DNA Nanostructures Coordinate Gene Silencing in Mature Plants

Huan Zhang[1,], Gozde S. Demirer[1,], Honglu Zhang[2,], Tianzheng Ye[1], Natalie S. Goh[1], Abhishek J. Aditham[1], Francis J. Cunningham[1], Chunhai Fan[3], and Markita P. Landry[1,4,5*]

[1] Department of Chemical and Biomolecular Engineering, University of California, Berkeley, CA 94720, USA.
[2] Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA.
[3] School of Chemistry and Chemical Engineering, Shanghai Jiao Tong University (SJTU), Shanghai, 200240, China.
[4] Innovative Genomics Institute (IGI), Berkeley, CA 94720, USA.
[5] Chan-Zuckerberg Biohub, San Francisco, CA 94158, USA.

Delivery of biomolecules to plants relies on *Agrobacterium* infection, biolistic particle delivery, and carbon nanotube-based DNA delivery (1, 2), the first of which is only amenable to DNA delivery . The difficulty in delivering functional biomolecules such as RNA to plant cells is due to the plant cell wall which is absent in mammalian cells and poses the dominant physical barrier to biomolecule delivery in plants. DNA nanostructure-mediated biomolecule delivery is an effective strategy to deliver cargoes across the lipid bilayer of mammalian cells (3) , however, nanoparticle-mediated delivery without external mechanical aid remains unexplored for delivery across the cell wall in plants.

Herein, we report a systematic assessment of different DNA nanostructures for their ability to internalize into cells of mature plants, deliver small interfering RNAs (siRNAs), and effectively silence a constitutively-expressed gene in *Nicotiana benthamiana* (Nb) leaves (4). We demonstrate that DNA nanostructures can be designed to internalize into plant cells through infiltration, and that siRNA can be controllably tethered to specific loci on the DNA nanostructures for effective gene silencing in *Nb* leaves. We show that siRNA delivered by DNA nanostructures silences a transgene more effectively than siRNA delivered alone. We further find that structural and mechanical properties (size, shape, compactness, and stiffness) of DNA nanostructures, and siRNA conjugation loci, affect not only nanostructure internalization into plant cells but also subsequent gene silencing efficiencies and pathways.

Our work demonstrates the feasibility of biomolecule delivery to plants with DNA nanostructures, and details both the design parameters of importance for plant cell internalization, and also assesses the impact of DNA nanostructure geometry for gene silencing mechanisms. These results further establish DNA nanostructures as a programmable toolset for the delivery of exogenous biomolecules such as siRNA to plants, and establishes guidelines for the design of DNA nanostructures for effective uptake into plant cells for various applications in plant biotechnology.

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A smart polymer for sequence-selective binding, pulldown and release of DNA targets

Elisha Krieg[1–5*], Krishna Gupta[4,6], Andreas Dahl[7], Mathias Lesche[7], Susanne Boye[4], Albena Lederer[4,5], William M. Shih[1–3*]

[1] *Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, USA*
[2] *Wyss Institute for Biologically Inspired Engineering at Harvard University, Boston, USA*
[3] *Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, USA*
[4] *Leibniz-Institut für Polymerforschung Dresden e.V., Germany*
[5] *School of Science, TU Dresden, Germany*
[6] *Biotechnology Center (BIOTEC), TU Dresden, Germany*
[7] *DRESDEN-concept Genome Center, Center for Molecular and Cellular Bioengineering, TU Dresden, Germany.*
**Correspondence should be addressed to E.K. (krieg@ipfdd.de) or W.M.S. (william.shih@wyss.harvard.edu)*

Selective isolation of DNA is crucial for applications in biology, bionanotechnology, clinical diagnostics and forensics. Despite the diverse variety of approaches, current methods have critical shortcomings that require trade-offs between material cost, ease of use, versatility and performance. To address this challenge, we have developed a smart methanol-responsive polymer (MeRPy) that can be programmed to bind and separate single- as well as double-stranded DNA targets [1]. MeRPy's development was inspired by SNAPCAR, a recently reported method for scalable production of kilobase-long single-stranded DNA [2]. MeRPy acts as a ready-to-use macroligand for affinity precipitation. Captured targets are quickly isolated and released back into solution by denaturation (sequence-agnostic) or toehold-mediated strand displacement (sequence-selective). The latter mode allows 99.8% efficient removal of unwanted sequences and 79% recovery of highly pure target sequences. We applied MeRPy for depletion of insulin cDNA from clinical next-generation sequencing (NGS) libraries. This step improved data quality for low-abundance transcripts in expression profiles of pancreatic tissues. Its low cost, scalability, high stability and ease of use make MeRPy suitable for diverse applications in research and clinical laboratories, including enhancement of NGS libraries, extraction of DNA from biological samples, preparative-scale DNA isolations [3], and sorting of DNA-labeled non-nucleic acid targets.

