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Computing in mammalian cells with nucleic acid strand exchange

Benjamin Groves^{#1}, Yuan-Jyue Chen^{#1}, Chiara Zurla^{#2}, Sergii Pochekailov¹, Jonathan L. Kirschman², Philip J. Santangelo^{2, **}, and Georg Seelig^{1,3, **}

¹Department of Electrical Engineering, University of Washington

²Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University

³Department of Computer Science & Engineering, University of Washington

These authors contributed equally to this work.

Abstract

DNA strand displacement has been widely used for the design of molecular circuits, motors, and sensors in cell-free settings. Recently, it has been shown that this technology can also operate in biological environments, but capabilities remain limited. Here, we look to adapt strand displacement and exchange reactions to mammalian cells and report DNA circuitry that can directly interact with a native mRNA. We began by optimizing the cellular performance of fluorescent reporters based on four-way strand exchange reactions and identified robust design principles by systematically varying the molecular structure, chemistry and delivery method. Next, we developed and tested AND and OR logic gates based on four-way strand exchange, demonstrating the feasibility of multi-input logic. Finally, we established that functional siRNA could be activated through strand exchange, and used native mRNA as programmable scaffolds for co-localizing gates and visualizing their operation with subcellular resolution.

“Biocomputers” able to sense, analyze and modulate molecular information in the cellular milieu would make a valuable contribution to medicine and biological research. Dynamic DNA nanotechnology has made important progress towards the goal of building such embedded cellular controllers by first establishing systematic methods for the design of complex molecular circuits that work reliably in *cell-free* settings¹. DNA realizations of Boolean logic circuits^{2–5}, finite state machines⁶, analog chemical reaction networks^{7,8}, linear control systems⁹, or neural networks¹⁰ have all been demonstrated. DNA

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*To whom correspondence should be addressed: ; Email: philip.santangelo@bme.gatech.edu or ; Email: gseelig@uw.edu

Author Contributions

P.J.S. and G.S. conceived the project, B.G., Y-J.C., C.Z., S.P., P.J.S. and G.S. designed experiments, B.G., Y-J.C., C.Z., and S.P. performed the experiments, J.L.K. and P.S. developed the monovalent MTRIPs, B.G., Y-J.C., C.Z., analyzed the data, B.G. Y-J. C., C.Z., P.J.S. and G.S. wrote the paper.

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No competing financial interest

nanotechnology has also resulted in molecular sensors and amplifiers^{11–15} that could provide inputs to such circuits, as well as molecular motors^{16–21} and switchable nanostructures^{19,22} that could be controlled by them.

Recent work has begun to demonstrate that DNA nano-devices can be compatible with complex biological environments²³. For example, molecular probes based on the hybridization chain reaction have enabled RNA imaging in fixed cells and tissues^{24,25}. A DNA nano-robot recognized cell surface markers on live cells and directed the delivery of a molecular payload to a subpopulation of cells²⁶. A similar nano-robot was also shown to be active in the bloodstream of live cockroaches²⁷. Antibody-guided DNA circuits were similarly applied for the analysis of plasma membrane targets²⁸. Delivery of complex DNA nanostructures to the interior of mammalian cells has been demonstrated^{29,30}, and intracellular DNAzyme-based logic gates and DNA pH sensors were shown to work reliably^{31,32}. Moreover, RNA-based structures reminiscent of DNA tiles have been expressed and assembled inside of bacteria³³. Recent work even suggested that DNA logic gates can detect microRNA in living cells³⁴. However, a systematic understanding for how to adapt DNA nanodevices to the cell is still missing.

To recreate in cells the full diversity of cell-free dynamic DNA devices there is a need to establish the design parameters that render intracellular systems as “engineerable” as their *in vitro* counterparts. Here, we address this challenge for DNA circuitry that relies on strand displacement and exchange reactions, which, owing to their simplicity, underlie the vast majority of dynamic DNA nano-devices. We focus on logic gates suitable for 4-way strand exchange which minimize crosstalk with other nucleic acids in complex environments because of the predominately double-stranded nature of components^{25,35}. Gates are chemically synthesized and, like siRNAs or antisense oligonucleotides, are transiently delivered to mammalian cells rather than genetically encoded and expressed within cells. For initial characterization experiments both the logic gate and inputs are exogenous since this approach provides a degree of quantitative control over all reactant concentrations.

To understand how design and delivery parameters affect gate operation (Fig. 1) we first characterized the effect of gate architecture (i.e the length and spatial arrangement of single and double-stranded domains) and chemical composition. Then, we compared different delivery methods that permit the observation of gate activation in cells and quantitatively characterized the relationship between reaction yield and subcellular distribution of the reactants. Finally, we demonstrated that strand exchange-based components can interface with endogenous cellular machinery, such as RISC and native mRNA, laying the foundation for future therapeutic or diagnostic applications.

4-way strand exchange mechanism and *in vitro* characterization

In a 4-way strand exchange reaction (Fig. 2a), the two reactants (the reporter, which carried a quenched TYE665 fluorophore, and the input) are predominately double-stranded (domain *a*) with forked single-stranded “toeholds” (domains *1*, *2*). The single-stranded toehold domains on the input are complementary to those on the reporter (*1* with *1**, *2* with *2**); hybridization of the reporter and input toeholds initiates the reaction. The reaction then

proceeds via a branch migration, which results in the exchange of strands between the complexes^{36,37}. Successful completion of the reaction yields two fully double-stranded complexes, one labeled with the fluorophore (the active reporter) and the other with the quencher (the waste).

