doi:10.1038/nature23271

Table of Contents

S1. Supplementary Methods

S1.1. Description of Plasmids and Ribocomputing Device Evaluation

S1.1.1. OR, AND, and Disjunctive Normal Form Circuits

OR, AND, and DNF circuit gate transcripts were expressed from a medium copy ColA origin plasmid with kanamycin resistance. For single OR gate systems, input RNAs, both cognate and non-cognate, were expressed from a high copy plasmid with the ColE1 origin of replication and ampicillin resistance. Different plasmid combinations were used for the dual OR gate systems (see Subsection S2.3.2 for details). For the AND and DNF circuits, the trigger RNAs were expressed from two high copy number plasmids: a plasmid with a ColE1 origin with ampicillin resistance, and a plasmid with a CDF origin with spectinomycin resistance.

AND gates with three or more input RNA species required at least one plasmid that expressed more than one input RNA. In these multi-input plasmids, all input RNAs were expressed using their own T7 promoter site, had their own transcriptional terminator, and were transcribed unidirectionally (i.e. in the same direction on the plasmids). Plasmids employing bidirectional transcription of the different input RNAs suffered from low assembly yields when both T7 promoters were placed adjacent to one another, likely as a result of the high secondary structure of the dual complementary promoters. Conversely, bidirectional constructs employing adjacent transcriptional terminators suffered from lower ON state expression levels from the gate RNAs, most likely because of transcriptional run-through effectively producing deactivating RNAs that silenced the complementary input RNA.

In order to prevent facile binding between two input RNAs as a result of their unidirectional transcription from the same plasmid, input RNAs programmed to bind to one another were never expressed from the same plasmid. This stipulation also reduced the likelihood of homologous recombination. Furthermore, transcriptionally adjacent input RNAs were expressed in the reverse order of their position inside the activated trigger RNA complexes. For instance, input A3 was placed upstream of A1 in the three-input AND gates. This configuration ensured that a run-through transcript would have the sequence 5'-A3-A1-3' rather than 5'-A1-A3-3', which could activate the gate RNA in the absence of the A2 input RNA.

OR circuits were tested using cells transformed with a gate-expressing plasmid and an input-expressing plasmid containing either a cognate or a non-cognate trigger RNA from the toehold switch library. Dual OR gate systems were tested using cells transformed with two plasmids for gate RNA expression and a third plasmid for expression of different input or decoy RNA combinations. AND and DNF circuits were tested in cells having three plasmids: a pair of high copy plasmids expressing input RNAs and a medium copy plasmid expressing the gate RNA. For all AND and DNF circuits, all input conditions were tested using cells triple-transformed with plasmids. At least one input RNA, either cognate or non-cognate, was expressed from each high copy plasmid for all truth table conditions tested for the circuits. This condition ensured that cells were tested under comparable metabolic loads and that the gate RNA had to confront similar numbers of potential RNA signals during circuit validation. Non-cognate RNAs tested in the circuits were the input or trigger RNAs from other orthogonal toehold switches or ribocomputing devices.

S1.1.2. A AND (NOT B) Circuits

A AND (NOT B) circuits were tested using a three-plasmid system similar to that used for the AND and DNF systems. The gate RNA was expressed from a kanamycin-resistant plasmid with a ColA origin of replication. The input trigger RNA was expressed from a chloramphenicol-resistant plasmid with a CDF origin. This plasmid was constructed by replacing the spectinomycin marker of pCDFDuet with the chloramphenicol marker of pACYCDuet and generated in order to transform the *E. coli* MG1655Pro strain, which is resistant to spectinomycin. The deactivating RNA was expressed from an ampicillin-resistant plasmid with a ColE1 origin. We found that transformation of MG1655Pro with this combination of three plasmids established three different copy number levels for each of the plasmids. Importantly, the copy number of the deactivating RNA plasmid was higher than that of the trigger RNA plasmid to ensure that the deactivating RNAs (input B) could bind to all copies of the trigger RNA (input A) and thus enable successful circuit operation.

The gate RNA was expressed constitutively from the ColA plasmid using the proD promoter[1]. The trigger and deactivating RNAs were inducibly expressed using the promoters/inducers[2] $P_{ltetO-1}$/anhydrotetracycline (aTc) and $P_{llacO-1}$/IPTG, respectively (see Supplementary Table S1 for promoter sequences).

The three plasmids for the circuit were transformed into *E. coli* MG1655Pro, which harbors a chromosomal copy of the tet-repressor for aTc inducible expression. Expression of the trigger and deactivating RNAs was induced with 50 ng mL^{-1} aTc and 1 mM IPTG, respectively. In truth table cases in which one or more inducers were missing, the volume contributed by either inducer was replaced by sterile water.

