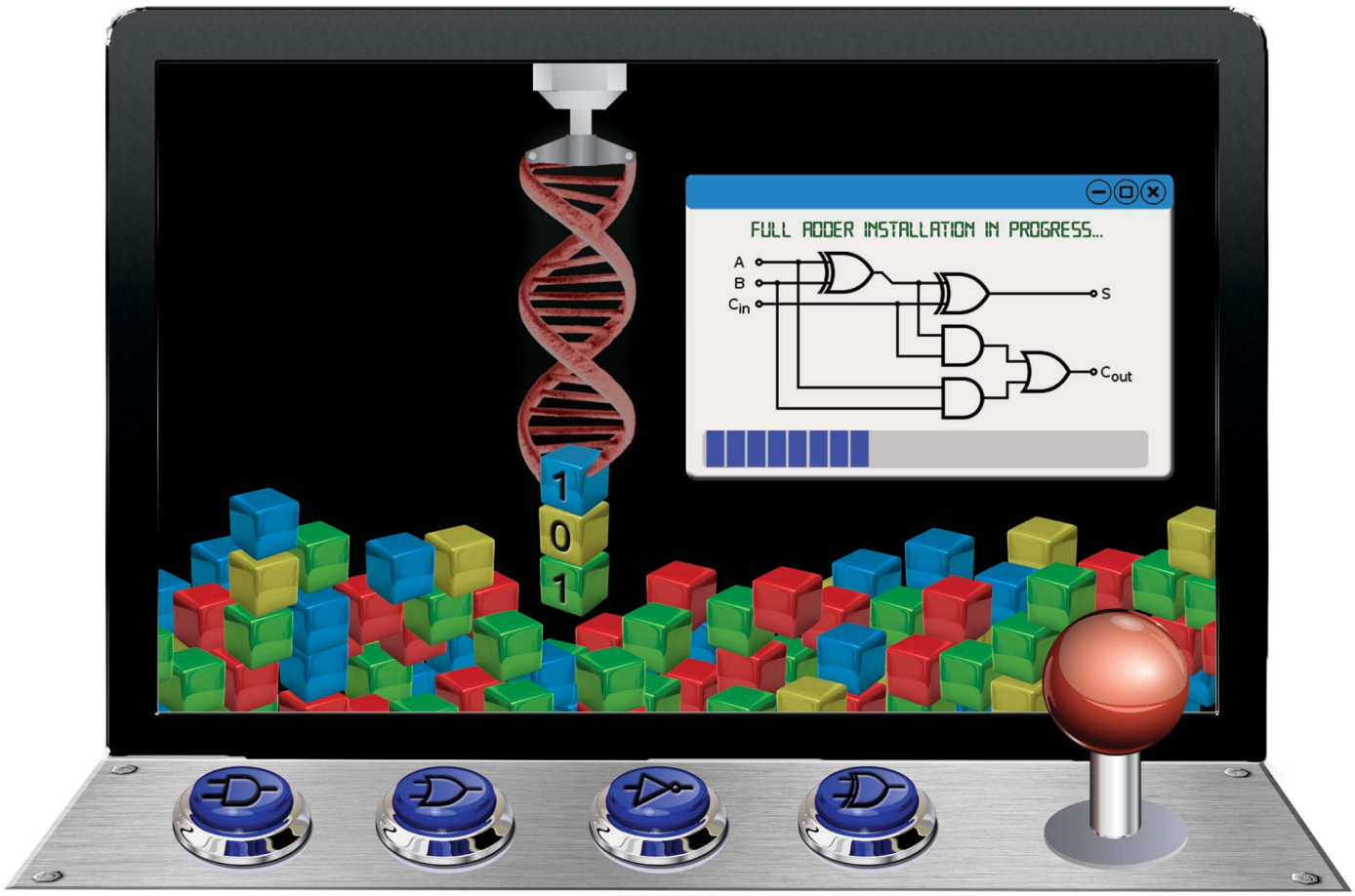

Showcasing research from Prof. Itamar Willner's Laboratory, The Hebrew University of Jerusalem, Jerusalem, Israel.

A Full-Adder Based on Reconfigurable DNA-Hairpin Inputs and DNAzyme Computing Modules

A library consisting of DNAzyme subunits provides a computing module for the operation of a full-adder system. DNA hairpin inputs are used for the guided assembly of DNAzyme subunits, and through their reconfiguration "sum" and "carry", fluorescence outputs are generated. The advantages of simultaneous three-inputs operation of the full-adder and the possibilities to implement the system for cascading full-adders are addressed.