We first characterized reaction kinetics in cell free settings and found that both the strengths of the toehold and the length of the double-stranded domain are determinants of reaction rate (Supplementary Fig. 1)³⁸. We chose to move forward with complexes containing 6 nucleotide (nt) toeholds and 16 or 22 base pair (bp) branch migration domains, parameters that result in a good compromise between desired fast kinetics and high thermodynamic stability.

We then compared the efficiency of strand exchange with DNA probes to that of probes made from nucleic acids with phosphorothioate (PS) bonds and/or 2'-O-methyl (2'OMe) ribonucleotides, whose increased stability against degradation by nucleases renders them better suited for live cell studies^{39,40}. We limited the PS modifications to seven bases at both ends of each oligonucleotide (Supplementary Table 4)⁴¹. Reaction kinetics were similar with 2'OMe RNA and DNA probes, while PS bonds appeared to slow down the reaction rates (Supplementary Fig. 2), likely because of the reduced thermodynamic stability of toehold binding interactions.

Characterization of 4-way strand exchange in cells

To visualize strand exchange reactions in mammalian cells, we first optimized the delivery regime. We initially focused on lipid-based transfection because this approach is experimentally straightforward and results in high cellular concentrations of nucleic acids. Moreover, to minimize the complexity of the experiment we aimed to package the reporter and input independently but deliver them to cells simultaneously. To ensure that the reporter and input did not interact before entering the cells, we tested several transfection reagents *in vitro* for their ability to stably package and insulate their nucleic acid cargo. We found that only Lipofectamine 2000 (L2K) prevented the interaction between the input and reporter complex when the pre-packaged complexes were mixed in a test tube (Supplementary Fig. 3)⁴².

Experiments in CHO K1 cells showed that reporter activation strongly depended on the probe chemistry. A DNA system achieved ~1.5 fold activation after 6 hours, and PS DNA performed only marginally better. Replacing DNA with 2'OMe RNA made a substantial improvement, with reporter activation increasing to ~7-fold. Further modification of the 2'OMe RNA with phosphorothioate bonds decreased reporter triggering (Fig. 2b). Confocal images of CHO K1 cells fixed after the delivery of 2'OMe RNA probes revealed a similar trend (Fig. 2c).

Lengthening the incubation time to 22 hours produced qualitatively similar results, but the difference between 2'OMe RNA (~4.5 fold activation) and PS 2'OMe RNA (~3.5-fold activation) was less pronounced (Supplementary Fig. 4). This may be due to the presence of PS modifications, which enhance the stability of 2'OMe RNA against nuclease

degradation⁴³⁻⁴⁶ while simultaneously reducing the reaction rate (Supplementary Fig. 2). A time course experiment suggests that the reaction with PS 2'OMe RNA duplexes is indeed still ongoing at 6 hours (Supplementary Fig. 5).

Probes with 22 bp duplex domains yielded slightly lower signal than probes with 16 bp duplex domains at six hours but slightly higher signal at 22 hours (Supplementary Fig. 4), consistent with cell-free kinetics measurements (Supplementary Fig. 2). Inputs with orthogonal (scrambled) toehold or double-stranded domains failed to activate the reporter (Supplementary Fig. 6). We also confirmed that a 4-way strand exchange reaction could be used to quench a fluorophore rather than unquench it (Supplementary Fig. 7). Together, these experiments suggest that the reaction proceeds through the intended toehold mediated strand exchange mechanism.

We further verified that all probes were stable under endosome-mimicking buffer conditions (Supplementary Fig. 8). We also investigated the cytotoxicity associated with nucleic acid modifications^{40,44}. Cells remained largely viable after treatment, with the transfection reagent having the largest impact (Supplementary Fig. 9).

Next, we tested an alternative, sequential, transfection regime. Cells were transfected with the reporter, washed, and following a two hour incubation, were transfected with the input. This approach ensured that reactions occur within cells. Indeed, upon co-transfection, reactions may occur during uptake. Overall, reporter activation was similar to that observed upon co-transfection, but with reduced on/off ratios and less pronounced differences between nucleic acid chemistries (Supplementary Fig. 10).

Impact of the inter- and intra-cellular distributions of reactants

In both CHO K1 and HeLa cells, adding an equal molar ratio or even an excess of input never fully triggered the reporter (Supplementary Fig. 11). We hypothesized that this incomplete activation is due to an uneven distribution of input and reporter among cells, such that in many cells there is insufficient input to activate all of the reporters. To track the intracellular concentrations of reporter and input we added a constitutively active fluorophore to both complexes (TYE563 and ATTO488, respectively) and co-transfected cells as described. Cells with a higher input:reporter ratio exhibited an increased 'efficiency of reporter activation', calculated by dividing reporter activation by reporter concentration (Fig. 2d). The same trend was seen when the reporter and input were transfected sequentially (Supplementary Fig. 10c).

The efficiency of reporter activation may be further limited by heterogeneities in the subcellular distributions of the gates. Even if the reactants are present in equal amounts in a given cell, they can interact only if their distributions overlap. Analyzing confocal images, the reporter signal was observed in the cell nucleus, cytoplasm and endosomal granules for both 2'OMe RNA or PS 2'OMe RNA reporters, but the distribution was not uniform (Supplementary Fig. 12).