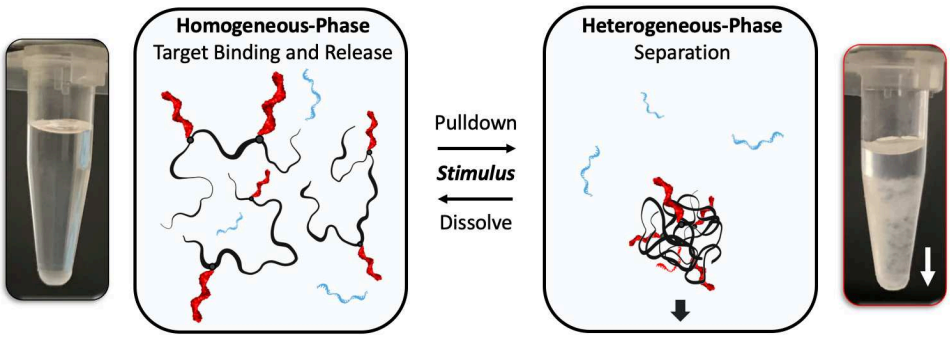

Figure 1: Methanol-responsive switching between homogeneous and heterogeneous phase enables MeRPy to rapidly bind, isolate and release DNA targets.

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Autonomous *in situ* generation of multi-stranded RNA complexes for synthetic molecular circuits

Wooli Bae[1], Thomas E. Ouldridge[1†], Guy-Bart V. Stan[1†]

[1] *Imperial College Centre for Synthetic Biology and Department of Bioengineering, Imperial College London, South Kensington Campus, SW7 2AZ, U.K.*

†Corresponding Authors. E-mail: t.ouldridge@imperial.ac.uk, g.stan@imperial.ac.uk

Abstract

Synthetic molecular circuits using DNA or RNA oligonucleotides can perform complex functions including logic-gate operations[1] and molecular computing.[2] In most synthetic molecular circuits, it is essential to have metastable complexes of oligonucleotide strands that can accept single-stranded input strands and give single-stranded output strands. Despite recent advances in methodology and theoretical concepts, realisation of oligonucleotide circuits *in vivo* still remains challenging. In part, this difficulty is due to a lack of methods to prepare their essential components – multi-stranded oligonucleotides complexes with designed sequences and domains – in a one-pot, autonomous process. Since neither RNA nor DNA polymerase can generate multi-stranded complexes directly, the primary method to produce such multi-stranded components for oligonucleotide circuits consists of synthesising each strand separately via chemical methods and then annealing the strands together. These individual complexes must then be mixed to demonstrate circuit function. Here we utilise naturally occurring self-digesting ribozymes to cut one long single-stranded RNA transcript into many short strands, forming multi-stranded complexes that are immediately ready to participate in strand displacement reactions, including multi-input multi-output reactions.

We first focus on engineering two hammerhead ribozymes (HHRs) from *chrysanthemum chlorotic mottle* viroid and *Schistosoma mansoni*, respectively. Each ribozyme exhibits cleavage yields close to ~95%, which is similar to previously reported studies. RNA oligonucleotides resulting from this cleavage can be displaced from their parent construct by an invading RNA strand, and subsequently trigger a DNA dye-quencher probe pair generating a fluorescence signal. Notably, this displacement process can be triggered and observed in real-time with RNA transcripts being generated by T7 RNA polymerases from DNA templates. We thus demonstrate strand displacement with inputs and gates produced autonomously and *in situ* for the first time.

Next, we designed a self-excising ribozyme module which consists of the two HHRs tested above. After successful cleavage reactions, this self-excising ribozyme module dissociates from the rest of the RNA transcript, leaving the multi-stranded RNA complex and its toehold ready to perform strand-displacement reactions without additional baggage. We found that our self-excising ribozyme module exhibits ~90% cleavage yield. Moreover, the self-excising ribozyme module spontaneously detaches from the rest of the RNA transcripts in non-denaturing conditions. After testing the self-excising ribozyme module, we designed a more complex RNA construct with two self-excising ribozyme modules (4 HHRs), which exhibited ~80% cleavage yield. This construct was used to implement a multi-input multi-output reaction where the second RNA output acts as an AND gate.

Overall, we show that HHRs can be used to generate multi-stranded complexes of RNA oligonucleotides directly from single transcription reactions. These RNA transcripts can be autonomously generated, processed and then used for strand displacement reactions, including complex multi-input multi-output reactions. Our design can be scaled and adapted to generate more complex sets of multi-stranded RNA oligonucleotides, which can serve as basis for the design of synthetic oligonucleotide circuits in live-cell environments.

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DNA Reaction-Diffusion Attractor Patterns

Phillip James Dorsey[a], Dominic Scalise[a], and Rebecca Schulman[a,b]

Living systems possess the ability to form and recover complex patterns in prescribed locations at length scales of hundreds of microns. In this study, we use oligonucleotide reaction networks, and leverage digital maskless photolithography and microfluidic delivery methods to program the shape of biomolecular attractor patterns in photopatterned poly(ethylene-glycol) diacrylate hydrogels. Linear and 'hill'-shaped DNA-based patterns formed and stabilized in 4 and 9 hours respectively. When patterns were perturbed in particular locations with varying dosages of UV light, they reformed their equilibrium profile over times ranging from 1 to 10 hours.

