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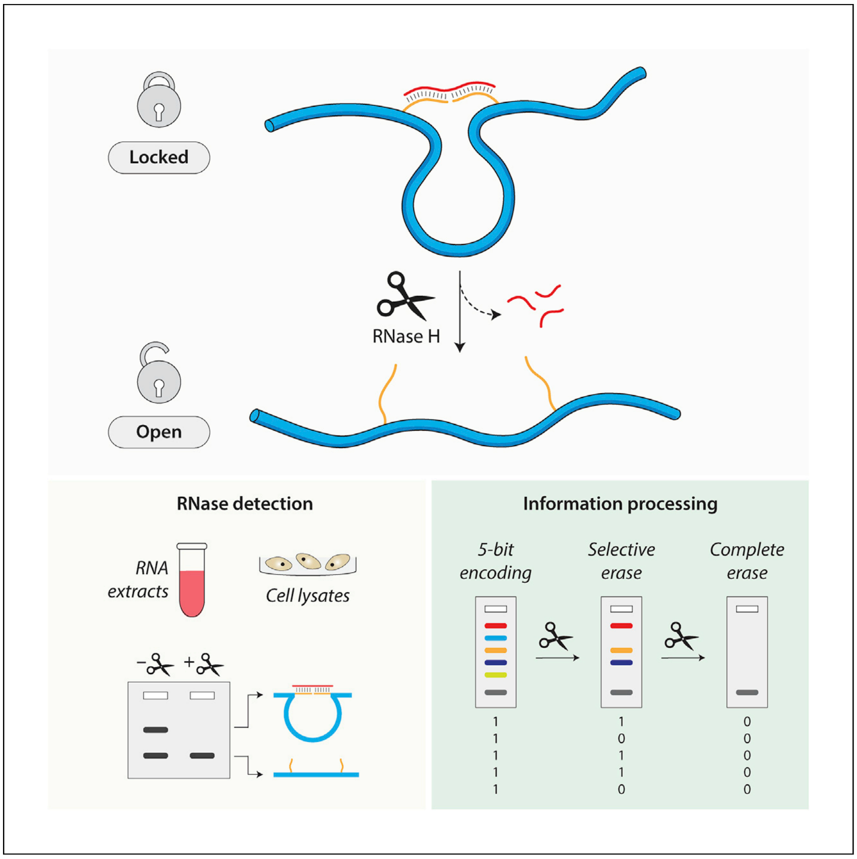
Ribonuclease-Responsive DNA Nanoswitches



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HIGHLIGHTS

DNA nanoswitches are useful tools in biosensing and molecular computation

DNA nanoswitches locked by an RNA strand are used in detecting RNase H activity

Looped nanoswitches act as "bits" for encoding information, with RNase-mediated erasing

Chandrasekaran et al. use reconfigurable DNA nanoswitches to detect ribonuclease (RNase) activity. Using RNase H as a molecular eraser, they also demonstrate ribonuclease-operated information processing using a combination of DNA and RNA inputs. The simplistic mix-and-read nature of this assay could facilitate its use in identifying RNase contamination in biological samples or for screening of RNase inhibitors.

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Report

Ribonuclease-Responsive DNA Nanoswitches

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SUMMARY

DNA has been used in the construction of dynamic DNA devices that can reconfigure in the presence of external stimuli. These nanodevices have found uses in fields ranging from biomedical to materials science applications. Here, we report a DNA nanoswitch that can be reconfigured using ribonucleases (RNases) and explore two applications: biosensing and molecular computing. For biosensing, we show the detection of RNase H and other RNases in relevant biological fluids and temperatures, as well as inhibition by the known enzyme inhibitor kanamycin. For molecular computing, we show that RNases can be used to enable erasing, write protection, and erase-rewrite functionality for information-encoding DNA nanoswitches. The simplistic mix-and-read nature of the ribonuclease-activated DNA nanoswitches could facilitate their use in assays for identifying RNase contamination in biological samples or for the screening and characterization of RNase inhibitors.

