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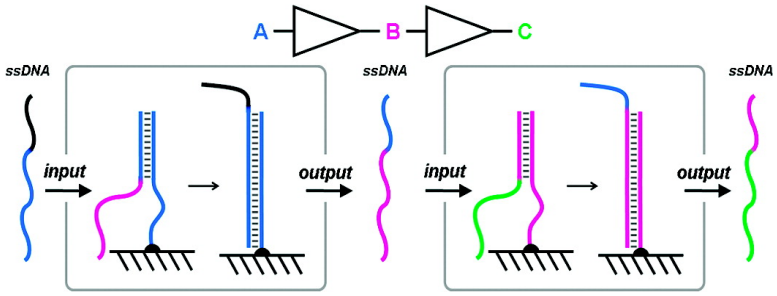

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Modular Multi-Level Circuits from Immobilized DNA-Based Logic Gates

Brian M. Frezza, Scott L. Cockroft, and M. Reza Ghadiri*

Contribution from the Departments of Chemistry and Molecular Biology and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Rd, La Jolla, California 92037

Received February 12, 2007; Revised Manuscript Received September 8, 2007; E-mail: ghadiri@scripps.edu

Abstract: One of the fundamental goals of molecular computing is to reproduce the tenets of digital logic, such as component modularity and hierarchical circuit design. An important step toward this goal is the creation of molecular logic gates that can be rationally wired into multi-level circuits. Here we report the design and functional characterization of a complete set of modular DNA-based Boolean logic gates (AND, OR, and AND-NOT) and further demonstrate their wiring into a three-level circuit that exhibits Boolean XOR (exclusive OR) function. The approach is based on solid-supported DNA logic gates that are designed to operate with single-stranded DNA inputs and outputs. Since the solution-phase serves as the communication medium between gates, circuit wiring can be achieved by designating the DNA output of one gate as the input to another. Solid-supported logic gates provide enhanced gate modularity versus solution-phase systems by significantly simplifying the task of choosing appropriate DNA input and output sequences used in the construction of multi-level circuits. The molecular logic gates and circuits reported here were characterized by coupling DNA outputs to a single-input REPORT gate and monitoring the resulting fluorescent output signals.

Introduction

In electronic integrated circuit architectures, logic gate components receive Boolean inputs representing true (1, high voltage) or false (0, low voltage) values and generate the appropriate Boolean output.[1] Since the output voltage of any gate can serve as the input to another, gates can be electrically wired together to form multi-level circuits that produce the desired functional and information processing characteristics. By analogy, in recent decades considerable efforts have been focused on devising chemical systems that implement the core tenets of information processing. As the result logic gates have been reproduced in numerous molecular settings, including supramolecular complexes and small molecules,[2] enzymatic biochemical networks,[3] peptide networks,[4] and other systems.[5] However, many molecular gates use incompatible input and output representations (e.g., ligand concentrations as inputs, and fluorescent emission as output), which pose significant challenges when attempting to connect these gates into multi-level circuits.[6]

The sequence-specific recognition properties of oligonucleotides provide intriguing possibilities for the construction of molecular logic gates and multi-level networks. For example, ribozyme-based logic gates that receive short single-stranded oligonucleotide inputs and cleave a fluorogenic substrate sequence in response to the appropriate Boolean combination of inputs have been studied extensively.[7−11] Since unique gates can be created by simply modifying the input sequences and the complementary regions within the ribozyme, this has allowed the construction of single-level ribozyme networks containing a large numbers of gates[8,11] and basic two-level ribozyme-based circuits.[8,9] In addition to addressable hybridization, sequence-

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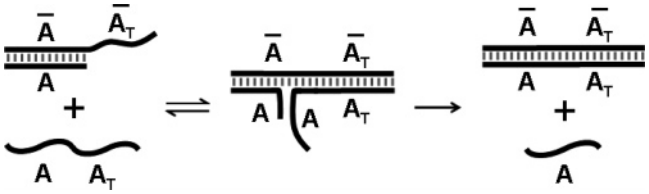

