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Biosensors

Construction of a Fuzzy and Boolean Logic Gates Based on DNA

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Logic gates are devices that can perform logical operations by transforming a set of inputs into a predictable single detectable output. The hybridization properties, structure, and function of nucleic acids can be used to make DNA-based logic gates. These devices are important modules in molecular computing and biosensing. The ideal logic gate system should provide a wide selection of logical operations, and be integrable in multiple copies into more complex structures. Here we show the successful construction of a small DNA-based logic gate complex that produces fluorescent outputs corresponding to the operation of the six Boolean logic gates AND, NAND, OR, NOR, XOR, and XNOR. The logic gate complex is shown to work also when implemented in a three-dimensional DNA origami box structure, where it controlled the position of the lid in a closed or open position. Implementation of multiple microRNA sensitive DNA locks on one DNA origami box structure enabled fuzzy logical operation that allows biosensing of complex molecular signals. Integrating logic gates with DNA origami systems opens a vast avenue to applications in the fields of nanomedicine for diagnostics and therapeutics.

1. Introduction

Logic gates are fundamental building blocks for performing complex operations out of abstract Boolean algebra. They need one or more external inputs and return each a single output. Logic gates exist in many types of material, both in nature and man-made devices. For instance, in silicon-based electronics, logic gates are components of integrated circuits and a computer processor will be made out of billions of them. By analogy, molecular logic gates are made of molecules enabling the development of molecular-scale computers and should find many applications in nanotechnology, biotechnology, and medicine. In 1994, Adleman demonstrated the use of DNA to perform logical operations by solving a complex mathematical problem using only DNA oligonucleotides.[1] This gave rise to the idea of DNA computing, and since then, biological logic gates in which both the inputs and the calculator are made of nucleic acids have been produced.[2–8] Nucleic acids are one of the best candidates to construct synthetic biocomputers, due to the unique predictability of Watson–Crick-base pairing, which makes them highly programmable.[9] The pioneering efforts of Seeman in DNA nanotechnology[10] and the revolutionizing work of Rothemund on DNA origami[11] also paved the way to enable rational design of nucleic acid nanostructures.[12] By utilizing the natural dynamics of these systems, one can develop reliable constructs that perform logical operations after addition of nucleic acid inputs where strand displacement drives the calculation and production of the output.

DNA-based logic gates have been reported to perform Boolean logic operations, such as XOR or AND, where the

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resulting output is either 0 or 1 depending on the applied inputs.[13–17] NOT, AND, and XOR logical operations were also accomplished by DNAzymes in which the presence or absence of a Förster/fluorescence resonance energy transfer (FRET) signal was used as output.[18] YES, NOT, and AND logic gates made of DNAzymes were combined to create a molecular automaton that could play the game Tic-Tac-Toe against a human opponent.[19] Fluorescence quenching was used as readout of the conformation of DNA tweezer structures that were combined for constructing logic systems .[20,21] In another study, Elbaz et al. utilized catenated DNA machines to specifically program the organization of gold nanoparticles that was used to produce the output signal.[22] DNA strand displacement was used to initiate the release of entrapped molecules through logic-based computation,[23] and in another study an automated DNA transporter was developed that delivered a DNA strand in a programed pattern.[24] The outputs from logical operations have also been used to control biological functions. For example, Benenson et al. produced a DNA-based YES automaton that rendered an output, which influenced gene expression levels.[25] In work by Douglas et al., AND logical operations were obtained by implementing aptamers into a hollow 3D DNA origami structure. In this study, the aptamers served as logic calculators and reacted to a variety of different inputs, leading to the exposure of a cloistered payload.[26] A very recent study showed that logic gates can function inside living cockroaches.[27]

