangle]^2 \rangle^{1/2}$, measured for each individual event. Consistent with Figure S8b, Figure S8c shows that for every measured voltage, the most probable σ_{META} value is $\approx 200\ pA$.

S8. Metastable State in Folded and Single-File Translocations

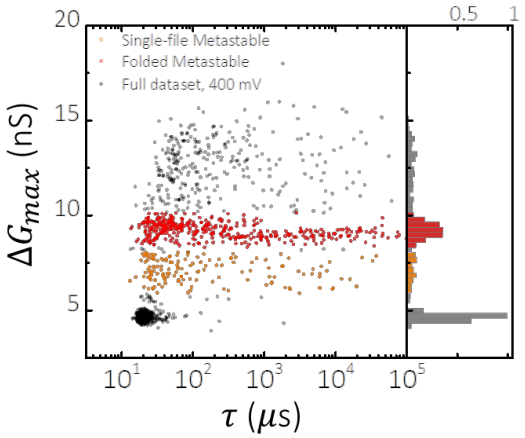

Figure S9. A scatter plot of maximum blockage versus translocation time and a histogram of maximum blockage distribution for 3HB molecules, N = 1330. Events with meta-stable states are highlighted: i) meta-stable followed by a single-file translocation in orange, N = 151; ii) meta-stable state followed by a folded translocation in red, N = 395. The experiment was perform using a 13.3 nm pore in 0.9 M LiCl, with an applied bias of 400 mV.

In addition to folded translocations, metastable states can also precede single-file translocations. Figure S9 shows the scatter plot of ΔG_{max} *versus* τ for 3HB passing through a 13.3 nm pore in 0.9 M LiCl solution under a 400 V bias. Under these conditions, the metastable states result in deeper blockages than single-file translocations, as demonstrated in Figure 3e of the main article. Events composed of a metastable state followed by a single-file translocation are therefore identifiable due to their ΔG_{max} values being between those of single-file translocations

(ΔG_{3HB}) and folded translocations ($2 \times \Delta G_{3HB}$). These events are highlighted in orange in Figure S9, whereas folded translocations are highlighted in red.

