

Flossing DNA in a Dual Nanopore Device

Xu Liu,[†] Philip Zimny,[†] Yuning Zhang,[‡] Ankit Rana,[†] Roland Nagel,[†] Walter
Reisner,^{*,‡} and William B. Dunbar^{*,†}

[†]*Ontera Inc., Santa Cruz, CA 95060, USA*

[‡]*Department of Physics, McGill University Montreal, QC H3A 2T8, Canada*

E-mail: reisner@physics.mcgill.ca; bill@ontera.bio

Abstract

Solid-state nanopores are a single-molecule technique that can provide access to biomolecular information that is otherwise masked by ensemble averaging. A promising application uses pores and barcoding chemistries to map molecular motifs along single DNA molecules. Despite recent research breakthroughs, however, it remains challenging to overcome molecular noise to fully exploit single molecule data. Here we present an active control technique termed “flossing” that uses a dual nanopore device to trap a protein-tagged DNA molecule and perform up to 100’s of back-and-forth electrical scans of the molecule in a few seconds. The protein motifs bound to 48 kb λ DNA are used as detectable features for active triggering of the bidirectional control. Molecular noise is suppressed by averaging the multi-scan data to produce averaged inter-tag distance estimates that are comparable to their known values. Since nanopore feature-mapping applications require DNA linearization when passing through the pore, a key advantage of flossing is that trans-pore linearization is increased to >98% by the second scan, compared to 35% for single nanopore passage of the same set of molecules. In concert with barcoding methods, the dual-pore flossing technique could enable genome mapping and structural variation applications, or mapping loci of epigenetic relevance.

19 **Keywords**

20 nanopore, DNA sensing, active control, single molecule, genome mapping

21 **Introduction**

22 A solid-state nanopore refers to a nanoscale hole formed in a solid-state membrane¹ or at the
23 tip of a glass pipette.² A voltage-clamp amplifier supplies a voltage that is concentrated across
24 the pore to electrically measure the trans-pore ionic current. When the voltage captures a
25 single DNA and drives it through the pore, the passing DNA produces a transient blockade
26 in the current that contains information about the molecule's chemical, conformational and
27 topological state. Nanopore sensing offers a simple and high-throughput electrical read-out³
28 with an instrument that can have a small foot-print at low cost.⁴

29 Recent research has showcased the potential of nanopores to detect molecular features
30 along a DNA carrier strand, including proteins such as anti-DNA antibodies⁵ and strep-
31 tavadin,^{6,7} single-stranded versus double stranded regions of a molecule,⁸ DNA-hairpins^{9,10}
32 and aptamers.^{11,12} Potential applications range from digital information storage,^{9,10} mul-
33 tiplexed sensing,^{9,11} and genomic and/or functional genomic applications including genome
34 mapping¹³ and epigenetics.^{14,15} Solid-state pores in particular can target a more diverse
35 analyte pool than protein pores¹⁶ (e.g. dsDNA, proteins, protein-DNA complexes, nucleo-
36 somes¹⁷) and thereby give access to a broad range of single-molecule applications.

37 A key challenge in performing multi-locus sensing of motifs along DNA is the inherent sen-
38 sitivity of single-molecule systems to noise. Unwanted conformations/topologies and molec-
39 ular fluctuations (both equilibrium and non-equilibrium in nature) create systematic and
40 random distortions in the electrical signal pattern of motifs resolved by the sensor. For exam-
41 ple, closely spaced features along DNA cannot always be resolved in a given single molecule
42 read even with state-of-the-art measurements performed with 5 nm diameter nanopores,¹⁰
43 requiring multiple independent reads from identical copies of different molecules to confi-

44 dently resolve the features. In addition, the stochastic nature of the translocation process
45 gives rise to broad distributions^{18,19} in tag spacings measured across a molecular ensemble;⁷
46 these broad distributions necessitate averaging over additional molecules to obtain precise
47 spacing estimates. A related challenge is providing independent genomic distance calibra-
48 tion along individual single-molecule reads, so that sensor output can be linked to sequence
49 position without a priori knowledge of the distance between motifs. While optics can pro-
50 vide high-resolution spatiotemporal data,^{20,21} nanopores can only infer spatial information
51 implicitly from temporal data. In order for nanopore technology to achieve its full potential,
52 it is essential that single-molecule reads have sufficient quality (e.g., contain sufficiently low
53 systematic and random errors), so that the requirement for further ensemble-level averaging
54 over different molecules is minimized or eliminated. The technology can then be applied to
55 the complex, heterogeneous samples reflective of applications where every molecule may have
56 a different number of bound motifs possessing a distinct spatial distribution. Nanochannel
57 technology, for example, harnesses the physics of nanofluidic polymer confinement to both
58 linearize DNA and suppress molecular fluctuations in barcoded molecules, giving rise to
59 powerful genome-scale applications in the area of DNA optical mapping.^{22,23}

60 Here we show that highly accurate spatial information that is correlative with motif
61 binding can be obtained from a *single* labeled carrier dsDNA strand via repeated back-and-
62 forth scanning of the molecule trapped in a nanopore device. Using a new active control
63 technique termed “flossing,” we are able to perform up to 100’s of scans of a given trapped
64 molecule within a few seconds. Flossing is showcased here using a model system consisting
65 of a 48.5 kbp double-stranded λ -DNA with a set of chemically incorporated sequence-specific
66 protein tags. Our approach complements existing carrier-strand DNA nanopore technology
67 by enhancing the quality of information that can be extracted from a single trapped molecule.
68 By taking a large number of statistically equivalent scans, we reduce stochastic fluctuations
69 through scan averaging, and show mean tag spacing estimates that are comparable to known
70 inter-tag distances, even while tolerating missed tag(s) within a subset of scans.

71 While prior work leveraged control logic for automated re-capture and sensing of molecules
72 with single nanopores,^{24,25} our approach is distinctive in a few ways. First, our device em-
73 ploys a dual-pore architecture.^{26–28} By having the dual pores sufficiently close, our prior
74 work shows the DNA can be captured simultaneously by both pores and exist in a ‘tug-of-
75 war’ state where competing electrophoretic voltage forces are applied at the pores.²⁸ During
76 tug-of-war, the molecule’s orientation and identity are maintained, the molecule speed is
77 regulated to facilitate tag sensing, and the likelihood of the molecule finding a linearized
78 conformation through the pore is increased to 70% compared to 30% with single pore data.
79 An advantage of flossing is that linearization through the pore is further increased to >98%
80 by the second scan, which in turn increases the throughput of nanopore feature-mapping
81 applications.

82 Another distinct feature of the presented approach is that the active controller cyclically
83 modulates the voltage at one pore by a real-time feedback on the sensing current of the
84 other pore. Specifically, during control, the cyclical application of unbalanced competing
85 voltage forces are used to drive the molecule’s motion in one direction and then, after real-
86 time detection of a set number of tags, in the reverse direction, thereby embodying the
87 concept of DNA “flossing.” Previously, the original concept of flossing DNA in a nanopore
88 was envisioned with single protein pores, and only by creating stoppers at the end of the DNA
89 to prevent escape.²⁹ This approach facilitated interesting research^{30,31} but did not provide
90 a means of speed control or of mineable data generation *during* the molecule’s motion in
91 either direction. Coupling the DNA to a stage has produced both speed control and mineable
92 data generation during the molecule’s motion through a solid-state pore, but at the price
93 of complex instrumentation, higher sensing noise and lower throughput.³² In the presented
94 flossing control method, an interrogated DNA molecule can be ejected from the pores and a
95 new DNA captured with the same throughput and ease of any single-nanopore based assay,
96 with no tethering of the molecule required.

97 Results and Discussion

98 The DNA Flossing Concept

99 Figure 1 introduces the general flossing concept, showing pictorially and with actual recorded
100 data the cyclical bidirectional scans of a co-captured molecule in a dual nanopore device.
101 Our dual nanopore device was fabricated as previously described,³³ with voltages V_1 and V_2
102 that can be independently applied at pore 1 and 2, respectively. Two currents (I_1 and I_2)
103 can also be independently measured at the pores. The tagged reagent features monovalent
104 streptavidin (MS) proteins bound along the DNA.

105 Figure 1a illustrates each step in a multi-scan cycle. Since the motion control is bidirec-
106 tional, we define a single transit with fixed direction as a “scan” and two sequential scans
107 of reversed polarity as a “cycle.” By convention, we define the left pore as pore 1 and the
108 right pore as pore 2. During the multi-scan control logic sequence, V_2 across pore 2 is kept
109 constant while V_1 is modulated in step-wise fashion. The signal I_2 is monitored in real-time
110 for tag-related events as a logic trigger for V_1 changes, as described next.

111 The flossing control logic begins by initially co-capturing a tagged DNA in both pores to
112 reach the tug-of-war state. Once co-capture occurs, a lower voltage is applied across pore 1
113 than pore 2 ($V_1 < V_2$) to direct the molecule motion towards pore 2 (left-to-right, or “L-to-
114 R”). While monitoring I_2 , the control logic readjusts the voltage at pore 1 so that $V_1 > V_2$
115 after a set number N of tags were detected translocating through pore 2. The readjustment
116 directs the molecule motion back towards pore 1 (R-to-L). After detecting the same set
117 number N of tags translocating through pore 2, the logic resets $V_1 < V_2$ to initiate another
118 L-to-R scan, and thus a new cycle begins. Figure 1b shows a recorded example of the first
119 two cycles and the final cycle of the I_2 signal for which the tag number setting was $N = 2$.
120 In this example, the multi-scan cycle begins with the molecule moving L-to-R for the first
121 9 ms and continues until 172 ms when the DNA escapes just after a V_1 modulation (escape
122 modes are discussed below and in detail in the SI). Details on the set of dual-pore chips

123 and voltage settings used in this paper are provided in Tables S1-S2. Having described the
 124 general flossing concept, we next present the method in greater detail and results obtained
 125 using the method.