Another potential application for molecular logic gates is biosensing. As molecular logic gates usually detect external inputs, which can for example be ions or small biological molecules, the logic gates in themselves can act as sensors for these inputs. Chuang et al. produced an enzymatic NOR gate that could sense the presence of either the explosive TNT, the nerve agent PAX, or both, resulting in a "hazard" output.[28] Logic AND gates based on DNA have been used for microRNA (miRNA) detection using the opening of a 3D DNA-origami box structure.[29] Logical operations have also been used for in vivo sensing in a study where adenosine triphosphate was sensed through configuration switching of a DNA octahedron structure in response to external stimuli.[30] A YES and an AND gate have recently been implemented on a DNA-origami 2D structure for miRNA detection yielding the outputs "+" or "-" written on the origami surface.[31] Indeed, specific miRNAs have been identified to be up- or down-regulated in different diseases and can provide signatures for six different solid cancer types.[32] As an example, the up-regulation of miR-21 and miR-191 was linked with lung cancer and an AND or NAND logic gate, which takes both these miRNAs as inputs, can be used as a biosensor to detect the expression level of these miRNAs. Extending the range of possible DNA-based logical operations to all Boolean functions as well as using fuzzy logical operations would allow the detection of more complex expression patterns and thereby potentially improve cancer cell diagnostics.

In contrast to Boolean logic that only accepts 0 and 1 as true values, fuzzy logic systems operate with analog logic where inputs and outputs have values between 0 and 1 or partial truth statements. Such devices are less studied in the context of nanotechnology but may, in many instances, reflect the behavior of gates found in nature. The importance of fuzzy logic arises from its role in fields such as medical diagnosis and medicine delivery,[33] bioinformatics,[34] artificial neural networks,[35] RNA secondary structure prediction,[36] image understanding, and processing by artificial intelligence,[37] or in industrial aspects such as fuzzy electronics and computation.[38] Moreover, most of the biological systems use fuzzy logic to perform their roles. For examples, in gene expression multiple transcriptional activators and silencers decide together on the level of transcription,[39] or in post transcriptional regulation, in which multiple protein and RNA factors bind to the messenger RNA and collaboratively decide on the fate of the transcript.[40] Understanding fuzzy logic and applying it to artificial synthetic biology, especially in combination with DNA origami, could empower our understanding of natural systems and additionally provide a new tool to perform soft computing.

In this report, we demonstrate successful construction of a universal Boolean logic gate system that can act as the complete set of Boolean logic gates (AND, NAND, OR, NOR, XOR, and XNOR gates). DNA oligonucleotides were used as inputs to trigger the desired reactions and outputs were quantified by fluorescence measurements through FRET between two fluorescent probes attached to the investigated system. Output values of 0 and 1 are obtained by low or high FRET efficiencies, respectively. These proof-of-concept principles can be utilized to construct de novo designed logic gate systems to achieve reliable programmable logical operations, which can be used in biocomputing and biosensing.[41] We also demonstrate that the designed gates can be incorporated into larger DNA structures by implementing the NOR logic gate in our previously reported 3D DNA-origami box.[42] Hence, the output is, in this way, directly coupled to a physical device that potentially affects downstream processes as logic gate operation may result in box opening. Several gates can be attached to one DNA box, which thus allows parallel operation of several logical gates in one system. In this configuration, we demonstrate construction of a fuzzy logic gate which is implemented in the DNA origami box.

2. Results and Discussion

We demonstrate here the design and construction of a fuzzy logic gate and of the Boolean logic gates AND, NAND, OR, NOR, XOR, and XNOR based on DNA, which are designed to operate with miRNA inputs. The output is a FRET signal, in which low and high FRET efficiencies correspond to an output of 0 and 1, respectively.

2.1. Simple Boolean Logic Gate Construct

The DNA logic gates all contain an overall similar structure comprised of six oligonucleotides (**Figure 1**): Two long strands (Strand 2 and Strand 3), which are connected through a shorter connector strand (Strand 1), and a longer DNA active strand. Two strands, modified with Cy3 or Cy5, respectively, connect through Strand 2 and Strand 3 to form the