S1.2. Description of AND-Computing Toehold Switches (ACTS)

S1.2.1. ACTS Design Rationale

We constructed a set of AND-computing toehold switches (ACTS) in order to reduce the likelihood of leakage from AND gates compared to those using our existing libraries of first- and second-generation toehold switches[3]. Previous studies have found that trigger RNAs with binding regions shortened from their 5' ends are capable of activating the original toehold switches, even if their lengths have been reduced by half. In designing these AND-computing systems, we aimed to reduce the potential for leakage from expression of either of the AND gate half-trigger sequences.

Nucleotide-level diagrams of the Type I and Type II ACTS systems described in this work are shown in Extended Data Fig. 1a. Although these systems are optimized for AND logic, the diagrams in Extended Data Fig. 1a depict the simplified case where the ACTS devices detect only one trigger RNA. For comparison, critical design parameters for ACTS systems versus previously reported toehold switches[3] are shown in Supplementary Table S10. Both ACTS designs feature lower stems extended by 2 to 3 bp compared to earlier toehold switches. This modification was used to decrease system leakage and to shift the binding site of the trigger to the lower stem of switch RNA. In addition, the ACTS riboregulators employ 16- and 15-nt toehold domains for Type I and Type II designs, respectively, in order to promote efficient trigger-switch binding. Loop lengths of 14- and 12-nts, which are in between those of the earlier toehold switches, are used to achieve high ON state gene expression while maintaining low system leakage.

A critical design feature of the ACTS systems that separates them from previous toehold switches is the extent that the switch RNA hairpin is disrupted upon binding of the trigger RNA. In first-generation toehold switch designs, binding between switch and trigger RNAs leads to complete disruption of the upper stem of the switch RNA hairpin. In the second-generation toehold switches, a 3-bp shift of the trigger-switch binding region causes the 3 bp at the top of the stem-loop to remain undisturbed upon switch activation. The low melting temperature of this 3-bp stem-loop region ensures that this sequence opens spontaneously under typical experimental conditions. Thus, in both previous toehold switch designs, trigger binding leads to complete exposure of the RBS for efficient translation of the output gene.

In contrast, ACTS riboregulators are designed so that trigger-switch binding leaves the upper stem and start codon bulge of the switch RNA undisturbed. This modification is essential to ensure that truncated trigger RNAs or input RNAs for AND logic are unable to attack the start codon bulge in the switch RNA and induce leaky gene expression. As shown in Extended Fig. 1b and Supplementary Table S10, the Type I and Type II ACTS systems retain 6- and 5-bp stem-loop regions upon trigger RNA binding. Despite the stem-loop secondary structure, these riboregulators are still able to activate gene expression (see Subsection S1.2.4) as a result of three factors. First, during the sequence design process (see Subsection S1.2.3), ACTS systems are selected to have relatively weak secondary structure in the stem-loop region after trigger binding. As a result, the library of ACTS devices have low GC content in the upper stem, which forms weak stem-loop structures. Second, the 5- and 6-bp stem-loop RNA structures are sufficiently unstable to open transiently at 37°C for RBS exposure. Third, ribosomal RNAs that recognize the Shine-Dalgarno sequence within the RBS can bind to exposed bases in the loop. This competition for binding can encourage disruption of the stem-loop to allow translation to begin.

S1.2.2. Implementation of ACTS Riboregulators as AND Logic Devices

ACTS riboregulators also incorporate several design features that are specific to AND logic systems. To reduce the space of potential designs, we focused on AND logic systems in which the trigger sequence that binds to the switch RNA is divided evenly between two input RNAs. We refer to the half-trigger RNA formed from the 5' region of the full trigger as the 5' half-trigger, and the other half-trigger formed from the 3' end as the 3' half-trigger. In effect, the length of both half-trigger RNAs is matched to the toehold and lower stem dimensions of the ACTS systems to ensure proper AND logic operation.

To completely eliminate leakage from expression of the 3' half-trigger, we asserted that the toehold of the switch RNA could not be shorter than the 3' half-trigger sequence. To reduce leakage from 5' half-trigger RNAs, we made use of the extra base pairs added to the lower stem and avoided base pairing to the start codon bulge (as described in Subsection S1.2.1). To encourage activation by a complex formed by the 5' and 3' half-triggers, we further stipulated that the junction between the two half-triggers had to be located within the toehold region of the switch RNA. This requirement was implemented to ensure that steric effects at the junction between the input RNAs would not impede the strand displacement reaction required to unwind the gate RNA hairpin.