[a] P.J. Dorsey, D. Scalise, Prof. R. Schulman
Department of Chemical and Biomolecular Engineering
Johns Hopkins University
3400 N. Charles Street, Baltimore, MD 21218
Email: rschulm3@jhu.edu

[b] Prof. R. Schulman
Department of Computer Science
Johns Hopkins University
3400 N. Charles Street, Baltimore, MD 21218

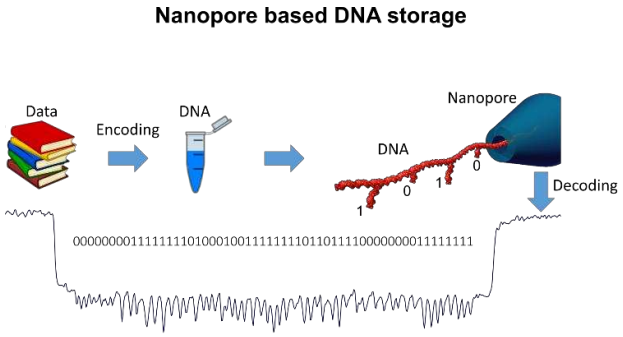
Solid-State Nanopore-Based DNA Data Storage and DNA Computing

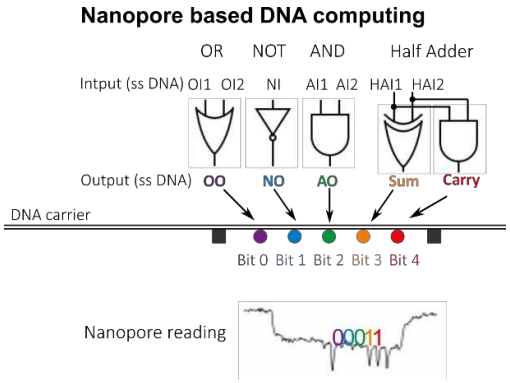
Kaikai Chen,[1] Jinglin Kong,[1] Jinbo Zhu,[1] Niklas Ermann,[1] Paul Predki,[2] and Ulrich F. Keyser[1*]

[1]Cavendish Laboratory, University of Cambridge, JJ Thomson Avenue, Cambridge, CB3 0HE, United Kingdom

[2]Iridia, Inc. 3156 Lionshead Ave, Suite 1, Carlsbad, CA 92010, USA

DNA is promising for information storage and processing due to the great potential for ultra-compact and long-term storage and highly efficient parallel computing. The challenge is the ease of access to the stored data and computing results. Here we demonstrate the storage and reading of up to 112-bit data on a double-stranded DNA molecule by encoding binary information into protruding DNA hairpins and decoding the information with nanopore measurements. This unit can be used for data storage containing address and data which allows for the libraries consisting of 2^{112} or 5×10^{33} different molecules to encode vast amounts of data. These molecules can be made by mixing oligos and read by solid-state nanopores, proving a potential alternative to the data storage in DNA sequence. Utilising DNA strand displacement we show writing, erasing and rewriting information on DNA strands demonstrating the flexibility of DNA for molecular storage. Finally, we show the potential of DNA nanotechnology integrated with nanopore sensing by reading multiple outputs from parallel DNA computing systems comprised of logic gates. Our method has the potential for miniature scale integration, interfacing with electrical measurements and paves the way for practical applications of DNA storage and DNA computing.





Email: kc494@cam.ac.uk, ufk20@cam.ac.uk

DNA Nanostructures that Self-Heal in Serum

Yi Li† and Rebecca Schulman†‡*

†Chemical and Biomolecular Engineering and ‡Computer Science, Johns Hopkins University, Baltimore, Maryland 21218, United States

*E-mail: rschulm3@jhu.edu

Keywords: DNA nanotubes; self-assembly; nuclease; self-healing; bionanotechnology

Abstract

Self-assembled DNA nanostructures have potential applications in therapeutics, diagnostics, and synthetic biology. A challenge in using DNA nanostructures in biological environments or cell culture, however, is that they may be degraded by enzymes found in these environments, such as nucleases. Such degradation can be slowed by introducing alternative substrates for nucleases, or by coating nanostructures with membranes or peptides. Here we demonstrate a means by which degradation can be reversed *in situ* through the repair of nanostructure defects. To demonstrate this effect, we show that degradation rates of DNA nanotubes, micron-scale self-assembled structures, are at least 4-fold lower in the presence of tiles that can repair nanotube defects during the degradation process. Micrographs of nanotubes show that tiles from solution incorporate into nanotubes, and that this incorporation increases nanotube lifetime to several days in serum. We use experimental data to formulate a simple model of nanostructure self-healing. This model suggests how introducing even a relatively low rate of repair could allow a nanostructure to survive almost indefinitely because of a dynamic equilibrium between microscale repair and degradation processes. The ability to repair nanostructures could thus allow particular structures or devices to operate for long periods of time and might offer a single means to resist different types of chemical degradation.