Figure 1. Sequence-directed DNA strand displacement. Full length complement strand (AA_T) binds to the "toe-hold" region (A_T) of the duplex leading to the irreversible displacement of strand (A) via a three-way branch migration mechanism.[13] The room-temperature kinetics of this process are limited by the initial hybridization to the toe-hold region when it is approximately seven nucleotides or longer.[15,21]

specific DNA recognition also has the potential to disrupt DNA interactions through the process of strand displacement.[12,13] In this process, a full-length complementary strand binds to a single-stranded overhang known as a "toe-hold" and proceeds to invade and displace shorter hybridized sequences via a three-way branch migration mechanism (Figure 1). This phenomenon has been used to produce DNA-based molecular machines ranging from molecular tweezers to "DNA walkers".[10,12,14,15] DNA strand displacements of this type are well-suited for use in logic circuits, since both the input and output of the process are single-stranded oligonucleotides. Boolean values can be represented by high (1) or low (0) concentrations of each single-stranded DNA species. The challenge then is to implement an experimental design that exploits the sequence-addressability of displaced single-stranded DNA outputs, while enforcing the correct communication, or wiring between different gates. One approach to addressing this challenge in DNA-based logic circuits was recently implemented by orchestrating a series of strand displacement events such that the toe-hold regions of later gates were inaccessible and could not be triggered until the preceding gates in the circuit had been executed.[16]

Here we describe an alternative approach to constructing multi-level DNA displacement-based logic circuits. Gates were constructed by annealing output strands to complementary regions within single-stranded DNA that was immobilized on streptavidin-sephrose beads via a 5′-biotin modification. By isolating each layer of logic gates on spatially separated supports, output strands are prevented from acting as inputs to later gates until they have been displaced from the solid support by the appropriate combination of inputs. The advantage of this modular approach is that gates can be wired together by simply appending the input sequence of the destination gate to the output of the source gate (Figure 2).

Experimental Methods

Logic Gate Preparation. Each DNA logic gate was prepared in SPSC buffer (50 mM sodium phosphate, 1 M NaCl, pH 7.5) by first annealing a biotinylated DNA to 1.1 equiv of its corresponding DNA output(s) by heating at 85 °C for 5 min and then slowly cooling down to ambient temperature over 6 h. Streptavidin-Sepharose High Performance (GE Healthcare, 34 μm mean particle size, highly cross-linked spherical agarose, loading: minimum 300 nmol biotin/mL medium) was washed with SPSC buffer ($5 \times 350\ \mu$L). An excess of streptavidin-sepharose suspension was then incubated with the preannealed gates ($\sim$100 equiv of streptavidin per biotinylated DNA gate complex) for 20 min with gentle shaking at room temperature. After washing the gates with SPSC buffer ($10 \times 350\ \mu$L) to remove unbound output DNA, all solid-supported gates were diluted with SPSC buffer to a final gate concentration of approximately 0.5 μM. The stock solutions of each gate were stored at 5 °C.

Circuit Execution. Our method of immobilizing DNA on streptavidin-sepharose beads resulted in high surface-exposure, which benefits the kinetics of logic gate operation. However, this did not completely prevent surface-exposed single-stranded regions of immobilized output strands from contacting and prematurely displacing the outputs of subsequent layers of gates (data not shown). Thus, we employed a more robust filtering procedure to ensure the complete isolation of each layer of gates, which improved the contrast between true (high) versus false (low) output levels.

AND, OR, AND-NOT to Report Circuits. Ten microliters of 0.5 μM input strands were incubated with 1 equiv (10 μL stock) of the corresponding gate for 1 h in a final volume of 30 μL SPSC buffer at room temperature with light stirring. Gates were filtered though an Ultrafree-MC Centrifugal 0.22 μm filter (Amicon Bioseparations). The resulting solution was incubated as before with the addition of 1 equiv of the reporter Gate and filtered again. The final solution was diluted to 300 μL with SPSC buffer to allow the measurement of the sample fluorescence.