INTRODUCTION

Dynamic DNA nanotechnology has yielded a variety of DNA devices and switches that can reconfigure in response to stimuli.[1] Such programmed conformational changes have been used in biosensing,[2] in mechanical motions,[3] and in directing site-specific chemical reactions.[4] Through chemical functionalities, DNA nanostructures have been designed to react to triggers such as light,[5] pH,[2] temperature,[6] ionic conditions,[7] and biological stimuli such as nucleic acids.[8] We have previously developed DNA nanoswitches that undergo a conformational change in response to an external trigger. These nanoswitches have been used for sensing nucleic acids and proteins,[9–13] biomolecular interaction analysis,[14] single-molecule experimentation,[15,16] and molecular memory.[17–19] The DNA nanoswitch is a long DNA duplex made from a single-stranded M13 (7,249 nt) and short complementary backbone oligos (Figures 1A and S1). Specific DNA[9] and RNA[10] sequences can be targeted by incorporating two complementary single-stranded extensions on the nanoswitch, causing target binding to reconfigure the nanoswitch to a looped ''locked'' state. The open and locked states of the DNA nanoswitch can be easily visualized on an agarose gel. Here, we report DNA nanoswitches that can be triggered by ribonucleases (RNases), and demonstrate two potential applications: (1) detecting RNase activity and (2) in ribonuclease-operated information processing encoded by DNA.

In contrast to our previous work with DNA nanoswitches, here, we use a signal-off strategy based on RNase-triggered reconfiguration of DNA nanoswitches. To accomplish this, we pre-hybridized the DNA nanoswitch with an RNA lock strand, forming a locked nanoswitch (Figure 1A, locked looped state). For proof-of-concept, we chose RNase H, a ribonuclease that specifically catalyzes the hydrolysis of RNA in an RNA-DNA duplex.[20] On the addition of RNase H, the RNA lock is digested, leading to the release of the DNA latches and thus opening of the nanoswitch (Figure 1B).

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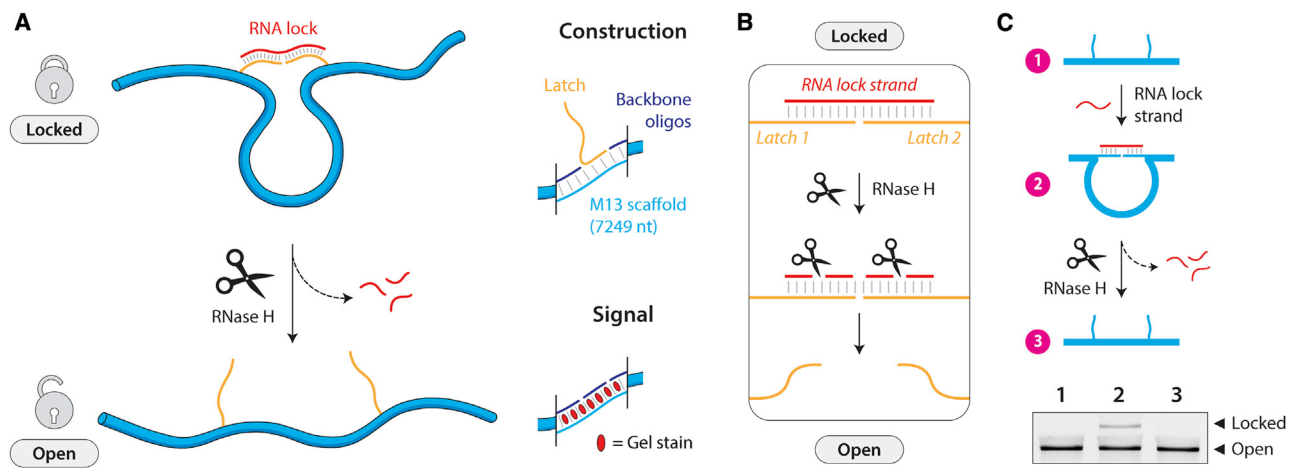

Figure 1. DNA Nanoswitch Design and Operation

(A) The nanoswitch is locked in a looped conformation with a pre-hybridized RNA lock strand. On the addition of RNase H, the lock strand is digested, resulting in unlooping the nanoswitch to the open state.