As featured in:

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Cite this: *Chem. Sci.*, 2014, **5**, 3381

A full-adder based on reconfigurable DNA-hairpin inputs and DNAzyme computing modules†

Ron Orbach,[a] Fuan Wang,[a] Oleg Lioubashevski,[a] R. D. Levine,[a] Francoise Remacle[b] and Itamar Willner*[a]

In nature, post-transcriptional alternative splicing processes expand the proteome biodiversity, providing means to synthesize various protein isoforms. We describe the input-guided assembly of a DNAzyme-based full-adder computing system, which mimics functions of the natural processes by increasing the diversity of logic elements by the reconfiguration of the inputs. The full-adder comprises the simultaneous operation of three inputs that yield two different output signals, acting as sum and carry bits. The DNAzyme-based full-adder system consists of a library of Mg^{2+}-dependent DNAzyme subunits and their substrates that are modified by two different fluorophore/quencher pairs that encode the sum and carry outputs. The input-guided assembly of DNAzyme subunits, formed by three inputs composed of nucleic acid hairpin structures, leads to computing modules that yield the sum and carry outputs of the full-adder. In the presence of a single input the DNAzyme computing module yields the sum fluorescence output. In the presence of two of the inputs, the reconfiguration of the input structures proceeds, leading to an input-guided computing module that yields the carry fluorescence output. By introducing all the three inputs the sequential inter-input hybridization leads to the reconfiguration of the inputs into polymer wires. These include binding sites for two types of DNAzyme and their substrates leading to the carry and sum fluorescence outputs. The advantages of the simultaneous three-input operation of the full-adder and the possibilities to implement DNAzyme-based computing modules for cascading full-adders are discussed.

Received 27th March 2014
Accepted 24th April 2014

DOI: 10.1039/c4sc00914b

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Computing with molecules and biomolecules has become an active research field in the past fifteen years. Different studies have implemented molecular and supramolecular systems,[1] as well as biomolecules, such as enzymes[2] or nucleic acids,[3] as functional elements that mimic the performance of Boolean logic gates in computer science. In these systems, light,[4] electrical,[5] and chemical agents (such as ions[6] and pH[7]) acted as inputs, whereas color,[8] light (fluorescence),[9] and electrical signal,[10] provided the output signals. Many different logic gates for instance AND, OR, XOR, NOR, NAND, INHIBIT have been constructed using molecular and biomolecular systems.[11]

The sequence-specific base-pairing of nucleotides, the cooperative stabilization of duplex nucleic acids by ions, the energetically-controlled formation of duplex DNA structures and their separation by strand-displacement, the catalytic properties of sequence-specific nucleic acids (DNAzymes), and the specific recognition functions of nucleic acids (aptamers), provide important tools to design logic gates and computing circuits. Indeed, many different DNA-based logic gates,[3] DNA-driven computational circuitries[12] and nucleic-acid-based automata[13] have been reported.

Although remarkable progress in the construction of logic gates and the assembly of automata have been demonstrated, the design of molecular and biomolecular systems performing arithmetic computing circuits of enhanced complexity is still challenging. One of the basic arithmetic operations in computers is the addition function (adders). The addition of two binary digits (bits) yields a half-adder, while the addition of three digits (bits) yields a full-adder. The full-adder is a computational element that adds together three binary digits to generate two binary outputs. One of the output digits represents the sum, whereas the second output digit acts as the carry. The cascading of full-adder units is the basis for multi-digit additions. A full-adder system may be constructed, in principle, by cascading two half-adders, consisting of two AND/XOR gates, Fig. 1. Indeed, several studies have used functional mixtures that respond to three binary inputs to construct half-adder gates and their cascading leads to a full-adder. For example, the pH-switching of fluorescein across different charged states enabled the reconfiguration of the molecule by three different pH-input triggers that allowed the binary readout of the full-adder system.[14] Nucleic-acid-based full-adder systems have also been

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† Electronic supplementary information (ESI) available: Gel electrophoresis analysis of the inter-interaction of the inputs. See DOI: 10.1039/c4sc00914b

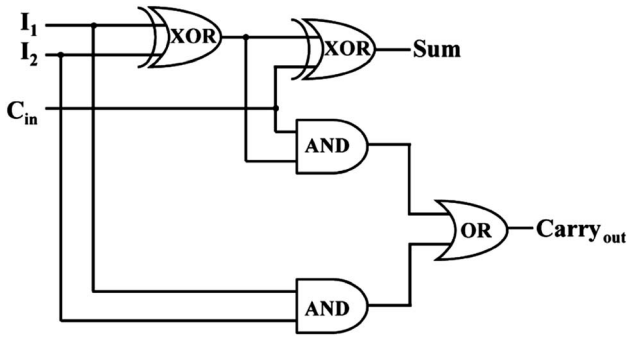


Fig. 1 Cascaded AND/XOR gates leading to a full-adder.

