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Parallel DNA circuits by autocatalytic strand displacement and nanopore readout†

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DNA nanotechnology provides a unique opportunity for molecular computation, with strand displacement reactions enabling controllable reorganization of nanostructures. Additional DNA strand exchange strategies with high selectivity for input will enable novel complex systems including biosensing applications. Herein, we propose an autocatalytic strand displacement (ACSD) circuit: initiated by DNA breathing and accelerated by a seesaw catalytic reaction, ACSD ensures that only the correct base sequence starts the catalytic cycle. Analogous to an electronic circuit with a variable resistor, two ACSD reactions with different rates are connected in parallel to mimic a parallel circuit containing branches with different resistances. Finally, we introduce a multiplexed nanopore sensing platform to report the output results of a parallel path selection system at the single-molecule level. By combining the ACSD strategy with fast and sensitive single-molecule nanopore readout, a new generation of DNA-based computing tools is established.

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1. Introduction

DNA computing has developed over the last several decades, making valuable contributions to computer science and biomedicine,^{1,2} such as neural network-like computations reported recently.^{3,4} In a complex computing system, the information loaded on DNA needs to be processed and transmitted among different logic units and circuits. In early studies, enzymes were mainly used, which increased the complexity of both the design and operation of these circuits.^{5,6} The emergence of toehold-mediated strand displacement (TMSD) greatly simplified the development of DNA computing and nanotechnology.^{7–9} TMSD is an enzyme-free reaction to release the prehybridized incumbent DNA strand in the duplex upon the binding of the invader strand from the toehold end, which plays a vital role in TMSD, as its length dictates the kinetics of the reaction.^{10–12} This tunability has enabled the use of TMSD in DNA computing, molecular machines, biosensing, gene regulation, *etc.*^{13–16} Single base differences in the input strand can be detected by commonly used TMSD based on three-way branch migration. However, robust discrimination of a single

base difference in the input strand is challenging due to inevitable crosstalk and leaks as the reaction can still proceed even with a single base mismatch.^{17,18}

The second important challenge for all complex DNA computing systems is the readout of multiple outputs at the same time, especially for parallel computing. While fluorescence is the main method to report on DNA computing systems,^{19,20} the cost of fluorescently labelled DNA is much higher than that of label-free assays and the overlap of the fluorescence wavelength limits the potential of multi-channel monitoring. Atomic force microscopy provides another solution for the readout of complex systems on surfaces, but is unsuitable for reactions in solution.^{21,22} One alternative readout system is based on single-molecule analysis using resistive-pulse sensing with solid-state nanopores.²³ The nanopore technique provides a simple and label-free approach for nucleic acid detection and structural analysis.^{24–28} Our recently proposed solid state nanopore biosensing assay based on digitally encoded DNA nanostructures supplies an ideal label-free multiplexed sensing platform.^{29,30} Our multiplexed nanopore method is ideally suited to decode a parallel DNA computing system as recently suggested.³¹

Herein, we build an autocatalytic strand displacement (ACSD) circuit initiated by DNA breathing instead of toehold binding (Fig. 1a). Without toehold triggering, the ‘toeless’ strand displacement reaction^{32,33} is much slower than TMSD, while the selectivity for the trigger strand and adjustability for the DNA circuit design are greatly improved. We show the impact of a single base change in the invader strand on the kinetics of the strand displacement and demonstrate the