We therefore measured the co-localization (Manders overlap coefficient) of two orthogonal fluorescent duplexes in CHO K1 cells. Each duplex carried a constitutively active

fluorophore (ATTO 488 or TYE 665) and was delivered by co- or sequential transfections. As a control, we transfected duplexes that had been incubated together *before* being mixed with L2K. Co-localization of the complexes was strongly influenced by the transfection method, decreasing from ~40% in co-transfections, to ~5% in the sequential transfections (Fig. 2e). These results give a physical explanation for the differences we observed between the two delivery methods and highlight an important consideration specific to cell-based experiments.

OR and AND logic in cells

To test more complex 4-way strand exchange systems in cells we designed logic OR and AND gates (Fig. 3a). The OR gate consists of three strands, a long bottom strand and two shorter top strands. Dye and quencher labels are attached to the short top strands such that the labels are co-localized at the center of the structure when the gate is intact. The forked toeholds on either side of the gate engage with either input A or B; input A displaces the fluorophore strand from the OR gate, while input B displaces the quencher strand. Both reactions result in high fluorescence. In a cell-free setting, the reaction mechanism worked as designed for gates made of either 2'OMe RNA or PS 2'OMe (Supplementary Fig. 13).

In cells, a 2'OMe RNA-based OR gate did not exhibit the expected behavior. Specifically, addition of input B did not result in high final fluorescence. We hypothesized that the fluorescent end product of that reaction, a nicked 38 base pair duplex with forked overhangs on one end only, was susceptible to degradation by cellular nucleases that bypass shorter duplex domains. To solve this problem, we tested 2'OMe RNA OR gates and inputs that also included at least some strands with PS bonds. Although all of the combinations behaved as expected *in vitro*, in cells the complexes containing PS 2'OMe RNA strands were less efficiently activated, but appeared to have more uniform stability (Supplementary Fig. 14). We replaced one of the strands of the input B with PS 2'OMe RNA, increasing the stability of the longer double-stranded product while allowing the OR gate to be activated ~2.5-3 fold by either input alone or both together (Fig. 3a); confocal images revealed activated gates localized throughout the cells (Supplementary Fig. 15).

The AND gate is composed of two strands with a set of forked toeholds at the end opposite of the dye and quencher label (Fig. 3b). Integral to the gate design is the 'hiding' of the second set of toeholds (1* and 2*) in looped DNA. Two inputs must interact with the AND gate consecutively to trigger fluorescence. Only following the interaction of the first signal (input B) with the gate are these available for binding to the second signal (input A). AND logic gates built of either 2'OMe RNA or PS 2'OMe RNA functioned as designed in cell free settings (Supplementary Fig. 16 & 17).

We measured AND gate activation in cells using both flow cytometry and live-cell confocal microscopy (Fig. 3b & Supplementary Fig. 19). Addition of both inputs resulted in >3.5-fold activation, while only negligible activation occurred when only one input or inputs with scrambled toeholds were used (Fig. 3b, Supplementary Fig. 17 & 18).

Activation of an siRNA through strand exchange

Next, we demonstrated that strand exchange reactions could generate RNA substrates recognized by the endogenous RNAi machinery. In cells, such substrates originate as double-stranded structures, and siRNA activity is sensitive to both the size of the duplex and its chemical composition^{40,47}. We designed a 4-way strand exchange reaction that yields an active siRNA from two inactive complexes. To optimize the design of the initial (inactive) and final (active) complexes, we built a GFP-based sensor for siRNA activity and tested RNA-RNA, RNA-DNA, RNA-PS DNA, RNA-2'OMe RNA and RNA-PS 2'OMe RNA duplexes for their ability to knock down GFP (Fig. 4a). Only RNA was able to serve as fully functional guide strands (Fig. 4b) but only when the passenger strand was composed of PS 2'OMe RNA was siRNA activity completely lost (Fig. 4c).

Based on these results, the inactive starting complexes were composed of an RNA-PS 2'OMe RNA hybrid. Following a 4-way strand exchange reaction, a double-stranded RNA product was formed that could enter the RNAi pathway (Fig. 4d). CHO K1 cells were co-transfected with the inactive starting complexes and 24 hours later, they were transfected with the reporter plasmid. Cells were analyzed 48 hours post-transfection via flow cytometry (Supplementary Fig. 19). The siRNA activated through strand exchange was approximately as efficient in knocking down GFP as a pre-assembled siRNA of the same sequence, reducing the fluorescent reporter signal by more than 60% (Fig. 4e).

Use of endogenous mRNA as a scaffold for strand exchange reactions

Above, we measured interactions between large numbers of gate complexes. To observe reactions that occur predominately within the cytosol between smaller numbers of complexes, we adapted our strand exchange reporter to a method first developed by Santangelo *et al.*, which relies on interactions with endogenous mRNAs to concentrate weak fluorescent signals⁴⁸. The method is based on the use of multiply-labeled, tetravalent imaging probes (MTRIPs), which can target endogenous mRNAs in living cells^{48,49}. Native mRNA can be long lived, allowing probes time to react, and they are often aggregated into large mRNP complexes (~100 nm), concentrating the reactants within the cytosol, and making reaction products easier to discern from background. Thus, endogenous mRNAs can be used as scaffolding for nucleic acid-based circuits to facilitate and visualize strand exchange reactions within the cytosol.

