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DNAzyme-based 2:1 and 4:1 multiplexers and 1:2 demultiplexer†

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Scaffolding proteins play a central role in many regulatory cellular networks, where signalling proteins trigger different, and even orthogonal biological pathways. Such biological regulatory networks can be duplicated by multiplexer/demultiplexer logic operations. We present the use of libraries of Mg^{2+}-dependent DNAzyme subunits as computational moduli for the construction of 2:1 and 4:1 multiplexers and a 1:2 demultiplexer. In the presence of the appropriate inputs, and the presence or absence of selector units, the guided assembly of the DNAzyme subunits to form active Mg^{2+}-dependent DNAzyme proceeds. The formation of the active DNAzyme nanostructures is controlled by the energetics associated with the resulting duplexes between the inputs/selectors and the DNAzyme subunits. The library subunits are designed in such a way that, in the presence of the appropriate inputs/selectors, the inputs are knocked-down or triggered-on to yield the respective multiplexer/demultiplexer operations. Fluorescence is used as the readout for the outputs of the logic operations. The DNAzyme-based multiplexer/demultiplexer systems present biomolecular assemblies for data compression and decompression.

Introduction

Data compression and decompression is a central theme of information theory[1] and computer science. Extensive research efforts are directed to the use of molecular or biomolecular assemblies to perform functions duplicating Boolean logic gates.[2] During the past few years ingenious supramolecular and biomolecular systems mimicking electronic logic gates have been developed. For example, different molecular systems that perform logic gate functions, such as AND, OR, XOR, NAND, INHIB or NOR, have been designed, and logic operations of enhanced complexity, such as adders, subtractors, encoder–decoders, keypad locks and multi-valued logic devices, have been reported.[3] Chemical signals, such as pH,[4] metal ions,[5] photonic stimuli[6] or electrical signals,[7] have been used as inputs for the logic operations. Also, biomolecules, such as enzymes[8] or nucleic acids[9] (DNA, aptamers or DNAzymes), have been extensively used as functional materials for tailoring logic gate operations and for biocomputing. Nucleic acid-based logic gate cascades or automata performing parallel logic operations have been reported.[10] For example, the construction of a library of subunits of the Mg^{2+}-dependent DNAzyme enabled the activation of a universal set of logic gates, cascaded gates and fan-out gates.[11] Similarly, by the construction of a composite library of two different metal-dependent DNAzyme subunits (Mg^{2+} and UO_2^{2+}), exhibiting pH-controlled catalytic activities, pH-programmable DNA logic arrays were demonstrated.[12] Also, the Toffoli and the Fredkin gates that exhibit logic reversibility were addressed using a library of DNAzyme subunits.[13] Molecular and biomolecular logic and computational circuits hold great promise for future nanoengineering and nanomedicine. Specifically, the incorporation of logic circuits into biological environments could yield the *in vivo* control of biotransformations. Indeed, such concepts have sparked the scientific imagination for the future applications of intracellular logic systems as autonomous sense-and-treat systems. For example, a nanostructure consisting of two origami clam-shaped sheets carrying active payloads was locked by aptamer chains and provided a nanorobotic reservoir for cellular control. Its incorporation into cells enabled the logic-gate-controlled opening of the reservoir by biomarker inputs, and the subsequent control of cell functions.[14] Also, a programmable library of DNAzyme subunits was introduced in cells and its activation by cellular biomarker inputs provided the basis for logic diagnosis and therapeutics.[15]

In many regulatory cellular networks scaffolding proteins play a central role in signaling common proteins to trigger different and even orthogonal, biological pathways.[16] Such biological regulatory networks may be duplicated by a multiplexer/demultiplexer logic operation that presents a route for data compression/decompression. A multiplexer is a logic circuit that has n selectors and 2^n inputs. It selects and directs one of several inputs into a single output. The demultiplexer represents a logic gate, which exhibits reversed function to the multiplexer, and has one input, n selectors and 2^n outputs. For example, a 2:1 multiplexer transforms two different inputs,