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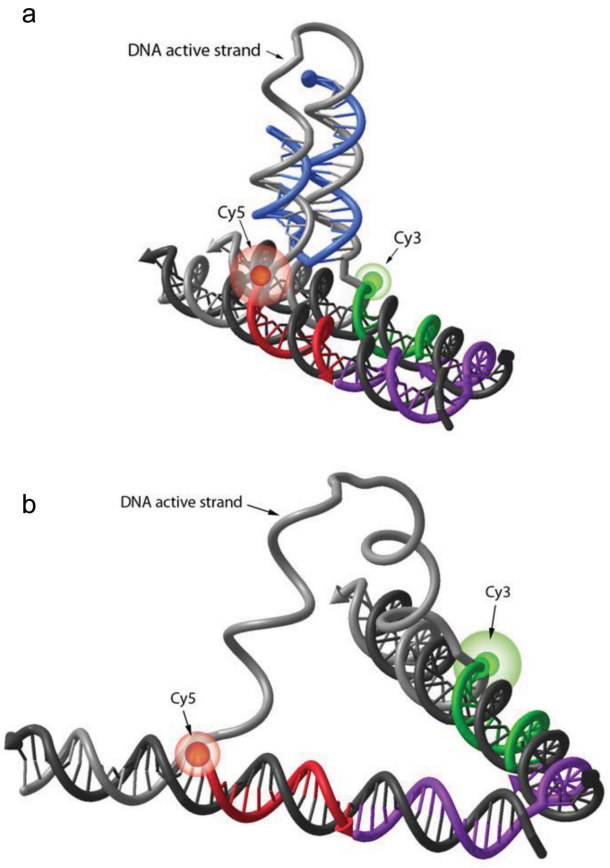

Figure 1. Design of the DNA logic gate complex. a) Closed complex giving rise to a high FRET signal. b) Open complex giving rise to a low FRET signal. The complex contains six oligonucleotides and in some instances a lock oligonucleotide (blue). The DNA active strand (light grey) responds to input strands and can either be in a closed conformation: the two fluorophores (Cy3, green; Cy5, red) are close to each other and this corresponds to an output of 1, or the DNA active strand can be in an open conformation: the fluorophores are further away from each other and this corresponds to an output of 0.

functional complex. This complex serves as a universal logic gate, and to produce all the different logic gates, simple additions of input oligonucleotides have been performed.

The active part of the logic gate complex resides in one oligonucleotide, the DNA active strand, which was designed to open and close the complex in response to the different logic gate inputs. A donor and acceptor FRET pair was strategically positioned in the complex, so that the FRET efficiency is low when the DNA active strand is open and high when it is closed. The inputs for each type of logic gate were carefully designed so that the active strand will open or close in response to the specified logical operations. The fluorescence emission spectra of the complex in closed and open conformation are shown in Figure S1, Supporting Information. A lock oligonucleotide was added to the complex when the logic gate output should be 1 in the absence of any inputs (NAND, NOR, XNOR) in order to prepare the active strand in an initial closed conformation.

The outputs from all six logic gates arise from a conformational change within a single DNA oligonucleotide, the DNA active strand, in response to two DNA inputs. In the AND gate (**Figure 2**a), the DNA active strand is initially in a single-stranded form in the absence of any inputs. The DNA logic gate has thus an open conformation which yields a low FRET signal. The two inputs are partially complementary to different parts of the DNA active strand, but individually added they did not cause the DNA active strand to close. However, if concurrently present, the two input strands can anneal through a 14 nucleotides (nts) long complementary overhang near the root of the active strand, causing the DNA active strand to close and enabling the production of a high FRET signal. The same design was used for the XNOR gate (Figure 2f), where the only difference was the presence of a lock oligonucleotide in the initial complex, causing the output to be 1 in the absence of any inputs.

The NAND and XOR gates (Figure 2b and 2e, respectively) were designed so that addition of one of the two inputs strands caused the DNA active strand to be closed due to hybridization of the input strand to both sides of the DNA active strand. The input strand had a short sequence complementarity to one side of the active strand while the other side was complementary to a longer part of the input. The same applied for the other input with reverse direction of the long and short complementary stretches on the DNA active strand. This means that when both inputs are present, only the long complementary parts of both inputs will base pair to the active strand causing the active strand to open in the NAND and XOR gate. The NAND gate was designed to be closed by a lock oligonucleotide in the absence of inputs, whereas the DNA active strand was in the open conformation before addition of input strands in the XOR gate.