We created monovalent MTRIPs (mMTRIPs), consisting of a neutravidin core combined with two types of 2'OMe RNA oligos, one that targets endogenous mRNAs, and the other modeled on the above strand exchange systems. The 'targeting' oligo, complementary to an endogenous mRNA sequence, is covalently bound to the neutravidin core; the four reporter gates are attached via the biotin-neutravidin linkage (Fig. 5a). mMTRIPs were delivered using streptolysin-O (SLO) mediated reversible permeabilization and activated with a separately delivered input, also via SLO. An advantage of this delivery method is the low level of cytotoxicity, making it amenable to sequential rounds of delivery without compromising cell viability, altering cell morphology or causing observable toxic effects^{48,50}.

We first compared the ability of mMTRIPs to target endogenous β -actin (ACTB) mRNA, c-myc mRNA or poly-adenylated (polyA+) mRNAs in A549 cells with that of the classical MTRIPs, and chose ACTB mRNA as a scaffold to observe reporter activation (Supplementary Fig. 20 & 21). We delivered mMTRIPs targeting five separate sequences of ACTB mRNA in A549 cells followed by either 1x or 3x of the input. We initially used this approach to observe the effect of sequential deliveries in a simple “capture” assay, where fluorescently labeled single strands used in place of the reporters were quenched approximately 7-fold by the addition of a complementary oligonucleotide carrying the quencher (Supplementary Fig. 22). Then we measured reporter activation in a 4-way strand exchange system, where the addition of inputs increased reporter signal by ~2-fold (Fig. 5b). Inputs with scrambled toehold domains failed to elicit a response (Supplementary Fig. 23). Importantly, when neutravidin-reporter complexes lacking the mRNA-targeting oligos were delivered to cells, no statistically significant activation was detected (Fig. 5c).

Scaffolding AND logic on endogenous mRNA

We next tested whether this approach was compatible with the AND gate design described above. We used mMTRIP-AND gates targeting three sites on endogenous ACTB mRNA. Only in the presence of both inputs A and B did we observe a ~2 fold activation of the AND gates, while only negligible activation occurred when a single input was used or in the presence of inputs with scrambled toeholds (Fig. 6). The use of 1x molar excess of inputs was not sufficient to detect reporter activation (data not shown). It should also be noted that the incubation time following the second delivery had to be reduced to 30 minutes to limit non-specific activation of the reporter.

Conclusion

In this work, we established design principles and experimental approaches that provide a path for adapting dynamic strand displacement devices to the cellular environment. We focused on the 4-way strand exchange mechanism and systematically explored how the interplay of molecular structure, chemistry and delivery method determines the performance of strand exchange-based logic gates and reporters in cells.

We found the chemical composition of the nucleic acid complexes to be a key determinant of system function. Among the modifications tested, 2'OMe RNA resulted in the highest degree of gate activation while also having minimal cytotoxicity. Moreover, we demonstrated that hybrid complexes consisting of RNA and PS 2'OMe RNA strands could be used to control the activation of a siRNA, thus connecting a bio-orthogonal system with a signal that is interpretable by the cell. However, we also observed that the interplay between gate structure and chemical modifications could affect performance in unforeseen ways. For example, we had to integrate PS modifications into one input used in the OR gate experiments in order to ensure correct operation. Thus, although the systematic use of 2'OMe RNA provides a good starting point for building strand exchange systems that function in cells, additional optimization is required on a case-by-case basis in order to properly control interactions with the cell.

The delivery method strongly influenced performance by controlling the distribution of nucleic acid devices between and within cells. Co-delivery of nucleic acids resulted in high cellular concentrations of the nucleic acid cargo as well as a high fold-change in reporter activation, but reactions likely occurred in endosomes during nucleic acid uptake. Delivering reactants sequentially with lipofectamine ensured that they only encounter one another in the cell. Not surprisingly, this approach resulted in a lower degree of co-localization and, consequently, lower fold-changes of activation. Sequential SLO delivery ensured that the nucleic acids are delivered directly into the cytosol, thus facilitating interactions with endogenous mRNA. However, the resulting nucleic acid concentrations in the cell are considerably lower than those achievable with lipid based delivery. To experimentally observe reporter activation, we co-localized multiple reporter gates on a native mRNA scaffold.

By characterizing elementary reporters and logic gates in cells and, importantly, demonstrating that such devices can be controllably interfaced with cellular mRNA, we have laid a foundation for the rational design of strand exchange-based control circuitry for mammalian cells. We believe that the unique advantages of strand exchange systems – e.g. the small nucleotide footprint, the feasibility of rational design at the sequence level and the ease with which additional, orthogonal instances of any device can be created simply by changing the sequence – will make them an important complement to existing approaches in RNA-based synthetic biology.

Methods

Cell free kinetics assays

All *in vitro* kinetics experiments were performed in Tris-acetate-EDTA buffer containing 12.5 mM Mg^{2+} (1x TAE/ Mg^{2+}) at 37°C or PBS. Kinetics measurements were performed in a total 600 μ l on a spectrofluorimeter (SPEX Fluorolog-3, Horiba) with 0.875 mL synthetic quartz cells (Starna catalog number 23-5.45-S0G-5). Excitation and emission values for different fluorophores were set as follows: ROX (588 nm/ 608 nm), Cy5 (648 nm/ 668 nm), TYE665 (645 nm/ 665 nm). The integration time was 10 sec for all experiments for every 60 sec time-point. A non-reactive 20 nucleotide poly-T “carrier” strand at the concentration of 1 μ M was used in all reactions to reduce non-specifically binding to pipette tips.