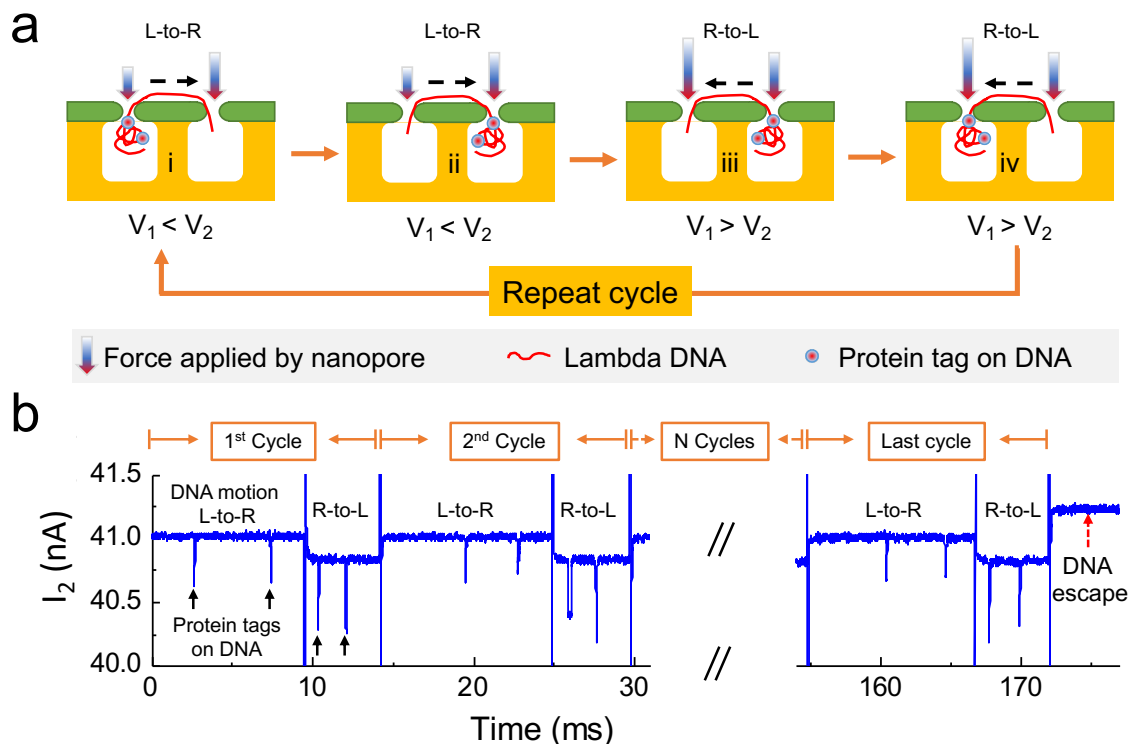


Figure 1: **Flossing DNA with competing voltage forces in a dual pore device.** **a** After DNA co-capture, the DNA molecule will be threaded from left-to-right (L-to-R) using a voltage $V_1 < V_2$, with V_1 and V_2 the voltages across pore 1 (left) and pore 2 (right), respectively. A single transit of DNA motion during this fixed polarity period is called a “scan.” After automated detection of a predefined number of tags, the direction of DNA motion is reversed with a voltage $V_1 > V_2$ triggered to move the molecule from right-to-left (R-to-L), giving rise to a second scan. The process is repeated in cyclical fashion until the molecule randomly exits the co-capture state. **b** A recorded multi-scan current trace I_2 from pore 2, using logic for which the predefined tag detection number is 2, after which the controller triggers the change in direction. The signal from 30-150 ms is truncated for visualization.

126 Initializing Tug-of-War and Identifying Scanning Voltages

127 The control logic that automates the flossing process shown in Figure 1 is described here in
 128 detail. The flossing method first uses active control to automate initializing co-capture and

129 tug-of-war on a single molecule. The control logic was run in real-time (MHz clock rate) on
130 a Field Programmable Gate Array (FPGA). We modified our previously designed tug-of-war
131 control process²⁸ to permit loading reagent in the common fluidic chamber above the two
132 nanopores and to screen out short-fragments (SI Section 2, Fig. S1). Once co-capture is
133 achieved the competing voltage forces at V_1 and V_2 lead to a tug-of-war and reduce the
134 DNA speed during sensing. The mean durations of all co-captured events are computed at
135 each of a set of different V_1 values while keeping V_2 constant, showing a bell curve with the
136 peak revealing the force-balancing voltages that maximize co-capture duration. Figure S1c
137 shows an example device with peak mean duration of 110 ms with V_1 and V_2 both set to
138 500 mV. This data is generated with bare DNA that has no tags.

139 While capturing a molecule in dual pore tug-of-war introduces speed and conformational
140 control, bare double-stranded DNA offers no detectable features with which to monitor the
141 molecule's motion. To enable *in-situ* feature monitoring, we developed MS-tagged DNA as
142 a model reagent, with up to 7 sites tagged on the DNA (SI Section 3, Fig. S2). The tag
143 features are used to make inferences on the speed and direction of each scan, which enables
144 identifying the scanning voltages as described next.

145 The scanning voltages are found after the force-balancing voltages that maximize co-
146 capture duration have been identified for a given chip. Specifically, the scanning voltage
147 values for V_1 are chosen above and below its force balancing value in order to promote un-
148 perturbed DNA motion toward pore 1 and pore 2, respectively, while keeping V_2 at its force
149 balancing value. The V_1 scanning values are chosen heuristically, using the following guid-
150 ance. A working scan speed should enable robust sensing of tag blockades at the recording
151 bandwidth (i.e., not too fast), while ensuring sufficiently high Peclet numbers so that translo-
152 cation time distributions are well-defined (i.e., not too slow). That is, broad translocation
153 time distributions increase the probability of fluctuations, which can undermine the inter-
154 tag time-to-distance mapping objectives described in a later section. In practice, achieving
155 a not-too-fast and not-too-slow scan speed is achievable for a broad range of scanning V_1

156 voltages. For the data in this paper, that range is as low as 150 and as high as 500 mV
157 away from the force-balancing voltage (Table S2). Figure S2 shows an example tug-of-war
158 experiment with MS-tagged DNA at a working scan speed, for which $V_1 = 200$ mV and V_2
159 = 500 mV, with a typical current traces for I_1 and I_2 from one representative event (Fig.
160 S2c). There are three downward spikes in both I_1 and I_2 , indicating three resolvable tags
161 along the DNA. The $V_1 = 200$ mV value that promotes L-to-R motion is 300 mV below the
162 force-balancing value (500 mV).

163 **Representative Flossing Event with Protein-Tagged DNA**

164 Once the scanning voltage values are identified, the full flossing multi-scan logic can be
165 applied. The full details of the FPGA-implemented logic are provided in supplementary
166 materials (SI Section 4, Figure S3). The output of the logic is conveyed here by representative
167 flossing data (Figure 2). Figure 2a shows the full signal trace of I_1 , V_1 , I_2 and V_2 from a
168 typical flossing event. We kept $V_2 = 300$ mV unchanged within the event. We set $V_1 =$
169 100 mV for L-to-R movement and $V_1 = 850$ mV for R-to-L movement. Note that the I_2
170 baseline varies when V_1 is adjusted even though V_2 is constant. This effect is caused by
171 cross talk between pore 1 and pore 2 that was previously characterized in Liu *et al.*²⁸ The
172 detectability of tags relative to the DNA baseline in I_2 is still robust, despite the baseline
173 change. As detailed in the previous section, we chose sufficiently large voltage differentials
174 between V_1 and V_2 to promote controlled motion in each direction during sensing.

175 Figure 2b,c give a magnified view of the signal for the first cycle that comprises the
176 1st and 2nd scans, and the last cycle comprising the 81st and 82nd scans. By convention,
177 since the multi-scan logic starts in the L-to-R direction, the odd scans correspond to L-to-R
178 movement and the even scans R-to-L movement. The event in Figure 2a includes 41 cycles
179 and 82 scans total, all in less than 0.66 sec. As depicted in Figure 1, the FPGA logic was
180 designed to switch V_1 once two tags were detected within I_2 . The FPGA detects a tag when
181 I_2 falls at least 70 pA below the untagged DNA baseline for at least 0.012 ms (based on the

182 distribution in Figure S2f). The data in Figure 2 shows two tags, A and B, in both I_1 and
183 I_2 when the molecule moved L-to-R from 9 to 19 ms. Following a 1.5 ms-delay after tag B
184 was detected, the FPGA set $V_1 = 850$ mV, driving the molecule to move R-to-L from 19 to
185 25 ms. The same tags, B and A, were detected in both I_1 and I_2 in reverse order. The same
186 logic continued until the FPGA failed to detect tag B in the last cycle (Figure 2c), which
187 was caused by the tag appearing too close to the voltage change for the FPGA to detect it.