Application of the above requirements led to toehold domains 2 nts longer than the half-trigger sequences for the Type I and Type II ACTS designs (Extended Data Fig. 1a). This relationship between toehold and 3' half-trigger length was successful in eliminating leakage from input A*N* for the *N*-input AND gates (see Fig. 2e-h, Fig. 3a-c, and Extended Data Fig. 4). Conversely, it

led to the 5' half-trigger having a 2-nt toehold domain that has the potential to induce leaky expression. Previous measurements have shown that a 2-nt toehold is insufficient for activation of toehold switches[3] and characterization of AND logic circuits confirm that 5' half-triggers are unable to fully activate ACTS devices. However, the 5' half-triggers do in some cases lead to noticeable leakage from the gate RNAs (Extended Data Fig. 4e-n). Reduction of this toehold length for the 5' half-trigger could reduce device leakage, provided the strand displacement reaction proceeds for different half-trigger junction sites.

S1.2.3. ACTS Sequence Design

The Type I and Type II ACTS devices were designed using NUPACK[4] with AAAA, CCCC, GGGG, UUUU, KKKKKK, MMMMMM, RRRRRR, SSSSSS, WWWWWW, and YYYYYY as prevented sequences. All systems were designed using RNA free energies from Mathews et al[5]. We did not provide any additional stipulations on the sequences of the trigger RNAs or the stem base pairs of the switch, and used the same 21-nt linker sequence (AACCUGGCGGCAGCGCAAAAG) and RBS sequence (AGAGGAGA) employed for the earlier toehold switch libraries[3].

The resulting designs were screened for in-frame stop codons both upstream and downstream of the start codon. Putative devices were then ranked according to the thermodynamic term $\Delta G_{RBS\text{-}linker}$ described previously[3]. This term was computed based on the switch RNA sub-sequence that started immediately downstream of the switch region bound to by the trigger RNA and ran through to the end of the 21-nt linker sequence (Extended Data Fig. 1a). Devices that had the largest values (i.e., lowest magnitude) of $\Delta G_{RBS\text{-}linker}$ were rated highest. Rating designs based on the $\Delta G_{RBS\text{-}linker}$ parameter effectively selected for switch RNAs that had the lowest secondary structure upon activation by the trigger RNA. Consequently, these designs are expected to have relatively unstable upper stem loops in the switch RNA to facilitate ribosome docking and enable efficient translation. Sets of devices were then screened for orthogonality by maximizing the net edit distance for trigger sequences in the library[3].

S1.2.4. Validation of ACTS Riboregulators

The ON/OFF ratios obtained for the ACTS systems used in the ribocomputing devices presented in this paper are shown in Extended Data Fig. 1c (see Supplementary Table S2 for sequences). These 10 devices were selected for their high ON/OFF ratios and thus have an average ON/OFF of 609 with values ranging from 250 for the weakest device to 1480 for the strongest device. All ON/OFF levels were obtained using GFPmut3b-ASV as the reporter in BL21 Star DE3 cells after 3 hours of induction with 0.1 mM IPTG.

S1.3. Design of Ribocomputing Device RNA Sequences

The synthetic ribocomputing device sequences were designed completely *de novo* using the NUPACK software package[4]. Different design strategies were used for generating each class of circuit components: gate RNA strands, input RNAs for AND computations, and deactivating RNA systems. All design methods used validated toehold switches as initial conserved sequences. NUPACK designs specified AAAA, CCCC, GGGG, UUUU, KKKKKK, MMMMMM, RRRRRR, SSSSSS, WWWWWW, and YYYYYY as prevented sequences to avoid long strings of repeated nucleotides. In addition, RNA energies from Mathews et al[5] were used for designing the RNA sequences. The design methods for each circuit component class are described in the subsections below.

S1.3.1. Multi-Input Gate RNA Design

Three different general design methods were employed for producing the gate strands depending on the number of RNA input species for the final circuit and the toehold switches used. All gate RNAs were screened for in-frame stop codons to eliminate any designs that would cause premature termination of translation. Sequences and information regarding the parental toehold switches used for the OR circuits are provided in Supplementary Tables S3, S6, S7, S8, and S9.

Two-Input OR Gates: Two-input gate RNAs are nearly fully defined once the two switch modules have been selected and require only the design of the spacer sequence between the two sensor elements. The two-input system employed a 12-nt spacer of arbitrary sequence specified to be completely unpaired in NUPACK (Extended Data Fig. 2a). Switches were obtained from the first-generation toehold switch library with the start codon positioned at the 48th nucleotide (or 51st nucleotide including the GGG leader sequence) from 5' end of the transcript. As a result, this start codon was not in the same frame as an upstream switch module for a 12-nt spacer. A single base was added to the 5' end of the loop of both switch RNA modules to expand the loop to 12-nts and enable both switches to be in-frame of any upstream sensor. Based on earlier studies[3], this modification is also expected to increase the output expression levels of the toehold switches in the gate. The toehold switches used in the two-input systems were also preselected to not have any stop codons upstream of their start codons to avoid any stop codon removal issues.