Cell lines and cell culture

CHO K1 cells were cultured in MEM/alpha media (Corning) + 10% FBS (Hyclone), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). HeLa cells and A549 cells were cultured in DMEM (Lonza) with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen). For flow cytometry experiments, cells were plated at 50,000 cell/well in 24 well plates. For microscopy, cells were plated on glass coverslips one day prior to experiments.

Co-transfection

When transfecting <150 nM of the indicated nucleic acid complex, 2 μ l/well of Lipofectamine 2000 (L2K; Life Technologies), was used, while 3 μ l was used when

transfecting 150 nM. In all cases, 1x refers to a 50 nM final concentration. Each complex was prepared with L2K independently and the resulting L2K/nucleic acid mixtures were then added to the cells together. Transfection were performed in pre-warmed Opti-MEM without phenol red. Transfections were allowed to proceed for 4 hours, following which cells were washed twice with 1x PBS. Finally, 500 μ l of MEM/alpha media + 10% FBS was added and the cells were incubated for an additional 2 hours.

Sequential transfection

The same ratios of transfection reagent to nucleic acid were used for co-transfection but but Lipofectamine RNAiMAX (Life Technologies) instead of L2K because of its lower toxicity. Transfection mixtures were prepared as above; the first transfection mixture was added and allowed to incubate with the cells for 2 hours. The cells were then washed twice with 1x PBS and the second transfection was performed. After proceeding for 2 hours, cells were washed twice again with 1x PBS and MEM/alpha media + 10% FBS was added. A final two hour incubation was performed before measurement.

Plasmid DNA transfection

Plasmid DNA was transfected using Lipofectamine LTX (Life Technologies). 500 μ g/well of plasmid DNA was used, along with 2 μ l of Lipofectamine LTX. Transfections were allowed to proceed for at least 6 hours. Measurements were taken the following day.

SLO delivery

mMTRIPs were sequentially delivered into A549 cells using reversible membrane permeabilization. Briefly, 4U/ml Streptolysin O (SLO) (Sigma) were first reduced using 7.5mM Tris (2-carboxyethyl) phosphine (TCEP) (Pierce) for 1 hr at 37°C. Cells were rinsed using PBS ($-\text{Ca}^{2+}$ $-\text{Mg}^{2+}$) (Thermo) and then incubated with delivery medium containing 0.4U/ml SLO and probes at 15 nM in OptiMEM (Gibco) for 10 min at 37°C. The delivery medium was then removed and replaced with DMEM for recovery for 1h. 1x or 3x molar excess of the appropriate input gate was subsequently delivered using SLO. Cells were fixed using 4% paraformaldehyde and nuclei were stained with DAPI and mounted for imaging.

siRNA sensor plasmids

The plasmids used in this study were created using the Gibson cloning method. Each plasmid contains a pUC1 origin, ampicillin resistance marker, hygromycin marker (without a promoter), H2B-mCherry driven by the CMV promoter, and pEF-1 α driving H2B-citrine with a modified version of the *mus musculus* Vamp3 3'UTR into which was inserted a target site for one or the other possible siRNA products. We independently created target plasmids for both possible mature siRNA that could result from the initial duplex, since either strand could potentially be inserted into the RISC complex. Maps are available as GenBank files online.

mMTRIP probes

Monovalent MTRIPs (mMTRIPS) consist of a Neutravidin core bound to an mRNA-targeting ligand via an aromatic hydrazine and aldehyde linkage (Hynic-4FB, Solulink) and

to four biotinlated and fluorescently labeled reporter oligonucleotides. The mRNA-targeting ligand – containing a 5' thiol modification (Biosearch Technologies) – are first conjugated to a 30x molar excess of maleimide HyNiC crosslinker (MHPH, Solulink). NeutrAvidin (Sigma) is labeled with 4FB groups (S-4FB, Solulink) to achieve a ratio of 2 4FB groups per molecule, as quantified by colorimetric reaction with 2-hydrazinopyridine (Solulink) by UV-Vis (Abs=350). The oligonucleotides and NeutrAvidin are separately filtered and buffer exchanged then combined according to the manufacturer protocol. The concentration of the resulting modified NeutrAvidin is quantified using a BCA Protein Assay Kit (Pierce). The reporter oligonucleotides contain a 5'-biotin modification and one dT-C6-NH₂ modification for conjugation with Cy3B-NHS ester (GE Healthcare) using manufacturer protocols. Free dye was removed using 3kD Nanosep spin columns (Pall Corp.). The purified ligands were resuspended in 1xPBS and mixed at a 5:1 molar ratio with the modified NeutrAvidin for 1 hour at RT. Free ligands were removed using 30 kD Nanosep spin columns.

Flow cytometry

To detach cells, each well was washed once with ~500 µl 1x PBS then added 100 µl of 0.25% trypsin + EDTA were added. Cells were incubated for ~5 min and the trypsin quenched using 200 µl 1x PBS + 2% FBS. Cells were triturated several times before being run on an Accuri C6 cytometer (BioRad). Gates were drawn using FSC-H vs. FSC-A and were kept the same throughout the experiments.