developed. A mixture of an array of seven DNAzyme constructs was reported to act as a full-adder.[15] In this system three of the constructs acted as two-input AND gates, whereas the other four constructs acted as three-input gates comprising one AND gate and three ANDNOTANDNOT gates. A different DNA-based full-adder was assembled by the sequence-specific photocleavage of crosslinked strands that included a non-natural 5-carboxyvinyl-2-deoxyuridine nucleotide.[16] However, the different molecular or biomolecular full-adder systems suffer from several basic limitations: (i) the gate components are often degraded while performing the full-adder operation; (ii) the nature of the three digit inputs are transformed into a different functional output (*e.g.*, pH to fluorescence). Under such conditions it is difficult to envisage the cascading of full-adders to yield serial adders of enhanced complexity; (iii) upon cascading molecular or biomolecular half-adders, undesired intermolecular perturbing interactions might occur. For example, partial hybridization (crosstalks) of nucleic acids in the cascaded half-adder systems may proceed. These phenomena are anticipated to introduce noise and to decrease the output signal.

A different approach to overcome part of these difficulties could involve the design of systems that simultaneously function as a full-adder, without the need to cascade separate half-adders. This was demonstrated by the three-bit laser photoexcitation of a single chromophore and its fragmentation at different molecular sites.[17] While the method introduces an important gating principle, the fragmentation of the chromophore prohibits cascading of the full-adder. Also, a fully CMOS compatible full-adder was implemented as a solid state single dopant transistor by utilizing charge and quantum degree of freedom as inputs, and the cascading of two such full-adders was demonstrated.[18]

In the past few years we have introduced a new paradigm for the construction of nucleic-acid logic gates and computing circuits by implementing catalytic nucleic acids, DNAzymes, as functional components.[19] According to this approach a library of DNAzyme subunits and their substrates is constructed, and the input-guided selection of the respective DNAzyme subunits yields the computing module that dictates the nature of the resulting gate. The cleavage of fluorophore/quencher-functionalized substrates provides the fluorescence output of the respective gates. Using this approach a universal set of logic gates was constructed, and by the appropriate design of the substrates, in the library ensemble, cascaded gates and fan-out gates were demonstrated.[20] By the assembly of libraries of two different ion-dependent DNAzyme subunits (Mg^{2+} and UO_2^{2+}), operating at different pH environments, field programmable logic arrays, and the cascading of the logic gates were demonstrated.[21] The concept of input-guided generation of computing modules by DNAzyme subunit libraries was further used to develop Toffoli and Fredkin gates[22] and to construct multiplexer and demultiplexer logic systems for data compression and decompression.[23] The input-guided operations of logic gates using a DNAzyme subunits library has been suggested as an important paradigm for future nanomedicine,[20] and indeed, recent cellular experiments supported this visionary link between DNA computing and nanomedicine.[24]

In the present study we apply a library of Mg^{2+}-dependent DNAzyme subunits and their substrates as a functional ensemble to construct computing modules that yield a full-adder (a computing module is defined as a supramolecular nanostructure generated by the input-guided assembly of functional DNA strands from a library of DNAzyme subunits/DNAzyme substrates leading to a dictated logic-gate operation). The uniqueness of the system is reflected by the fact that it does not involve the sequential cascading of two half-adders (AND/XOR gates), but involves a one-pot computational system that is triggered by three inputs to yield two outputs acting as sum and carry bits. In contrast to the approach to construct a full-adder by cascading several logic elements, Fig. 1, we present a single-layer design of a full-adder. We emphasize that the functional DNAzyme units are not degraded during the computation process and discuss the possibility to cascade the system to serial full-adders.

Results and discussion

The construction, and novelty, of the full-adder is based on the use of hairpin nucleic acids that act as reconfigurable inputs for the system, and a library consisting of Mg^{2+}-dependent DNAzyme subunits and their substrates, Fig. 2(A). The input-guided selection of the DNAzyme subunits from the library yields the respective computing module. This computing module is composed of the input-assembled DNAzyme structure that selectively binds the appropriate fluorophore/quencher-functionalized ribonucleobase-modified substrate. The DNAzymes cleave the respective substrates leading to two fluorescence outputs acting as the sum and carry digits. Fig. 2(B) exemplifies schematically the input-guided assembly of a DNAzyme structure. The input includes a single-stranded loop, x, and a duplex stem region consisting of the complementary b/b′ sequences. The hairpin stem is elongated at its 5′-end and 3′-end with the single-stranded sequences e, a and c′, f, respectively. The input selects from the library the subunits L_1 and L_2 that include complementary "arms" a′ and c to the respective sequences associated with the input. In the presence of the substrate, S_1, functionalized with the fluorophore/quencher units, the input-guided assembly of the Mg^{2+}-dependent DNAzyme proceeds. Thus, the input and substrate are hybridized with the respective