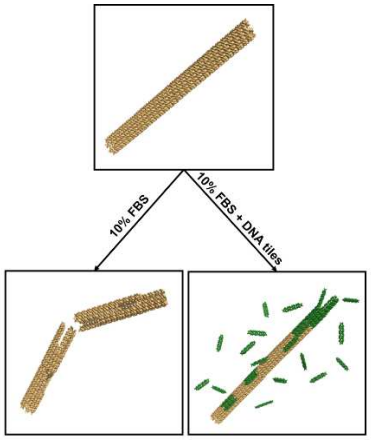


Figure 1. Schematic showing when DNA tiles that make up the DNA nanotubes are present in solution, damaged nanotubes can be repaired and have extended lifetimes.

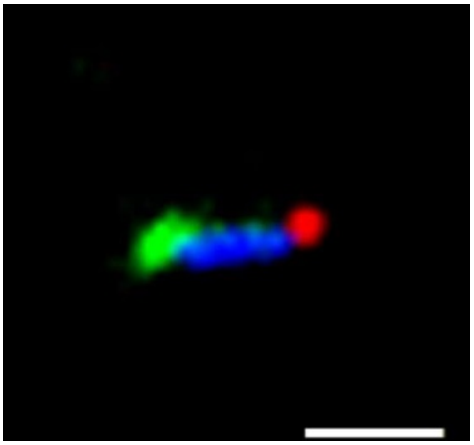


Figure 2. Monomer tiles (Cy3, green) can self-heal a DNA nanotube (atto647, blue); the DNA origami "seed" was used to create the nanotube. Scale bar, 2 μm.

Automated Design of Curved DNA Origami Nano-Capsules with Specific Shape and Variable Multilayer-Reinforced Rigidity

Daniel Fu[1], Raghu Narayanan Pradeep[2], Fei Zhang[2], John Schreck[3], Hao Yan[2], John Reif[1*]
[1] Department of Computer Science, Duke University, Durham, NC 27516, USA
[2] School of Molecular Sciences and Biodesign Institute, Arizona State University, Tempe, AZ 85287, USA
[3] Department of Chemical Engineering, Columbia University, New York, NY 10027, USA
* reif@cs.duke.edu

Within a decade of research, the shape variety of DNA origami nanostructures has become increasingly saturated, but the availability of structures has outpaced research into their usages. The design criteria of DNA origami must evolve to more heavily prioritize properties that were previously secondary to shape such as mechanical or biomimetic properties that would enhance lifetime or effectiveness in vivo or functionality in conjunction with other nanomaterials in situ. For instance, archetypal capsule structures, such as viral capsids or cell membranes, possess many beneficial properties to applications in nanoscience. Yet, despite its structural versatility, DNA origami has had limited success emulating capsule functionality. In addition, as a soft material, DNA nanostructures are susceptible to degradation and deformation, emphasizing a need for generalized structural reinforcement strategies to improve the robustness of structures for reliable usage.

We present a set of software modules that automate the design (and generate the staple sequences) for curved enclosing DNA origami nanostructures, which we term DNA Nano-Capsules, that are of axially symmetric 3-D geometry. Our software also includes the rapid design of multiple layer structures to explore reinforcement strategies for enhancing rigidity and shape accuracy. The resulting designs for our DNA Nano-Capsules are analyzed by molecular dynamics simulations provided by oxDNA as well as experiments to validate our methods and investigate the physical properties of the structures. Our results demonstrate consolidated design principles that can be applied to consistently create DNA Nano-Capsules and reinforced variants, and our subsequent investigations of reinforcement strategies show improvements in shape accuracy, mechanical rigidity, and formation of small or weak features for these complex DNA origami nanostructures.

The DNA Nano-Capsules resemble naturally occurring capsule structures, such as viral capsids or cell membranes, that are found widely in biology, and could have biomimetic applications similar to these natural counterparts. The stabilized cavity of the DNA Nano-Capsule may be more reliably used in downstream applications including drug delivery, cargo transport, or metallic nanocasting due to combined control over all of shape, structural rigidity, and membrane permeability.

Molecular program for the isothermal digital droplets detection of microRNA

Guillaume Gines[1], Roberta Menezes[2], Thomas Jet[2], Valérie Taly[2] and Yannick Rondelez[1]