(B) Mechanism of cleavage of the RNA lock strand by RNase H and release of the DNA latches.

(C) The DNA nanoswitch (1) is locked by an RNA lock strand into a looped configuration (2). RNase H causes cleavage of the RNA lock, causing the nanoswitch to unlock and reconfigure into the linear state (3). This conformational change can be read out on an agarose gel to detect the presence of RNase H (inset).

This DNA nanoswitch conformational change provides a direct gel-based readout of the RNase H activity (Figure 1C). The signal is provided by the intercalation of thousands of dye molecules (from regular DNA gel stains) into the nanoswitch, thus providing a huge signal, even for a single molecular event such as enzymatic cleavage of an RNA strand (Figure 1A, inset). The use of a long scaffold DNA such as the M13 provides higher numbers of intercalation sites for these DNA stains and provides an enhanced signal compared to shorter scaffolds.

RESULTS AND DISCUSSION

DNA Nanoswitches for RNase Detection

For our first application, we demonstrate the detection of RNase activity. RNases are involved in many biological processes, including neurotoxicity, genome replication and maintenance, angiogenic activity, immunosuppression, and antitumor activity.[21] In retroviruses such as HIV-1, an RNase H activity associated with the viral reverse transcriptase is required for replication, making RNase H inhibitors potential drugs for AIDS.[22] RNases are also potential biomarkers for neoplastic diseases such as pancreatic cancer and in cystic fibrosis.[23] In a laboratory setting, RNases are important for some molecular biology protocols, but can also be the source of frustrating contaminations that degrade biological RNA samples. Detection of RNases and their inhibition have therefore become increasingly important, and various RNase detection kits are commercially available. Early methods developed to determine RNase activity include renaturation gel assays,[24] high-performance liquid chromatography (HPLC),[25] colorimetry,[26] and fluorometry.[27] These methods suffer from limitations such as complexity, high cost, and low sensitivity,[28] spurring recent detection approaches using catalytic hairpin assembly,[27] gold nanoparticle conjugates,[29] magnetic nanoparticles,[30] and DNA walkers.[31] These assays have higher sensitivity but include multiple wash steps, additional amplification, indirect quantitation, and specific equipment for readout. Our RNase detection assay uses reconfigurable DNA nanoswitches as a simple mix-and-read strategy.