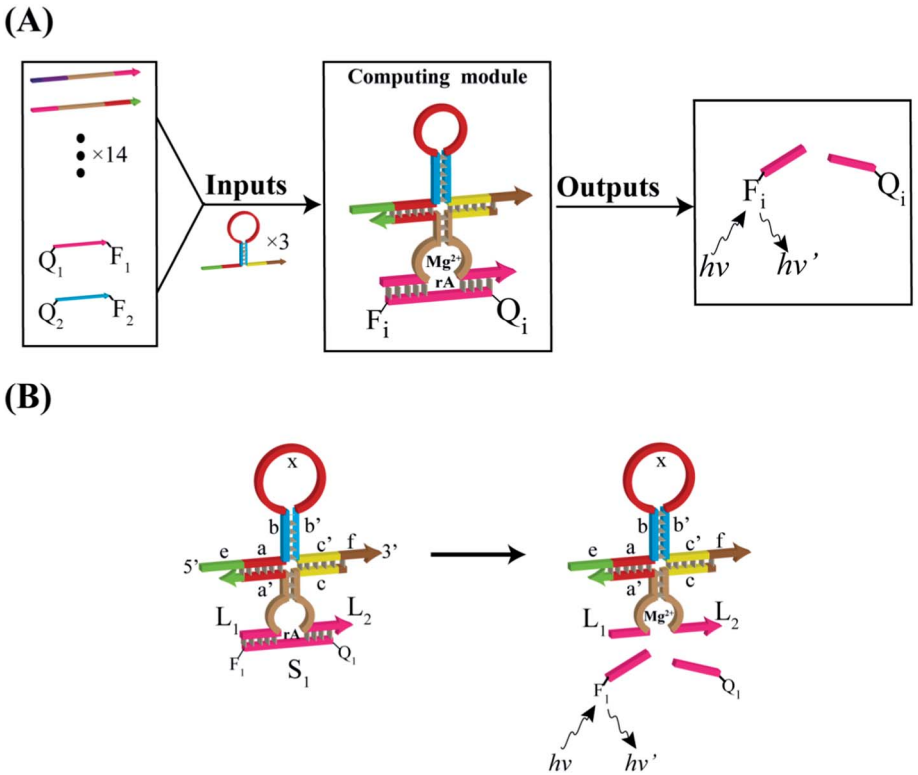


Fig. 2 Schematic assembly of the Mg^{2+}-dependent DNAzyme computing module based on a hairpin nucleic acid acting as input: (A) the system includes a library of fourteen DNAzyme subunits and two fluorophore/quencher-functionalized substrates. The input-guided selection of the DNAzyme subunits dictates the structure and functions of the computing module. The DNAzyme-mediated cleavage of the respective substrate leads to the fluorescence output. (B) Schematic programmed sequences and base-complementarities of the hairpin nucleic acid input and the respective DNAzyme subunits/substrate components leading to an input-guided computating DNAzyme module that yields F_1 as output.

two DNAzyme subunits, and cooperatively stabilize the catalytic DNA nanostructures. The assembly of the DNAzyme causes the cleavage of the ribonucleobase-modified substrate, a process that leads to the fluorescent fragmented substrate (fluorescence of F_1). Note that the complementarity between the substrate, S_1, and the DNAzyme subunits L_1 and L_2 is insufficient to form stable duplexes, whereas in the presence of the hairpin inputs the cooperative stabilization of the supramolecular input/DNAzyme subunits/substrate proceeds resulting in the active DNAzyme structure.

According to this principle, the full-adder system was constructed as outlined in Fig. 3. The system consists of a library of DNAzyme subunits (1) to (14) that are capable of generating seven different Mg^{2+}-dependent DNAzyme structures. Also, the two substrates, S_1 (ROX/Black Hole Quencher-2) and S_2 (FAM/Black Hole Quencher-1) are included in the system. The hairpin inputs, I_1, I_2, I_3, activate the computing modules. The sequences comprising the different domains are marked on the DNAzyme subunits/inputs, where regions i/i′ represent complementary domains. Subjecting the DNAzyme subunits/substrates library to input I_1, I_2 or I_3 yields the input-guided assembly of three different Mg^{2+}-dependent DNAzyme structures, P_1, P_2 or P_3, as computing modules. Each of these catalytic structures leads to the cleavage of substrate S_1 and to the generation of F_1 as fluorescent output and no fluorescence of F_2, Panel I. Subjecting the subunits/substrates library to the combination of two inputs, $I_1 + I_2$, $I_1 + I_3$ or $I_2 + I_3$ leads to an interesting phenomenon; The inter-interaction between the respective inputs yields, energetically-stabilized bi-loop supramolecular structures (for gel electrophoresis experiments supporting the formation of the bi-loop structures see Fig. S1†). These supramolecular structures include recognition sequences for the guided selection of the DNAzyme subunits from the library leading, in the presence of the substrate S_2, to the cooperative stabilization of active Mg^{2+}-dependent DNAzyme structures that act as computing modules. Accordingly, in the presence of the inputs $I_1 + I_2$, $I_1 + I_3$ or $I_2 + I_3$ the DNAzyme configurations P_4, P_5, P_6 are formed, respectively. The cleavage of the substrate S_2 yields a fluorescent fragmented product that provides the output signal F_2, and no fluorescence of F_1, Panel II. Finally, in the presence of the three inputs, $I_1 + I_2 + I_3$, and only in the presence of all three inputs, the partial complementarities of the stem regions and loop regions of the different hairpins results in sequential inter-hairpin hybridization leading to the reconfiguration of the inputs into polymer wires, Panel III (for gel electrophoresis experiments supporting the formation of polymer wires see Fig. S1†).