[1] *Laboratoire Gulliver, CNRS, ESPCI Paris, PSL Research University (France)*

[2] *INSERM UMR-S1147, CNRS SNC5014, Paris Descartes University (France)*

Email: guillaume.gines@espci.fr

Molecular programming is an emerging discipline that involves the design of artificial biomolecular circuits. These architectures, capable of information-processing tasks, have been implemented in Boolean computations, reaction-diffusion systems or matter shaping and are now expected in biosensing applications. Here, we report a molecular program performing isothermal target-triggered signal amplification. As a proof of concept we demonstrate the ultrasensitive detection of microRNAs, short RNA strands involved in the regulation of gene expression and emerging as promising disease-specific biomarkers. We used a versatile molecular programming language named PEN-DNA toolbox (Polymerase Exonuclease Nickase Dynamic Network Assembly) [1],[2]. It employs a set of short oligonucleotides (template) encoding the topology of a network of reactions, which are catalyzed by a mixture of enzymes. Each template thus senses the presence of input strands an in turn produces output strands that act on other nodes of the system. The connectivity of the microRNA detection circuit is presented in Figure 1a: the universal signal amplification occurs in the form of a bistable node composed of two templates: an autocatalytic template (aT), made of a dual-repeat sequence catalyzing the exponential replication of a 12-mer oligonucleotide (trigger) via a polymerization/nicking process; and a pseudotemplate (pT) that absorbs the trigger produced by spurious reactions (leak) on the aT, essential to avoid nonspecific amplification [3]. Upon binding of the microRNA target, a converter template (cT) linearly produces a 12-mer strand, which in turn triggers the autocatalytic reaction on the aT. A reporting template (rT) captures the amplified trigger to produce a fluorescence signal. Real-time monitoring of the amplification reaction allows for the detection of microRNA concentration down to the femtomolar range (Figure 1b). The background-free amplification switch makes possible the detection of single molecules compartmentalized in water-in-oil droplets and thus enables digital absolute quantification (Figure 1c). In perspective, we present a multiplex extension of our digital detection platform, which uses the compartmentalization of DNA-grafted particles that are specific for each targeted molecule.

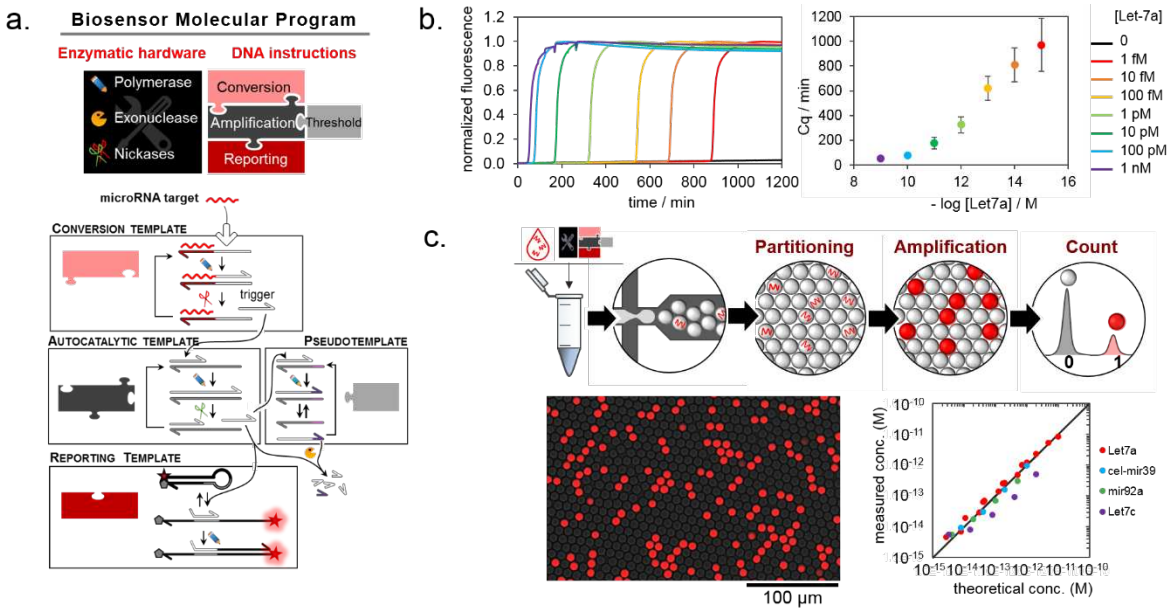


Figure1. Molecular programming for the digital detection of microRNA biomarkers. **a.** Molecular program design. **b.** Background-free detection of the microRNA Let7a. **c.** Droplet digital format enables absolute target quantification.

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Thioflavin-T as a Fluorogenic Small-Molecule Probe for DNA Circuits

Peter B. Allen*, Tulsi R. Damase, Md. Mamunul Islam, Mason Shipley

**pballen@uidaho.edu*

University of Idaho, Dept. of Chemistry, 001 Renfrew Hall, 875 Perimeter Dr, Moscow, ID 83844-2343

ABSTRACT: *DNA-DNA reactions can be monitored with a label-free fluorogenic reaction between a new DNA oligonucleotide reporter and the small molecule dye, thioflavin-T (TFT). Covalent dyes and quenchers are often used to monitor DNA-DNA reactions. This approach suffers from high price and purity requirements. Synthesis impurities and degradation products produce high background due to inefficient quenching. We present an alternative: oligonucleotides with specific sequences induce small molecules to become fluorescent. We discovered a novel DNA sequence that produces fluorescence upon binding to the widely available and inexpensive dye, TFT. We denote this oligonucleotide TFTSignal. We present data on the behavior and structural properties of TFTSignal. We use TFTSignal as a label-free reporter for the activity of several designed DNA-DNA reactions. The DNA-DNA reactions conditionally produce the TFTSignal oligonucleotide by association or by liberating the TFTSignal oligonucleotide from a double-stranded DNA duplex. This strategy offers label-free, cost-effective and sensitive fluorogenic detection of the molecular beacon reaction, split reporter reaction, one-step strand displacement reaction, and the entropy-driven amplifier reaction.*