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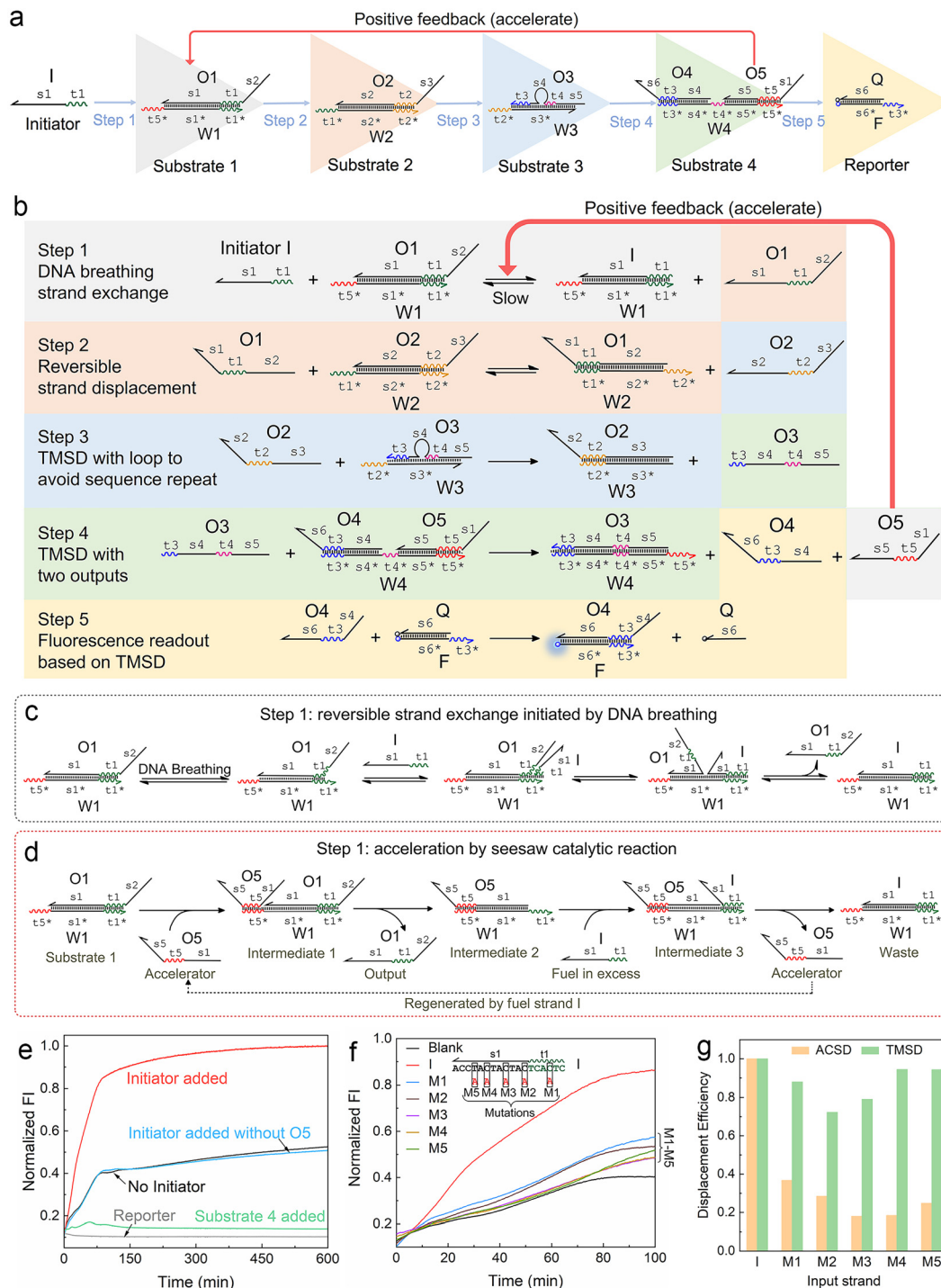


Fig. 1 Mechanism of the autocatalytic strand displacement (ACSD)-based cascade DNA circuit. (a) Basic constitution of the ACSD circuit from the initial input strand *I* (Initiator) to the final fluorescent reporter (DNA complex F-Q). A series of YES gates are connected linearly to construct the ACSD circuit. Names of DNA strands are given in uppercase letters and domains in each strand are labelled in lowercase letters. (b) Reaction details of the whole ACSD circuit. (c) Details of the reaction in step 1 initiated by DNA breathing at the beginning before strand O5 is released. (d) Details of the reaction in step 1 accelerated by O5. (e) Kinetics measurements of the cascade DNA circuit under different conditions. Data of 'No Initiator' is from the mixture of DNA substrates in (a) without strand *I*. The blue curve shows another negative control, in which strand *I* is added but O5 is replaced by O5s (domain s1 is removed, Table S1†). The green curve shows the change in fluorescence when the reporter is mixed with substrate 4. (f) Kinetic measurements of the ACSD circuit triggered by different initiator strands. In groups of M1–5, strand *I* is replaced with its mutant strands M1–5, as shown above the curves, to investigate the effect of single base mutation on the ACSD circuit. No initiator is added in the blank. The fluorescence intensity (FI) is normalized by the FI of the 'Initiator added' group at 600 min. (g) Comparison between the ACSD circuit and the TMSD reaction for distinguishing single base mutation. The strand displacement efficiency (DE) is calculated based on the kinetics data at 100 min in (f) for the ACSD circuit and at 30 min in Fig. S3† for the TMSD reaction.