The OR gate was designed to give a 0 as output in the absence of inputs and an output of 1 in the presence of either one or two inputs (Figure 2c). The two input strands were complementary to 11 and 22 nts, respectively, on each side of the DNA active strand with a crossover near the root of the active strand. Thus, the structure was in closed conformation after addition of either one of the inputs strands. When both inputs were present, the longer input strand formed a more stable complex with the DNA active strand due to more extended base pairing. Hence, in the presence of both inputs, the DNA active strand will be closed.

The NOR gate was assembled in the presence of a lock oligonucleotide to obtain an output of 1 in the absence of inputs (Figure 2d). Both inputs are complementary to either side of the active strand, and each shared more base pairs with the DNA active strand than the lock oligonucleotide. Therefore, the lock oligonucleotide is displaced by addition of input strands, which caused the DNA active strand to open in the presence of either one or both of the inputs.

All Boolean logic gates produced the expected 0 or 1 values corresponding to low and high FRET, respectively, in response to all combinations of inputs, confirming their rational designs. To achieve this, a threshold value of 0.4 was chosen, so that FRET efficiencies below 0.4 corresponded to an output of 0, whereas FRET efficiencies above 0.4 corresponded to an output of 1. However, since the logic gate system is a de novo design, the threshold value can be adjusted to fit the desired system. The low FRET values, corresponding to output of 0, were in most cases below 0.2, but

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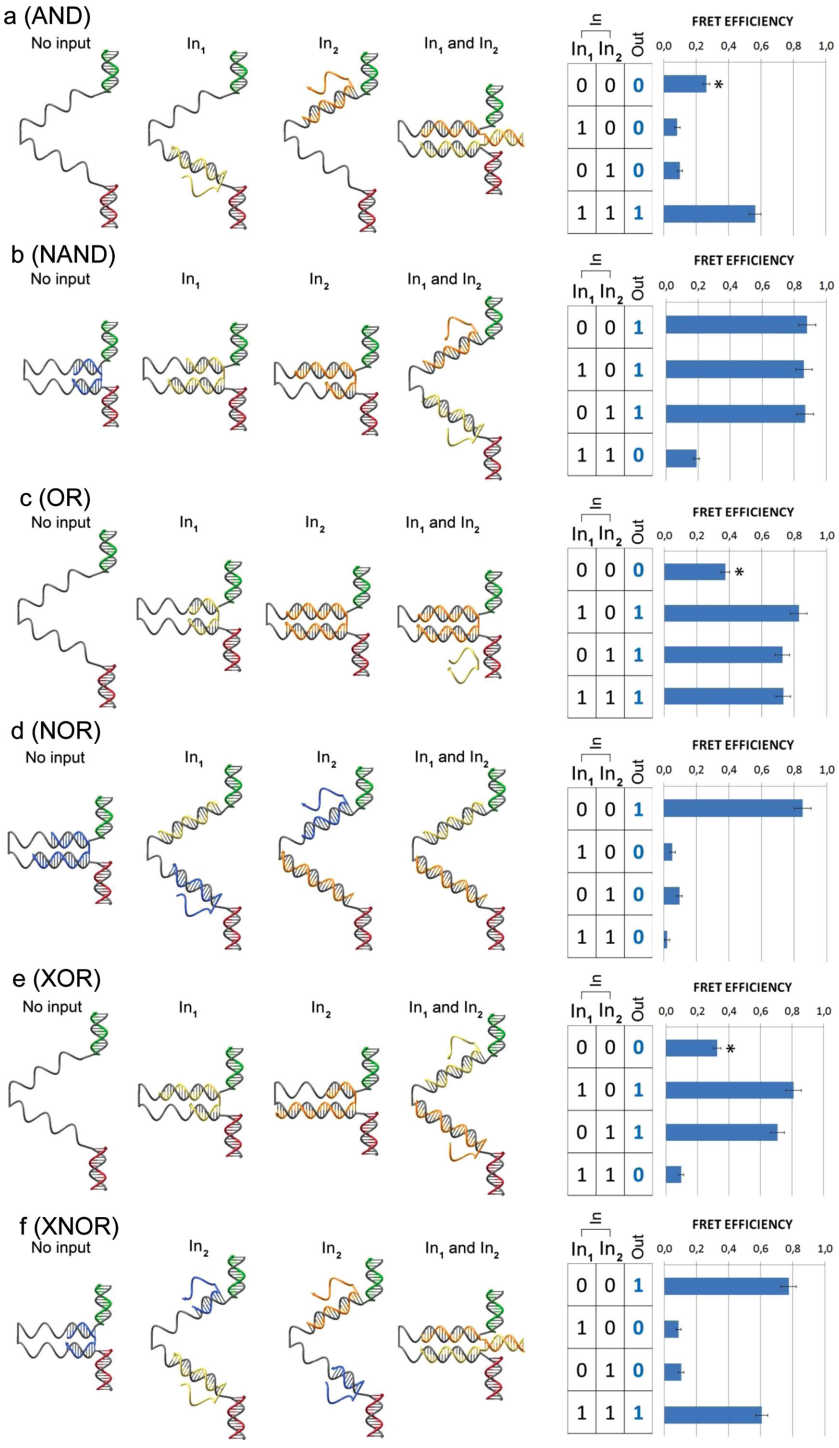