Sequence selective activation of conditional small interfering RNAs by RNA transcripts in mammalian cells

Si-ping Han, [1, 2†*] Lisa Scherer,[2†] Ane Miren Salvador-Garicano,[4] Matt Gethers,[1] Marwa Ben Haj Salah, [2] Rebecca Mancusi,[2] Sahil Sagar, [2] Robin Hu, [2] Julia DeRogatis, [2] Ya-Huei Kuo, [3] Guido Marcucci,[3] Saumya Das,[4] John Rossi,[2*] and William A. Goddard III[1*]

[1]Materials and Process Simulation Center, California Institute of Technology, Pasadena, California
[2]Department of Molecular and Cellular Biology,
[3]Gehr Family Center for Leukemia Research[3], City of Hope, Duarte, California
[4]Cardiovascular Research Center, Massachusetts General Hospital, Boston, MA

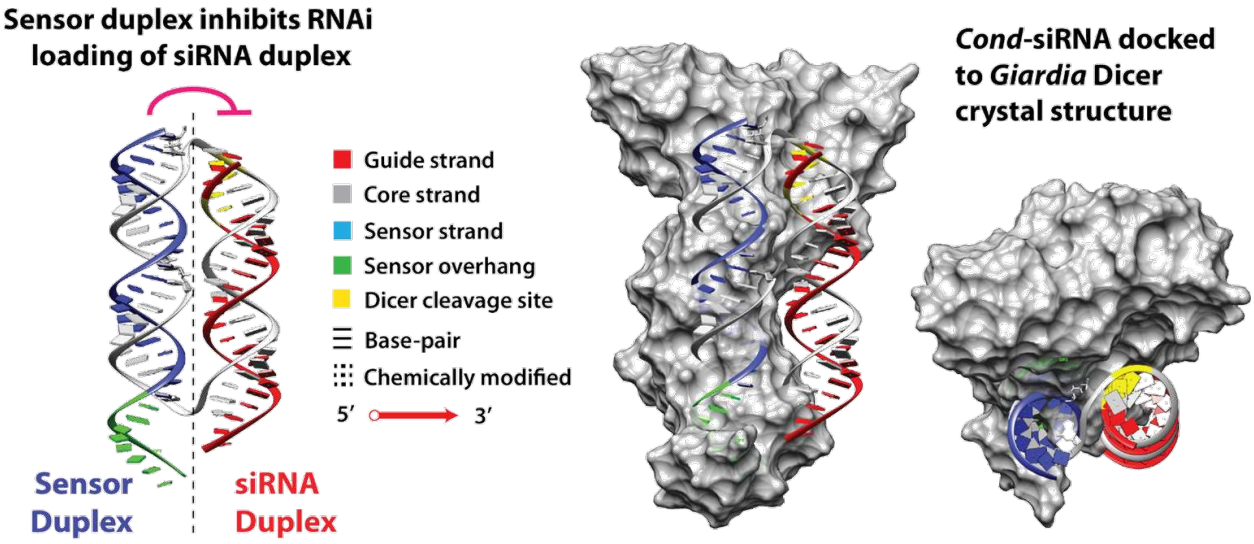


Figure 1: Basic design of *Cond*-siRNA, and docking to *Giardia* Dicer crystal structure showing steric hindrance of RNAi processing.

A long-term goal for dynamic nucleic acid nanotechnology is the algorithmic regulation of cellular biological activities using enzyme free nucleic acid circuits. In mammalian cells, strand displacement switches can currently read RNA transcripts or produce cell regulating output, but not both. Current challenges for connecting RNA detection with translational regulation include decoupling input and output sequences, ensuring correct device operation over biologically important durations (days) and device concentrations (<10 nM), and controlling the biological activities of output molecules such as small interfering RNAs (siRNA).

We have now developed a programmable, conditionally activated siRNA (*Cond*-siRNA). This device outputs an active siRNA against a target gene only upon detection of RNA transcripts from a different input gene. Optimized *Cond*-siRNAs display effective suppression of OFF state RNAi activity, sequence specific detection of RNA transcripts, and effective activation of RNAi silencing in a variety of mammalian cell lines for a number of different input: output sequence combinations.

We will discuss design principles and chemical modification patterns that enable strand displacement switches to operate with low background and effective performance in the mammalian cytosol and control RNAi. We will also present preliminary data on the biological activity of *Cond*-siRNAs in a primary cell line model of heart disease.

* wagoddard3@gmail.com, jrossi@coh.org, hansiping@gmail.com
†These authors contributed equally to this work

DNA Punch Cards: Encoding Data on Native DNA Sequences via Topological Modifications

S Kasra Tabatabaei[1], Boya Wang[2], Nagendra Bala Murali Athreya[3], Behnam Enghiad[5], Alvaro Gonzalo Hernandez[4], Jean-Pierre Leburton[3], David Soloveichik[2], Huimin Zhao[1,5,6], Olgica Milenkovic[3]

[1] Center for Biophysics and Quantitative Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois, 61801, USA,
[2] Department of Electrical and Computer Engineering, University of Texas at Austin, Austin, Texas, 78712, USA,
[3] Department of Electrical and Computer Engineering, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, USA,
[4] Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign, Urbana, Illinois, 61801, USA,
[5] Departments of Chemical and Biomolecular engineering, University of Illinois at Urbana-Champaign, Urbana, Illinois, 61801, USA,
[6] Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois, 61801, USA.