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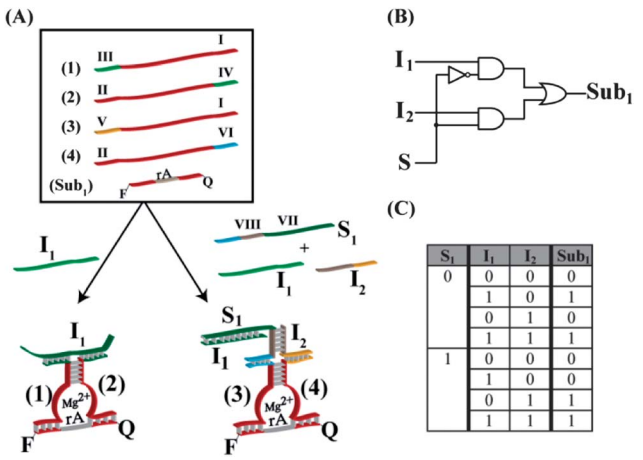
using one selector, into one common output. In contrast, a 1:2 demultiplexer transforms one input, using a selector, into two different output signals. Naturally, the multiplexer function performs an information compressing mechanism, whereas the demultiplexer function leads to an information decompressing path. Different molecular and supramolecular structures have been reported to perform multiplexer and demultiplexer functions.[17] For example, the pH-switchable absorbance properties of a methoxyquinoline were implemented as a selector system for the design of a multiplexer or a demultiplexer, using appropriate excitation wavelengths as inputs and fluorescence signals as outputs.[18] Also, tri-chromophoric supramolecular structures have been reported to act as 2:1 multiplexer or 1:2 demultiplexer systems using photonic signals as inputs and selector units.[19] Similarly, enzyme-based 2:1 multiplexer and 1:2 demultiplexer systems have been reported.[20] Finally, the genetic engineering of *Escherichia coli* enabled the demonstration of an intracellular multiplexer that flips a promoter and allows expression to be toggled between two genes operating in opposite directions.[21] While significant advances in the design of molecular- and biomolecular-based multiplexer or demultiplexer systems have been demonstrated, to the best of our knowledge, no synthetic DNA-based multiplexer or demultiplexer system has been reported. Furthermore, all reported systems represent 2:1 (multiplexer) and 1:2 (demultiplexer) functions, and systems of enhanced degrees of multiplexing are unknown. Here we wish to report on the development of a 2:1 multiplexer system or a 1:2 demultiplexer system using the Mg^{2+}-dependent DNAzyme[22] as a functional computing module. We further demonstrate the versatility of the DNAzyme concept by constructing a 4:1 multiplexer system.

Results and discussion

Fig. 1(A) depicts the concept to construct the 2:1 multiplexer. The system consists of a library composed of Mg^{2+}-dependent



S_1	I_1	I_2	Sub_1
0	0	0	0
	1	0	1
	0	1	0
	1	1	1
1	0	0	0
	1	0	0
	0	1	1
	1	1	1

Fig. 1 (A) Schematic composition of the computation modulus for the 2:1 multiplexer, using a Mg^{2+}-dependent DNAzyme subunits library and the inputs I_1 and I_2 in the absence/presence of the selector S_1. (B) Logic circuit scheme of the 2:1 multiplexer. (C) Resulting truth table of the experimental results corresponding to the 2:1 multiplexer. (F = ROX; Q = BHQ2).

DNAzyme subunits (**1**), (**2**), (**3**) and (**4**), and the respective DNAzyme substrate (**Sub_1**), modified by fluorophore/quencher pairs. The regions I and II of the DNAzyme subunits (**1**)/(**3**) and (**2**)/(**4**) are identical, and hence the substrate is common for the two possible DNAzyme structures. The arms III and IV associated with the DNAzyme subunits (**1**) and (**2**) are complementary to input I_1, whereas the arms V and VI associated with (**3**) and (**4**) are complementary to parts of the selector S_1 and I_2, respectively. The selector S_1 includes additional encoded information, the domain VII, which is complementary to I_1, and the sequence VIII that is complementary to a part of I_2. It should be noted that the input/selector-guided assembly of the respective structures is controlled by cooperative base-pair stabilization of the components. While the separate base-pair domains are insufficient to form stable duplexes, the cooperative interactions lead to stable and active DNAzyme structures. Consequently, in the presence of I_1, and in the absence of the selector, the assembly of the (**1**)/(**2**) DNAzyme subunits proceeds, thus, leading to the activation of the DNAzyme that results in the cleavage of the substrate (**Sub_1**), and to the formation of a fluorescence signal as output. Treatment of the system with input I_2, in the absence of the selector, does not lead to any active DNAzyme structure. Furthermore, the treatment of the system subjected to I_1, in the presence of the selector S_1, prohibits the assembly of the (**1**)/(**2**) DNAzyme due to the energetically preferred hybridization of I_1 to domain VII of the selector, S_1. In the presence of the input I_2 and the selector S_1, the cooperatively stabilized DNAzyme nanostructure, consisting of the subunits (**3**) and (**4**), is formed. In this structure the co-hybridization of the domain VIII of S_1 with I_2 stabilizes the hybridization of domain V of (**3**) and domain VI of (**4**) with S_1 and I_2, respectively. The hybridization of I_1 with sequence VII of S_1 knocks-down I_1. The stabilized (**3**)/(**4**) Mg^{2+}-dependent DNAzyme leads, then, to the cleavage of (**Sub_1**) and the resulting fluorescence of the fluorophore as output. Accordingly, the system performs the functions of a 2:1 multiplexer, Fig. 1(B). The truth-table of this logic device is shown in Fig. 1(C). The experimental results of the system are shown in Fig. 2. In the absence of the selector a true output (fluorescence) is obtained in the presence of I_1 or I_1 + I_2, Fig. 2(A). However, in the presence of the selector true outputs are observed in the presence of I_2 and I_1 + I_2, Fig. 2(B). The fluorescence intensities corresponding to the outputs formed in the presence of the different combinations of input(s)/selector, in the form of a bar presentation, are shown in Fig. 2(C). The results follow the truth-table shown in Fig. 1(C), thus confirming that the system functions as a 2:1 multiplexer. It should be noted that the baseline fluorescence of the system, in the absence or presence of the selector S_1, are slightly different. Presumably, the interaction of S_1 with the subunits (**3**)/(**4**) and the substrate leads to a bent configuration of the substrate that leads to a slightly enhanced quenching. Although this difference in the baseline fluorescence intensities is small, it suggests that it is essential to normalize the fluorescence intensities of all states with respect to the baseline fluorescence features of the respective states.