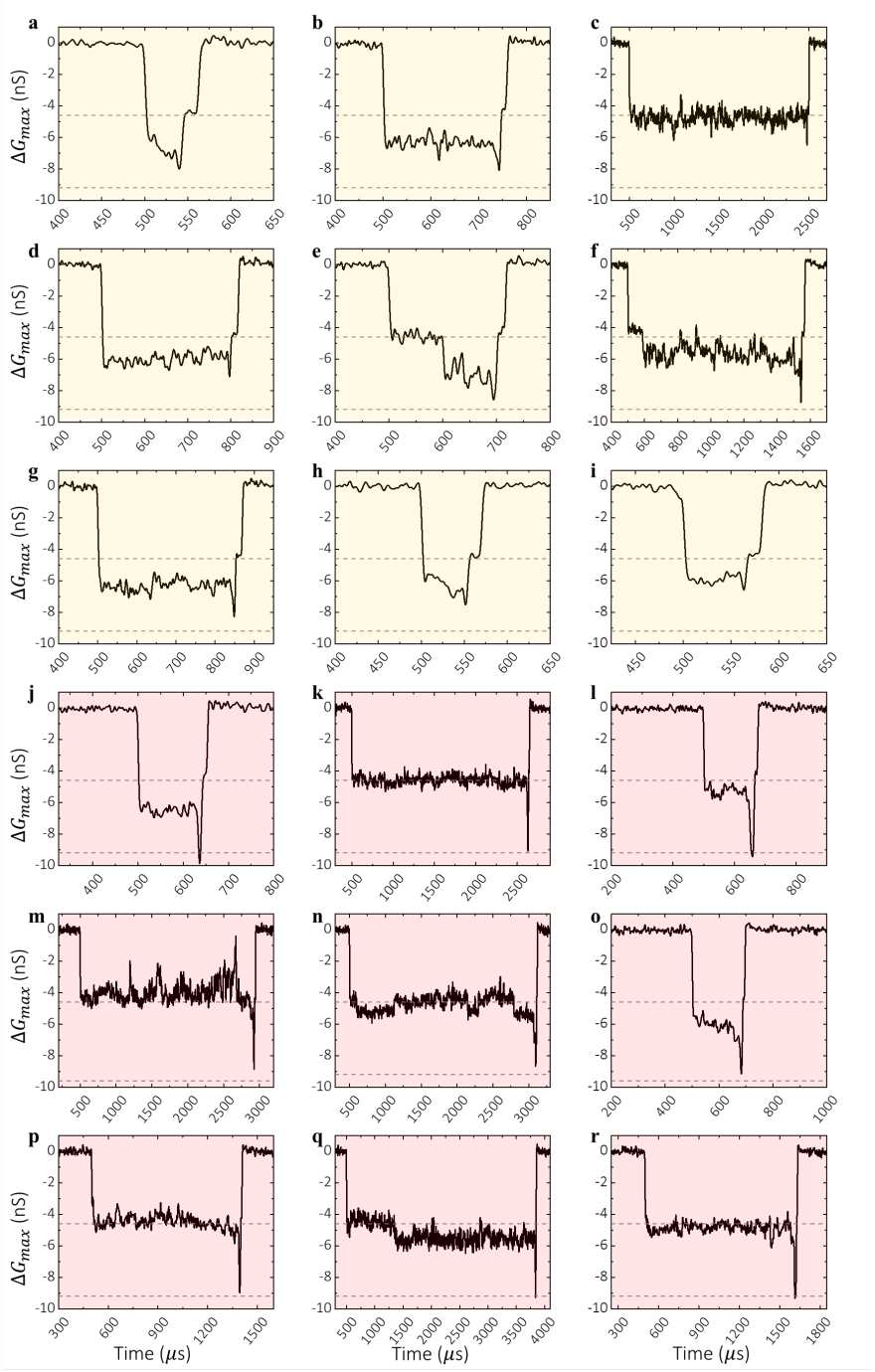

Figure S10. Current traces of 3HB with meta-stable events. a-i) 3HB current traces of meta-stable state followed by single-file translocation. j-r) 3HB current traces of meta-stable state followed by folded translocation. All experiments performed in 0.9 M LiCl, in a 13.3 nm nanopore with an applied bias of 400 mV. The grey dash lines correspond to $\Delta G_{max} = \Delta G_{3HB}$, and $\Delta G_{max} = 2 \times \Delta G_{3HB}$.

Figures S10a-i show the current traces of nine single-file translocations preceded by metastable states, whereas Figures S10j-r show folded translocations. For almost all events, transition from metastable state to the single file blockage state is characterized by a rapid spike, slightly deeper than the metastable state. This spike could either be a folded state too rapid to be well resolved, as sometimes observed, but it could also be an orientation feature, as Wu *et al.* observed when passing the rigid rod-shaped tobacco mosaic virus through nanopores, wherein a rigid molecule enters the pore at an angle and needs to re-orient with the pore to fully enter and traverse.[1] The presence of non-aligned molecules inside nanopores results in deeper blockages than aligned conformations due to the slightly increased cross-sectional area and would therefore be expected from a laterally diffusing 3HB molecule finding and entering by an end.