Recent implementations of synthetic DNA-based data storage systems have demonstrated several promising applications of macromolecular recorders. However, the proposed systems suffer from high cost, read-write latency and error-rates that render them non-competitive with traditional silicon-based devices. One means to avoid synthesizing DNA is to use readily available, naturally occurring DNA. As the nucleotide sequences of native DNA are fixed, they cannot be easily edited to accommodate arbitrary user-defined content. Hence, instead of changing the sequence content, one may adopt an alternative recording strategy -- akin to card punching -- that modifies the topology of native DNA to encode desired information. Here we report the first macromolecular storage paradigm in which data is written in the form of "nicks" at predetermined positions on the sugar-phosphate backbone of double–stranded native DNA. The platform accommodates parallel nicking on one or multiple "orthogonal" genomic DNA fragments, and paired nicking and disassociation for creating "toehold" regions that enable single-bit random access and strand displacement. Our approach also provides a large mass of inexpensive DNA that may be used for a large number of error-free readout cycles. As a proof of concept, we used the multiple-turnover programmable artificial restriction enzyme *Pyrococcus furiosus* Argonaute, *Pf*Ago, to punch both text and image files into the PCR products of *Escherichia coli* genomic DNA fragments *in vitro*. The encoded data was reliably reconstructed through sequence alignment and read coverage analysis without the use of error-control codes (**Figure 1**).

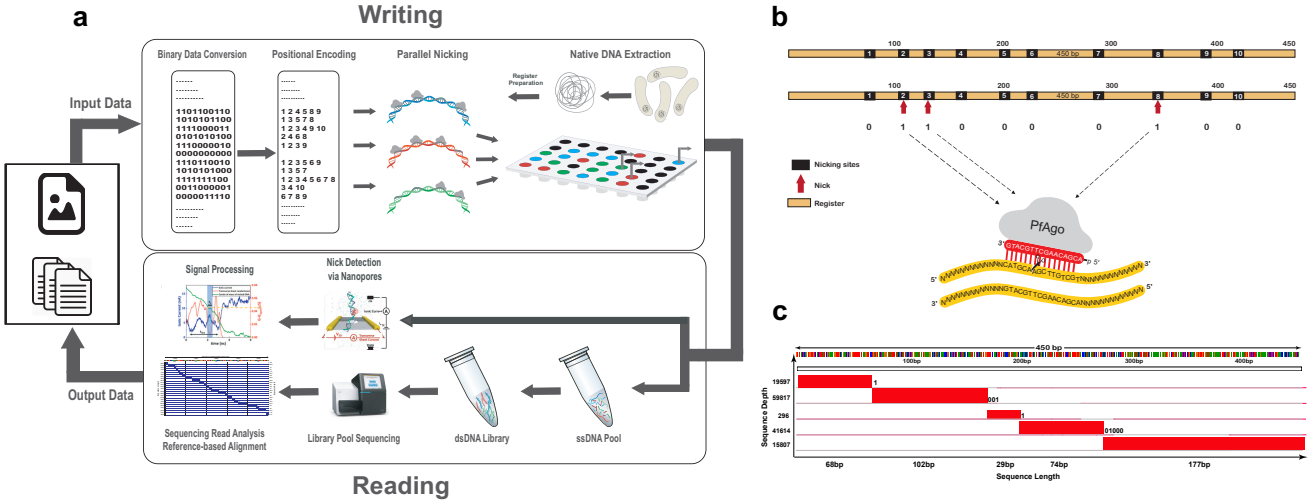


Figure 1| The native DNA-based data storage platform. **a)** In the **Write component**, arbitrary user content is converted into a binary message. The message is then parsed into blocks of *m* bits, where *m* corresponds to the number of nicking positions on the register (for the running example, *m* = 10). Subsequently, binary information is translated into positional information indicating where to nick. Nicking reactions are performed in parallel via combinations of *Pf*Ago enzymes and guides. In the **Read component**, nicked products are purified and denatured to obtain a pool of ssDNAs of different lengths. The pool of ssDNAs is sequenced via MiSeq. The output reads are processed by first performing reference-based alignment of the reads, and then using read coverage to determine the nicked positions. **b)** *Pf*Ago can nick several pre-designated locations. The chosen register is a PCR product of a 450 bp *E. coli* genomic DNA fragment with 10 pre-designated non-uniformly spaced nicking positions (**top**). The positional code 238 corresponds to the binary vector 0110000100 (**bottom**). **c)** The MiSeq read analysis of a randomly chosen 10-bit string. Based on the sequenced (retrieved) reads and their lengths, one can detect the nicked sites as well as the sites that were not nicked and hence, reconstruct the 10-bit string stored in each register; here: 1001101000.