Six-Input OR Gate with First-Generation Toehold Switches: Many of the high-performance first-generation toehold switches have in-frame stop codons upstream of the start codon. To construct the six-input OR gate, we selected three high-performance systems with only one upstream stop codon and made small sequence adjustments to eliminate each stop codon. New cognate trigger expression plasmids were also constructed in order to activate the modified devices. The remaining three switches for the six-input device were already free of stop codons. A single base was added to the 5' sequence of the loop for each of the switches to ensure that each remained in the same reading frame. The six-input gate RNA was designed as a single, long transcript in NUPACK. The six switch hairpins were separated by 12-nt spacers of arbitrary sequence specified to have no secondary structure (Extended Data Fig. 2b). The gate RNA with the lowest ensemble defect[6] and with no in-frame stop codons was selected for *in vivo* testing.

Four- and five-input OR Gates with ACTS Systems: The AND-computing systems were designed to have lengths that were multiples of three to ensure they remained in the same reading frame as long as they were separated by spacers that were also multiples of three in length. Furthermore, they were pre-screened at the initial design stage to be free of any in-frame stop codons (see Section S1.2). These design features enabled us to employ a more modular approach in designing this set of gate RNAs.

After identifying a set of high-performance ACTS and performing a study of ACTS integration into gate RNAs (see Subsection S2.1.3), we used NUPACK to rapidly design a library of spacer sequences with lengths of 9-, 12-, and 15-nts selected to be free of stop codons and provide unpaired structures between combinations of two different switch modules. This library enabled us to select a set of input RNAs to employ for a given gate RNA and model the ensemble defect of different gate permutations having different orders of the switch modules and varying spacer lengths. Promising designs were selected based on their ensemble defect

and the order of switch RNA modules used in the gate transcript. In general, we found that device performance was improved by using switches that were arranged from highest dynamic range to the lowest dynamic range from 5' to 3' end of the transcript, likely because switches with higher intrinsic activity were better able to compensate for losses in translational output as the ribosome encountered multiple downstream stem-loops. Weaker switch stems to promote ribosome processivity were generated simply by modifying a base near the 3' end of the stem to create a bulge without requiring changes to the trigger sequence (see Subsection S2.1.3). Although this overall design approach may have yielded systems with less than optimal secondary structures, it is advantageous in its modularity since libraries of gate RNAs can be constructed using a common set of primers that links different pairwise combinations of switch modules together.

S1.3.2. AND Circuit Input Sequence Design

Input sequences for the AND circuits were generated in two NUPACK design stages. First, the 26-nt or 28-nt minimal trigger RNA sequence for a given ACTS system was divided evenly into either 14-nt or 13-nt halves for Type I and Type II systems, respectively. For an *N*-input gate, the 5' half-trigger was used for input 1 and the 3' half-trigger was used for input *N*. Input 1 was generally designed to feature a 3-nt spacer region immediately after the half-trigger sequence followed by a 21-nt hybridization domain for binding to input 2. Similarly, input *N* was designed to have a 21-nt hybridization domain for binding to input *N-1* followed by a 3-nt spacer and the half-trigger sequence. The 3-nt spacer was used for inputs 1 and *N* in order to compensate, at least in part, for the extra distance between trigger halves created when forming an *N*-RNA complex and its effect on switch activation. Inputs 2 to *N-1* were then designed to have 21-nt hybridization domains to their two binding partners within the input RNA complex. The hybridization and spacer domains were allowed to take on any sequence.

Within NUPACK, the network of interacting and non-interacting RNAs was modeled using two-RNA complexes to reduce computational load. The complex formed between input RNA *n* and input RNA *n*+1 was explicitly specified for binding pairs in the network. For $N > 2$, inputs 1 and *N* were also specified to not bind to one another in order to avoid direct binding between the outermost input RNAs activating the switch in the absence of the internal inputs. Similarly, the internal input RNAs 2 to *N-1* were also specified to not interact with the switch RNA to avoid unwanted input/switch interactions. To designate these non-interacting RNAs, a complex in which no base pairs existed between the two RNAs in question was explicitly specified as a target structure in NUPACK. The NUPACK test tube design package[7] was not used for any of the RNA circuits reported in this work.

After this first stage design, the resulting minimal input RNA sequences were then linked to designed 5' and 3' sequences to facilitate their expression *in vivo*. Each input RNA was given a 5' hairpin sequence and a short 3-nt spacer between the end of the minimal trigger and the transcriptional terminator. Base-pair-level schematics of the two-input AND gate system are shown in Extended Data Fig. 2c and three-, four-, and five-input AND gate input RNA complexes are shown in Extended Data Fig. 4b, i, l. Sequences, parental toehold switches, and the transcriptional terminators used for the AND gate RNAs are provided in Supplementary Table S4. DNF circuits, which combine AND, OR, and NOT expressions, employ the sequences listed in Supplementary Table S9.