188 The total flossing time and distribution of the number of scans per event are shown in
189 Figure 2d for a total of 309 flossing events in an experiment, including the 82 scan event in
190 Figure 2a. Total flossing time increases with more scans, while all events terminated in less
191 than a few seconds, even for the largest scan count of 157 for this data. While individual
192 scans last 5-10 ms (Figures 1b and 2b-c), the total time data shows a significant increase
193 (up to 100X more) in time spent interrogating each molecule by using the flossing method.

From the probability plot in Figure 2d, 37% of the events had less than 5 scans, and events with higher scan count are less likely. We examined the probability $P(n)$ of seeing a specific total number of scans n , where the probability of any intermediate scan has correct detection probability p and missed detection probability $(1 - p)$. An event with n total scans indicates the system successfully catches the initial $(n - 1)$ scans but fails to catch the n th scan. Thus its probability $P(n)$ is

$$P(n) = p^{n-1}(1 - p). \quad (1)$$

194 Fitting the data to equation (1) results in $p = 0.89$ for this specific data set (Figure 2d).
195 To determine why the molecule exits co-capture, we studied the last cycles and found four
196 common cases: missing a tag in the n th scan (Figure S4a); a false positive spike detected
197 in the $(n - 1)$ st scan (Figure S4b); a false negative spike in the n th scan (Figure S4c); and
198 the molecule exits the pore during the FPGA delay state (Figure S4d). A discussion on
199 how to increase the total number of scans per event can be found in SI section 5. Naturally,

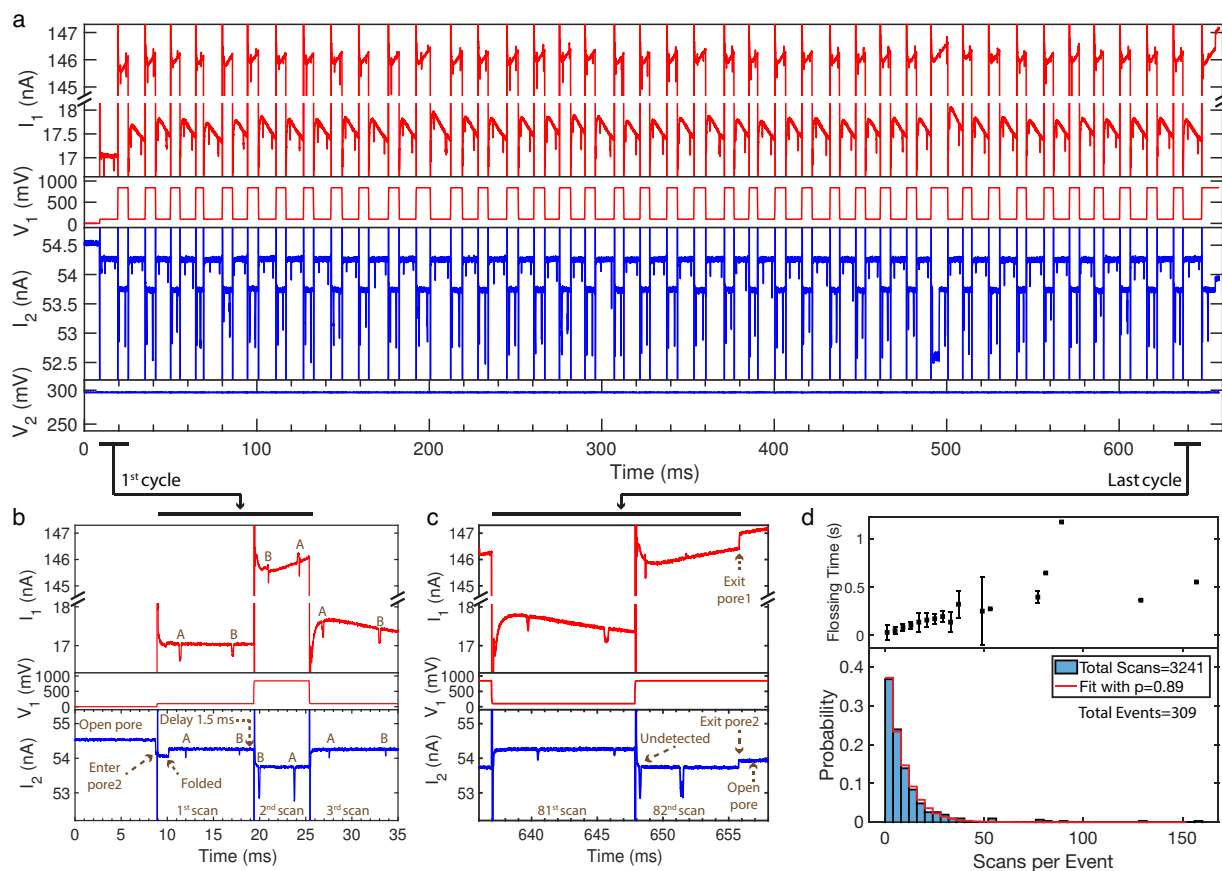


Figure 2: **Representative dual current signals and scan count statistics generated during a flossing experiment with MS-tagged DNA.** **a** Full signal traces for I_1 , V_1 , I_2 and V_2 are shown for a representative multi-scan flossing event. The vertical-axis break in the I_1 signal permits vertical-scale zooming on the low and high ranges during the lower and higher V_1 values. **b** Zoom in of the 1st cycle where two-tag logic shows resolvable tags A and B in both signals. **c** Zoom-in of the 41st and last cycle, showing the end of co-capture due to an undetected tag. **d** The total flossing time (mean \pm standard deviation) and probability distribution versus scan counts across all co-captured events for the device used (bin width = 4). The red line on the probability data is the fitted model equation (1), with $p = 0.89$ the probability of correctly detecting two tags in each scan. The chip used had a pore-to-pore distance of $0.61 \mu\text{m}$, 27 nm pore 1 diameter, and 25 nm pore 2 diameter (Chip C, Table S1).

200 changing the voltage settings will affect event duration between tags, which in turn will affect
 201 tag detection probabilities.

202 A dependence of tag amplitude on scan direction is observed in Figure 2a-c, with MS tags
 203 showing relatively shallower and faster spikes when passing through the pore with the higher
 204 voltage of the two. Seeing a faster and shallower tag event at higher voltage is consistent
 205 with single pore results, and is in part an artifact of the low-pass filter (10 kHz bandwidth)

206 preventing the tag events from hitting full depth (i.e., the faster the event, the shallower).

207 A multi-scan experiment using a three tag triggering setting was also performed (Figure
208 S5). It is harder to get a higher scan count using three tag triggering than with two. In part,
209 this arises because fewer molecules (<20%) show three or more tags (Figure S2e), which
210 is a limitation of our current reagent preparation methods. Also, even when the system
211 co-captures one molecule with three tags in both pores, the probability of correct detection
212 of all three tags is lower (Figure S5c), and failing to detect any one of the tags moves the
213 molecule to a new region, thereby lowering the scan count for the originally scanned three tag
214 region. To generate more data with higher scan counts, we therefore focused the experiments
215 on two-tag triggering in this initial presentation.

216 **Flossing Increases the Fraction of DNA with Mappable Data**

217 Nanopore feature-mapping applications require DNA linearization when passing through the
218 sensing pore. As such, we explored the fraction of DNA that can be linearized by the flossing
219 technique, where linearization refers to the removal of DNA folds that are initially in the
220 pore when co-capture is initialized. By example, the molecule in Figure 2a-c was partially
221 folded at around 10 ms in the 1st cycle (Figure 2b), which was eliminated in the 2nd cycle
222 and thereafter, demonstrating the tendency of tug-of-war control to induce and maintain
223 DNA linearization. We examined the probability of complete linearization over the scanning
224 cycle through pore 2 as a function of scan number to see if the trend in Figure 2a-c was
225 representative of the population. Indeed, Figure 3 shows that the probability of linearization
226 is increased to 98% by the second scan. In the data, a folded event is identified if the current
227 blockage is larger than 1.5 times the unfolded blockage amount, and lasts more than 180 μ s.
228 Figure 3a,b show representative single pore and multi-scan events with observable folding
229 examples.

230 We propose a qualitative mechanism of the progression of unfolding during flossing in
231 Figure 3c. Going L-to-R, motion and the field force at pore 2 are aligned, which promotes

232 folds eventually moving through pore 2 and into channel 2. Subsequently, R-to-L motion pulls
233 only the region of DNA that is under tension via Tug-of-War back through pore 2, despite
234 the counter field force at pore 2, while folds not under Tug-of-War tension experience only
235 the field force and thus remain in channel 2 and away from pore 2. Figure 3d shows the ratio
236 of unfolded events for the progression of translocation types that each of the 309 events went
237 through. Thus the statistics in each column are from exactly the same group of molecules,
238 and are the same data as Figure 2. The 35% unfolded probability through the initial single
239 pore capture (pore 1 in our device) is consistent with other single pore studies.^{1,9,33} Following
240 co-capture, 66% of 1st scans are unfolded, which is consistent with tug-of-war data without
241 flossing.²⁸ By the 2nd scan, only 6 molecules out of 309 (2%) remain folded, and only 5
242 remain folded by their last scan. Thus the flossing process effectively linearizes the DNA
243 molecules through the nanopore sensors at high probability.