Kinetic control of spontaneous nucleation in uniquely-addressed multifarious self-assembly

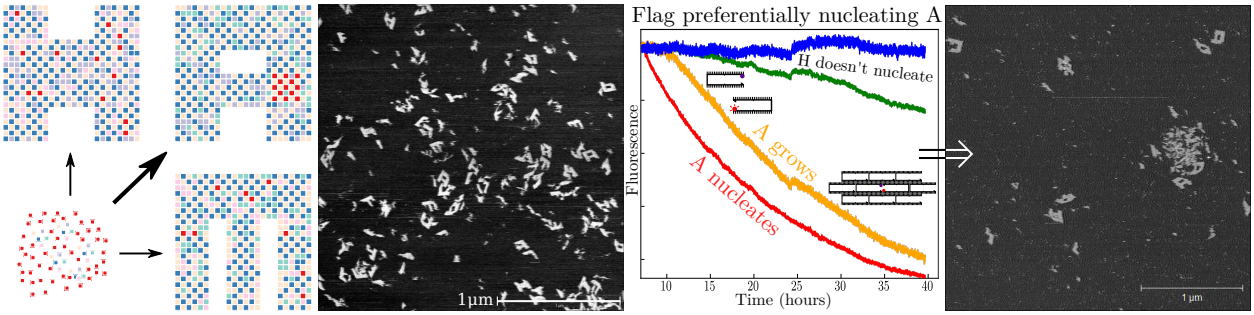
Constantine Glen Evans, Jackson O'Brien, Erik Winfree, Arvind Murugan

Uniquely addressable components can be programmed to assemble complex structures with high fidelity.[1] The highly-constraining specific interactions between these components usually code for a single desired structure as the unique equilibrium state of the system. However, in theory, a single set of components with promiscuous interactions can also assemble into multiple distinct structures, each containing the same components but in different geometrical arrangements and thus different neighborhood relationships.[2] The design of such multifarious systems is reminiscent of the training of a Hopfield Associative Memory[3] in the sense that defining the promiscuous interactions follows directly from the set of target shapes (memories), that this programs deep energy minima corresponding to those shapes (memories), and that randomness statistically ensures sufficient orthogonality that prevents stabilizing alternative shapes (memories) so long as not too many shapes (memories) are stored. Intriguingly, multifarious systems can—still in theory—perform pattern recognition, associating a pattern of component concentrations with nucleation of a target shape.[4]

Here we experimentally created a multifarious assembly mixture of 917 single-stranded DNA tiles with the ability to assemble three distinct structures, shaped as H, A and M, taking advantage of recently developed sequence design methods.[5] We then kinetically selected for the assembly of each of H, A and M, on command, by enhancing the concentrations of a few tiles that are spatially proximal in one structure (e.g., H) but are far apart in others (e.g., A or M). Such concentration patterns cannot change the relative equilibrium yields of these structures but can enhance nucleation rates for one select structure.

We confirmed such nucleation-based selectivity, using concentration profiles that select for each of H, A and M, by imaging the resulting mixture using Atomic Force Microscopy (AFM). To quantitatively track selective nucleation, we incorporated distinct fluorophore-quencher pairs in different parts of the structures H, A and M. We confirmed selective nucleation of a target structure (e.g., H) but not others (A or M) using the quenching of specific fluorescence signals.

Thus, through a combination of fluorescence signals during an anneal and AFM imaging after, we confirmed kinetically-controlled selective assembly of any one of three distinct structures from one set of tiles. Further, these structures were assembled in response to concentrations patterns of tiles present in all three structures. Our work shows the potential for multifarious self-assembly to serve as a nucleation-based Hopfield Associative Memory that recognizes concentration patterns.



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Modular design of functional RNA origami scaffolds

Cody Geary[1,2], Guido Grossi[1], Ebbe S. Andersen[1], Paul W.K. Rothemund[2]

1. Interdisciplinary Nanoscience Center and Department of Molecular Biology and Genetics, Aarhus University, Aarhus C, 8000, Denmark.
2. Bioengineering, Computer Science, and Computation and Neural Systems, California Institute of Technology, Pasadena, CA 91125, USA

Single-stranded RNA origami is an architecture enabling the design of genetically expressible RNA scaffolds that assemble cotranscriptionally. Through the addition of RNA aptamer modules, such scaffolds can control the relative position and orientations of small molecules and proteins in three dimensions (3D). The design of large and complex RNA origami has been limited by a lack of modeling and design tools. Here we expand the complexity of RNA origami architecture, and provide software to automate the 3D modeling of structures and thermodynamic design of sequences. This enables the scale and diversity of RNA origami scaffolds to be greatly extended, as exemplified by a series of 14 different shapes of increasing size and complexity. Nanometer-scale positioning of small-molecule and protein binding modules is demonstrated using fluorescence energy transfer for both light-up aptamers and fluorescent proteins. This study opens the door for the modular design of functional RNA-scaffolded systems ranging from signaling pathways to enzyme cascades.