S9. Dependence of Metastable State on Experimental Conditions

Pore size

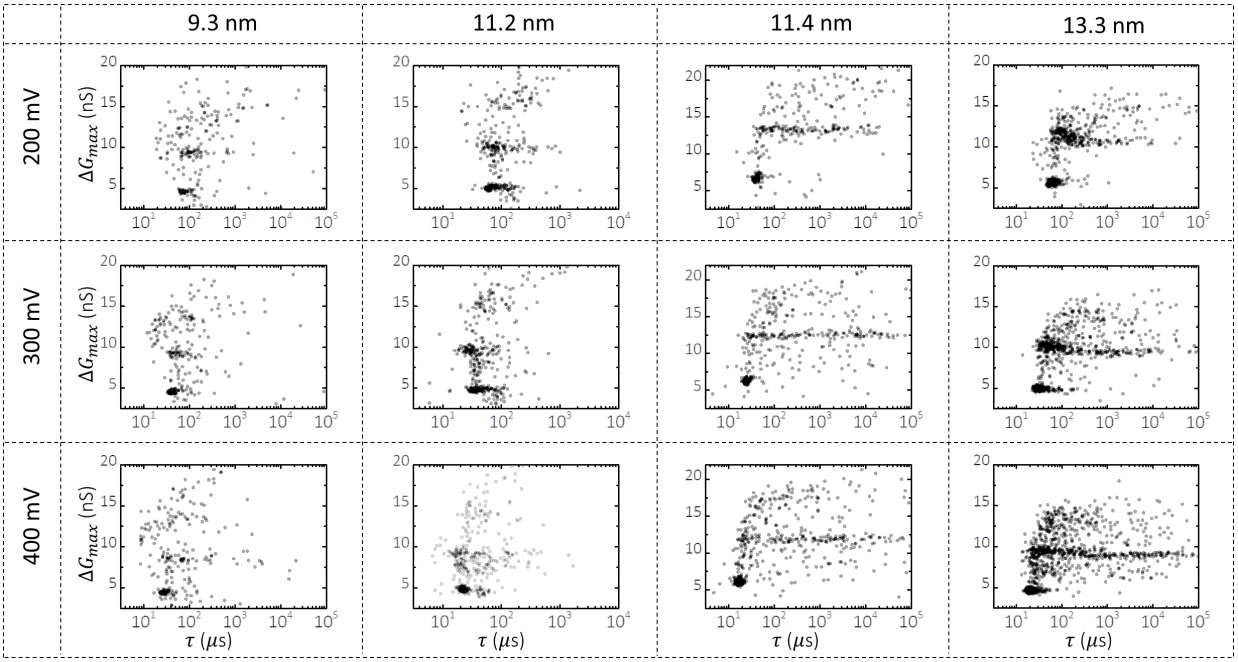

Figure S11. Scatter plots of 3HB translocation events in 0.9 M LiCl under various applied biases and using nanopores of different sizes.

Salt concentration

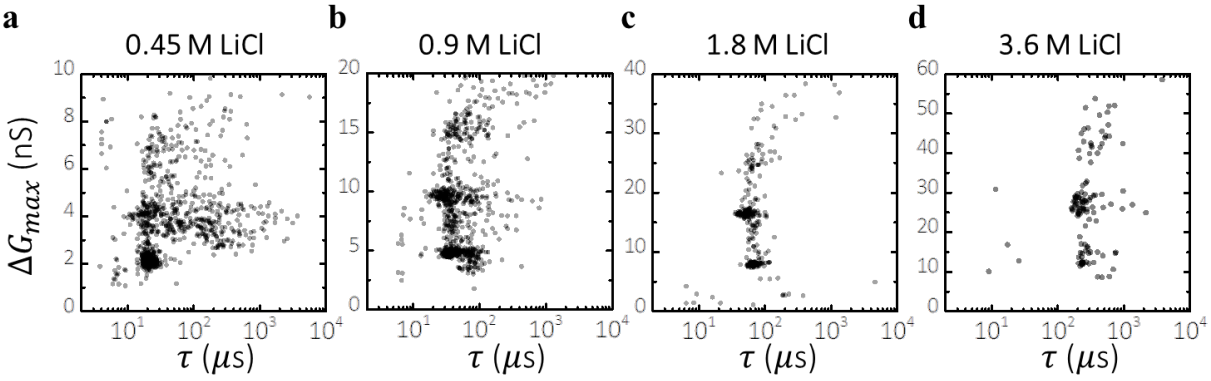

Figure S12. Scatter plots of 3HB translocation events using a single 11.2 nm pore in different LiCl concentrations. a-c) Under 300 mV in 0.45, 0.9, and 1.8 LiCl salt, N = 948, 885, and 327. D) Under 200 mV in 3.6 M LiCl salt, N = 321.