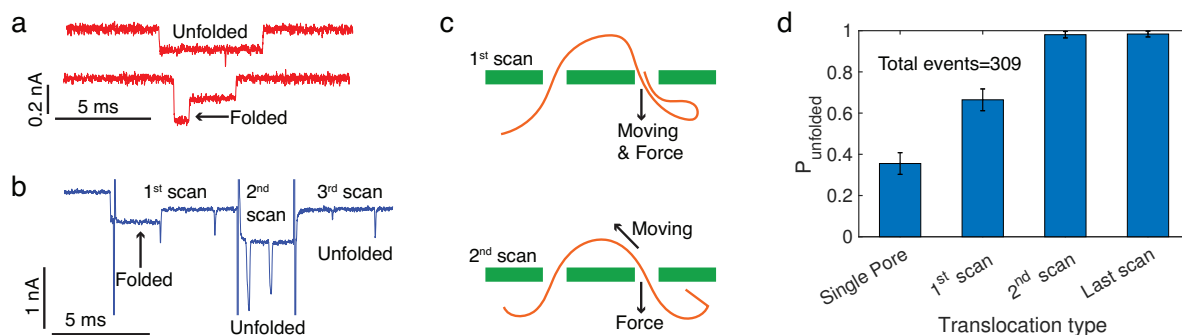


Figure 3: **Flossing increases linearization of DNA in dual pore device.** **a** Typical I_1 traces of single pore events, including both unfolded folded examples. Only single pore events that resulted in eventual co-capture were included in subsequent probability calculations (pre-i step events in Figure S1). **b** Typical I_2 trace of a multi-scan event in which scan 1 shows folding and subsequent scans do not. **c** Illustration of a mechanism by which the folded part (initially only in I_2) gets removed by the 2nd scan when the molecule moves R-to-L, as described in the text. **d** The probability P ($\pm 95\%$ error bar) is the fraction of events that are unfolded, for the different translocation types. A total of 309 events experienced all four types in sequential order.

244 **Tag Data Analysis: A Single Scan View**

245 The multi-scan data set (constituting L-to-R and R-to-L scans for each pore) contains rich
246 information regarding the underlying tag binding profile and translocation physics. Each
247 individual scan taken from the two pores provides a snapshot of the translocation process
248 for the portion of DNA being scanned. There are two ways to assess the translocation
249 velocity from the tag. The first approach is to quantify the speed of a tag as moves through
250 a single-pore ('dwell time estimation'), which is based on dividing the tag blockade duration
251 (width-at-half-maximum) into the membrane thickness (35 nm). The second approach is
252 unique to dual nanopore technology, and is to assess tag speed as it moves from the first
253 pore (Entry) to the second pore (Exit). This entry-to-exit time is in reference to the time
254 the tag resides in the common chamber above both pores, and is also referred to as the
255 pore-to-pore time. In pore-to-pore speed estimation, the pore-to-pore time is divided into
256 the measured distance between the pores for each chip (Table S1). By definition, the pore-
257 to-pore approach utilizes correlation between the two current signals I_1 and I_2 , since the
258 time starts when the tag leaves the Entry pore and ends with the tag enters the Exit pore.
259 We note that, at the voltages applied, DNA in the common reservoir is expected to be fully
260 stretched between the pores²⁸ (0.34 nm/bp).

261 Figure 4 shows an example of an adjacent pair of scans in a multi-scan event, and demon-
262 strates how inter-tag separation distances can be estimated from each scan. For the L-to-R
263 scan (Figure 4a,c), tag A then tag B move through pore 1, and about 1 ms later they move
264 through pore 2. The signal pattern reveals that tags A and B are spatially closer than the
265 distance between the pores (0.64 μm , chip E, Table S1). The FPGA is monitoring I_2 for
266 two tags during the control logic. During the waiting period after detecting A and B in I_2 ,
267 a third tag C passes through pore 1. Visually, it is also clear from the pore-to-pore transit
268 times of A and B that tags B and C are roughly two times farther apart than the distance
269 between the pores. Upon changing V_1 to promote motion in the reverse direction R-to-L
270 (Figure 4b,d), the first observable tag in I_1 is C passing back through pore 1. Again, the

271 logic detects A and B in I_2 , and this time both tags pass through pore 1 before the logic
272 triggers the V_1 to promote L-to-R motion. Seeing three tags within I_1 was a result of three
273 physical tag separations that are close enough to accommodate 3-tag pore 1 transit within
274 the 2-tag pore 2 detection time window of I_2 , as implemented on the FPGA. More common
275 was to see two tags reliably in both pores, as detailed in the next section.

276 For an L-to-R scan, a tag blockade in I_2 corresponds to a tag exiting the common reservoir,
277 making pore 2 the Exit in L-to-R, whereas moving R-to-L means a tag in I_2 is entering the
278 common reservoir. While I_2 shows two tags, the third tag present in I_1 for both scan directions
279 yields an opportunity to quantify two tag-pair separation distances. For the signals shown,
280 the number of tags between the pores are plotted, and the tag speeds based on dwell-time
281 and pore-to-pore speeds are also plotted. These speed values versus time provides a glimpse
282 into how the molecule is moving during co-capture tug-of-war, along with the illustrations
283 added for visualization (Figure 4a,b). The pore-to-pore speed is modestly faster for an R-
284 to-L direction (0.8 vs. 0.6 $\mu\text{m}/\text{ms}$), which is consistent with a larger voltage differential for
285 R-to-L motion ($V_1 = 600 \text{ mV}$, $V_2 = 400 \text{ mV}$) than for L-to-R motion ($V_1 = 250 \text{ mV}$, $V_2 =$
286 400 mV).

287 We estimate tag-to-tag separation distances (Figure 4c,d bottom) by multiplying the
288 mean pore-to-pore speed within a scan by the tag-to-tag times recorded within that scan,
289 and adding the membrane thickness. Membrane thickness is added to account for the added
290 spatial separation that is equivalent to either tag passing the length of a pore, since tag-to-
291 tag times are computed from the rising edge of a detected tag blockade to the falling edge
292 of the next detected tag blockade. The tag-to-tag distance estimates are shown for both
293 the L-to-R scan and the R-to-L scan. The results suggest that each separation prediction
294 will vary across the two different scan directions. Tag-pair separation distance predictions
295 will also vary due to differences between the tag pore-to-pore speed and the true speed
296 profile during the tag-to-tag time for a given pore. That is, the assumption that the speed
297 between the tags is constant and equal to the pore-to-pore speed is not exactly correct. It