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the tip side of the glass nanopore. An Axon Axopatch 200B amplifier (Molecular Devices) connected to the electrodes, applied a voltage (600 mV) to drive the DNA through the nanopore. The current signal was recorded using an amplifier, filtered with an external Bessel filter (Frequency Devices) at 50 kHz and digitized at a 250 kHz sampling rate with a data card (PCI-6251, National Instruments). The introduction of nanopore data analysis can be found in the ESI S1.5.†

3. Results and discussion

3.1 Design of the ACSD circuit

We firstly designed a DNA breathing initiated ACSD cascade circuit as shown in Fig. 1a: this can be simply seen as a series connection of five YES gates, and each gate is a DNA strand exchange (steps 1 and 2, reversible) or displacement reaction (steps 3, 4 and 5 irreversible) as shown in Fig. 1b. To identify different functional domains in each strand, toehold domains are named *tx* and represented by wavy lines, and binding domains are named *sx* and represented by straight lines. The asterisk after the domain name means a complementary sequence and all strand sequences are given in Table S1.†

In step 1, there is no free toehold end in substrate 1 (DNA complex W1·O1) for initiator strand *I* to quickly trigger the reaction, and thus the DNA circuit starts from a slow reversible strand exchange initiated by DNA breathing^{32,33} as shown in Fig. 1c. A small amount of strand O1 output from substrate 1 will consequently interact with substrate 2 (complex W2·O2) to trigger a reversible seesaw strand exchange reaction and release strand O2 (Fig. 1b). step 3 is a classic TMSD reaction, where strand O3 will be replaced by O2 quickly as long as free O2 is present in solution. The loop in the middle of substrate 3 (complex W3·O3) is designed to avoid a sequence repeat. Rapid consumption of free O2 by substrate 3 will break the equilibrium of reactions in steps 2 and 1 and further promote the reactions to move forward. The released O3 interacts with substrate 4 in step 4 by binding with the middle toehold domain *t4* to displace O4 and O5 simultaneously. Finally, O4 will be received by the reporter (complex F·Q) to yield a fluorescence signal, and strand O5 acts as positive feedback to accelerate the reaction in step 1. Therefore, the whole reaction gets accelerated with the accumulation of released O5 and the fluorescence intensity reaches the maximum much earlier (group 'Initiator added') than the control groups as shown in Fig. 1e. Gel electrophoresis was applied to investigate the reactions in the ACSD circuit. In Fig. S1,† the clear band of O4 is only observed when initiator *I* is added.

The acceleration mechanism of strand O5 in step 1 is based on a seesaw catalytic reaction. As illustrated in Fig. 1d, O5 interacts with substrate 1 to partly displace prehybridized O1 through the pre-reserved toehold end *t5** on W1. When O1 is released from W1, the excess initiator *I* will work as fuel to exchange O5 on W1 and produce the waste (W1 *I*), and then the regenerated O5 can be reused in a cycle to catalyse the whole reaction. As the reaction progresses, the more O1 is

output, the more O5 is fed back from step 4 to speed up the reaction in step 1. Thus, O5 plays a key role in guiding the reaction to completion. These prehybridized DNA complexes were not further purified after annealing, hence a small amount of unhybridized single strands, such as O5, O4, *etc.*, may leak from the complexes and cause the background signal as observed from the blank group ('No Initiator') in Fig. 1e. Given the specific effect of O5 on the kinetics of the forward reaction in step 1, we infer that the strand leak, as the green curve shown in Fig. 1e, should be another factor to cooperate with strand *I* to initiate and accelerate the reaction. To demonstrate the significance of O5 in the ACSD circuit, we removed the domain *s1* from O5 to generate the control strand O5s (Table S1†), which loses the ability to displace O1 in substrate 1. The resulting fluorescence signal ('Initiator added without O5' in Fig. 1e) is nearly the same as the blank, which indicates that the accelerator O5 is essential for the whole circuit to function properly. A similar result is gained by gel electrophoresis (Fig. S2†).