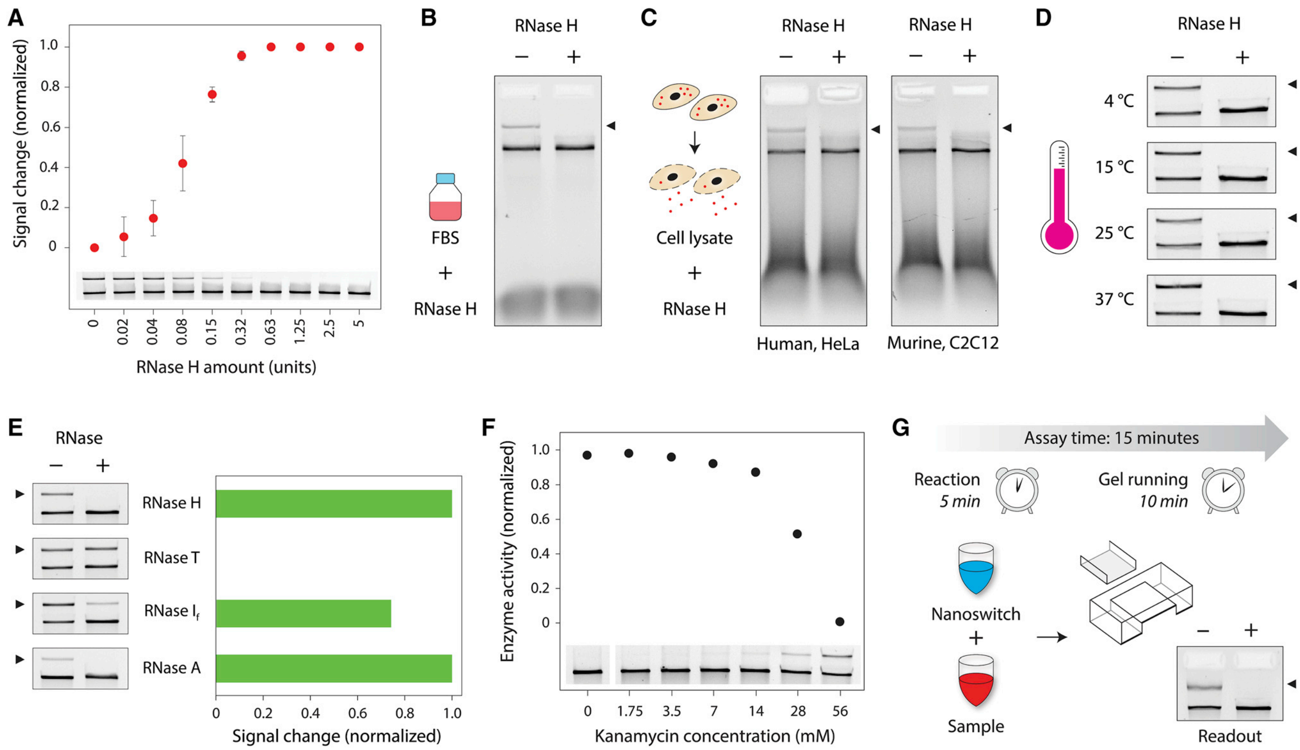

Figure 2. RNase H Assay

(A) Sensitivity plot showing nanoswitch unlocking with different enzyme concentrations (gel image shown as inset). Error bars represent standard deviation obtained from triplicate experiments.

(B and C) Detection of RNase H in 10% FBS (B) and human (HeLa) and murine (C2C12) cell lysates (C).

(D) The nanoswitch-based RNase assay works at a range of temperatures.

(E) Activity of different RNases (H, T, I_f, and A) in the nanoswitch assay.

(F) Inhibition efficiency of kanamycin on RNase H activity.

(G) Rapid readout can be obtained by mixing nanoswitches with the sample containing RNase H and a quick gel run.

Along with demonstrating the basic operation of the DNA nanoswitches by RNase H in Figure 1, we performed controls to show that looped nanoswitches were not affected at the optimal RNase H temperature of 37°C and that nanoswitches locked with DNA strands were not affected by RNase H (Figure S2). We tested the looping efficiency of the nanoswitches with different concentrations of the RNA lock at different incubation times, with average looping yields of 60% (Figure S3). We tested the sensitivity of the assay with different amounts of RNase H in a 1-h assay and showed corresponding variation in unlooping the nanoswitch (Figures 2A and S4). By quantifying the reduction in the looped band of the nanoswitch, we reliably detected as low as 0.02 U RNase H in a 10-μL reaction.

As a step toward testing ribonuclease presence in biological samples, we spiked in a known concentration of RNase H in fetal bovine serum (FBS) and cell lysates extracted from human (HeLa) and murine (C2C12) cell lines. We confirmed that RNase detection was preserved in 10% FBS and cell lysates with the unlocking of the DNA nanoswitch in both biofluids (Figures 2B and 2C). We also demonstrated the assay under different temperatures ranging from 4°C to 37°C, suggesting that the assay could be performed without any temperature control (Figures 2D and S5). To further generalize the assay, we tested a panel of other RNases: RNase T, RNase I_f, and RNase A. The response of the locked nanoswitches varies between these RNases,