XOR Circuit. Two equivalents of the AND gate (20 μL stock) and 1 equiv of the OR gates (10 μL stock) were incubated with the input strands in parallel in 30 μL of SPSC buffer for 5 h at room temperature. Two equivalents of the AND gate and its associated inputs were used to compensate for its lower output yields compared to the OR gate (see Results and Discussion). Both gates were filtered, and the output solution from the AND gate was preincubated with 1 equiv of the AND-NOT gate (10 μL stock). After 5 h, 1 equiv (10 μL stock) of the output solution from the OR gate was also added to the AND-NOT gate and

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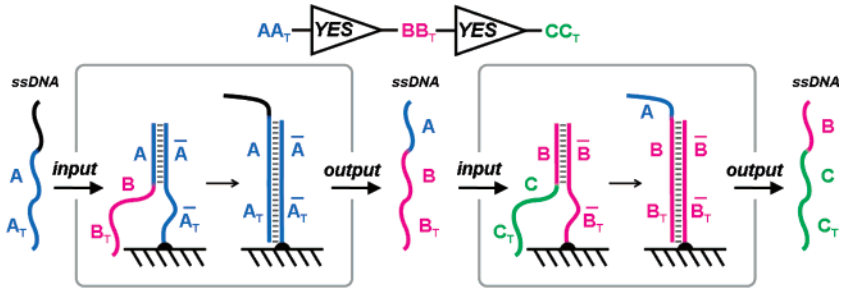

Figure 2. Any single-stranded sequence containing AA_T can serve as an input to the first gate by binding the A_T toehold and subsequently displacing ABB_T into solution via a three-way branch migration mechanism. The displaced terminal BB_T sequence is then able to serve as the input to the second gate, which in turn mediates the output of BCC_T into solution. By immobilizing the first and second gates on isolated solid supports, outputs are unable to trigger downstream gates until they are displaced into solution by the appropriate inputs. This modular approach allows gates to be chained together to form multi-level circuits.

incubated for one further hour. The combined output solution was filtered as before to remove the gates and added to 1 equiv (10 μL stock) of the reporter gate. The resulting solution was incubated with 1 equiv (10 μL stock) of the reporter gate for 1 h and filtered again. The final solution was diluted to 300 μL with SPSC buffer to allow the measurement of the sample fluorescence.

DNA Sequences. DNA sequences were generated using a custom code based on the algorithm reported by Fledkamp and co-workers.[17] Sequences were analyzed with the DINAMelt online server folding/hybridization tools to minimize the number of undesirable stable secondary structures.[18] The sequences used in this study are specified in the Supporting Information. DNA was purchased from Sigma Genosys and purified by preparative PAGE. DNA stock solutions (3.5−7 μM) were prepared in ultrapure water using extinction coefficients calculated by the nearest neighbor method with Ambion's web-based script[19] and stored at 5 °C.

Fluorescence Data. Fluorescence data were acquired on a Series 2 Luminescence Spectrometer (Sim-Aminco) with the AB2 (v5.5) software package (Thermo Electron). Each sample was diluted to a final volume of 300 μL and was excited at 525 nm (8 nm band-pass) with emission readings at 565 nm (8 nm band-pass) at a photomultiplier tube sensitivity (gain) of 835 V (OR->reporter) or 900 V (for the other circuits). One-hundred data points were taken for each sample at a rate of 1 reading per second and averaged to obtain a final value. AND->Report, OR->Report, AND-NOT->Report circuits were independently executed 5 times to estimate experimental errors. Five baseline recordings were taken for each sensitivity level and the average values subtracted from the raw RFU to correct signal baseline. After adjusting the baseline, data were normalized by setting the lowest value (buffer only) to 1, to determine the relative fluorescence of each recording.