The tailoring of the 4:1 multiplexer is presented in Fig. 3. The functional library consists of eight Mg^{2+}-dependent DNAzyme

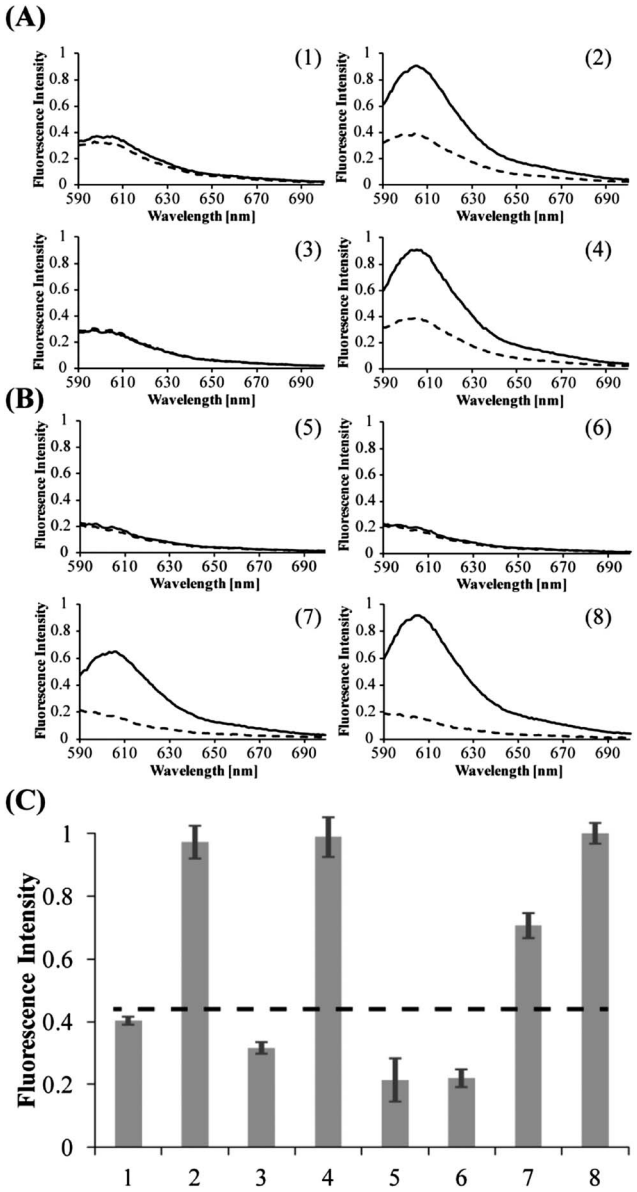


Fig. 2 Fluorescence intensities corresponding to the output of the 2:1 multiplexer. (A) In the absence of S_1 ($S_1 = 0$): (1) $I_1 = 0$; $I_2 = 0$ (2) $I_1 = 1$; $I_2 = 0$ (3) $I_1 = 0$; $I_2 = 1$ (4) $I_1 = 1$; $I_2 = 1$. (B) In the presence of S_1 ($S_1 = 1$): (5) $I_1 = 0$; $I_2 = 0$ (6) $I_1 = 1$; $I_2 = 0$ (7) $I_1 = 0$; $I_2 = 1$ (8) $I_1 = 1$; $I_2 = 1$. (C) Fluorescence intensities of the resulting output in the form of a bar presentation. The numbers on the *x*-axis correspond to the systems presented in panels (1)–(8) in (A) and (B). The dashed line represents the threshold fluorescence level for defining outputs "0" or "1". Error bars were derived by using $n = 4$ experiments. Dashed curves correspond to background fluorescence prior to the addition of inputs. Solid curves correspond to fluorescence spectra after the addition of the inputs.