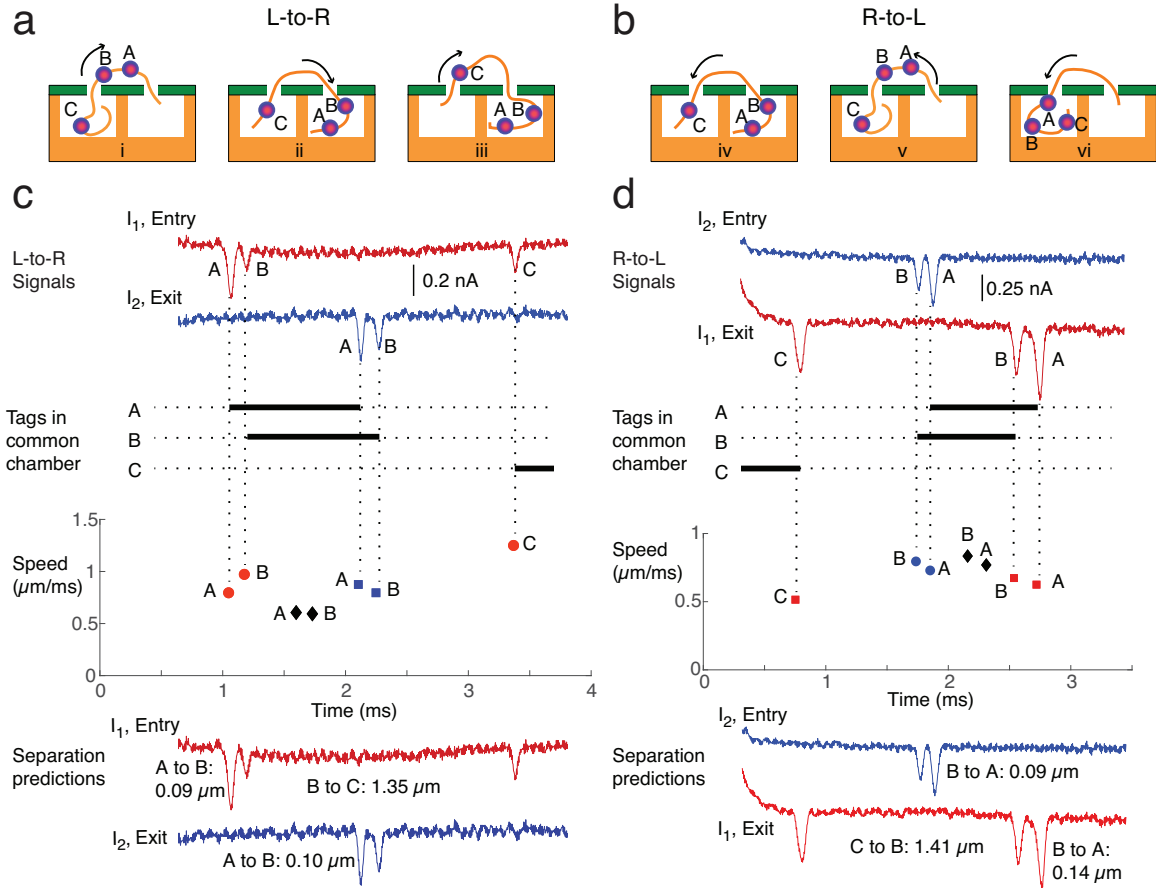


Figure 4: Estimating inter-tag separation distances from dual current signals generated during a multi-scan experiment. The **a** L-to-R and **b** R-to-L illustrations help visualize the relative tag locations that are revealed by the scan signals. The **c** L-to-R and **d** R-to-L signals were from adjacent scans of a co-captured molecule that was scanned for 48 cycles. In L-to-R, pore 1 is the Entry pore for a tag while pore 2 is the Exit pore. In R-to-L, pore 2 is the Entry pore for a tag while pore 1 is the Exit pore. Entry and Exit are thus relative to the direction of motion of a tag as it passes from pore to pore. The signals and inferred number of tags in the common chamber between the pores versus time are plotted. Illustration (ai) visualizes the period when A and B are in the common chamber, while (aiv) visualizes the period after B exits but before C enters the common chamber, etc. The Speed plots shows the computed tag speeds at the Entry and Exit pores, based on tag duration divided into membrane thickness, and tag pore-to-pore speeds computed as the known distance between the pores divided by the pore-to-pore time. Inter-tag separation distance predictions are computed by multiplying the mean pore-to-pore speed within a scan by the time between detected tag pairs, and adding the membrane thickness as a correction (main text). The voltages were set to $V_1 = 250 \text{ mV}$ for L-to-R and 600 mV for R-to-L, with $V_2 = 400 \text{ mV}$ held constant (Chip E, Table S1). The FPGA monitored I_2 for $N = 2$ tags (exit signal L-to-R, entry signal R-to-L), though 3 tags were visible in I_1 in both directions.

298 likely that this assumption is better when the tag-to-tag times are shorter than the pore-to-
299 pore times, which is the case for tags A and B but not tags B and C in Figure 4. In any
300 case, if the assumption that the DNA is traveling at the constant pore-to-pore speed is true
301 on average, we would expect the average of many re-scans to predict accurate separation
302 distances between any two sequential tags. We next compute the average of multiple scan
303 predictions and test how well the predictions line up with the known separations from the
304 model tagged-DNA reagent.

305 **Combining Scans to Improve Tag-to-Tag Distance Predictions**

306 We examined the error-reduction performance of averaging the distances obtained from in-
307 dividual scans within a multi-scan event. Five different multi-scan events with at least 30
308 cycles are reported in Table 1. For each event, the averaged distance estimates are shown for
309 each scan direction, and using pore 2 estimates alone as well as merging the pore 1 and pore
310 2 estimates. The table reports the number of cycles, which is equal to the number of scans
311 in each direction, and the number of tag-pairs that contributed to each separation distance
312 estimate. In all cases, there are fewer distance estimates than scans. For example, for event
313 (v) that had 65 scans in each direction, and for the R-to-L direction, 57 scans produced
314 detectable tag pairs in I_2 while 62 tag pairs were detected in I_1 for a total of 119 separation
315 estimates. The attrition is because the probability of a missing a tag within any one scan
316 increases with cycle count, as described by equation (1).

317 We can assess the correspondence between the averaged separation distances in Table 1
318 and the known inter-tag separations that are possible from the position map (Figure S6).
319 Known distances are computed using the conversion 0.34 nm/bp, which assumes the DNA in
320 the common reservoir is fully stretched between the pores. For event (i), only the R-to-L scan
321 directions were combined and reported for event (i), since the L-to-R data showed significant
322 variation in the pore-to-pore speed (described in SI Section 11). Note that the two scans
323 shown in Figure 4 are from event (i), which pathologically generated two tag-pair estimates

324 in pore 1 current for the reason described in that figure. If we assume tag 6 is absent for the
325 molecule and that tags 4, 5 and 7 are present, the adjacent tag-pair separations for event (i)
326 have their closest match among all possible adjacent tag-pair permutations that are possible
327 according to the position map. Specifically, the map shows 0.1 and 1.5 μm adjacent distance
328 between tags 4-5 and 5-7. It is reasonable to assume that a tag (i.e., tag 6) is absent, as
329 discussed previously (Figure S2e).

330 Events (ii-v) in Table 1 show very consistent results across both scan directions, and
331 between both pores when comparing Pore 2 results with Combined results. For these events,
332 only a single separation distance estimate was produced, which is most common for control
333 logic that uses $N = 2$ tag detection in I_2 to trigger direction switching. In terms of compar-
334 ing averaged separation distances and the known inter-tag separations, events (ii) and (iii)
335 correspond most closely to 1.5 μm and 1.3 μm distances between 5-7 and 6-7, respectively,
336 with the 1.5 μm value possible if the tag 6 position is assumed vacant for the molecule of
337 event (ii). And events (iv) and (v) correspond most closely to 0.3 μm and 0.2 μm distances
338 between 4-6 and 5-6, respectively, with the 0.3 μm value possible if the tag 5 position is
339 assumed vacant for the molecule of event (iv).

340 Consistent with the thesis of this work, the correspondence between distance estimates
341 and map-possible permutations is generally not as clean for the individual scans (e.g., Fig. 4)
342 as it is for the averaged scans, and is impossible for scans where tags are missed (represen-
343 tative examples are shown in Figs. S11, S13 and S14). This demonstrates the value of error
344 reduction by averaging across a multi-scan data set generated for each molecule. Additional
345 data on the velocity profiles for events (i-v) in Table 1 and data for four additional multi-scan
346 events are reported in Table S3. The error on each separation estimate is obtained as the
347 error on the mean over the group of estimates, with significant reduction of error achieved
348 through averaging.

349 The data in Table 1 show the power of the flossing approach when the events have
350 tag-to-tag times in I_2 that are unambiguously attributable to the same physical set of tags

Table 1: **Summary of separation statistics from five multi-scan events**

Event ^{a)}	Number of cycles	Scan direction	Pore 2 tag-to-tag separations [μm] ^{b)}	Combined tag-to-tag separations [μm] ^{b)}
(i) ^{c)}	48	R-to-L	a. 0.10 ± 0.005 (32)	a. 0.12 ± 0.006 (56)
			b. — (0)	b. 1.4 ± 0.031 (21)
(ii)	30	L-to-R	1.5 ± 0.12 (28)	1.5 ± 0.12 (48)
		R-to-L	1.5 ± 0.026 (15)	1.5 ± 0.017 (38)
(iii)	70	L-to-R	1.3 ± 0.045 (68)	1.3 ± 0.034 (116)
		R-to-L	1.4 ± 0.017 (32)	1.3 ± 0.011 (96)
(iv)	31	L-to-R	0.31 ± 0.022 (9)	0.31 ± 0.022 (9)
		R-to-L	0.3 ± 0.005 (28)	0.31 ± 0.004 (55)
(v)	65	L-to-R	0.2 ± 0.008 (18)	0.2 ± 0.009 (20)
		R-to-L	0.21 ± 0.003 (57)	0.21 ± 0.002 (119)

^{a)} Events (i-iii) and (iv)-(v) are from chips E and F (Table S1), respectively.

^{b)} Mean \pm standard deviation of the mean (number of samples).

^{c)} Three tags were detected to produce two adjacent separations, listed as (a) and (b). L-to-R results had larger errors due to larger pore-to-pore speed fluctuation (coefficient of variation is 83%, Table S3), and so were excluded.

351 (representative scans with both I_1 and I_2 signals are provided in Figures S7-S14). In other
352 data, however, when a tag is missed in I_2 within a scan, the two-tag scanning logic will
353 eject the molecule or subsequently shift to a new physical tag pairing on the same molecule,
354 which creates a register-shift in the tag-to-tag time data. An example of this is event (vi) in
355 Table S3 with the register-shift scan signals shown in Fig. S11. While this complexity can
356 be visually observed in the data and accommodated manually, we next sought to develop an
357 alternative approach that could detect and automate analysis for such register shifts.

358 The alternative method presented next is based on aligning the signal in the time domain
359 based on tag blockade proximity, with the aim of automating the binning of tag-pair times,
360 particularly where there is greater ambiguity in assigning such times across scans. Our
361 own prior work has shown that time-based signal alignment of nanopore data can increase
362 the value of multiple nanopore reads in the context of sequencing through homopolymer
363 regions.³⁴ The time-based signal alignment strategy is described below, as applied to two

364 multi-scan events shown in Figure 5. The first in Figure 5a provides an example where
365 tag-pair matching from scan to scan is not ambiguous, while Figure 5b provides an example
366 where tag-pair matching from scan to scan is ambiguous due to the aforementioned register-
367 shifting effect (Figure 5a,c events are events (vii),(vi) in Table S3, respectively).

368 In the time-bases signal alignment method, the temporal position of each tag relative to
369 the starting time of each scan is first computed. To facilitate alignment, the method must
370 tolerate potentially large differences in tag event shape, and so the tag analysis procedure
371 was modified and based on fitting a model function to each peak based on the convolution of
372 a box with a Gaussian function (SI Section 10). This model can characterize tag blockades
373 that are both broad/rectangular in character or narrow/Gaussian-like (see Figure S15 for
374 examples of fitted scans). The extracted tag positions in time are plotted versus scan number,
375 in Figure 5b,e. The scan to scan signals show a relative translational offset, arising from the
376 fact that different portions of the molecule are observed in each scan. There is also stochastic
377 variation in molecular motion, arising from Brownian fluctuations. These effects complicate
378 correct association of tags across multiple scans, that is ensuring that the tags observed in a
379 given scan correspond to tags at the same sequence position in adjacent scans for the same
380 scan direction.