Confocal microscopy of fixed cells

In all imaging experiments where the gates were introduced using the Lipofectamine 2000 transfection reagent, cells were imaged using a laser scanning confocal microscope Zeiss LSM 700 with a 40x objective 1.3NA. Laser combinations were as follows: DAPI/TYE665 = 405/639 for two-color simultaneous acquisition, DAPI/Alexafluor 488/TYE665 = 405/488/639 for co-localization measurements. In all cases we used the ZEN 2012 “best compromise” feature that optimizes filters for maximum acquisition speed with minimum crosstalk. mMTRIP AND gates were imaged using a Zeiss Axiovert 200 M microscope with a Perkin Elmer Ultraview spinning disk confocal using a 63xNA 1.4 Plan Apochromat primary objective and a Hamamatsu Flash 4.0v2 camera.

Fluorescence imaging of fixed cells

Images for 4-way experiments were taken on a Zeiss Axiovert 200M epifluorescence microscope with a 63x NA 1.4 Plan Apochromat primary objective and a Hamamatsu ORCA-ER AG camera. The Chroma Sedat ET filter set was used. All imaging experiments were performed using the Volocity acquisition software (Improvision, PerkinElmer). Image z-stacks were acquired in 200nm steps. All images were deconvolved using the iterative algorithms in Volocity. Images are extended views, which compress all of the imaging planes in the z direction into one plane. The mean sum of intensity was used to determine changes in fluorescence intensity in all experimental conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Zhang DY, Seelig G. Dynamic DNA nanotechnology using strand-displacement reactions. *Nature Chem.* 2011; 3:103–113. [PubMed: 21258382]
2. Qian L, Winfree E. Scaling up digital circuit computation with DNA strand displacement cascades. *Science.* 2011; 332:1196–1201. [PubMed: 21636773]
3. Seelig G, Soloveichik D, Zhang DY, Winfree E. Enzyme-free nucleic acid logic circuits. *Science.* 2006; 314:1585–1588. [PubMed: 17158324]
4. Benenson Y, Gil B, Ben-Dor U, Adar R, Shapiro E. An autonomous molecular computer for logical control of gene expression. *Nature.* 2004; 429:423–429. [PubMed: 15116117]
5. Elbaz J, et al. DNA computing circuits using libraries of DNAzyme subunits. *Nature. Nanotech.* 2010; 5:417–422.
6. Benenson Y, et al. Programmable and autonomous computing machine made of biomolecules. *Nature.* 2001; 414:430–434. [PubMed: 11719800]
7. Chen Y-J, et al. Programmable chemical controllers made from DNA. *Nature. Nanotech.* 2013; 8:755–762.
8. Soloveichik D, Seelig G, Winfree E. DNA as a universal substrate for chemical kinetics. *Proc. Natl. Acad. Sci. U. S. A.* 2010; 107:5393–5398. [PubMed: 20203007]
9. Oishi K, Klavins E. Biomolecular implementation of linear I/O systems. *IET Syst. Biol.* 2011; 5:252–260. [PubMed: 21823756]
10. Qian L, Winfree E, Bruck J. Neural network computation with DNA strand displacement cascades. *Nature.* 2011; 475:368–372. [PubMed: 21776082]
11. Dirks RM, Pierce NA. Triggered amplification by hybridization chain reaction. *Proc. Natl. Acad. Sci. U. S. A.* 2004; 101:15275–15278. [PubMed: 15492210]
12. Yin P, Choi HMT, Calvert CR, Pierce NA. Programming biomolecular self-assembly pathways. *Nature.* 2008; 451:318–322. [PubMed: 18202654]
13. Zhang DY, Turberfield AJ, Yurke B, Winfree E. Engineering entropy-driven reactions and networks catalyzed by DNA. *Science.* 2007; 318:1121–1125. [PubMed: 18006742]
14. Seelig G, Yurke B, Winfree E. Catalyzed relaxation of a metastable DNA fuel. *J. Am. Chem. Soc.* 2006; 128:12211–12220. [PubMed: 16967972]
15. Turberfield AJ, et al. DNA fuel for free-running nanomachines. *Phys. Rev. Lett.* 2003; 90:118102. [PubMed: 12688969]
16. Venkataraman S, Dirks RM, Rothmund PW, Winfree E, Pierce NA. An autonomous polymerization motor powered by DNA hybridization. *Nature. Nanotech.* 2007; 2:490–494.
17. Omabegho T, Sha R, Seeman NC. A bipedal DNA Brownian motor with coordinated legs. *Science.* 2009; 324:67–71. [PubMed: 19342582]
18. Green S, Bath J, Turberfield AJ. Coordinated chemomechanical cycles: a mechanism for autonomous molecular motion. *Phys. Rev. Lett.* 2008; 101:238101. [PubMed: 19113596]
19. Andersen ES, et al. Self-assembly of a nanoscale DNA box with a controllable lid. *Nature.* 2009; 459:73–76. [PubMed: 19424153]
20. Yurke B, Turberfield AJ, Mills AP Jr, Simmel FC, Neumann JL. A DNA-fuelled molecular machine made of DNA. *Nature.* 2000; 406:605–608. [PubMed: 10949296]
21. Green SJ, Lubrich D, Turberfield AJ. DNA hairpins: fuel for autonomous DNA devices. *Biophys. J.* 2006; 91:2966–2975. [PubMed: 16861269]
22. Zhang DY, Hariadi RF, Choi HMT, Winfree E. Integrating DNA strand-displacement circuitry with DNA tile self-assembly. *Nature Commun.* 2013; 4:1965. [PubMed: 23756381]