S1.3.3. Deactivating RNA Circuit Design

A two-stage approach was also used for generating deactivating RNAs and complementary trigger RNAs for A and (NOT B) circuits. First, extended trigger and deactivating RNA sequences were produced using the minimal trigger sequence from the selected toehold switch. The extended trigger RNA was formed by adding flanking 16-nt regions to either side of minimal trigger core. A corresponding deactivating RNA of identical length was prepared by placing the reverse complement of the minimal trigger at the midpoint of the RNA. The complex between the two RNAs was then specified to be completely double stranded, except for a pair of bulges just immediately adjacent to trigger core (Extended Data Fig. 2d). After NUPACK had generated the extended trigger and deactivating RNA sequences, a second design cycle was used to add a 5' hairpin and a 3-nt spacer between the RNA and the transcriptional terminator. A 5' hairpin sequence was also added to the switch RNA to promote greater transcript stability when expressed from the endogenous *E. coli* RNA polymerase. Sequences for the A AND (NOT B) circuits are provided in Supplementary Table S5.

S1.3.4. Deactivating Input RNA within AND Gate Design

A two-stage approach was also used for generating deactivating input RNAs for AND gate design. After functional verification of two-input AND gates, one of the half-triggers was selected to be extended and its complement sequence was designed. As in Subsection S1.3.3, the minimal core sequence of half trigger was extended by adding flanking 16-nt regions to either side, and the corresponding deactivating input was designed to be the reverse complement of the extended trigger except for a pair of bulges. A second design cycle was used to add a 5' hairpin and a 3-nt spacer between the RNA and the transcriptional terminator. DNF circuits, which combine AND, OR, and NOT expressions, are shown in Fig. 4 with the sequences listed in Supplementary Table S9.

S2. Supplementary Data

S2.1. Selection of Ribocomputing Device Domain Lengths and Secondary Structures

S2.1.1. Systematic Study of AND Input RNA Overlap Domains

In the early stages of this work, a systematic study was conducted to determine optimal dimensions for overlap between RNA inputs for the two-input AND gates. These circuits employed first-generation toehold switch designs and divided the 30-nt trigger RNA sequence evenly between the A1 and A2 inputs. A 30-nt u* domain was added to the 3' end of input A1, while nine different u' domain lengths to hybridize to u* were investigated for input A2. Both input RNAs did not employ any spacer sequences between the half-trigger sequence and the u* and u' domains (see Extended Data Fig. 3a). Experiments expressing the three circuit RNAs, A1, A2, and the gate RNA in *E. coli* BL21 Star DE3 revealed that AND gate activation occurred as the overlap length shifted from 10- to 14-bp corresponding to a predicted RNA duplex melting temperature[8] that transitioned from 34°C to 50°C (see Extended Data Fig. 3b). This behavior is consistent with the 37°C temperature used for the experiments. As the overlap length increased beyond 22-bps, output from the gate decreased substantially, likely as a result of the increased probability of misfolding for longer RNAs that interfered with their hybridization to one another. Given the observed 22-nt optimum and the decrease in output for longer u' domains, we employed slightly shorter 21-nt overlap domains between inputs for the other AND gates reported in this work (see Subsection S1.3.2).

S2.1.2. Rationale for u and v Domain Lengths in Deactivating Circuit Designs

In the deactivating circuits, hybridization between the trigger RNA and deactivating RNA can occur via two main pathways: (1) the trigger RNA and deactivating RNA can bind directly to each other, and (2) the deactivating RNA can bind to exposed u and v toeholds in the trigger-gate RNA complex to outcompete the gate RNA for trigger binding (Fig. 2i). In the first pathway, the prescribed single-stranded structure of both trigger and deactivating RNAs should enable hybridization to occur rapidly. However, as described in Subsection S2.1.1, increasing the lengths of these RNAs can promote strand misfolding and decrease hybridization rates. In the second pathway, the reaction proceeds through a toehold-mediated strand-displacement interaction analogous to that employed in the toehold switches. Accordingly, we decided to employ 15-nt toeholds for both the u-u* and v-v* interactions with an extra nucleotide added to form matching bulge sites (see Subsection S1.3.3 and Extended Data Fig. 2d). These bulge sites were incorporated into the structure to reduce the potential for degradation of the trigger-deactivating RNA complex by RNase III. Fifteen-nucleotide toeholds were selected to provide substantial free energy for RNA hybridization, while avoiding the misfolding problems possible with longer trigger and deactivating RNAs.

S2.1.3. Study of Gate RNAs with Varying Secondary Structure

When the ribosome initiates translation at hairpins nearer to the 5' end of the gate RNA, it must process through one or more downstream hairpins before producing the output protein. Translation through these regions can be hindered by the strong secondary structure of the gate RNA hairpins. To counter this effect, one alternative is to decrease the strength of the hairpins within the gate RNA. However, this modification leaves the RBS and start codon regions within each hairpin more accessible for ribosome binding, which in turn can increase OFF state gene expression.