The polymer wires include, however, recognition sequences for the specific binding of the DNAzyme subunits (7)/(10), (8)/(11), (9)/(12) to the respective domains of inputs I_1, I_2, and I_3, and for the guided association of subunits (13) and (14). By the cooperative hybridization of the substrates S_1 and S_2 to the

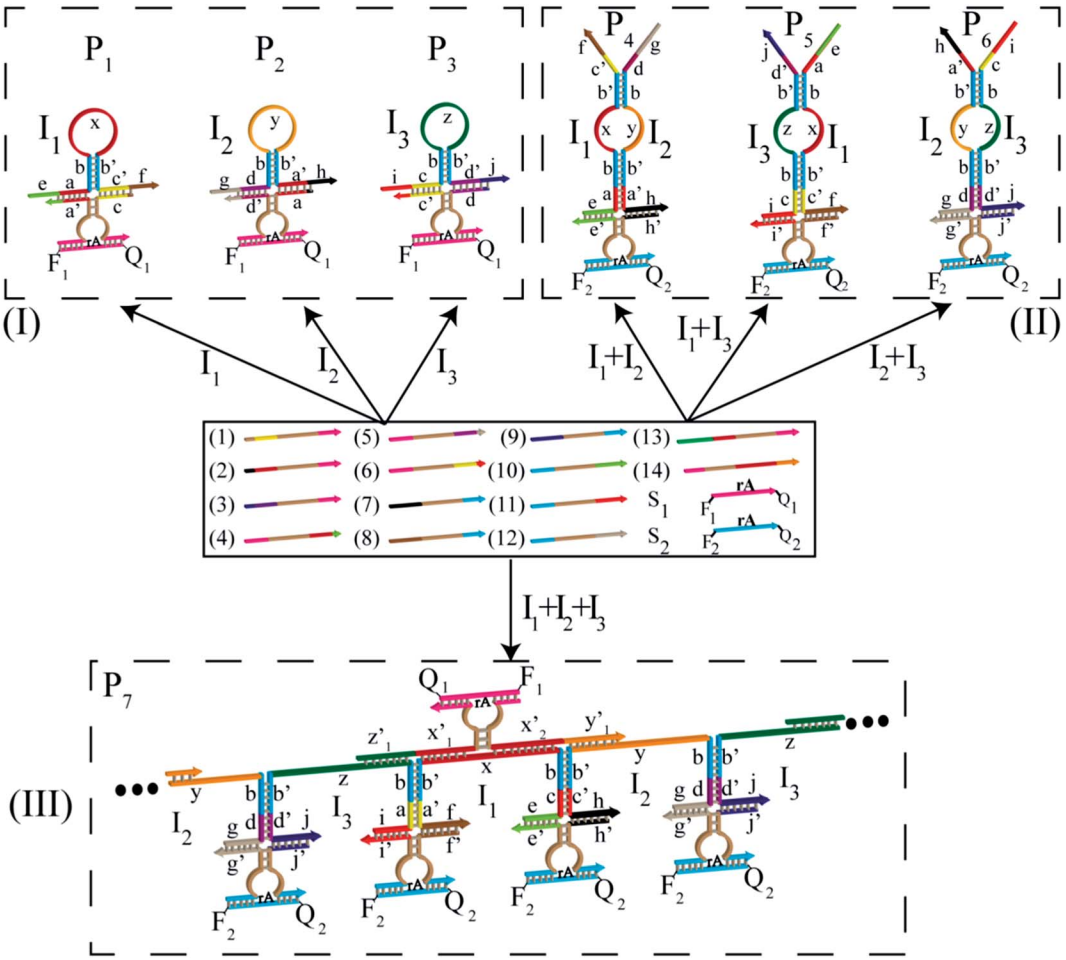


Fig. 3 Scheme for the input-guided assembly of the computing modules leading to the "sum" and "carry" fluorescence outputs using a library of Mg^{2+}-dependent DNAzyme subunits, two different fluorophore/quencher-modified substrates and three different hairpin inputs, I_1, I_2 and I_3. Panel I – computing modules generated by inputs I_1, I_2 or I_3 leading to the "sum" fluorescence output F_1. Panel II – computing modules formed by the reconfiguration of the hairpin inputs $I_1 + I_2$, $I_1 + I_3$ or $I_2 + I_3$ leading to the "carry" output F_2. Panel III – computing module consisting of polymeric DNAzyme wires, generated by the sequential inter-input hybridization, leading to the "sum" and "carry" outputs, F_1 and F_2.

respective DNAzyme subunits, active Mg^{2+}-dependent DNAzymes associated with the input-generated recognition wires are formed. The cleavage of the respective substrates, S_1 and S_2, leads to the fluorescence output signals F_1 and F_2, Panel III.

Fig. 4 demonstrates the experimental results of the full-adder functions of the systems. Fig. 4(A) shows low fluorescence intensity changes in the absence of any input, Panel I. In the presence of any of the three inputs, I_1, I_2 or I_3 the fluorescence change of F_1 is intensified, while the fluorescence change of F_2 stays low (Panels II–IV), consistent with the output associated with the configurations P_1, P_2 and P_3 outlined in Fig. 3. Subjecting the system to the combination