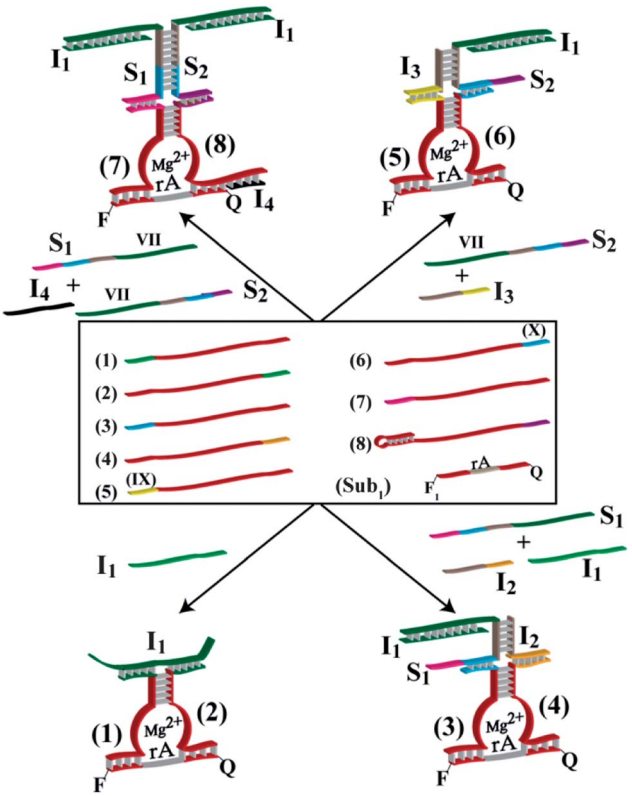
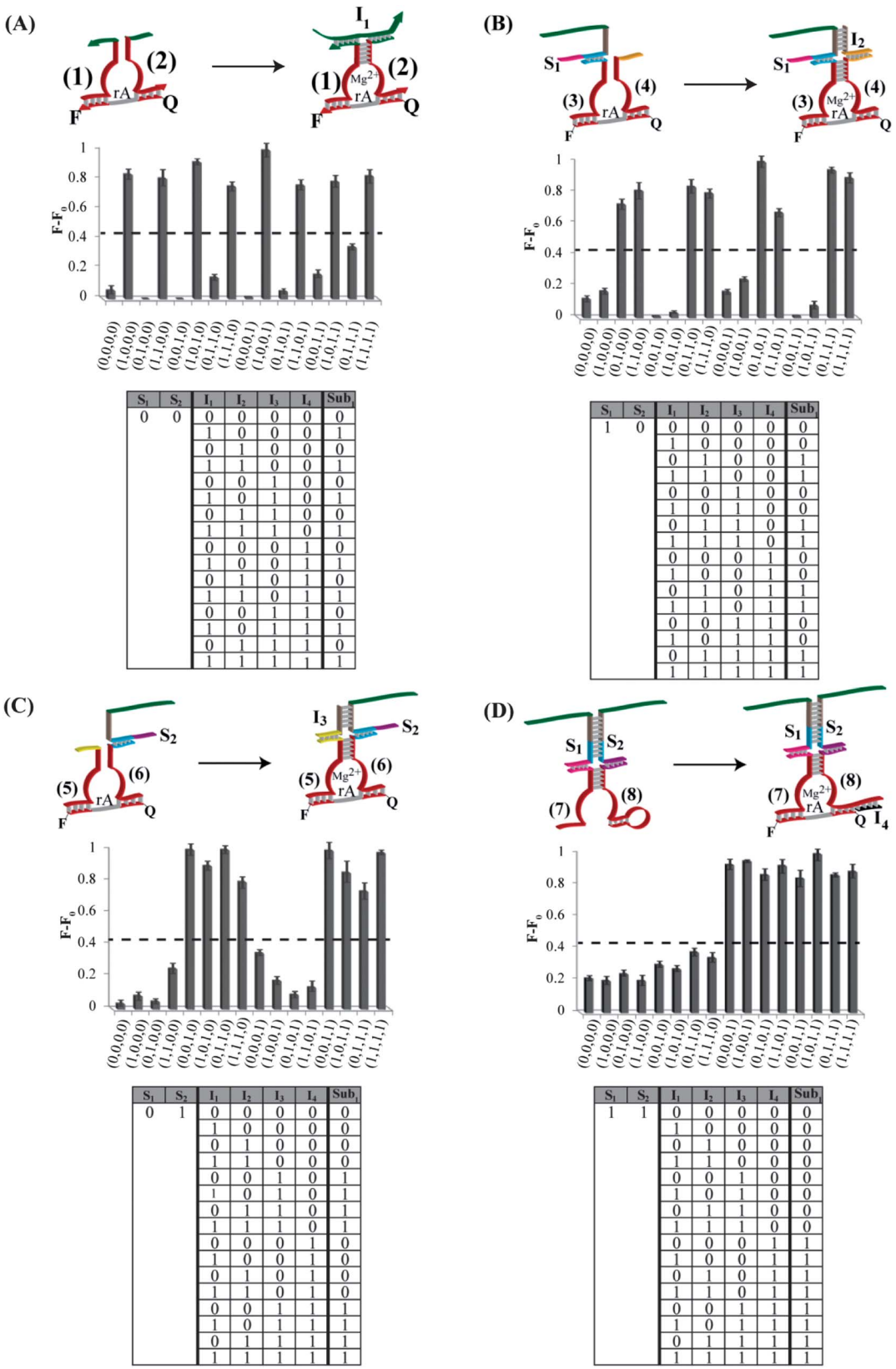