23. Chen Y-J, Groves B, Muscat RA, Seelig G. DNA nanotechnology from the test tube to the cell. *Nature. Nanotech.* 2015; 10:748–760.
24. Choi HMT, et al. Programmable in situ amplification for multiplexed imaging of mRNA expression. *Nature Biotech.* 2010; 28:1208–1212.
25. Duose DY, et al. Configuring robust DNA strand displacement reactions for in situ molecular analyses. *Nucleic Acids Res.* 2012; 40:3289–3298. [PubMed: 22156404]
26. Douglas SM, Bachelet I, Church GM. A logic-gated nanorobot for targeted transport of molecular payloads. *Science.* 2012; 335:831–834. [PubMed: 22344439]
27. Amir Y, et al. Universal computing by DNA origami robots in a living animal. *Nature. Nanotech.* 2014; 9:353–357.
28. Rudchenko M, et al. Autonomous molecular cascades for evaluation of cell surfaces. *Nature. Nanotech.* 2013; 8:580–586.
29. Jiang Q, et al. DNA origami as a carrier for circumvention of drug resistance. *J. Am. Chem. Soc.* 2012; 134:13396–13403. [PubMed: 22803823]
30. Schüller VJ, et al. Cellular immunostimulation by CpG-sequence-coated DNA origami structures. *ACS Nano.* 2011; 5:9696–9702. [PubMed: 22092186]
31. Modi S, Nizak C, Surana S, Halder S, Krishnan Y. Two DNA nanomachines map pH changes along intersecting endocytic pathways inside the same cell. *Nature. Nanotech.* 2013; 8:459–467.
32. Kahan-Hanum M, Douek Y, Adar R, Shapiro E. A library of programmable DNAzymes that operate in a cellular environment. *Sci. Rep.* 2013; 3:1535. [PubMed: 23525068]
33. Delebecque CJ, Lindner AB, Silver PA, Aldaye F. a. Organization of intracellular reactions with rationally designed RNA assemblies. *Science.* 2011; 333:470–474. [PubMed: 21700839]
34. Hemphill J, Deiters A. DNA computation in mammalian cells: microRNA logic operations. *J. Am. Chem. Soc.* 2013; 135:10512–10518. [PubMed: 23795550]
35. Chen SX, Zhang DY, Seelig G. Conditionally fluorescent molecular probes for detecting single base changes in double-stranded DNA. *Nature Chem.* 2013; 5:782–789. [PubMed: 23965681]
36. Radding CM, Beattie KL, Holloman WK, Wiegand RC. Uptake of homologous single-stranded fragments by superhelical DNA. *J. Mol. Biol.* 1977; 116:805–824. [PubMed: 592402]
37. Thompson BJ, Camien MN, Warner RC. Kinetics of branch migration in double-stranded DNA. *Proc. Natl. Acad. Sci. U. S. A.* 1976; 73:2299–2303. [PubMed: 1065879]
38. Zhang DY, Winfree E. Control of DNA strand displacement kinetics using toehold exchange. *J. Am. Chem. Soc.* 2009; 131:17303–17314. [PubMed: 19894722]
39. Santangelo PJ. Molecular beacons and related probes for intracellular RNA imaging. *Wiley Interdiscip. Rev. Nanomedicine Nanobiotechnology.* 2010; 2:11–19. [PubMed: 20049827]
40. Bramsen JB, et al. A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity. *Nucleic Acids Res.* 2009; 37:2867–2881. [PubMed: 19282453]
41. Amarzguioui M, Holen T, Babaie E, Prydz H. Tolerance for mutations and chemical modifications in a siRNA. *Nucleic Acids Res.* 2003; 31:589–595. [PubMed: 12527766]
42. Afonin KA, et al. Activation of different split functionalities on re-association of RNA-DNA hybrids. *Nature. Nanotech.* 2013; 8:296–304.
43. Dias N, Stein CA. Antisense oligonucleotides: basic concepts and mechanisms. *Mol. Cancer Ther.* 2002; 1:347–355. [PubMed: 12489851]
44. Harborth J, et al. Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. *Antisense Nucleic Acid Drug Dev.* 2003; 13:83–105. [PubMed: 12804036]
45. Tyagi S. Imaging intracellular RNA distribution and dynamics in living cells. *Nature Methods.* 2009; 6:331–338. [PubMed: 19404252]
46. Lennox K, et al. Characterization of modified antisense oligonucleotides in *Xenopus laevis* embryos. *Oligonucleotides.* 2006; 16:26–42. [PubMed: 16584293]
47. Czauderna F, et al. Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucleic Acids Res.* 2003; 31:2705–2716. [PubMed: 12771196]

48. Santangelo PJ, et al. Single molecule-sensitive probes for imaging RNA in live cells. *Nature Methods*. 2009; 6:10–14.
49. Sivaraman D, Biswas P, Cella LN, Yates MV, Chen W. Detecting RNA viruses in living mammalian cells by fluorescence microscopy. *Trends Biotechnol*. 2011; 29:307–313. [PubMed: 21529975]
50. Lifland AW, Zurla C, Yu J, Santangelo PJ. Dynamics of native β -actin mRNA transport in the cytoplasm. *Traffic*. 2011; 12:1000–1011. [PubMed: 21518164]

Figure 1. Empirical design parameters determine in-cell performance

Decisions made at the design level, such as the choice of gate architecture, nucleic acids modifications and delivery method have a strong impact on reaction kinetics, stability against nuclease degradation and subcellular localization of strand exchange-based logic gates. In turn, these properties determine how well a logic gate can fulfill its intended function including the ability to controllably interface with native mRNAs or the RNAi pathway.