In view of these opposing effects, we studied three gate RNAs containing ACTS hairpin variants to find those with optimal performance and incorporate their features into subsequent designs. The three gate RNAs each detected the same four input RNAs and employed the same order of the hairpins: two Type II ACTS hairpins followed by two Type I ACTS hairpins in the 5' to 3' direction (Extended Data Fig. 5a). Linkers separating the toehold switch modules were each 33 nts. The three different gate RNA versions differed systematically in the secondary structure of their constituent hairpins, while leaving the binding site sequences for the input RNAs unchanged. Version 1 featured ACTS hairpins with the same secondary structure as those shown in Extended Data Fig. 1a. In gate RNA version 2, a single bulge was formed at the mid-point of the lower stem of each hairpin and, in two cases, a single base was changed in the hairpin to establish a full 3-nt bulge in the start codon region. Since NUPACK designs toehold switches based on their overall ensemble defect, which takes into account secondary structure of the trigger, switch, and trigger/switch complex, the software can return designs that do not provide the exact specified structure for each strand. The modified bases are shown as red circles in the schematics of Extended Data Fig. 5a. In gate RNA version 3, each of the hairpins was further weakened by breaking a second base pair in the lower stem. This modification was done by taking gate RNA version 2 and altering the blue bases marked in Extended Data Fig. 5a. These modifications required changes to only 10 nucleotides between the version 1 and version 3 gate RNAs. Apart from these specific locations, the sequences of the three gate RNAs were identical.

We measured the GFP output level from the gate RNAs in *E. coli* BL21 Star DE3 under control of the T7 promoter. Despite the close similarity in sequence, substantial differences in ON and OFF state expression levels were observed for all three gate RNAs. Extended Data Fig. 5b, c shows the GFP fluorescence levels after three hours of induction of the four-input OR gates when tested against the same panel of input and decoy RNAs. The gate RNAs all display a general trend of increasing GFP output for input RNAs that bind closer to the 3' end of the gate. Compared to gate RNA version 2, gate RNA version 1 displays sizable signal attenuation for all inputs. Gate RNA version 3, with its weakened hairpins, suffers from substantial OFF state GFP signal leakage compared to the other gate RNAs. Across the four decoy RNAs, average signal leakage was 3.2- and 2.7-fold higher for version 3 compared to versions 1 and 2, respectively. Although it is difficult to precisely identify the source of all the observed changes in GFP fluorescence, we attributed the sharp attenuation in signal for 5' input RNAs to decreases in translational efficiency caused by strong hairpin structures in the gate RNA open reading frame. The large decrease in GFP expression for input A in gate RNA version 3, in contrast, could be due to changes in the sequence of the output protein, since its signal attenuation behavior otherwise closely matches that of gate RNA version 2 for inputs B, C, and D. ON/OFF expression changes were also calculated for the three gate RNA variants using the decoy RNA with the lowest fluorescence as the OFF state (Extended Data Fig. 5d). Gate RNA versions 1 and 2 provide ON/OFF levels of at least 10-fold for all inputs, while the performance of version 3 suffered from the increased leakage and weak output for input A. Overall, gate RNA version 2, with a single bulge added to each hairpin stem, provided the best performance with ON/OFF ratios ranging from 60- to 1600-fold and low leakage for all decoy RNAs. Consequently, we used the secondary structures of the version 2 hairpins in all the OR gate ribocomputing devices based on ACTS systems.

S2.2. Additional Multi-Input Synthetic Ribocomputing Devices

RNA-only genetic circuits are advantageous compared to protein-based circuits since one device design can be used to construct multiple orthogonal logic gates with different sequences. The capabilities of synthetic RNA circuits are demonstrated in the disjunctive normal form expressions evaluated in the main text (Fig. 4). To further show that the ribocomputing devices can be used to make multiple functional gates, we include additional experimental data acquired from other multi-input AND, OR, and DNF gates.

S2.2.1. Multi-Input AND Gates

Six additional AND gate circuits with three, four, and five inputs are shown in Extended Data Fig. 4. The set of four three-input AND circuits provide worst-case fold-changes ranging from 8.5- to 25-fold and ON/OFF levels up to 560-fold compared to the null-input case (Extended Data Fig. 4a-g). An additional four-input AND system provides GFP expression increases of up to 43-fold compared to the null-input case (Extended Data Fig. 4h-j). A five-input AND gate circuit was also tested and formed the five RNA complex illustrated in Extended Data Fig. 4k-l. This ribocomputing circuit provided an ON/OFF ratio of up to 65-fold in the logical TRUE case compared to the null-input case; however, it displayed substantial signal leakage in cases where input A1 was expressed (Extended Data Fig. 4m-n). This leakage, likely caused by invasion of input A1 into the stem of the gate RNA, led to a worst-case fold-change of only 1.65. Despite the high leakage, application of Welch's t-test led to p-values below 0.028 for all logical FALSE conditions compared to the logical TRUE condition.