Figure 2. Design principles of the DNA logic gates. The logic gates are displayed in closed or opened configuration with no input, in the presence of either input In_1 or input In_2, or both In_1 and In_2 together, according to the rules specified by each gate. The predicted values and FRET efficiency results of the gates are displayed to the right. An asterisk indicates that the DNA active strand is single stranded. a) AND gate. b) NAND gate. c) OR gate. d) NOR gate. e) XOR gate. f) XNOR gate.

values around 0.3 were observed in the initial state of the AND, OR, and XOR gates, which may be explained by the fact that the DNA active strand was completely single stranded in the absence of any inputs. Single stranded DNA, in the open state, is flexible and thus the DNA logic gate complex may have a number of different conformations and the measured FRET value is thus an average of all such conformations. Incorporating the AND, OR, and XOR DNA logic gates in a larger, more rigid structure is expected to yield a lower 0-value FRET efficiency for the initial open state. Low FRET values have indeed been previously obtained for the open conformation of the DNA origami box structure when using single stranded locks.[42] High FRET efficiencies between 0.56 and 0.85 corresponded to outputs of 1. In most designs, the closing mechanism was a single oligonucleotide that bridges the roots of the DNA active strand, thereby holding the two fluorophores close to each other and giving high FRET values around 0.85. In this case, we observe a minor inverse dependence of the DNA input length on FRET efficiency. Indeed, an input that can anneal with 11 base pairs on each side of the DNA active strand yielded a higher FRET efficiency than an input that can anneal with 22 base pairs on each side (Figure 2c). The AND and XNOR gates exhibited lower FRET efficiencies (0.56 and 0.61, respectively) in the closed state (Figure 2). In these two cases, the closing mechanism was different and accomplished by hybridization of two complementary overhangs near the roots of the DNA active strand. Hence, an additional DNA double helix with a diameter of 2 nm was bridging the two fluorophores in the AND and XNOR gates, which is likely the explanation of the smaller FRET efficiencies. In all cases, the closed and open states of the DNA logic gate complex displayed FRET values above and below the threshold, respectively, allowing a non-ambiguous readout of the outputs as 0 and 1. The response time of the logic gates was furthermore studied and found to be less than 30 s which is compatible with real-time detection for potential biosensing applications (Figure S4, Supporting Information).