of two inputs, results in the high fluorescence intensity changes shown in Fig. 4(B), Panels V–VII. The results reveal that any of the two inputs leads to the fluorescence F_2 as output signal, while the fluorescence change of F_1 stays low. These results are consistent with the states P_4, P_5 and P_6, outlined in Fig. 3. Finally, subjecting the computing modules to all three inputs, $I_1 + I_2 + I_3$, yields the intense fluorescence change of F_1 and F_2, consistent with the state P_7 shown in Fig. 3. The fluorescence intensity changes of the system, upon applying the different inputs, in the form of a bar-presentation, are presented in Fig. 5(A), and the resulting truth-table is detailed in Fig. 5(B). Evidently, the system acts as a binary full-adder, where the output F_1 provides the sum, and the output F_2 acts as the carry digit. Atomic force microscopy (AFM) experiments further supported that the interactions of $I_1 + I_2 + I_3$ lead to the polymer wires as computing modules, Fig. 6. Micrometer-long wires exhibiting a height of *ca.* 1.2 nm consistent with duplex DNA structures are observed. The uniqueness of the present full-adder system is reflected by the cooperatively programmed interactions between the inputs *and* with the library components. These cooperative interactions lead to the reconfiguration of the catalytic DNAzyme structures generated upon the guided selection of the DNAzyme subunits. The input-guided reconfigured structures drive allosterically the formation of the catalytic DNAzyme units that provide the computing modules.

To conclude, the present study has introduced a new paradigm for the construction of a DNA-based full-adder system. The system is based on several new principles: (i) the

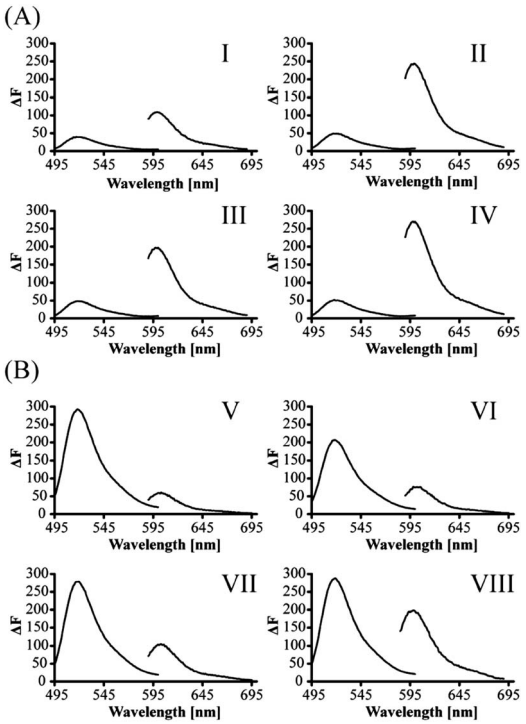
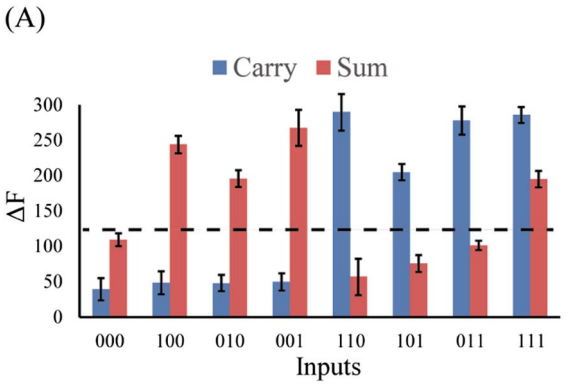