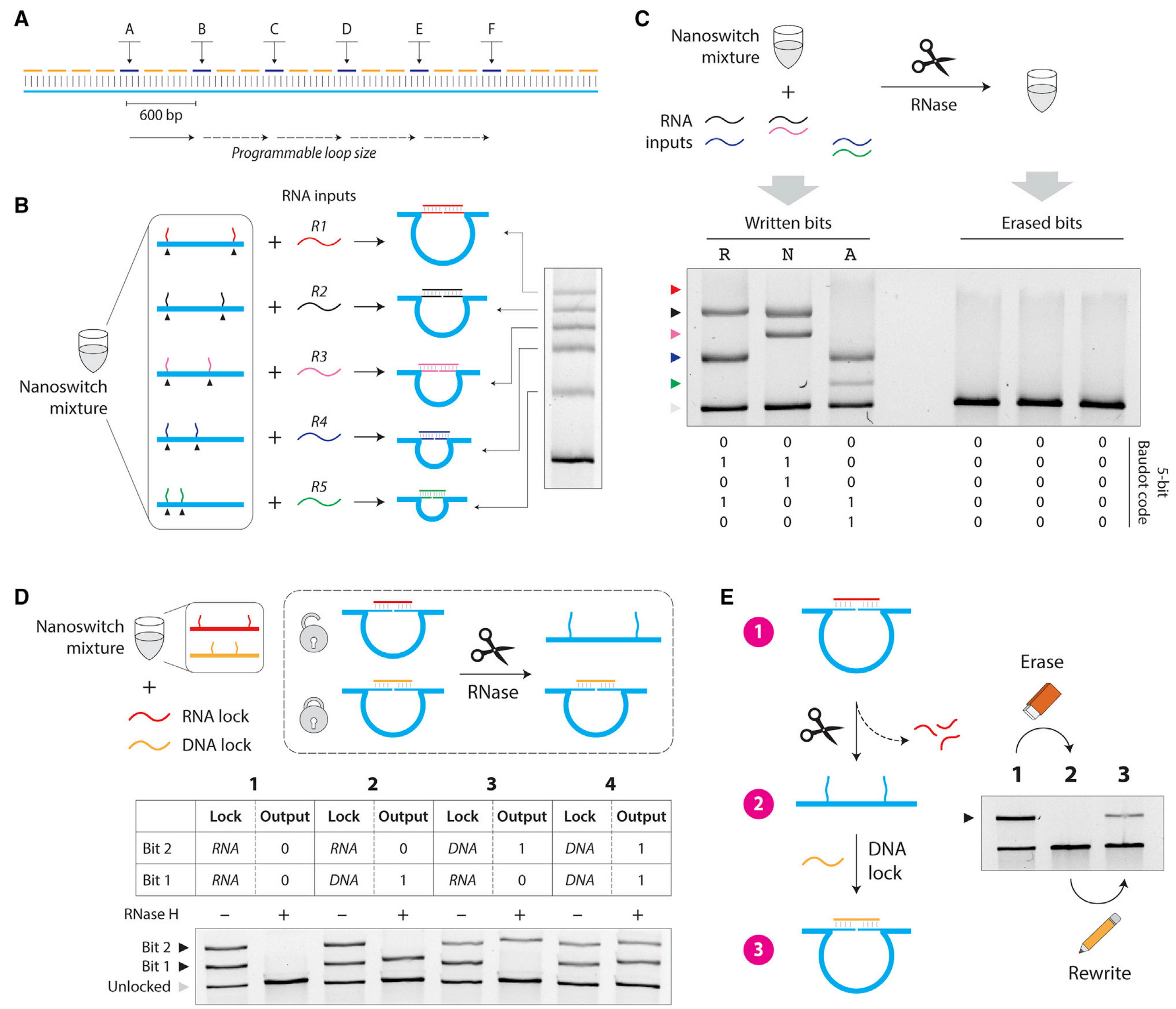

Figure 3. Multi-input DNA Nanoswitches and Information Processing

(A) DNA latches can be placed at specific locations on the scaffold, resulting in different loop sizes.