2.2. Logic Gate Operation Within a DNA Origami Box

To demonstrate that the logic gates can be implemented into larger systems, we introduced the NOR gate as the lock in our previously reported $18 \times 18 \times 24$ nm^3 DNA origami box (**Figure 3**a).[42] The box was prepared in a closed state with one lock acting as a NOR logic gate. Addition of keys corresponding to inputs 1 and 2 led to the opening of the otherwise closed box (Figure 3b),

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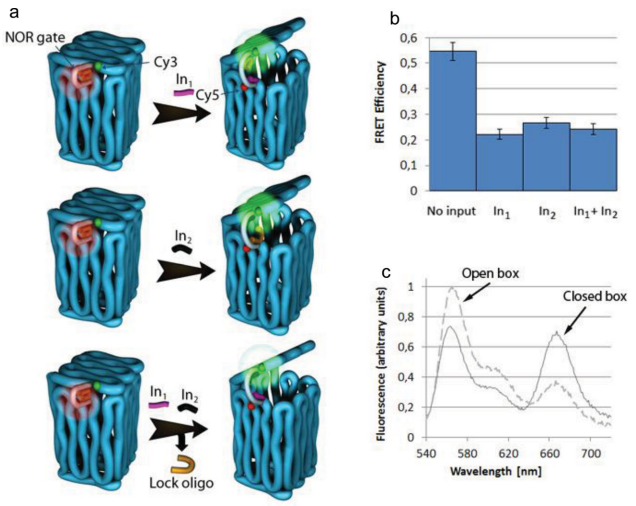

Figure 3. Logic operations of a 3D DNA origami box with a NOR gate as lock. a) Model of the DNA origami box showing NOR logic gate operation. b) FRET efficiencies of the box in closed (no input) and opened states (In_1, In_2, or In_1 and In_2 as inputs). c) Fluorescence spectra obtained for the closed box (full line) and open box (dashed line), where both inputs were added.

as expected for the operation of a NOR gate (Figure 2d). The closed state yielded a high FRET efficiency of 0.55 and the open states lower FRET efficiencies around 0.25 leading to clear readouts above and below the threshold of 0.4 of the open and closed states, respectively, of the NOR logic gate implemented in the DNA origami box. These FRET values are slightly different from those obtained for the NOR logic gate incorporated in the more simple DNA complex (Figure 2d). This is likely due to different fluorophore positioning in the box and in the simple logic gate complex. Indeed the placement of the donor and acceptor on the side of the box (see Figure 3) yields a FRET value of ≈0.2–0.3 in the opened state as the distance between the fluorophores does not exceed ≈8 nm for any position of the box lid. The NOR logic gate behaved as predicted inside the DNA origami box implying that the constructed Boolean logic gates can also function when integrated in larger DNA systems. By implementing the logic gates in these larger DNA systems, and in particular in a DNA origami box, the logical operations could potentially control the release or exposure of a cargo hidden inside the box. This could for example be an antibody or nucleic acids that can target biological processes associated with disease once a logic gate had responded to external stimuli and opened the box.

2.3. Fuzzy Logic Gates

Unlike the Boolean logic, fuzzy logic by definition is a multivalued logic, where the outputs can vary between 0 and 1.[43,44] FRET is an example of fuzzy logic, as values can vary between 0 and 1 corresponding to partial energy transfer depending on the distance between the donor and acceptor fluorophores. We demonstrate here production of a fuzzy logic gate implemented in the DNA origami box. To achieve fuzzy logic three distinct FRET pairs were positioned at the three edges of the DNA origami box and four active DNA locks were placed on the structure (**Figure 4**a). By having three FRET pairs, opening of either of the locks on the box would be sensed by at least one of the three FRET pairs, leading to a decrease in FRET, whereas having only one FRET pair would not necessarily allow such detection. Addition of the respective keys for each lock in the order that is represented in Figure 4a lead to a concomitant change in the distance of each distinct FRET pair and thus a change in the total FRET value (Figure 4b), which is interpreted as the transition of the structure from "Locked" state to "Partially Locked" state to "Not-Locked" state. Measured FRET efficiencies were above 0.6 in the initial closed state of the box lid and decreased with the sequential addition of each key to a final FRET efficiency of ≈0.1 corresponding to an open box (Figure 4b). These observations confirm the fuzzy logic gate behavior of the DNA origami box in response to the added keys as inputs. Fuzzy logic systems like this one could help improve our understanding of natural controlling mechanisms. For instance, one can adapt such a system to mimic natural mechanisms inside cells, like for example the binding of multiple miRNAs to the 3′-untranslated region of an messenger RNA to down regulate protein expression in a cooperative manner.[45]