Results and Discussion

Using the methods described above, we constructed a set of 2-input logic gates (AND, OR, and AND-NOT) and a single-input REPORT gate that produces a fluorescent signal in response to an input sequence (Figure 3). When the reporter gate is triggered by a ZZ_T input sequence, the fluorescent Cy3-labeled strand is displaced into solution, freeing it from contact-mediated quenching by the dabcyl group on the immobilized strand (Figure 3a). The fluorescent response of the reporter gate was used to validate the function of the 2-input gates (AND, OR, and AND-NOT) by appending the ZZ_T sequence to the output sequences of each 2-input gate.

The DNA-based AND gate (Figure 3b) was designed such that the addition of either the XX_T or the YY_T input sequence

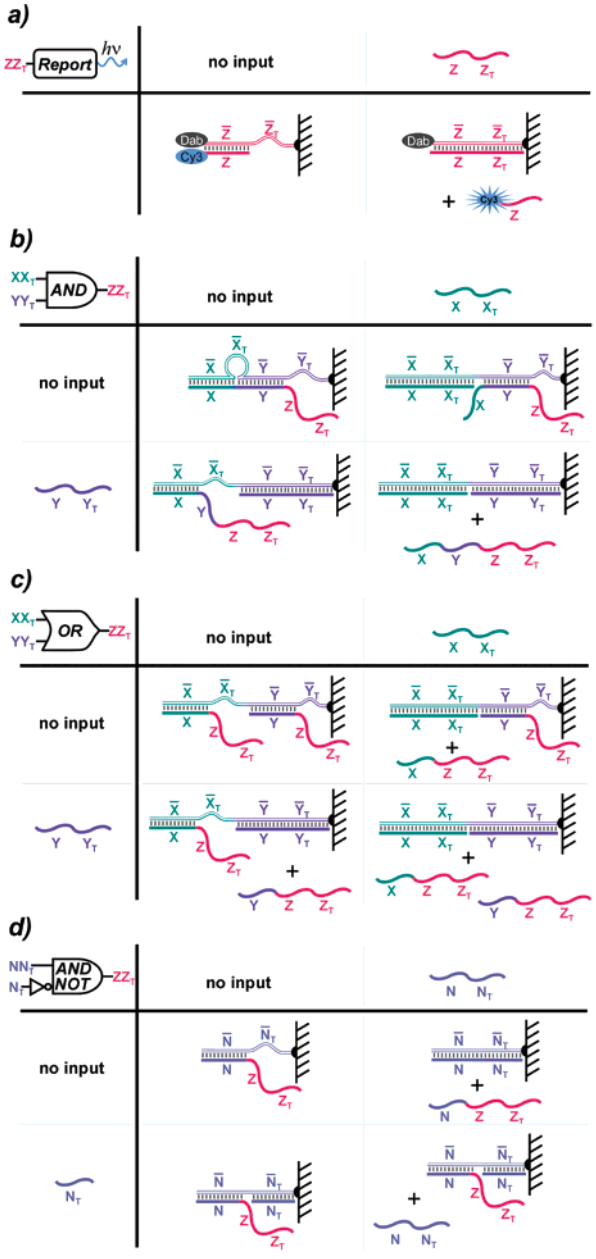

Figure 3. Mechanisms of DNA-based logic gate operation. The first row and column of each truth table show the inputs and the associated output states. (a) Reporter gate. (b) AND gate. (c) OR gate. (d) AND-NOT gate. Letters A, B, etc. represent unique, noncomplementary nucleotide sequences with X_T,Y_T, etc. designating "toehold" regions 25 nucleotides in length.