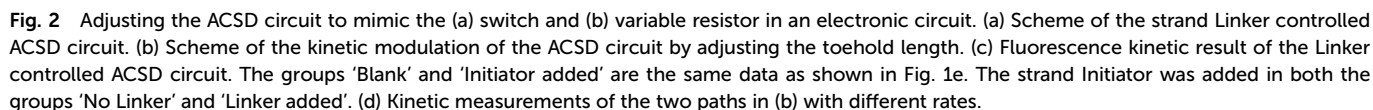
3.2 Detecting single base mutation

The ability of the ACSD circuit to detect single base mutation in the input strand is compared with the widely used TMSD reaction. As shown in Fig. 1f, initiator *I* and its mutant strands M1–M5 are applied to trigger the reactions in ACSD (Fig. 1a) and TMSD (Fig. S3†). For the ACSD circuit, the reaction is much slower, and hence we choose the time point of 100 min to determine the displacement efficiency (DE) for comparison (ESI S1.4†). At this point, the strand *I*-initiated ACSD reaction is nearly complete and the difference between *I* and its mutants is clear (Fig. 1f). For TMSD, the reaction quickly reaches the steady state even for the mutants (Fig. S3†), and we thus determine the DE at 30 min. As shown by comparison in Fig. 1g, a clear difference of the DEs with mutant inputs between ACSD and TMSD indicates that the ACSD circuit is more sensitive to single base mutation than TMSD due to the DNA breathing-based initiation mechanism of the ACSD circuit. The ability of the ACSD circuit to distinguish single base mutations within the input sequence renders it suitable for selective biosensing or constructing complex systems with multiple input strands and simultaneous computations.

3.3 Modulating the circuit by adjusting step 2

Another feature of the ACSD circuit is that the reaction can be flexibly modulated by changing the design of substrate 2 in step 2, which is a reserved section that allows for easy adaptability of the circuit. For instance, by splitting W2 into two halves in step 2 (Fig. 2a), an external input strand Linker can be used to control the reaction, which is based on a four-way junction driven strand displacement reaction.³⁴ Analogous to a switch within an electronic circuit, the strand displacement reaction only proceeds when the Linker is present in the solution (Fig. 2c). This stimuli-responsive feature of the ACSD circuit will be critical for the flexible control of DNA computers as well as biosensing applications based on DNA, such as using a DNA aptamer as the linker.³⁵





For the development of efficient computing systems, multiple ACSD circuits need to operate in parallel. As illustrated in Fig. 3a, two parallel ACSD circuits triggered by the same initiator strand I are designed. The main difference between them is the toehold length in step 2, which is adjusted to mimic two circuits with different resistance values. In common TMSD reactions, the effective rate constant would