(B) A nanoswitch mixture with 5 different nanoswitches can recognize specific RNA lock strands to reconfigure and yield specific bands on a gel. The presence or absence of these 5 bands can be used as a 5-bit code to encode information in DNA nanoswitches.

(C) Combination of different locked states of the 5 nanoswitches is used as a 5-bit code to encode information (e.g., "R," "N," "A"), and the information can be erased by the addition of RNase H.

(D) By using DNA locks, specific nanoswitches can be protected from unlocking, providing a write-protection feature for information encoding.

(E) Information (locked bit) erased using RNase H can be rewritten using a DNA lock of the same sequence as the previously used RNA lock.

with RNase H and RNase A showing high levels of unlooping, RNase I_f showing partial unlooping, and RNase T showing no activity on the nanoswitch (Figure 2E).

We then tested the potential of our assay for screening RNase H inhibitors. Since HIV-1 reverse transcriptase is known to have RNase activity, HIV drug development includes the screening of small molecules and antibiotics that can inhibit this enzymatic activity. As a proof-of-concept demonstration, we tested a known RNase H inhibitor, kanamycin, for its inhibitory effects on RNase H.[32] We incubated the nanoswitch with different concentrations of kanamycin and then added RNase H (0.5 U) (Figure 2F). Quantified results show that kanamycin inhibits the RNA cleavage activity of the enzyme, with a half-maximal inhibitory concentration (IC_{50}) value of 30.6 mM. The inhibition efficiency of kanamycin reported in the literature has varied in levels from weak to strong inhibition of RNase H,[32,33] and the inhibition level reported here is within the extremes of the reported numbers. To further make a rapid readout, we performed a start-to-finish assay within 15 min by incubating the nanoswitches with RNase H for 5 min followed by agarose gel electrophoresis for 10 min (Figure 2G). Our assay can also be used in point-of-care settings with existing bufferless electrophoresis units (e.g., E-Gel from Thermo Fisher).

RNase-Triggered DNA Nanoswitches for Information Processing

For our second application, we demonstrate the use of RNase H in processing information encoded using DNA nanoswitches. In our previous studies, we have used nanoswitches of different loop sizes to encode bits of memory.[17,18] Here, we expand the control of erasable and rewritable memory using a ribonuclease. By incorporating DNA latches at defined locations on the scaffold, the resulting loop size of the locked conformation can be changed (Figures 3A and S6). We created a nanoswitch mix containing nanoswitches that can form five different loop sizes. Specific lock strands bind sequence specifically to corresponding nanoswitches in the mixture and cause reconfiguration to form loops of different sizes based on the distance between the DNA latches (Figure 3B). The location of the DNA latches along the scaffold in each nanoswitch was designed to provide well-separated locked bands on the gel when all five RNA locks are present (Figure S7). In previous work, we showed that the kinetics of the writing process can be tuned by changing the loop size of the DNA nanoswitches, with shorter loops forming faster than longer loops.[17] In the present study, we reacted the nanoswitches with RNA inputs (20–23 nt) to obtain the highest looping for each of the 5 nanoswitches in the mix (typically overnight) (Figure S3; sequences in Table S1). Our previous work has shown that this length regime for target nucleic acid results in better looping of nanoswitches.[9]

Using the 5 different locked states of the nanoswitches, we created a memory system that can encode 5 bits of information per gel lane, which can be translated into alphabet characters using the 5-bit Baudot code.[34] We used specific RNA lock strands as inputs to trigger specific nanoswitches, with each loop acting as a bit in a 5-bit code (shortest loop is bit 1 and longest loop is bit 5; Figure S7). On a gel-based readout, we treated each lane as a 5-bit encoder, with multiple consecutive gel lanes providing a string of characters. To demonstrate this, we encoded information using RNA lock strands to display the characters "RNA" on a gel (Figure 3C). We used the RNase H to act as a molecular eraser, cleaving the RNA in a DNA-RNA hybrid to erase the written information.