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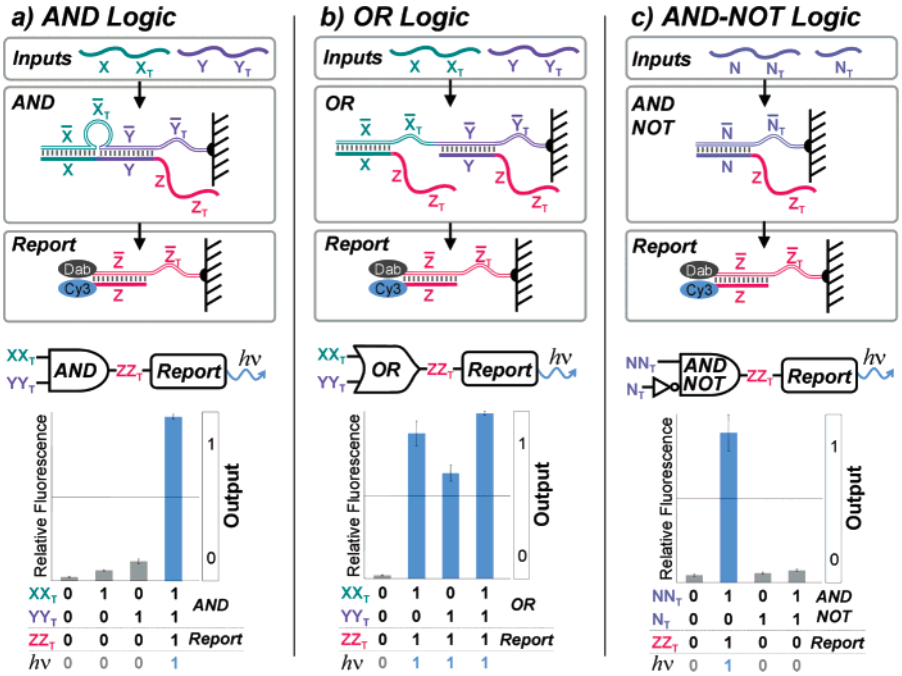

Figure 4. Diagrammatic representation of each 2-input logic gate wired to a fluorescent reporter (top) and the experimentally observed fluorescence output from execution of each simple circuit with every combination of inputs (bottom). Circuits were executed as described in the experimental section. Each gate was executed with each set of inputs five times to produce error bars (±1 standard deviation).

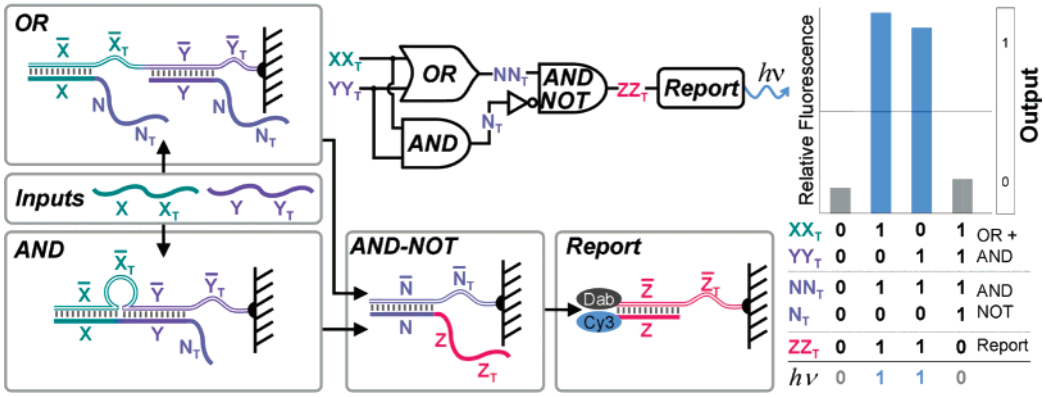

Figure 5. Multi-level circuit built from an OR, AND, and AND-NOT gates of gates that performs a net XOR (exclusive-OR) analysis on the inputs. The function of the circuit was assayed using the fluorescent readout from the reporter gate and is described in the experimental section.

displaces only one duplex region (X or Y), leaving the $XYZZ_T$ output anchored to the stationary strand. However, addition of both XX_T and YY_T inputs displaces both anchor points and the $XYZZ_T$ output strand is released into solution.