S10. Gel Electrophoresis for Free-Solution Mobility Extraction

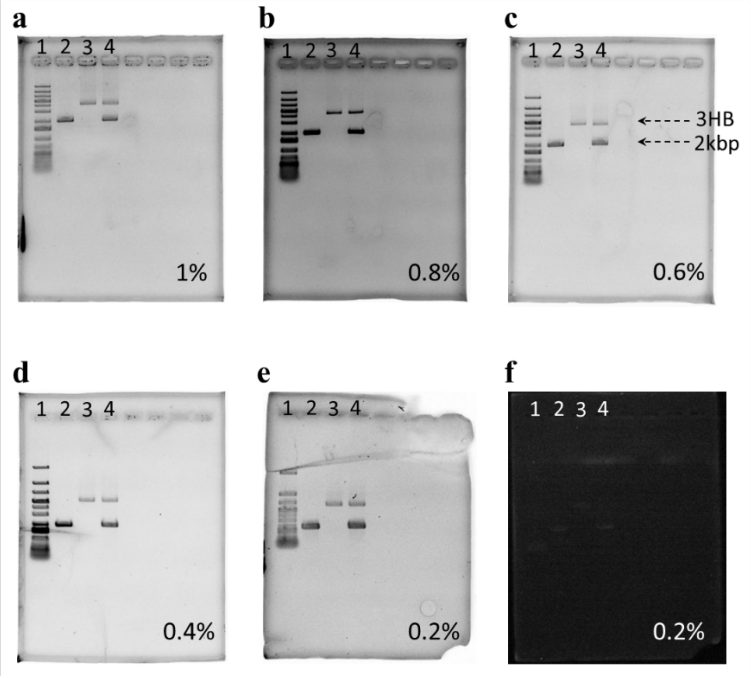

Figure S13. A-e) Gel electrophoresis of 2kbp dsDNA fragments (ThermoFisher Scientific, SM1701) and 3HB in 0.2 – 1 % Agarose gels, post-stained using 1x GelRed (Biotium, #41003). F) Gel electrophoresis of 2kbp dsDNA fragments and 3HB in 0.2 % Agarose gel with no staining. Lane 1: Generuler 1 kb plus DNA Ladder (ThermoFisher Scientific, SM1331). Lane 2: 2kbp dsDNA Fragments. Lane 3: 3HB molecules. Lane 4:

mixture of 3HB and 2kbp dsDNA fragments. All experiments were performed in 1xTAE buffer (40 mM Tris, 20 mM acetic acid, and 2 mM EDTA) under 70 V for an hour.

S11. Nanopore Analysis of 3HB Thermal Degradation

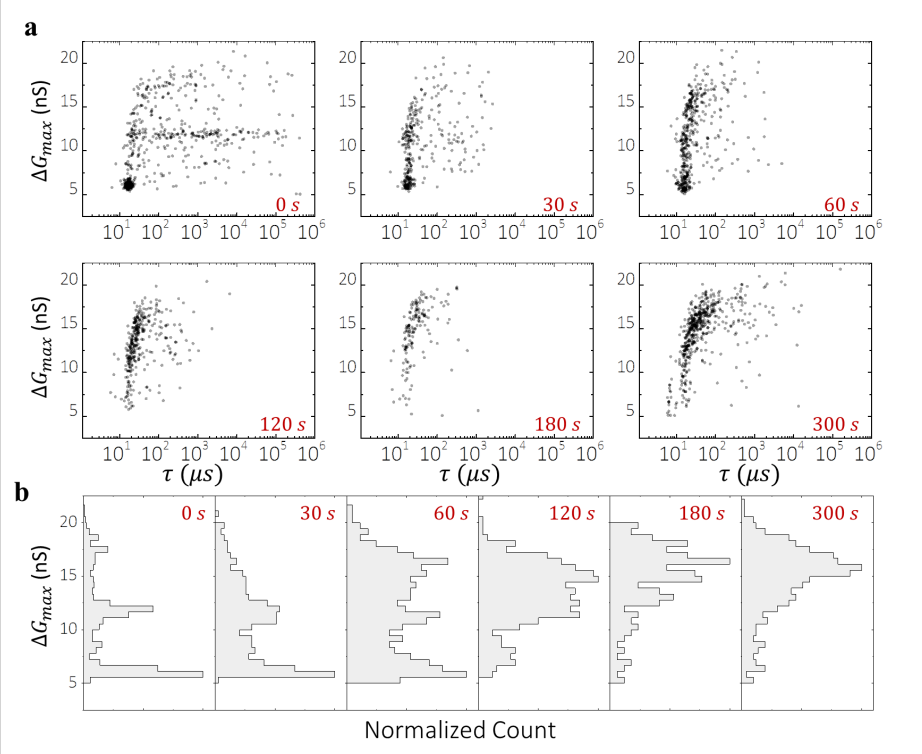

Figure S14. A) Scatter plots of ΔG_{max} versus τ for 3HB structures heated at 65 C for $0, 30, 60, 120, 180,$ and 300 seconds, with N = 599, 313, 629, 314, 150, and 493. b) Distributions of ΔG_{max} for translocations of 3HB thermally degraded for different amounts of time, corresponding to the data of a). Single-file 3HB translocations produce a maximum blockage level of $\sim 6\ nS$. All nanopore experiments are performed using a 11.3 nm pore, in 0.9 M LiCl with an applied bias of 400 mV.