Fig. 3 Schematic composition of the computation modulus for the 4:1 multiplexer, using a Mg^{2+}-dependent DNAzyme subunits library and the inputs I_1–I_4 in the absence/presence of the selectors S_1 and S_2. (F = ROX; Q = BHQ2).

subunits (**1**) to (**8**), and the substrate (**Sub$_1$**) that is common for all inputs/selector-generated DNAzymes. The library is subjected to four different inputs, I_1, I_2, I_3 and I_4 and two selectors, S_1 and S_2. In the presence of I_1 the guided assembly of the subunits (**1**) and (**2**) proceeds, leading to the cleavage of the substrate and to a "true" output. In the presence of I_2 and the selector S_1, the guided assembly of the DNAzyme subunits (**3**) and (**4**) proceeds. In this structure the subunits (**3**)/(**4**) form the active DNAzyme through the linkage of the inter-hybridized S_1 and I_2 with the "arms" of (**3**) and (**4**). Note that in this structure the functions of I_1 are blocked through the hybridization with S_1. Formation of the (**3**)/(**4**) DNAzyme leads to the cleavage of the substrate and to a "true" fluorescence output in the presence of I_2/S_1. In the presence of I_3 and selector S_2, the guided assembly of the DNAzyme consisting of (**5**) and (**6**) proceeds. In this structure, the inter-hybridization between I_3 and S_2 cooperatively stabilizes the hybridization of domain IX and X of (**5**) and (**6**) with the respective domains of I_3 and S_2, leading to the active DNAzyme structure. Note that S_2 includes a domain VII that is complementary to I_1, and thus, the formation of the (**5**)/(**6**) nanostructures blocks any activity of I_1 (prohibits the formation of the (**1**)/(**2**) DNAzyme). Finally, in the presence of I_4, the two selectors, S_1 and S_2, participate in the multiplexing process, leading to the guided assembly of the (**7**)/(**8**) DNAzyme subunits. S_1 and S_2 are designed in such a way that the two selectors form a tight inter-selector duplex that is energetically stabilized as compared to the duplexes formed between I_2/S_1 and I_3/S_2. The resulting S_1/S_2 duplex includes the domain VII that hybridizes with I_1, thus knocking-out this input. Note, however, that the DNAzyme subunit (**8**) is designed to include a hairpin structure that is opened by input I_4. Its opening deprotects a sequence that is essential to hybridize the substrate of the DNAzyme with the two DNAzyme subunits. Thus, in the presence of I_4 and S_1 and S_2 the cooperative



S_1	S_2	I_1	I_2	I_3	I_4	Sub
0	0	0	0	0	0	0
		1	0	0	0	1
		0	1	0	0	0
		1	1	0	0	1
		0	0	1	0	0
		1	0	1	0	1
		0	1	1	0	0
		1	1	1	0	1
		0	0	0	1	0
		1	0	0	1	1
		0	1	0	1	0
		1	1	0	1	1
		0	0	1	1	0
		1	0	1	1	1
		0	1	1	1	0
		1	1	1	1	1

S_1	S_2	I_1	I_2	I_3	I_4	Sub
1	0	0	0	0	0	0
		1	0	0	0	0
		0	1	0	0	1
		1	1	0	0	1
		0	0	1	0	0
		1	0	1	0	0
		0	1	1	0	1
		1	1	1	0	1
		0	0	0	1	0
		1	0	0	1	0
		0	1	0	1	1
		1	1	0	1	1
		0	0	1	1	0
		1	0	1	1	0
		0	1	1	1	1
		1	1	1	1	1

S_1	S_2	I_1	I_2	I_3	I_4	Sub
0	1	0	0	0	0	0
		1	0	0	0	0
		0	1	0	0	0
		1	1	0	0	0
		0	0	1	0	1
		1	0	1	0	1
		0	1	1	0	1
		1	1	1	0	1
		0	0	0	1	0
		1	0	0	1	0
		0	1	0	1	0
		1	1	0	1	0
		0	0	1	1	1
		1	0	1	1	1
		0	1	1	1	1
		1	1	1	1	1