S2.2.2. Multi-Input OR Gates

Four- and five-input OR gates constructed from the ACTS systems are shown in Extended Data Fig. 6 after a 3-hour induction by IPTG and expression via T7 RNA polymerase. These multi-input OR gates are the same ones employed for the DNF circuits shown in Fig. 4, Extended Data Figs. 9 and 10 and share four of the same source ACTS systems. They employ 9-nt linkers between switch RNA modules. The four-input gate in Extended Data Fig. 6a provided ON/OFF levels of at least 47-fold for all cognate input RNAs and fold changes below 2.4 for four non-cognate RNAs. The five-input gate provided substantial increases in ON-state GFP expression for logical TRUE cases compared to three non-cognate RNAs tested (Extended Data Fig. 6b).

S2.2.3. 8-input and 10-input DNF Gates

We applied the four-input OR gate in Extended Data Fig. 6a to evaluate the 8-input DNF expression (A1 AND A2) OR (B1 AND B2) OR (C1 AND C2) OR (D1 AND D2). This circuit was tested using 16 different combinations of input RNAs representing the full truth tables for each of the two-input AND expressions evaluated by the four-input OR gate RNA. Extended Data Fig. 9 displays the results of the ribocomputing device computation showing strong GFP expression for the four logical TRUE combinations of inputs and low GFP levels for the remaining 12 different FALSE combinations of inputs. Similarly, we applied the five-input OR gate in Extended Data Fig. 6b to evaluate the 10-input DNF expression (A1 AND A2) OR (B1 AND B2) OR (C1 AND C2) OR (D1 AND D2) OR (E1 and E2). Extended Data Fig. 10 shows the results of the ribocomputing device computation showing strong GFP expression for the 5 logical TRUE states and low leakage for the 15 logical FALSE states. These DNF ribocomputing circuits were evaluated 6 hours after induction with IPTG and expression via T7 RNA polymerase.

S2.3. Ribocomputing Devices with Different Output Proteins

S2.3.1. Gate RNAs with Alternative Outputs

Sequences for mCherry and cerulean proteins were inserted downstream of the five-input ACTS OR gate RNA to confirm gate RNA functionality with new output proteins (see Subsection S2.2.2 and Extended Data Fig. 6b for GFP results). Gates were tested in identical conditions to those used in Subsection S2.2.2 except with a longer 4-hour induction time. Extended Data Fig. 7a-d provide the ON/OFF levels recorded for mCherry and cerulean regulation. In both systems, all five cognate input RNAs provide at least 10-fold increases in gene expression and show low output signal from three non-cognate decoy RNAs. mCherry and cerulean exhibit a substantial reduction in ON/OFF ratios compared to GFP. Similar decreases in ON/OFF ratios versus GFP have previously been observed for three different toehold switches regulating the mCherry and cerulean output genes[3]. Consequently, the observed effect is likely due to differences in the translational efficiencies of the output gene itself or its interaction with the 3'-most toehold switch, rather than the gate RNA design. The mCherry and cerulean ON/OFF ratios exhibit a less pronounced decrease in magnitude as the input binding site moves toward the 5' end of the gate RNA compared to GFP. This behavior leads to inputs A, B, and C providing ON/OFF ratios that fall within a factor of 1.5 to 3 across the GFP, cerulean, and mCherry outputs. We expect that further tuning of the 3'-most toehold switch and the early nucleotides in the output protein sequence will be helpful in increasing the dynamic range of gate RNAs regulating different genes.

S2.3.2. 11-Input Dual OR Gate Circuit

To confirm that more than one gate RNA can be active in a cell at the same time, we evaluated an 11-input circuit that featured a six-input OR gate RNA regulating GFP and a five-input OR gate RNA regulating mCherry (Extended Data Fig. 7e). *E. coli* BL21 Star DE3 were used to evaluate the circuits with a three-plasmid system, employing two plasmids for gate RNA expression and a third for expression of different input or decoy RNA combinations. A plasmid with a ColA origin and kanamycin resistance was used to express the GFP six-input OR gate RNA, while a plasmid with a P15A origin and chloramphenicol resistance was used for expression of the mCherry five-input ACTS OR gate RNA. Either one or two input/decoy RNAs were expressed unidirectionally from the third plasmid, which had a ColE1 origin and ampicillin resistance. The sequences used for the 11-input circuit are listed in Supplementary Table S8.