The DNA-based OR gate (Figure 3c) was designed such that the addition of either the XX_T or the YY_T input displaces either the XZZ_T or the YZZ_T output strands, respectively. When both inputs are present, the effective output of ZZ_T is doubled since the immobilized OR gate DNA possesses two ZZ_T-containing outputs. However, equimolar quantities of the gate complexes are used throughout an assembled circuit, so the maximum output of each gate is limited to a single equivalent even in the presence of excess inputs.

The DNA-based AND-NOT gate (Figure 3d) was designed such that the addition of the NN_T input displaces the NZZ_T output into solution, whereas addition of the N_T input only binds the toe-hold region of the immobilized gate DNA. Thus, if the N_T input is bound to the gate prior to addition of the NN_T input, then the N_T strand occupies the toe-hold and prevents NN_T from displacing the output strand. Thus, the AND-NOT gate requires two incubation cycles to ensure correct Boolean execution.

Each of the 2-input logic gates were wired to the reporter gate as described above, and executed with all possible combinations of initial inputs as described in the experimental section. Fluorescent analysis of the resulting output solutions revealed proper implementation of Boolean behavior in all cases (Figure 4).

This particular combination of logic gates (AND, OR, AND-NOT) was selected because it represents one example of a "complete" set of logic gates, meaning that they can be theoretically assembled into multi-level circuits capable of performing any imaginable Boolean function.[20] To demonstrate the emergence of novel function through rational gate networking, we set about rewiring the gates to into a multi-level circuit that enforced a net XOR (exclusive OR) Boolean behavior

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(Figure 5). Rewiring was accomplished by simply modifying the output sequences of each logic gate. The terminal ZZ_T sequence of the OR gate, which had previously directed it to the reporter, was replaced with a terminal NN_T sequence directing it to the activating input of the AND-NOT gate. Similarly, the terminal ZZ_T sequence of the AND gate output was replaced with the N_T sequence to direct it to the inhibiting input of the AND-NOT gate. The AND-NOT gate was left unchanged and remained wired to the reporter gate via the terminal ZZ_T sequence so that overall circuit operation could be verified using fluorescence. However, when attempting to construct logic networks, it is important to remember that our molecular implementations of logic gates are by nature analog approximations of their electronic digital counterparts. Sources of signal discrepancies arise from nonideal displacement yields, with different sequences and gate architectures leading to unique output levels. Small differences in gate output levels accumulate at each level of circuit execution, and without correction can cause improper network behavior. For instance, we noted that the output level of the AND gate was ~30% lower than the OR gate in the case when both Boolean inputs were present. Therefore, 2 equiv of the AND gate and longer incubation times were used to compensate for these differing output levels in our implementation of the XOR gate (see Experimental Section). After taking these precautions, the circuit demonstrated proper XOR behavior for all combinations of input (Figure 5, right).

Conclusion

In summary, we have constructed a complete set of immobilized 2-input logic gates (AND, OR, AND-NOT) that release single-stranded DNA outputs in response to the appropriate Boolean combination of single-stranded DNA inputs. We validated the function of these gates using a single-input reporter gate that releases a fluorescently labeled output in response to a specific single-stranded DNA input. The immobilization of gates allows for simple gate designs and facilitates the construction of modular logic circuits. These gates can be wired together to form multi-level circuits that use the solution as a communication medium, since the output of one gate can serve as the input to another. We demonstrated this capability by modifying output sequences to redirect the wiring between gates to create a circuit that possesses XOR Boolean behavior as an emergent property of the network. One notable drawback of this approach is that our circuits are analog approximations of digital circuits. Thus, imperfect strand displacement yields result in a minor loss in the input/output signal at each level of circuit operation, which fundamentally limits the size of circuits that can be constructed. Implementation of methods that amplify and threshold strand concentrations would overcome this limitation by producing a truly digital system, thus facilitating the construction of multi-level circuits of arbitrary depth. Work is currently underway to explore these possibilities.

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Supporting Information Available: DNA sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

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