S_1	S_2	I_1	I_2	I_3	I_4	Sub
1	1	0	0	0	0	0
		1	0	0	0	0
		0	1	0	0	0
		1	1	0	0	0
		0	0	1	0	0
		1	0	1	0	0
		0	1	1	0	0
		1	1	1	0	0
		0	0	0	1	1
		1	0	0	1	1
		0	1	0	1	1
		1	1	0	1	1
		0	0	1	1	1
		1	0	1	1	1
		0	1	1	1	1
		1	1	1	1	1

Fig. 4 Fluorescence intensity change ($F - F_0$) in bar presentation, corresponding to the output of the 4:1 multiplexer. (A) In the absence of both selectors ($S_1 = 0$; $S_2 = 0$) (B) In the presence of S_1 ($S_1 = 1$; $S_2 = 0$) (C) In the presence of S_2 ($S_1 = 0$; $S_2 = 1$) (D) In the presence of both selectors ($S_1 = 1$; $S_2 = 1$). The dashed lines represent the threshold fluorescence level for defining outputs "0" or "1". Error bars were derived by using $n = 4$ experiments.

stabilization of the Mg^{2+}-dependent DNAzyme proceeds, leading to the generation of the fluorescence output. (Note, that in the presence of S_1, S_2 and I_4 inputs I_2, I_3 are knocked-out due to the favored stabilization of S_1 and S_2, and I_1 is knocked-out through the energetically favored hybridization with the free tethers of S_1 and S_2).

Fig. 4 presents the experimental fluorescence intensity changes of the system, confirming that the library of DNAzyme subunits functions as a 4:1 multiplexer in the presence of the respective I_1 to I_4 inputs and the two selectors. In Fig. 4(A) the fluorescence intensity changes generated by the system, upon its challenging, in the absence of the selectors S_1 and S_2, with the inputs I_1–I_4, are presented in the form of a bar presentation. Evidently, the fluorescence signal is formed in all systems, where I_1 is present and the DNAzyme structure (**1**)/(**2**) is the origin of the fluorescence. The resulting truth-table of this set of reactions is summarized in the lower part. In Fig. 4(B) the fluorescence intensity changes (bar presentation) of the system subjected to the different inputs and S_1 are presented. Evidently, only the S_1/I_2 guided assembly of the DNAzyme subunits, (**3**)/(**4**), leads to the true output fluorescence signal, and all other inputs yield incomplete, inactive, DNAzyme structures. The resulting truth-table is presented in the lower part of Fig. 4(B). In the presence of S_2 and the different inputs I_1–I_4, the guided DNAzyme structure (**5**)/(**6**) is formed, Fig. 4(C), and this results in the fluorescence intensity changes generated in the presence of the different inputs. The figure shows also the resulting truth-table using S_2 as selector. Finally, Fig. 4(D) shows the fluorescence intensity changes of the system subjected to the two selectors S_1 and S_2 and the respective inputs, I_1–I_4. These fluorescence intensities correspond to the S_1/S_2 and I_4-guided assembly of the functional DNAzyme structure (**7**)/(**8**). This leads to the truth-table shown in Fig. 4(D). The different truth-tables shown in Fig. 4(A) to (D) can be formulated in the form of the logic network shown in Fig. 5 that follows the map of the 4:1 multiplexer (for example of the raw data corresponding to the 4:1 multiplexer system see Fig. S1†).

The Mg^{2+}-dependent DNAzyme subunits library approach was also implemented to construct a logic modulus that functions as a 1:2 demultiplexer network. In this system the logic network subjected to a single input, I_1 yields, in the absence or presence of a selector unit S_1, two distinct, and different, outputs **Sub$_1$** or **Sub$_2$**, Fig. 6(A). The construction and mode of operation of the 1:2 demultiplexer are shown in Fig. 6(B). The computational modules consist of the three Mg^{2+}-DNAzyme subunits (**9**)–(**11**). Two different fluorophore/quencher-functionalized substrates (**Sub$_1$**) and (**Sub$_2$**), as substrates for the DNAzymes, and their catalytic cleavage by the respective DNAzymes, yield the two fluorescence outputs, F_1 or F_2. In the presence of I_1, the input-guided assembly of active (**9**)/(**10**) Mg^{2+}-dependent DNAzyme proceeds, leading to the cleavage of (**Sub$_1$**) and the formation of F_1 as one output. In the presence of I_1 and the selector S_1, the inter-hybridization between I_1 and S_1 leads to the energetically stabilized assembly of the subunits (**9**) and (**11**), leading to the catalytic cleavage of (**Sub$_2$**) and the generation of the fluorescence of F_2 as the second output. Note that in the presence of the selector S_1, the formation of the (**9**)/(**10**) DNAzyme structure is prohibited since the duplex formed between I_1 and S_1 yields an energetically favored DNAzyme nanostructure consisting of (**9**) and (**11**). Fig. 6(C) depicts the fluorescence spectra of the two fluorophores, F_1 and F_2, in the presence or absence of the input (I_1) and selector (S_1); in the absence of I_1 and S_1 no fluorescence is generated, (panel 1) consistent with the lack of formation of an active DNAzyme structure. Similarly, in the absence of I_1 and the presence of the selector S_1, no fluorescence changes are observed, since no DNAzyme structure is formed (panel 2). In the presence of I_1 and