Fig. 4 Fluorescence intensity changes generated upon subjecting the DNAzyme subunits/substrates library to: (A) Panel I: no input; Panels II–IV: inputs I_1, I_2 and I_3, respectively. (B) Panel V: inputs $I_1 + I_2$; Panel VI: inputs $I_1 + I_3$; Panel VII: inputs $I_2 + I_3$. Panel VIII: inputs $I_1 + I_2 + I_3$. Fluorescence at $\lambda = 605$ nm corresponds to F_1, fluorescence at $\lambda = 516$ nm, corresponds to F_2. It should be noted that all inputs in Panels V–VIII are applied simultaneously.



(B)

I_1	I_2	I_3	Carry	Sum
0	0	0	0	0
1	0	0	0	1
0	1	0	0	1
0	1	1	1	0
0	0	1	0	1
1	0	1	1	0
1	1	0	1	0
1	1	1	1	1

Fig. 5 (A) Fluorescence intensity changes corresponding to the "sum" and "carry" outputs, generated by the different inputs. (B) Truth-table corresponding to the full-adder system. Error bars were derived from a set of $N = 5$ experiments.

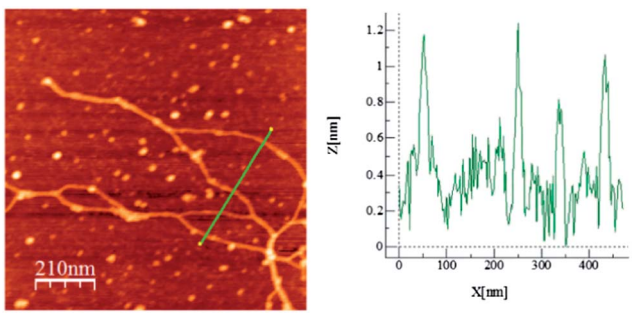


Fig. 6 AFM image and cross-section analysis corresponding to the polymer DNA wires generated by the three inputs, $I_1 + I_2 + I_3$. The sequential inter-input hybridization leads to the catalytic wires according to Fig. 3, Panel III (the white dots on the surface correspond to salt clusters formed upon drying on the surface).

construction of a library consisting of Mg^{2+}-dependent DNAzyme subunits and substrates for the respective DNAzyme. (ii) The essence of the logic device is the input-guided selection of the respective Mg^{2+}-dependent DNAzyme subunits. The selected DNAzyme subunits dictate the selectivity of the binding of the substrate to the respective DNAzyme structure and these binding phenomena control the nature of the outputs. There is *no* direct interaction between the inputs and the outputs, and these are interlinked through the DNAzyme subunits. This decreases perturbations arising from crosstalks between the components, and simplifies the design of the system. (iii) The uniqueness of the full-adder system is the involvement of intimate interactions between the inputs that lead to functional supramolecular structures that actively participate in the computing process.

Finally, the implementation of the DNAzyme subunits and their substrates as a library for the guided assembly of the computing modules introduces important new elements beyond the present art in the DNA computing field: (a) in a previous report,[20] we demonstrated that logic gates relying on the Mg^{2+}-dependent DNAzyme enable the cascading of gates and the fan-out of gates. This was achieved by the use of DNAzyme substrates that are cleaved, and one of the resulting fragmented sequences acted as input for the cascaded gate. This concept could be further implemented to cascade full-adders, a fundamental challenge in molecular or biomolecular computing. (b) There are different metal ion-dependent DNAzymes,[25] and these allow the complexity of the library to be increased, thus allowing the increase of the diversity of the computing modules. Specifically, different metal ion-dependent DNAzymes operating at diverse pH environments are available, these DNAzymes have been implemented, by us, to design pH field-programmable logic gates by libraries composed of different DNAzyme subunits.[21] This concept can now be extended to develop pH-programmable full-adder systems. Despite the unique features of the system, the limitations of the approach should be mentioned too. The fact that the substrate