381 In order to align scans in a systematic way, the algorithm automatically groups tags
382 and removes the translational offset across the scans. Figures 5c,f show examples of aligned
383 events and SI section 10 provides a detailed description of the approach. Essentially, the
384 algorithm works by assuming that at least two tags are shared between two successive scans.
385 In order to identify one of the shared or “common” tags, the algorithm brings each potential
386 tag pair in the two scans into alignment by shifting one scan relative to the other. Note
387 that only translational offsets are applied, i.e., there is no overall dilation of the time-scale.
388 For each one of these possible test alignments, the algorithm computes a measure of align-
389 ment error based on the summed squared difference between distinct tag pairs in the test
390 alignment. The algorithm identifies the pairing that yields minimum error as true common

391 tags and implements the translational shift that brings this pair into alignment. Note that
392 this approach yields both the translational offset between the scans and a correspondence
393 table of shared tags between the scans. Working iteratively across all scans in a set the
394 tags observed across the scan can be grouped together and translational offsets removed.
395 The tag group with the largest set of scans is defined to be the origin tag and each scan is
396 shifted so this origin is set to zero. This algorithm outputs a final barcode, or set of averaged
397 relative tag positions, for each set of single molecule scans. The final barcode estimates for
398 the two multi-scan events considered are shown in Figure 5c,f, showing values that are very
399 close to those reported using the method of combining distances presented above (see events
400 (vii),(vi) in Table S3). The error on a tag position in the barcode is obtained as the error
401 on the mean over the group of scans associated with the tag, with significant reduction of
402 error achieved through averaging of positional information over multiple scans.

403 The outputted barcodes are in units of time. In order to calibrate the scans to units
404 of distance, we first use an aggregate translocation velocity corresponding to the scan set.
405 The aggregate translocation velocity is computed as the mean of a subset of the pore-to-
406 pore speeds measured within a multi-scan event, using only those speeds for which the scan
407 displayed a conserved number of tags in both pores. The barcodes are then calibrated in
408 units of distance by multiplying by the mean pore-to-pore velocity. The reported error on
409 the final calibrated tag positions incorporates the error on the velocity and the error in
410 separation via standard propagation. The set of possible tag separations extracted from
411 these barcodes shows good reproducibility between the pores (Fig. 5g) and correspondence
412 with the expected separations from the λ -DNA tag map (Fig. 5h). Note that the sharpness
413 of the plateaus indicate the precision achieved through averaging of multiple scans. In
414 particular, when we sort the extracted tag separations by size, we find that the data shows
415 distinct plateaus that correspond to the expected separations in the map. Separations that
416 fall off the expected spacings could arise from non-specific binding (e.g., tags attached at
417 random nicks present non-specifically in λ -DNA), or offsets caused by imperfections in the

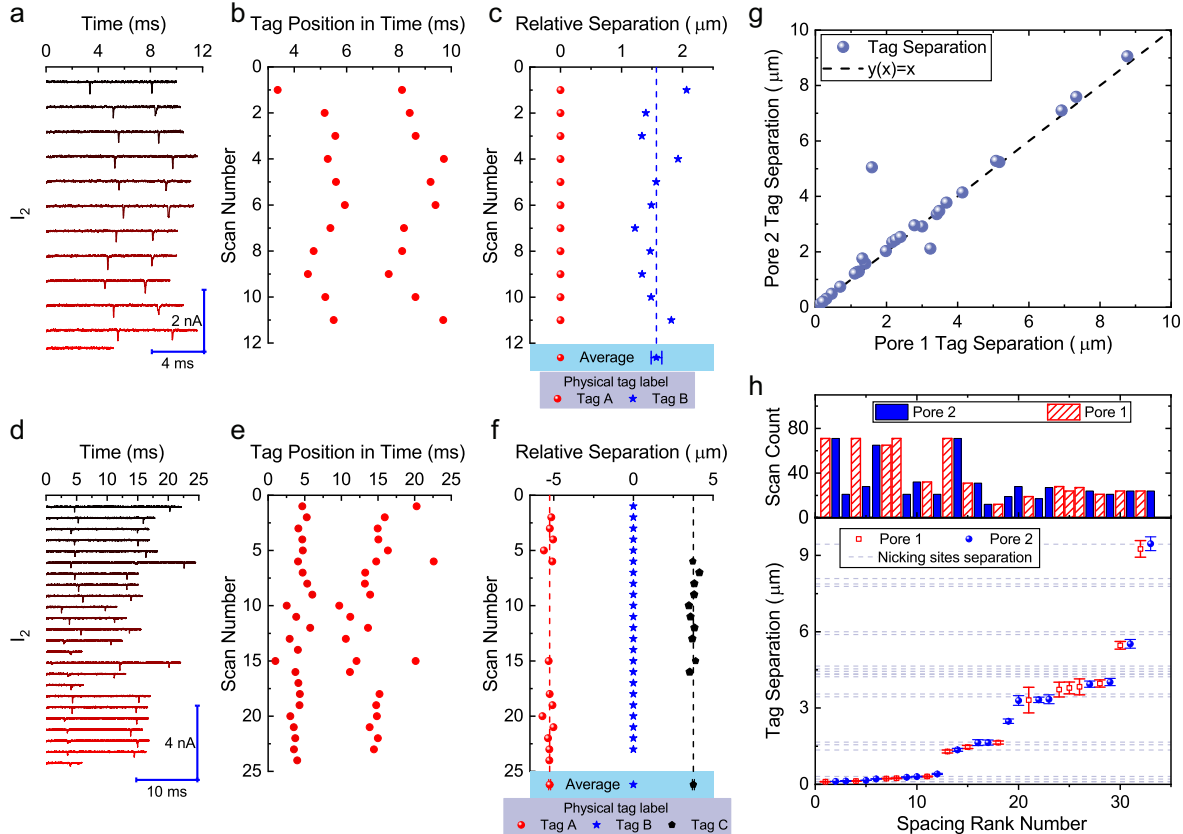


Figure 5: Tag position rescaling and tag spacing distribution using time-based signal alignment. **a** Raw L-to-R scans from pore 2 for a multi-scan event with control logic triggered to probe two tagged sites. The time-axis is plotted relative to the beginning of each scan. **b** Temporal positions for each tag obtained from **a**. **c** Aligned relative tag separations from **b** with resultant average separation distances between denoted tags A-B. **d** Raw L-to-R scans from pore 2 for a multi-scan event with control logic triggered to probe two tagged sites, but produced data covering three tagged sites due to an intermediate missed tag at scans 6 and 15 (offline analysis detects all 3 tags even where online control does not). **e** Temporal positions for each tag obtained from **d**. **f** Aligned relative tag separations from **e** with resultant average separation distances between denoted tags A-B and B-C. **g** Averaged separation distances between tags obtained from pore 2 versus pore 1. The linear correlation of the data indicates strong measurement reproducibility between the pores. **h** Calibrated tag separations arranged in ascending order using pore 1 and pore 2 estimates, with tag relative separation plotted versus separation ranking by size (spacing rank number). The plateaus are close to the expected permutations of tag-pair separations that are possible for the model MS-tagged λ -DNA reagent (horizontal lines, Figure S6). Tag separations with at least 10 scans were included, with the histogram above shows the scan count per data point. **a-f** data are from chip A and **g-h** data are from chips A, E and F (Table S1).

418 tag blockage analysis algorithm. Figure 5g,h shows data from the L-to-R portion of 33
 419 separate multi-scan estimates with an average velocity of $v = 0.56 \pm 0.04 \mu\text{m}/\text{ms}$ and a

420 minimum of 10 scans per tag pair. We also plotted the scan count aligned with each data of
421 the 33 estimates, with higher scan counts reducing the variance of each data point.

422 The data in Figures 4 and 5h show that the closest spacing available with the model
423 reagent (300 bp or 100 nm) is well resolved. In single-pore work, sub-4 nm diameter membrane-
424 based pores that hug PNA-based motifs (15 bp footprint) have demonstrated 100 bp inter-tag
425 distance resolution,³⁵ while pipette-based pores have resolved at low as 200 bp.⁶ We did not
426 explore the lower limit of inter-tag distance resolution here, but observe that since our peaks
427 are well resolved for the 300 bp separation (e.g., visible space between tags in Figure 4
428 traces), and since slower passage and higher bandwidth are knobs that can be turned in our
429 setup, we anticipate a lower limit below 300 bp should be achievable. It should be possible
430 to resolve tags that are spaced modestly farther apart than the membrane thickness, in prin-
431 ciple (~ 105 bp for the current chips). This can be examined in future work with different
432 model reagents.