Next, we showed that by using specific RNA or DNA lock strands, we could "protect" certain bits from being erased. We used a mix containing two nanoswitches with different loop sizes and used combinations of DNA or RNA locks to demonstrate this write-protection feature. Once the inputs were added, there were two written bits corresponding to the bands for two loop sizes (Figure 3D). On adding RNase H, only the RNA locked strand was cleaved, changing that nanoswitch to the open state (Figure 3D, inset). Even within the same mixture, nanoswitches locked by a DNA strand were not affected by the RNase H. We demonstrated all four possible combinations of the DNA and RNA lock strands and showed that the bits written using a DNA input strand are "protected" against erasing.

In addition to writing and erasing capabilities, molecular memory systems also often require a rewriting functionality. Once the RNA locks are cleaved by the enzyme, the

DNA latches on the nanoswitches are again available for hybridization. To demonstrate rewriting, we erased the bits using RNase H and rewrote the bits using DNA locks of the same sequence (Figure 3E). For erasing using RNase H, we performed a time series and show that erasing prewritten bits can be completed in under a few minutes with >1 U/μL RNase H (Figure S8). This processing time is faster than our prior work, in which we showed erasing encoded bits using toehold-based DNA strand displacement[17] or light activation of photocleavable locks.[19] These experiments show that we can controllably encode information using DNA nanoswitches, erase information using an enzyme such as RNase H, and further rewrite information as required.

Our DNA nanoswitch is a versatile biomolecular platform with broad applications.[10,12–14,17] The use of RNase provides an additional tool to manipulate DNA nanoswitches. Our DNA nanoswitch assay adds to the suite of available techniques for monitoring RNase activity. Most of these assays require fluorescently labeled probes and depend on a separate signal amplification step to enhance the signal produced by RNA cleavage.[27,29] In contrast, our ~7-kbp-long nanoswitch provides an inherent signal by the intercalation of thousands of dye molecules from regular DNA stains, providing a high signal even for the cleavage of a single RNA lock by RNases (unlooping the nanoswitch causes the shift of thousands of dye molecules on a gel). For use in a biological context, the biostability of DNA nanoswitches is an important factor. In previous work, we and others have demonstrated the detection of nucleic acid and protein biomarkers in 10%–20% serum,[9,11] as well as in human urine,[12] showing the inherent stability of DNA nanoswitches for use in real-life applications. Results in FBS and cell lysates demonstrated in this work further establish the potential of the DNA nanoswitch assay in *in vitro* applications. In molecular computing, this enzyme-based operation of DNA nanoswitches adds to our suite of nanoswitches that are responsive to light or DNA,[17,19] opening up avenues to create DNA devices that can be orthogonally operated by physical (light), biological (RNA/DNA/enzymes), or chemical (pH) triggers. For RNase detection, our assay provides a simple and effective mix-and-read strategy with a gel-based readout. Our strategy does not require labeling and amplification, thus being easy to adapt by any lab without the need for expensive equipment. We believe it could fill an important need for identifying RNase contamination in biological samples and for characterizing RNase inhibitors.

EXPERIMENTAL PROCEDURES

Resource Availability

Lead Contact

Further information and reasonable requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ken Halvorsen (khalvorsen@albany.edu).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Datasets generated during this study are available within the paper and the Supplemental Information.

Materials

Oligonucleotides were purchased from Integrated DNA Technologies (IDT) with standard desalting. M13 circular DNA, RNase H, RNase T (Exo T), RNase I_f, and BtsCI enzymes were purchased from New England Biolabs (NEB). GelRed nucleic acid stain was purchased from Biotium. Molecular biology grade agarose was purchased from Fisher BioReagents. We have used the viral genome M13mp18 (7,249 nt) for this and previous constructions of our nanoswitches due to its commercial availability and frequent use in DNA origami.