Figure 2. Strand exchange reactions work in mammalian cells

a. 4-way strand exchange reaction mechanism. Nucleic acid strands are depicted as lines, with a half-arrow denoting the 3' end. Short (6 nt), single-stranded toehold domains are numbered, while longer (16 bp) double-stranded domains are marked with letters. An asterisk indicates complementarity. 4-way strand exchange reactions are initiated when matching toeholds in different complexes hybridize. Branch migration then results in the formation of two new complexes and unquenching of a fluorophore (TYE665). **b.** Dependence of reporter activation on chemistry. Reporter and input were co-transfected into CHO K1 cells, and fluorescence was measured using flow cytometry. The reporter was transfected at 1x (50nM) and the control RNA or input at 3x. For each chemistry, the fluorescent values of reporter & input (+) are normalized to the inactive reporter & control RNA (-). Error bars represent the standard deviation of at least two experiments. **c.** Representative confocal images of 2'OMe RNA reporter activation in fixed CHO K1 cells. Signal intensity is represented by color, with blue signifying low intensity and red high intensity. Total fluorescent signal was measured using confocal images of at least 10 cells from each condition. The signal was normalized to the reporter & control RNA data. Scale bars are 15 μ m. **d.** Input and reporter concentrations were tracked using unquenched fluorophores (ATTO488 and TYE563, respectively). The signal intensity of the input and reporter gates from cells transfected with 1x of both gates is plotted along the x- and y-axes. The signal from reporter activation is normalized to the reporter signal (i.e. TYE665/TYE563) to give a measure of the efficiency of reporter activation. **e.** Two non-interacting 2'OMe RNA complexes labeled with TYE665 (red) and ATTO488 (green) were mixed before being incubated with L2K in the same tube (Pre-mixed; **i.**), mixed with L2K independently (Co-transfection; **ii.**), or mixed with RNAiMAX independently and added to cells separately with a two hour gap (Sequential transfection; **iii.**). Confocal images of fixed cells were used to determine the overlap coefficient of the two complexes in all conditions. Scale bars are 10 μ m. Error bars are standard deviation.

Figure 3. Strand exchange-based OR and AND logic gates work in mammalian cells

a. OR logic gate, b. AND logic gate. i. Gate design and reaction schematic. ii. Flow cytometry results. Cells were co-transfected with the indicated logic gate at 1x (50nM) and input (or control RNA) at 3x. The fluorescence signal was measured using flow cytometry. Left: Each histogram displays the cell counts versus fluorescence due to fluorophore labeling on the logic gate (the black line indicates the mean value). The inputs are indicated on the left, where less than two inputs are indicated a control RNA complex was added as well. Right: bar graphs of average fluorescence. Inputs are indicated on the x-axis. Error bars represent the standard deviation of at least two separate experiments. Quantitative analysis of confocal images is shown in Figures S16 & S19.

Figure 4. A functional siRNA can be activated through 4-way strand exchange

a. Nucleic acid hybrids were investigated for their ability to enter the RNAi pathway. Putative 'siRNAs' were tested against two sensor plasmids, one sensitive to the top – blue – strand (**b**) and the other to the bottom – green – strand (**c**). The columns in both graphs correspond to cells treated with a negative control siRNA, a positive control anti-GFP siRNA, a positive control active siRNA, either one of the two hybrid PS 2'OMe RNA/RNA complexes (along with a control siRNA) or both hybrid complexes. Twenty-four hours later, the cells were transfected with a plasmid encoding mCitrine bearing a target sequence for the siRNA in the 3'UTR. mCitrine fluorescence was assayed using flow cytometry after ~48 hours. Error bars represent the standard deviation of at least two experiments.

ACTB mRNAs (**b**) and un-targeted mMTRIPs (**c**). **i.** Details of the reactants. **ii.** Representative epifluorescence microscopy images. Images were deconvolved and analyzed to quantify the fluorescence signal of individual puncta and the mean fluorescence intensity was determined. **iii.** Mean fluorescence intensity, normalized to the quenched reporter. * indicates statistically different data according to Kruskal-Wallis One Way Analysis of Variance on Ranks. Scale bars are 10 μm . Error bars indicate one standard deviation.

Figure 6. ACTB mRNA-scaffolded mMTRIP AND logic gates work in cells

Cells were first transfected using SLO with three mMTRIP probes targeting three distinct sites on the ACTB mRNA, they were subsequently transfected with the indicated input species (“scr” stands for scrambled toehold). **a.** Representative confocal images of the mMTRIP AND logic gates in cells. **b.** Quantification of the signal from confocal microscopy images. The values of bars marked with a single asterisk are significantly different from those marked with two asterisks as determined by a Kruskal-Wallis One Way Analysis of Variance on Ranks. Scale bars are 10 μ m. Error bars are standard deviation. Microscopy data was collected from at least 10 cells.