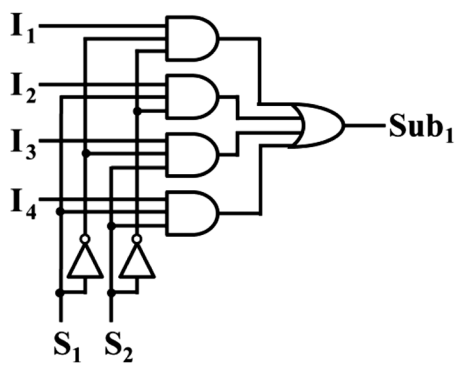


Fig. 5 Logic circuit scheme of the 4:1 multiplexer.

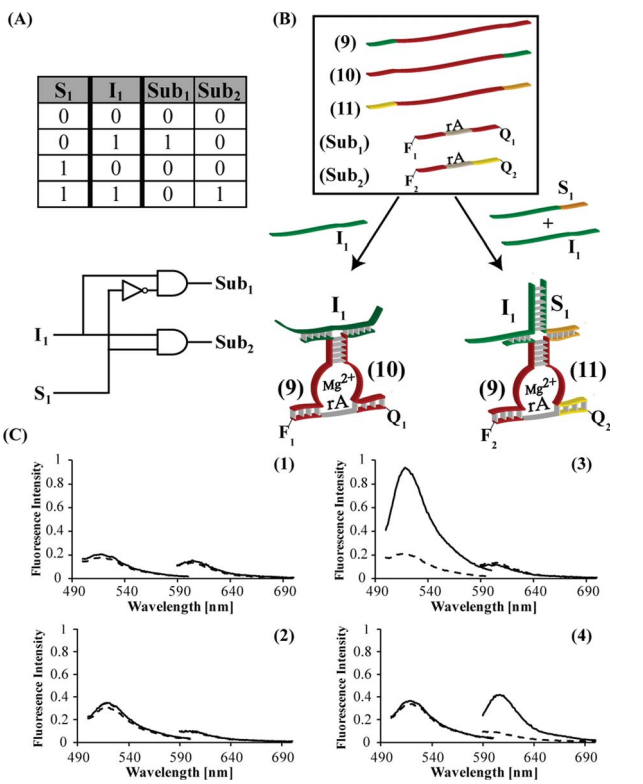


Fig. 6 (A) Logic circuit scheme of the 1:2 demultiplexer and the resulting truth table. (B) Schematic composition of the computation modulus for the 1:2 demultiplexer, using a Mg^{2+}-dependent DNAzyme subunits library in the absence/presence of the selector S_1. (C) Fluorescence intensities corresponding to the output of the 1:2 demultiplexer. (1) $S_1 = 0$; $I_1 = 0$ (2) $S_1 = 1$; $I_1 = 0$ (3) $S_1 = 0$; $I_1 = 1$ (4) $S_1 = 1$; $I_1 = 1$. (F_1 = ROX; F_2 = FAM; Q_1 = BHQ2; Q_2 = BHQ1).

without the selector S_1 the output fluorescence of F_1 is observed, due to the formation of the (**9**)/(**10**) DNAzyme structure (panel 3) and in the presence of I_1 and S_1, the fluorescence of F_2 as second output proceeds, while the fluorescence of F_1 is switched off (panel 4). The fluorescence results are consistent with the operation of a 1:2 demultiplexer logic circuit, where one input is transformed into two outputs in the presence of an appropriate selector.

It should be noted that throughout this study we have implemented equal concentrations of the selector(s)/inputs in the different multiplexer and demultiplexer systems. Under conditions where the selector(s) concentration(s) are equal to or higher than the concentrations of the inputs, the performance of all multiplexer and demultiplexer systems will be unaffected. In turn, under conditions where the selector(s) concentration(s) are lower than the concentration of I_1 the logic schemes would be perturbed. For example, in the 2:1 multiplexer, Fig. 1, if the concentration of S_1 is lower than the concentration of I_1, in addition to the selector/I_2-induced activation of the (**3**)/(**4**) DNAzyme, the I_1-stimulated activation of the (**1**)/(**2**) DNAzyme proceeds too. That is, upon the activation of the different multiplexer and demultiplexer schemes, the relative concentrations of the components must be kept as prerequisite conditions. Also, for all multiplexer and demultiplexer systems we selected a time interval of two hours for monitoring the output fluorescence intensities. This time-interval was selected in view of the time-dependent fluorescence profile that showed saturation after this time period (*cf.* Fig. S1†) Nonetheless, the time-dependent fluorescence changes were already *ca.* 80% of the saturation value after 60 min, implying that the output fluorescence signals could be monitored at shorter time-intervals.