433 Conclusion

434 We have developed an approach that first traps and linearizes an individual, long DNA
435 molecule in a dual nanopore device, and then provides multi-read coverage data using auto-
436 mated “flossing” control logic that moves the molecule back-and-forth during dual nanopore
437 current sensing. From the point-of-view of dual pore technology development, our rescanning
438 approach overcomes a key challenge: while maximally long trapping-times can be achieved
439 by balancing the competing forces at each pore, sub-diffusive dynamics will persist as speed
440 is reduced,²⁸ undermining mapping-based approaches that rely on a correspondence between
441 the time at which tags are detected and their physical position along a DNA. In our approach
442 sub-diffusive dynamics are avoided during bidirectional scanning of the molecule by using
443 speeds that permit reliable tag detection while being high enough to avoid broad translo-
444 cation time distributions. By enhancing the quality of information that can be extracted

445 from a given DNA carrier strand, we feel this is a step towards addressing a key challenge
446 in single-molecule technology: genome-scaling. Genome scaling is the potential to move
447 beyond experiments with short DNA constructs and tackle complex, heterogeneous samples
448 containing fragments in the mega-base size drawn from Gbp scale genomes. For genome
449 scaling to be feasible, single-molecule reads must have sufficient quality (e.g. contain suf-
450 ficiently low systematic and random errors) to enable alignment to reference genomes and
451 construct contigs from overlapping reads drawn from a shared genomic region. This is the
452 only way to identify long range structural variations that are masked by short read methods
453 (i.e., via NGS), and in some cases masked even by long-read sequencing.³⁶ Future work will
454 include developing a new model reagent with improved tagging efficiency, and optimizing
455 the multi-scanning logic for increased scan number. We also plan to apply the approach to
456 longer molecules with more complex tagging patterns, possibly using repetitive scanning at
457 targeted regions to gradually explore the barcode structure. In the applied context of epige-
458 netics, our technique can combine sequence-specific label mapping, using the same chemistry
459 here or other nanopore-compatible schemes that have a low spatial footprint per label,^{13,37}
460 with methylation-specific label detection.¹⁴ This would help meet the need for technologies
461 that perform long-range methylation analysis.³⁸

462 **Experimental Section**

463 **Preparation of mono-streptavidin tagged λ DNA reagent**

464 5 μ g of commercially prepared λ DNA (New England Biolabs) was incubated with 0.025 U of
465 Nt.BbvC1 in a final volume of 100 μ l of 1 X CutSmart buffer (New England Biolabs) to in-
466 troduce sequence specific nicks in λ DNA. The nicking reaction was incubated at 37°C for 30
467 minutes. The nicking endonuclease Nt.BbvC1 has the recognition sequence, 5'- CC↓TCAGC
468 -3' and there are 7 Nt.BbvC1 sites in λ DNA. Nick translation was initiated by the addition
469 of 5 μ l of 250 μ M biotin-11-dUTP (ThermoFisher Scientific) and 1.5 U of E.coli DNA poly-

merase (New England Biolabs) and incubated for a further 20 minutes at 37°C. The reaction was quenched by the addition of 3 μ l of 0.5M EDTA. Unincorporated biotin-11-dUTP was removed by Sephadex G-75 spin column filtration. To create mono-streptavidin tagged λ DNA complex, mono-streptavidin was added to the G-75 purified biotin labeled DNA to a final concentration of 50 nM and incubated at room temperature for 5 minutes to allow the biotin - mono-streptavidin interaction to saturate. The mono-streptavidin tagged λ DNA complex was then used directly in nanopore experiments.

Fabrication process of the two pore chip

We described the fabrication protocol in our previous work.³³ Briefly, we prepared the micro-channel on glass substrate and SiN membrane on Si substrate separately. The all-insulate architecture minimized the system capacitance. Thus the noise performance is optimized. We initially dry-etch two “V” shape, 1.5 μ m-deep micro-channel on the glass in a 8 mm \times 8 mm die, with the tip of the “V” 0.4 μ m away from each other. Considering we can not grow SiN membranes on glass, we then deposit 400 nm-thick LPCVD SiN, 100 nm-thick PECVD SiO₂, and 30 nm-thick LPCVD SiN on Si substrate. To transfer the 3-layers film stack to glass substrate from the Si substrate, we anodic-bonded the two substrates, with the micro-channel on glass facing the 3-layers film stack on Si. To remove the Si substrate, we first dry-etched away the 430 nm SiN on the backside of Si. Then we etched away the Si substrate using hot KOH, revealing the 3-layers films stack on the glass. The 3-layers films stack provides mechanical support to cover the micro-channel on glass, while it is too thick for nanopore sensing. So we had to open a window in the center for nanopore. To achieve that, we dry-etched a 10 μ m \times 10 μ m window in the center through the 400 nm-thick SiN mask into the 100 nm-thick SiO₂ buffer layer. Then we etched away the leftover 100 nm-thick SiO₂ layer using hydrofluoric acid, revealing the single 35 nm-thick SiN membrane layer. At last, we drilled two nanopores through the membrane using Focused Ion Beam at the tip of the two “V” shaped channels.

496 Nanopore experiments

497 We performed all the nanopore experiments at 2 M LiCl, 10 mM Tris, 1 mM EDTA, pH=8.8
498 buffer. The two pore chip was assembled in home-made micro-fluidic chunk, which guide the
499 buffer to channel 1, channel 2, and the center common chamber. Ag/ AgCl electrodes were
500 inserted to the buffer to apply voltage and measure current. The current and voltage signal
501 was collected by Molecular Device Multi-Clamp 700B, and was digitized by Axon Digidata
502 1550. The signal is sampled at 250 kHz and filtered at 10 kHz. The tag-sensing and voltage
503 control module was built on National Instruments Field Programmable Gate Array (FPGA)
504 PCIe-7851R and control logic was developed and run on the FPGA through LabView.

505 Data analysis

506 All data processing was performed using custom code written in Matlab (2018, MathWorks).
507 The start and end of each scan and event are extracted from the FPGA state signal (SI) for
508 offline analysis. During real-time tag detection on the FPGA, the presence of tag is detected
509 in the control logic if any sample falls 70 pA below the baseline and lasted at least 12 μ s.
510 During off-line analysis, for the analysis reported in Figure 4 and Table 1, tag blockade
511 quantification during each scan is performed as follows: the open pore baseline standard
512 deviation is calculated using 500 μ s of event-free samples (σ); the DNA co-capture baseline
513 I_{DNA} is determined using the mean of 100 tag blockade-free samples; a tag blockade candidate
514 is detected where at least one sample falls below $I_{DNA} - 6\sigma$, i.e., sufficiently below the DNA
515 co-capture baseline; a tag blockade is quantitated where the blockade candidate has samples
516 that return within 1σ below I_{DNA} , and the tag duration is computed as the full width at half
517 minimum (FWHM), where the half minimum is halfway between the lowest sample below
518 the DNA baseline and the DNA baseline. The alternative tag profile characterization via
519 least-squares fitting that was utilized for the alignment strategy data in Figure 5 is described
520 in SI section 12. Tag-to-tag times are computed from rising edge to falling edges using the
521 FWHM time transition (edge) points, and pore-to-pore times use the rising edge of the tag

522 blockade at the entry pore, to the falling edge of the corresponding tag blockade at the
523 exit pore. Pore-to-pore times were computed by assigning entry tags to have one matching
524 exit tag, utilizing the first exit tag not previously assigned and within a time limit of 10
525 ms. Cases where missed tags in analysis produced incorrectly assigned pore-to-pore times
526 occurred $\sim 9\%$ of the time (see tag-pair and pore-to-pore time counts in Table S3), and were
527 manually trimmed. Pore-to-pore times were utilized to compute pore-to-pore speed on a per
528 scan basis (Figure 4, Table 1, Table S3) or in the aggregate with the alignment strategy data
529 (Figure 5) as described in the main text and SI section 12. Compensation of transient decay
530 in I_1 following step changes in V_1 is described in SI section 12.

531 **Supporting Information**

532 Supporting Information is available from the Wiley Online Library or from the author.

533 **Acknowledgement**

534 This work done in Santa Cruz was performed and financially supported by Ontera, Inc.
535 The work done at McGill University was financially supported by the Natural Sciences and
536 Engineering Research Council of Canada (NSERC) Discovery Grants Program (Grant No.
537 RGPIN 386212).

538 **Competing Financial Interests Statement**

539 The authors declare competing financial interests: X.L., P.Z., A.R., R.N., and W.B.D. are
540 employees of Ontera, Inc., which has exclusively licensed the dual pore device patent from
541 the University of California, Santa Cruz, for commercialization purposes.