Molecular logic gates can be used to perform programmable logical operations and biosensing. Our DNA-based logic gates have been designed to recognize and respond to different miRNA inputs, such as miR-191 and miR-34a. miR-191 is upregulated in, e.g., colon, lung, and prostate cancer,[32] whereas miR-34a is down-regulated in high-grade glioma-tissues.[46] Therefore, combining several of these logic gates that perform simple yes/no operation together in a sensor, for example in a DNA origami box system, would enable the detection and identification of specific cancer types. The sequence of the DNA active strand can be varied for each logic gate complex and combining several DNA active strands with different sequences in one biosensor would allow complex simultaneous detection of different miRNA inputs. Furthermore, the fuzzy logic gate setup in the 3D origami box was designed to

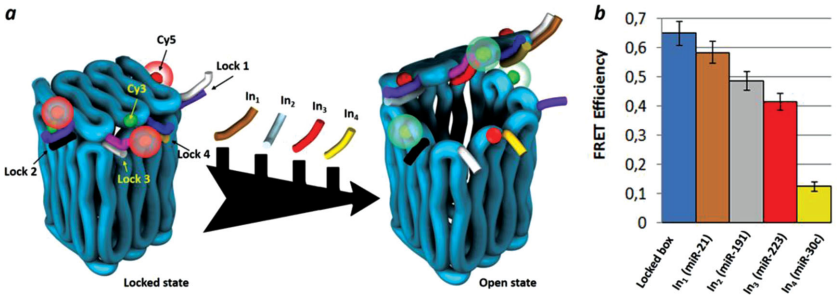

Figure 4. a) Schematic drawing of the fuzzy logic gate functionalized DNA origami box. Three FRET pairs are localized at different sides of the lid and the four locks are used to close the lid and serve as fuzzy logic gate operators. b) FRET efficiencies of the fuzzy logic gate. Upon addition of each input, the value of the logic gate output changes according to the conformational changes of the box structure.

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respond to miR-21, miR-30c, miR-191, and miR-223. In this system, a small decrease in FRET efficiency corresponds to the presence of one miRNA, whereas a larger decrease of FRET corresponds to the presence of several or all four miRNAs. If all miRNAs are present, a near complete loss of FRET would indicate that the cancer type was colon, pancreas, or prostate, since all four miRNAs are upregulated in these cancer types.

3. Conclusion

In 1994, Leonard Adleman showed that DNA can be used to solve the "traveling salesman" problem.[1] Since then, DNA has been used in a number of studies for performing biocomputation and molecular logic operations. A universal DNA logic gate that performs all Boolean logic operations can be used to conduct even more complex computations by threading or parallelizing the calculations into combinatorial logic gates. We have successfully demonstrated the operation of all the Boolean logic gates, AND, NAND, OR, NOR, XOR, and XNOR as well as of a fuzzy logic gate system with high fidelity and reliability. These Boolean DNA logic gates were all designed from a universal structure that can be readily implemented into more complex structures. As a proof-of-concept, we have implemented a NOR DNA-based logic gate into our previously reported DNA origami box. Integration of logic gates into more complex self-assembled structures offers the possibility of controlling the operation of multifunctional systems for new sophisticated biocalculators and nanomedical devices. The proposed systems may be able to perform the calculations in vivo and potentially be used for combined cancer diagnostics and treatment at the molecular level. Indeed, we propose that combining these DNA logic gates can be used to detect and respond only to the specific miRNA profiles signifying different cancer types. Incorporating the logic gates in a DNA origami box would, in principle, allow opening of the lid in response to the specific RNA inputs leading to exposure of a therapeutic agent such as an antibody, toxin, enzyme or nucleic acid caged in the DNA origami box.