Table 1

Name	Sequence
1	5′-GGTTTGGTCGCTACACAAGCACCCATGTACAGTCA-3′
2	5′-GTCATTCAGCGATCTTGAAGATGCCCTGGTAG-3′
3	5′-TCGAGCGGGCATCTTTTCTACACCCATGTACAGTCA-3′
4	5′-GTCATTCAGCGATTAGAACGCATCAAGTCGTCG-3′
5	5′-GGTCTACTTGATGCGATTTGCACCCATGTACAGTCA-3′
6	5′-GTCATTCAGCGATCAAATTGTAGCGACGTCAGG-3′
7	5′-AGTACTCAGCGATTCAAATGGTAGGATGAG-3′
8	5′-AGTACTCAGCGATGACAGTCAGGTTCTCC-3′
9	5′-AGTACTCAGCGATCTGGTCGTCGATTCTG-3′
10	5′-CATGTTTCGAGCTTTGACACCCATGTTATCCTA-3′
11	5′-GTATCTGGTTTGTGTCCACCCATGTTATCCTA-3′
12	5′-ACTTTTGGTCTACCAGCACCCATGTTATCCTA-3′
13	5′-GGTAAGGAAGGTTGTGTGCACCCATGTACAGTCA-3′
14	5′-GTCATTCAGCGATCACACGAGGACGGAAG-3′
S_1	5′-ROX-TGACTGTTRAGGAATGAC-BHQ-2-3′
S_2	5′-FAM-TAGGATATRAGGAGTACT-BHQ-1-3′
I_1	5′-CTCATCCTACCAGGGCATCTTGTAATAGCCGTCCTCAACCTCG GCTATTACTGTAGCGACCAAACCAGATAC-3′
I_2	5′-CAGAATCGACGACTTGATGCGGTAATAGCCGACTACTTCCGC GGCTATTACAAGATGCCCGCTCGAAACATG-3′
I_3	5′-GGAGAACCTGACGTCGCTACAGTAATAGCCGTCCTTACCACC GGCTATTACCGCATCAAGTAGACCAAAAGT-3′

reporting on the computing operation is being cleaved implies that the system is not reversible and resettable. Nonetheless, as long as the substrate is not fully consumed or by the addition of new substrate the computing module can be switched OFF/ON by the addition of anti-input strands (strand complementary to the inputs) and input strands, respectively. In nature, post-transcriptional alternative splicing processes expand the proteome biodiversity, thus providing the means to synthesize various protein isoforms. Our system mimics some functions of the natural process by providing means to increase the diversity of logic elements (genes) by the reconfiguration of the inputs and the guided selection of different computing modules.

Experimental section

All reactions were performed in NEBuffer2 (50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) at a final DNA concentration of 1 μM. The samples, without the inputs, were heated to 80 °C for 10 min, and then cooled down to room temperature for 1 h. Fluorescence spectra were recorded, for each of the states, prior and two hours following the addition of the inputs.

Materials

NEBuffer2 was purchased from New England Biolabs Inc. (Ipswich, MA). HPLC purified DNA primers were purchased from Integrated DNA Technologies Inc. (Coralville, IA). Ultrapure water from a NANOpure Diamond (Barnstead) source was used in all of the experiments.

Instrumentation

Light emission measurements were performed using a Cary Eclipse fluorimeter (Varian Inc.). The excitation of FAM, carboxy fluorescein, and ROX, carboxy-X-rhodamine, was performed at 480 nm and 570 nm, respectively. The quenchers used in the systems were: BHQ1 (Black Hole Quencher-1) and BHQ2 (Black Hole Quencher-2).

DNA oligonucleotides

All DNA sequences were designed to minimize undesired cross-hybridization using NUPACK (http://www.nupack.org/).[26] The sequences given in Table 1 were used for the full-adder.

Microscopy

AFM image was recorded using a Nanoscope V controller (Bruker Probes, Plainview, NY) with NSC 15 AFM tips (Mikromasch, Tallinn, Estonia) by the tapping mode at their resonant frequency. The DNA sample was deposited on a freshly cleaved mica surface (Structure Probe Inc., West Chester, PA) and dried in air. Images were analyzed using the WsXM SPIP software (Nanotec, Inc., Madrid, Spain).

Polyacrylamide gel electrophoresis

The gels consisted of 12% polyacrylamide (acrylamide–bis-acrylamide, 29 : 1) in a Tris–borate–EDTA (TBE) buffer solution that was purchased from Biological Industries Israel BEIT HAEMEK LTD. (Kibutz Beit-Haemek, Israel). The buffer included Tris base (89 mM, pH 7.9), boric acid (89 mM), and EDTA (ethylenediaminetetraacetic acid, 2 mM). A portion (2 μL) of each of the reaction mixtures was mixed with the loading dye and loaded onto the gel. The gels were run on a Hoefer SE 600 electrophoresis unit at 10 °C (250 V, constant voltage) for 5 h in 0.5× TBE buffer. After electrophoresis, the gels were stained with SYBR Gold nucleic acid gel stain (Invitrogen) and imaged.

Acknowledgements

This research is supported by the Future Emerging Technologies project, MULTI (317707), of the European Community

Seventh Framework Programme. F.R. is a Director of Research at Fonds National de la Recherche Scientifique, Belgium.

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