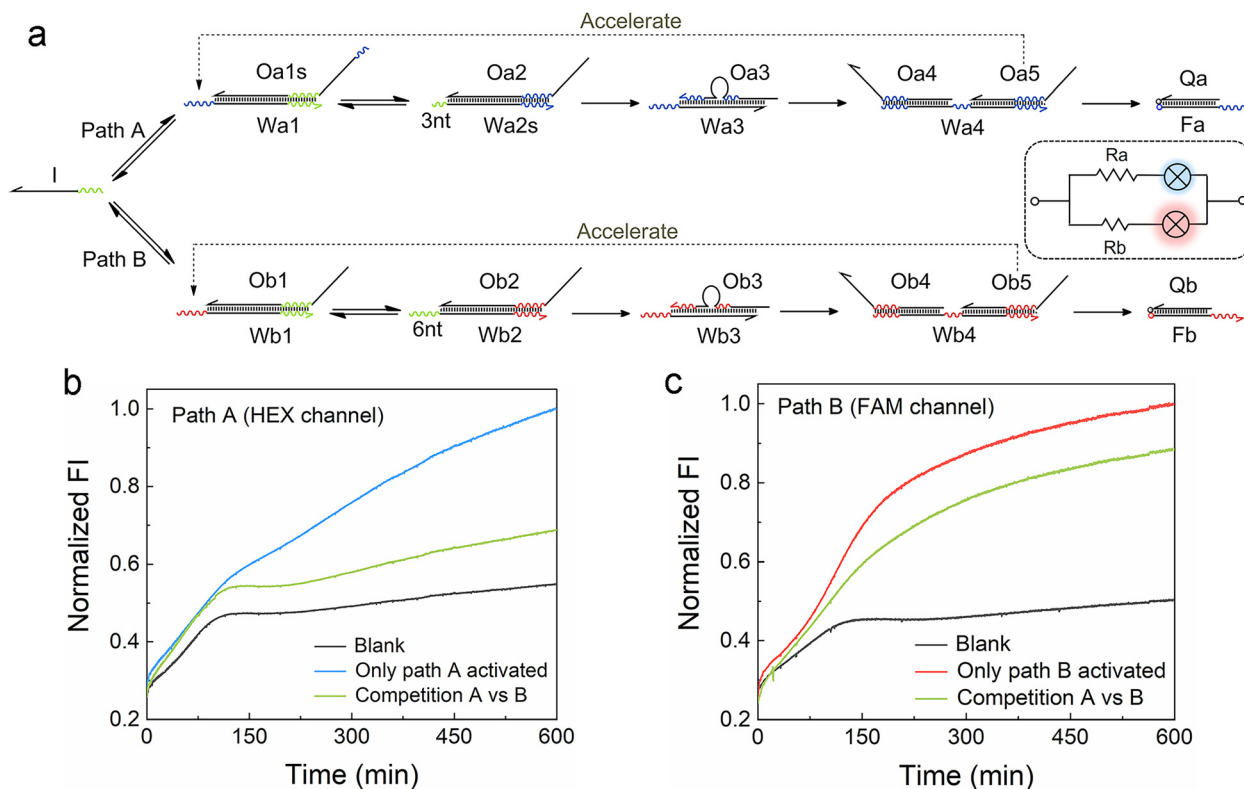


Fig. 3 (a) Scheme of two ACSD circuits connected in parallel and triggered by the same initiator strand *I*. Fluorescent labels HEX and FAM are applied for paths A and B, respectively. (b and c) Kinetic measurements of the two branch paths of the parallel circuit to show the distribution of strand *I* in two paths. DNA substrates of the two paths were mixed with strand *I* in the competition group and without strand *I* in the blank. The complex of Wb1-Ob1 was absent when only path A was activated and the complex of Wa1-Oa1s was absent when only path B was activated. FI is normalized by the FI of the highest group at 600 min.

increase by a factor of about 10^3 when the toehold length increases from 3 nt to 6 nt (ref. 12) and is nearly irreversible. Herein, we adjust the design into two reversible seesaw reactions, so the dynamic distribution of strand *I* between the two paths is possible. The toeholds with different lengths are used to make the reaction rates of the two paths different. Since path B has a longer toehold (*i.e.*, low resistance, Rb), it will be faster than path A. Two different fluorescent dyes are used to identify the report signals from different paths (HEX for path A and FAM for path B). The strand *I* randomly attempt to trigger the reactions in both circuits at the beginning, comparable to a case in a parallel circuit where the voltage across each branch path is the same. Due to the longer toehold end in the fast path B, more Ob5 will be released than Oa5 as the reactions progress to step 4 earlier. Then the Ob5 supplies more strand *I* to participate in the reactions in path B. Finally, more strand *I* will be involved in the reactions in path B and a high fluorescence output signal at 600 min was obtained from the FAM channel (green curve, Fig. 3c). Meanwhile, a low fluorescence signal was reported from the HEX channel (green curve, Fig. 3b) indicating only a small part of strand *I* was consumed in path A. In this case, strand *I* is like the current in a parallel circuit that splits into different branches based on the resistance value in each path, and then the bulbs showed