Linearization of M13 DNA

A total of 5 μL of 100 nM circular single-stranded M13 DNA, 2.5 μL of 10× Cut Smart buffer, 0.5 μL of 100 μM BtsCI restriction-site complementary oligonucleotide, and 16 μL of deionized water were mixed and annealed from 95°C to 50°C in a T100 Thermal Cycler (Bio-Rad, Hercules). Added to the mixture was 1 μL of the BtsCI enzyme (20,000 U/mL) and incubated at 50°C for 15 min. The mixture was brought up to 95°C for 1 min to heat deactivate the enzyme, followed by cooling down to 4°C.

Construction of Nanoswitches

Linearized single-stranded M13 DNA (20 nM) was mixed with a 10-fold excess of the backbone oligonucleotides and DNA latches and annealed from 90°C to 20°C at 1°C min^{-1} in a thermal cycler. Constructed nanoswitches were diluted in 1× PBS to a concentration of 400 pM. To form loops, 2 μL nanoswitches were mixed with the RNA lock strand (typically 2.5 nM final concentration) and incubated at 20°C overnight.

RNase H Activity Assay

Locked nanoswitches were first mixed with RNase H buffer (1× final) and placed at 37°C. A total of 1 μL RNase H (different units per microliter) was added to 10 μL of the locked nanoswitches and incubated at 37°C. For sensitivity experiments, samples were incubated for 1 h at 37°C. For time series experiments, RNase H enzyme was added at different time points (from 16 min to 0 min). After incubation, samples were mixed with 1 μL GelRed (1× final) and 2 μL loading dye (15% Ficoll, 0.1% bromophenol blue), and run on 0.8% agarose gels. A similar protocol was used for the other enzymes.

Detection in FBS and Cell Lysates

Locked nanoswitches were first mixed with RNase H buffer (1× final), and FBS or cell lysates were added to a final concentration of 10%. For positive controls, 1 μL RNase H (5 U/μL) was added to 10 μL nanoswitch-biofluid mix and incubated at 37°C for 15 min. After incubation, samples were mixed with 1 μL GelRed (1× final) and 2 μL loading dye (15% Ficoll, 0.1% bromophenol blue), and run on 0.8% agarose gels.

Gel Electrophoresis

Nanoswitches were run in 0.8% agarose gels, cast from molecular biology grade agarose (Fisher BioReagents) dissolved in 0.5× Tris-borate EDTA (TBE). Gels were typically run at 75 V (constant voltage) at room temperature. Samples were prestained by mixing 1× GelRed stain with the samples before loading. Gels were imaged with a Bio-Rad Gel Doc XR+ gel imager and analyzed using ImageJ.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xcrp.2020.100117.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.R.C.; Methodology, A.R.C. and K.H.; Investigation, A.R.C. and R.T.; Formal Analysis, A.R.C.; Visualization, A.R.C.; Writing – Original Draft, A.R.C.; Writing – Review & Editing, A.R.C. and K.H.; Funding Acquisition, K.H.; Supervision, K.H.

DECLARATION OF INTERESTS

A.R.C. and K.H. are inventors on patents and patent applications covering aspects of the DNA nanoswitch design and applications. K.H. is an inventor on three patents or patent applications related to this work, filed by the President and Fellows of Harvard College and the Children's Medical Center Corporation (US patent no. 9914958, issued March 13, 2018; US patent application no. 20170369935, published December 28, 2017; and US patent application no. 20180291434, published October 11, 2018). A.R.C. and K.H. are inventors on an additional patent application related to this work, filed by Children's Medical Center Corporation, the Research Foundation for The State University of New York, and the President and Fellows of Harvard College (US patent application no. 20180223344, published August 9, 2018). A.R.C. is also an inventor on one additional patent application related to this work, filed by Vital Biosciences (US patent application no. 20200150083, published May 14, 2020).

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