References

- 542 1. Storm, A. J.; Chen, J. H.; Zandbergen, H. W.; Dekker, C. Translocation of double-strand
543 DNA through a silicon oxide nanopore. *Physical Review E* **2005**, *71*, 051903.
- 544 2. Freedman, K. J.; Otto, L. M.; Ivanov, A. P.; Barik, A.; Oh, S.-H.; Edel, J. B. Nanopore
545 sensing at ultra-low concentrations using single-molecule dielectrophoretic trapping. *Nature*
546 *Communications* **2016**, *7*, 10217.
- 547 3. Wanunu, M.; Sutin, J.; McNally, B.; Chow, A.; Meller, A. DNA translocation governed
548 by interactions with solid-state nanopores. *Biophysical journal* **2008**, *95*, 4716–4725.
- 549 4. Morin, T. J.; McKenna, W. L.; Shropshire, T. D.; Wride, D. A.; Deschamps, J. D.;
550 Liu, X.; Stamm, R.; Wang, H.; Dunbar, W. B. A handheld platform for target protein
551 detection and quantification using disposable nanopore strips. *Scientific Reports* **2018**,
552 *8*, 14834.
- 553 5. Plesa, C.; Ruitenber, J. W.; Witteveen, M. J.; Dekker, C. Detection of Individual
554 Proteins Bound along DNA Using Solid-State Nanopores. *Nano Letters* **2015**, *15*, 3153–
555 3158.
- 556 6. Chen, K.; Juhasz, M.; Gularek, F.; Weinhold, E.; Tian, Y.; Keyser, U. F.; Bell, N. A. W.
557 Ionic Current-Based Mapping of Short Sequence Motifs in Single DNA Molecules Using
558 Solid-State Nanopores. *Nano Letters* **2017**, *17*, 5199–5205.
- 559 7. Kong, J.; Bell, N. A. W.; Keyser, U. F. Quantifying Nanomolar Protein Concentrations
560 Using Designed DNA Carriers and Solid-State Nanopores. *Nano Letters* **2016**, *16*, 3557–
561 3562.
- 562 8. Liu, K.; Pan, C.; Kuhn, A.; Nievergelt, A. P.; Fantner, G. E.; Milenkovic, O.; Raden-
563 ović, A. Detecting topological variations of DNA at single- molecule level. *Nature Com-*
564 *munications* **2019**, *10*.
- 565

- 566 9. Bell, N. A. W.; Keyser, U. F. Digitally encoded DNA nanostructures for multiplexed,
567 single-molecule protein sensing with nanopores. *Nature Nanotechnology* **2016**, *11*, 645–
568 651.
- 569 10. Chen, K.; Kong, J.; Zhu, J.; Ermann, N.; Predki, P.; Keyser, U. Digital Data Storage
570 Using DNA Nanostructures and Solid-State Nanopores. *Nano Letters* **2019**, *19*, 1210–
571 1215.
- 572 11. Sze, J. Y. Y.; Ivanov, A. P.; Cass, A. E. G.; Edel, J. B. Single molecule multiplexed
573 nanopore protein screening in human serum using aptamer modified DNA carriers. *Nature*
574 *Communications* **2017**, *8*, 209–10.
- 575 12. Kong, J.; Khu, J.; Chen, K.; Keyser, U. Specific Biosensing Using DNA Aptamers and
576 Nanopores. *Advanced Functional Materials* **2019**, *29*, 1807555.
- 577 13. Singer, A.; Wanunu, M.; Morrison, W.; Kuhn, H.; Frank-Kamenetskii, M.; Meller, A.
578 Nanopore based sequence specific detection of duplex DNA for genomic profiling. *Nano*
579 *Letters* **2010**, *10*, 738–742.
- 580 14. Shim, J.; Humphreys, G. I.; Venkatesan, B. M.; Munz, J. M.; Zou, X.; Sathe, C.; Schul-
581 ten, K.; Kosari, F.; Nardulli, A. M.; Vasmatzis, G. *et al.* Detection and quantification of
582 methylation in DNA using solid-state nanopores. *Scientific Reports* **2013**, *3*, 1389.
- 583 15. Shim, J.; Kim, Y.; Humphreys, G. I.; Nardulli, A. M.; Kosari, F.; Vasmatzis, G.; Tay-
584 lor, W. R.; Ahlquist, D. A.; Myong, S.; Bashir, R. Nanopore-Based Assay for Detection
585 of Methylation in Double-Stranded DNA Fragments. *ACS Nano* **2015**, *9*, 290–300.
- 586 16. Albrecht, T. Single-Molecule Analysis with Solid-State Nanopores. *Annual Review of*
587 *Analytical Chemistry* **2019**, *12*, 371–387.
- 588 17. Langecker, M.; Ivankin, A.; Carson, S.; Kinney, S. R. M.; Simmel, F. C.; Wanunu, M.

- 589 Nanopores Suggest a Negligible Influence of CpG Methylation on Nucleosome Packaging
590 and Stability. *Nano Letters* **2014**, *15*, 783–790.
- 591 18. Ling, D. Y.; Ling, X. S. On the distribution of DNA translocation times in solid-state
592 nanopores: an analysis using Schrodinger first-passage-time theory. *Journal of Physics:
593 Condensed Matter* **2013**, *25*, 375102.
- 594 19. Sarabadani, J.; Ala-Nissila, T. Theory of pore-driven and end-pulled polymer transloca-
595 tion dynamics through a nanopore: an overview. *Journal of physics. Condensed matter
596 : an Institute of Physics journal* **2018**, *30*, 274002.
- 597 20. Miller, H.; Zhou, Z.; Shepherd, J.; Wollman, A. J. M.; Leake, M. C. Single-molecule
598 techniques in biophysics: a review of the progress in methods and applications. *Reports
599 on Progress in Physics* **2018**, *81*, 024601.
- 600 21. Jeffet, J.; Kobo, A.; Su, T.; Grunwald, A.; Green, O.; Nilsson, A. N.; Eisenberg, E.;
601 Ambjörnsson, T.; Westerlund, F.; Weinhold, E. *et al.* Super-Resolution Genome Map-
602 ping in Silicon Nanochannels. *ACS Nano* **2016**, *10*, 9823–9830.
- 603 22. Reisner, W.; Pedersen, J. N.; Austin, R. H. DNA confinement in nanochannels: physics
604 and biological applications. *Reports on Progress in Physics* **2012**, *75*, 106601.
- 605 23. Lam, E. T.; Hastie, A.; Lin, C.; Ehrlich, D.; Das, S. K.; Austin, M. D.; Deshpande, P.;
606 Cao, H.; Nagarajan, N.; Xiao, M. *et al.* Genome mapping on nanochannel arrays for
607 structural variation analysis and sequence assembly. *Nature Nanotechnology* **2012**, *30*,
608 771–776.
- 609 24. Gershow, M.; Golovchenko, J. A. Recapturing and trapping single molecules with a
610 solid-state nanopore. *Nature Nanotechnology* **2007**, *2*, 775–779.
- 611 25. Plesa, C.; Cornelissen, L.; Tuijtel, M. W.; Dekker, C. Non-equilibrium folding of individ-

- 612 ual DNA molecules recaptured up to 1000 times in a solid state nanopore. *Nanotechnology*
613 **2013**, *24*, 475101.
- 614 26. Cadinu, P.; Campolo, G.; Pud, S.; Yang, W.; Edel, J. B.; Dekker, C.; Ivanov, A. P.
615 Double Barrel Nanopores as a New Tool for Controlling Single-Molecule Transport.
616 *Nano Letters* **2018**, *18*, 2738–2745.
- 617 27. Pud, S.; Chao, S.-H.; Belkin, M.; Verschueren, D.; Huijben, T.; van Engelenburg, C.;
618 Dekker, C.; Aksimentiev, A. Mechanical Trapping of DNA in a Double-Nanopore System.
619 *Nano Letters* **2016**, *16*, 8021–8028.
- 620 28. Liu, X.; Zhang, Y.; Nagel, R.; Reisner, W.; Dunbar, W. B. Controlling DNA Tug-of-War
621 in a Dual Nanopore Device. *Small* **2019**, *26*, 1901704.
- 622 29. Kasianowicz, J. J. Nanopores: flossing with DNA. *Nature Materials* **2004**, *3*, 355–356.
- 623 30. Sánchez-Quesada, J.; Saghatelian, A.; Cheley, S.; Bayley, H.; Ghadiri, M. R. Single DNA
624 Rotaxanes of a Transmembrane Pore Protein. *Angewandte Chemie International Edition*
625 **2004**, *43*, 3063–3067.
- 626 31. Wang, H.; Hurt, N.; Dunbar, W. B. Measuring and modeling the kinetics of individual
627 DNA-DNA polymerase complexes on a nanopore. *ACS Nano* **2013**, *7*, 3876–3886.
- 628 32. Trepagnier, E. H.; Radenovic, A.; Sivak, D.; Geissler, P.; Liphardt, J. Controlling DNA
629 Capture and Propagation through Artificial Nanopores. *Nano Letters* **2007**, *7*, 2824–
630 2830.
- 631 33. Zhang, Y.; Liu, X.; Zhao, Y.; Yu, J.-K.; Reisner, W.; Dunbar, W. B. Single Molecule
632 DNA Resensing Using a Two-Pore Device. *Small* **2018**, *14*, 1801890.
- 633 34. O'Donnell, C. R.; Wang, H.; Dunbar, W. B. Error analysis of idealized nanopore se-
634 quencing. *ELECTROPHORESIS* **2013**, *34*, 2137–2144.

- 635 35. Atas, E.; Singer, A.; Meller, A. DNA sequencing and bar-coding using solid-state
636 nanopores. *ELECTROPHORESIS* **2012**, *33*, 3437–3447.
- 637 36. Moll, K. M.; Zhou, P.; Ramaraj, T.; Fajardo, D.; Devitt, N. P.; Sadowsky, M. J.; Stu-
638 par, R. M.; Tiffin, P.; Miller, J. R.; Young, N. D. *et al.* Strategies for optimizing BioNano
639 and Dovetail explored through a second reference quality assembly for the legume model,
640 *Medicago truncatula*. **2017**, 1–16.
- 641 37. Morin, T. J.; Shropshire, T.; Liu, X.; Briggs, K.; Huynh, C.; Tabard-Cossa, V.;
642 Wang, H.; Dunbar, W. B. Nanopore-based target sequence detection. *PloS ONE* **2016**,
643 *11*, e0154426–21.
- 644 38. Pollard, M. O.; Gurdasani, D.; Mentzer, A. J.; Porter, T.; Sandhu, M. S. Long reads:
645 their purpose and place. *Human molecular genetics* **2018**, *27*, R234–R241.