Conclusions

The present study has introduced the concept of input-guided assembly of Mg^{2+}-dependent DNAzyme subunits into functional catalytic units that perform multiplexer and demultiplexer logic networks. The successful nucleic acid-triggered activation of multiplexer/demultiplexer logic functions adds new dimensions to the area of DNA computing since it highlights the possibility of compressing the information encoded in different genes in the form of a single output (multiplexers), and to fan-out the information encoded in one input in the form of two output paths. Particularly interesting is the complexity of multiplexing that can be reached with sequence-tailored DNAzyme subunit libraries. While all previous molecular/biomolecular multiplexers demonstrated 2:1 multiplexing functions, we were able to highlight 4:1 multiplexing. Furthermore, one of the interesting features of multiplexer/demultiplexer systems consisting of nucleic strands is the possibility to reset the computing module by implementing nucleic acid strands complementary to the selector units (anti-selector). For example, by applying the anti-S_1 strand, the $S_1/I_2/$(**3**)/(**4**) nanostructure is separated, leading to the I_1-assembled (**1**)/(**2**) nanostructure, *cf.* Fig. 1(A). However, while the system reveals important advances in the use of DNA for logic networking, important challenges are still ahead of us: (i) it is essential to demonstrate the utility of such DNA logic networks in controlling intracellular processes and, eventually, applications for future nanomedicine. (ii) At present, the multiplexer and demultiplexer logic networks are separated units. The synchronous coupling between the multiplexer and demultiplexer could provide new opportunities in DNA computing for controlling intracellular processes.

Experimental

Materials

Phosphate buffer, NaCl and $MgCl_2$ were purchased from Sigma-Aldrich. DNA oligonucleotides were HPLC-purified and 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

Table 1 Sequences used for the multiplexer

Name	Sequence (5′ → 3′)
1	5′-ACCTCACCTCAATCCTAATAGCACCCATGTACAGTCA-3′
2	5′-GTCATTCAGCGATCTATTAACCTACCTCATCCAT-3′
3	5′-AACTAAGAAATAGCTCCGAGCACCCATGTACAGTCA-3′
4	5′-GTCATTCAGCGATCTCGGCGGTAAAGGTATAGG-3′
5	5′-CATTCATCATTTCCAGCCAGCACCCATGTACAGTCA-3′
6	5′-GTCATTCAGCGATCTGGCAGCTATTTCTTAGTT-3′
7	5′-TGATCCTAACATAGCTCAGCACCCATGTACAGTCA-3′
8	5′-GAATGAACTGACAATGTCATTCAGCGATCTGAGGTA CTCATACCAAC-3′
Sub$_1$	5′-ROX/TGACTGTTrAGGAATGAC/BHQ2-3′
I_1	5′-GTGAGGATGGATGAGGTAGGTGGATTGAGGTGAGG TAGGTCT-3′
I_2	5′-CCTATACCTTTACCGTACAGTCAGCGGCACG-3′
I_3	5′-AATGCCGCTGACTGTATGGAAATGATGAATG-3′
I_4	5′-TTGTCAGTTCATTC-3′
S_1	5′-AGACCTACCTCACCTCAATCCACCTACCTCATCC ATCCTCACCGTGCCG CTGACTGTAAGCTATTTCTTAGTTCTATGTTAGGATCA-3′
S_2	5′-GTTGGTATGAGTACAACTAAGAAATAGCTTAC AGTCAGCGGCATTAGACCTACCTCAC CTCAATCCACCTACCTCATCCATCCTCAC-3′

Table 2 Sequences used for the demultiplexer

Name	Sequence (5′ → 3′)
9	5′-CTCACTCACCTACTCATCTTCACCCATGT TATCCTA-3′
10	5′-AGTACTCAGCGATAAGATCACTCACTCCACTCC-3′
11	5′-AAGTGACAGCGATAAGATAGAAGTGGAGGTAAG-3′
Sub$_1$	5′-ROX/TAGGATATrAGGTCACTT/BHQ2-3′
Sub$_2$	5′- FAM/TAGGATATrAGGAGTACT/BHQ1-3′
I_1	5′-TGGTTAGGAGTGGAGTGAGTGGAGTAGG TGAGTGAGTGGAGG-3′
S_1	5′-CTTACCTCCACTTCTCACTCACTCCACTCCTAACCA-3′

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/).[23] The sequences in Table 1 were used for the multiplexer. The sequences in Table 2 were used for the demultiplexer.

Sample preparation

All reactions were performed in phosphate buffer (50 mM, 500 mM NaCl) at a final DNA concentration of 1 μM and 50 mM of $MgCl_2$. The samples, without the inputs, were heated to 95 °C for 5 min, then cooled to 30 °C. Fluorescence spectra were recorded, for each one of the states, before adding the inputs and after two hours.

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