4. Experimental Section

DNA Logic Gate Complex Production: A single step assembly of the DNA logic gate complex was performed in a single tube. The assembled complex was purified twice on Illustra MicroSpin™ S-300 columns. Gel electrophoresis was performed to confirm high yield of formation of the complex (see Supporting Information).

The logic gate complex was assembled by 14 h annealing of 20 pmol of the DNA complex in 1× TAE-Mg^{2+} buffer (*tris*-acetate (40×10^{-3} M, pH 8.0), EDTA (1×10^{-3} M, pH 8.0), 12.5×10^{-3} M $MgCl_2$). In the case where the logic gate output needed to be 1 in the absence of any input (NAND, NOR, XNOR), an additional lock oligonucleotide was added. Illustra MicroSpin™ S-300 HR columns (GE Healthcare Life Sciences) were used to purify the assembled complex from the unincorporated oligonucleotides. Detailed information can be found in the Supporting Information.

DNA Origami Assembly and Purification: A small DNA box was assembled as described previously[42] with the locking system being either one DNA logic gate or several gates for fuzzy logic. Detailed information can be found in the Supporting Information. DNA origami assembly was performed in a 100 µL $1 \times$ TAE-Mg^{2+} buffer containing a final concentration of 9×10^{-9} M of the DNA origami template and 10× excess of staple strands. The annealing of the small DNA origami box was performed over a fast heating of the mixed material to 95 °C for 5 min followed by fast cooling to 80 °C at 1 °C per 10 s and slower cooling to 60 °C at 1 °C per 1 min followed by very slow cooling to 20 °C at 1 °C per 1 h. Illustra MicroSpin™ S-300 HR columns were used to purify the assembled origami structures from the unincorporated staple strands.

Performing Boolean Logic Operations with the DNA Logic Gate Complex: Samples for each logic gate were diluted in 1× TAE-Mg2+ to the final concentration of 10×10^{-9} M and divided into four. One of the four samples was kept as is, 50×10^{-9} M of Input 1 or of Input 2, or of both Input 1 and 2 were added to the rest of the samples, respectively. The DNA logic gate samples containing input strands were heated to 45 °C and cooled to 25 °C over 2 h annealing at 1°C per 6 min.

Performing NOR Logic Gate Operation Within the DNA Origami Box: The NOR logic gate DNA origami box sample was diluted to approximately 7×10^{-9} M in 1× TAE-Mg^{2+} and steady-state fluorescence measurements were performed. The sample was distributed in two. On the first half sample, measurements were performed first on the sample as is and then following sequential addition of Input 1 and Input 2. On the other half of the sample, inputs were added in the reverse order, i.e. Input 2 first, and then Input 1. The concentration of the input DNA strands was ~200 times that of the DNA origami box structure.

Performing Fuzzy Logic Gate Operation Within the DNA Origami Box: The Fuzzy logic origami sample was diluted to approximately 5×10^{-9} M in 1× TAE-Mg^{2+} buffer and steady-state fluorescence measurements were performed on the sample in absence of inputs and with addition of Input 1 followed by Input 2 and 3 and 4, to finally have all inputs present in the sample.

Steady-State Fluorescence Measurements and FRET Experiments: A Fluoromax 3 fluorometer [Horiba Jobin–Yvon] was used to conduct FRET experiments. The samples were exited at 530 and 600 nm to excite the Cy3 and Cy5 fluorophores, respectively. Monochromator slits were set to allow a spectral resolution of 5 nm and the integration time was set to 0.5 s per measured point. FRET efficiencies were calculated using the $ratio_A$ method as described previously.[42] The relative values used for ε_{AA} and ε_{DA} for determing the FRET efficiency were 0.07 ± 0.015 and 1.03 ± 0.06, respectively. Statistical analysis on FRET efficiency values was done by using error propagation in the determination of FRET efficiency and by performing statistics on independent repeats of the same experiment. The first method gave larger error bars and these are the ones plotted in Figures 2,3, and 4.

Supporting Information

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

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