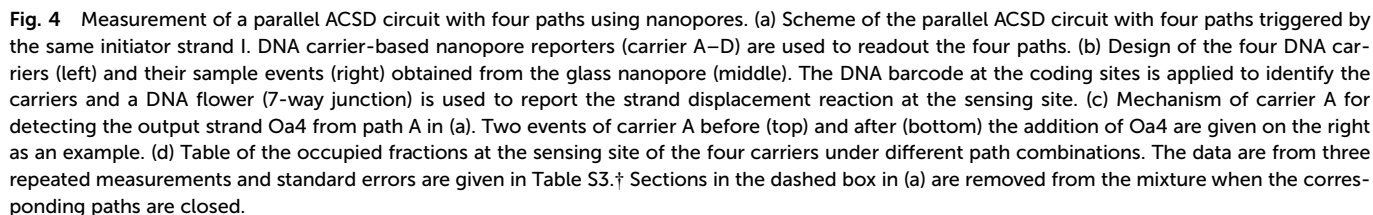
different brightness (*i.e.*, fluorescence intensity). As a positive control, DNA complex Wb1-Ob1 in step 1 of path B was removed to break it when only path A was activated, and it was the same for path A when only path B was activated. The FI ratio of the competition group to the positive control can indicate the relative extent of the reaction (RER, ESI S1.4†). 30.9% and 76.9% were obtained for the RERs of path A and path B, respectively, at 600 min in the competition group. Our results show that parallel DNA circuits can be constructed and the current distribution in parallel circuits can be imitated by the ACSD circuit.

3.5 Monitoring the multiple DNA circuits using a nanopore sensing platform

We now extend the competition model into a more complex path selection system with four parallel ACSD circuits (Fig. 4a). By changing the sequences of relative domains, the number of paths can be easily increased from one to four. The sequence of a binding domain in step 1 remained the same in all paths, so they all can be initiated by strand *I*. In specific path combinations, sections in the dashed boxes in Fig. 4a are removed to close the corresponding paths.

For multiplexed nanopore sensing, the other main change in the design of a parallel system with four branches is repor-





dumbbells (11 for each group) are used to construct a barcode system at the coding sites. The first and fifth groups of DNA dumbbells are set as reference structures, and the 3-bit binary codes between them are used to encode the carriers. At the other side of the carrier, a DNA flower (7-way junction, 7WJ) is bound by hybridizing with the overhang at the sensing site. Unlike the ordinary DNA junctions used in our recent work,³⁶ a long strand with a small T-loop in the 7WJ can enhance the

ity in the DNA base pairs. The unique initiation mechanism of the ACSD circuit makes the reaction extremely sensitive to single base mutations at multiple sites in the target strand, which reduces crosstalk compared to the classic TMSD.

Additionally, the ACSD circuit can be tuned to mimic specific electronic components. Firstly, step 2 was redesigned like a switch to be controlled by external stimuli. This feature is relevant for applications in biosensing and diagnosis. Secondly, toehold ends with different lengths were utilized in step 2 to adjust the reaction rates of the circuits. The possibility to connect ACSD circuits in parallel with different rates shows the equivalency to electronics. In addition, the initiator strand I interacts with the circuits according to their reaction rates, which is like the current distribution in branch paths with different resistance values in a parallel circuit.

Extending the path competition network, a four parallel path selection system is established and monitored with a nanopore multiplexed sensing platform. On the DNA carrier, the DNA dumbbells are used to encode the binary barcode to distinguish them, and the DNA flowers are used to detect the output strands from the circuits by calculating their OFs at the sensing site. Based on the circuit connection conditions, strand I can always correctly choose the activated paths and lead to the removal of DNA flowers on the relative carriers. Breaking the limitation of the wavelength overlap in the fluorescence assay, hundreds of carriers with different barcodes are possible to be simultaneously measured with the nanopore platform. The number of barcodes on a carrier can be extended *via* adding more coding sites on a carrier, increasing the number of digital codes on each coding site by using multi-level structures,^{30,36} increasing the coding sites by linking two carriers,³⁷ or improving the resolution of the nanopore to read more bits in the same range.³⁷ Multiplexed nanopore sensing and ACSD provide a promising approach for any parallel computing systems based on DNA.

J. Z, U. F. K and E. W conceived the project. J. Z designed and performed the experiments. J. Z and J. K analysed the data. All authors discussed the results and wrote the manuscript.

There are no conflicts to declare.

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In summary, we introduce a novel DNA breathing initiated DNA ACSD circuit. Unlike the ordinary TMSD, the initial strand displacement reaction is initiated by temporary instabil-