Extended Data Fig. 7f-g shows the ON/OFF ratios obtained from *E. coli* after 4 hours of IPTG induction. In cases with only a single input or decoy RNA expressed, the gate RNAs respond as expected. Low GFP and mCherry levels are obtained for all the decoy RNAs and they also remain low when the alternate gate is activated. Furthermore, cognate input RNAs yield substantial increases in gene expression, with mCherry increasing between 20- to 80-fold with its cognate inputs and GFP increasing between 200- to 900-fold via its inputs.

Systems in which two input or decoy RNAs are expressed also yield the expected behavior. In conditions where an input RNA is co-expressed with a decoy RNA, we often observed decreases in gene expression in both gates compared to that observed with a single input RNA. This behavior occurred for all input RNA cases in the mCherry gate and for inputs D1, E1, and F1 for the GFP gate. This phenomenon could be due to the increased metabolic load placed on the cell when expressing the additional RNA, and interactions between the activated gate RNA and decoy RNA that hinder translation. In conditions where input RNAs for both gates are expressed, substantial increases in mCherry and GFP production were observed. For mCherry, co-expression with GFP typically led to similar or higher mCherry levels than those

observed for cases with an mCherry input RNA and a decoy RNA. In contrast, GFP levels were typically lower under co-expression with mCherry. These decreased GFP levels could be caused by the increased metabolic load incurred by mCherry translation and translation-inhibiting interactions between the GFP gate RNA and the mCherry input. Despite the observed variations in ON state gene expression, in all cases the gate RNAs increased protein output by at least 10-fold and 50-fold for mCherry and GFP, respectively.

S2.4. Ribocomputing Devices in Different *E. coli* Strains

Most of the ribocomputing devices presented in the main text and in the subsections above have been characterized in the *E. coli* BL21 Star DE3 strain, which is RNase deficient, and employed RNAs transcribed using T7 RNA polymerase in the cell. We have also tested several circuits in non-RNase-deficient strains and using the endogenous *E. coli* RNA polymerase. These measurements indicate that the ribocomputing devices can function in cells with the full complement of RNases and using other RNA polymerases. Device performance does vary depending on the strain used and thus design modifications will be required to achieve optimal performance when moving to a new strain.

S2.4.1. Two-Input AND Gates

We evaluated two different two-input AND gate devices in *E. coli* BL21 DE3 and *E. coli* BL21 Star DE3. The first device was the circuit shown in Fig. 2e-h of the main text, which provided an ON/OFF ratio of up to 900-fold in *E. coli* BL21 Star DE3. Measurements in both strains for this circuit are shown for comparison in Extended Data Fig. 3c-d. We found that in the non-RNase-deficient strain there was a substantial ~5-fold decrease in ON/OFF ratio. Despite this effect, the circuit still provided good performance in BL21 DE3, with an ON/OFF level for the TRUE state of 175 and low leakage for the three FALSE states (Extended Data Fig. 3c-d).

We tested a second two-input AND circuit with an identical design to the first but using a different toehold switch from the ACTS Type II library. In this case, the AND gate displayed a ~2-fold increase in ON/OFF ratio in the non-RNase-deficient strain (Extended Data Fig. 3e-f), with a BL21 Star DE3 ON/OFF level of 125 and BL21 DE3 at 260. Taken together, these results indicate that low RNase levels are not required for successful operation of the AND ribocomputing devices.

S2.4.2. Six-Input OR Gates

We transformed *E. coli* BL21 DE3 with the six-input OR gate characterized in BL21 Star DE3 in Figure 3d-f. This circuit also provided good performance in the non-RNase-deficient strain. The six input RNA yielded ON/OFF ratios between 300- and 1300-fold while a panel of five decoy RNAs did not strongly activate the gate (Extended Data Fig. 8a-b). In comparison, the same gate RNA provided lower ON/OFF ratios between 110- and 900-fold in RNase-deficient BL21 Star DE3 under the same conditions.

We characterized the same six-input OR gate in *E. coli* MG1655Pro using the endogenous *E. coli* RNA polymerase to transcribe all RNAs for the ribocomputing circuit. Extended Data Fig. 8c-d shows OR device with input and gate RNAs expressed from the endogenous *E. coli* RNA polymerase. The gate RNA was expressed using the P_{lacO-1} promoter[2] with IPTG as the inducer and input RNAs were expressed using the strong constitutive PN25 promoter. Four hours after induction of the gate RNA with 1 mM IPTG, the six cognate RNA inputs led to at least 10-fold increases in GFP expression compared to the panel of five non-cognate RNAs.

S3. Supplementary Discussion

The performance of our circuits demonstrates that RNA alone can be used for constructing complex *in vivo* regulatory networks. We attribute the capabilities of the synthetic ribocomputing devices to four main factors. First, ribocomputing circuits take full advantage of programmable and predictable RNA-RNA interactions to direct self-assembly *in vivo*. Second, RNA provides a large sequence bandwidth for information processing. This allows many signals at the same time to be transmitted within the cell, and *in silico* design enables us to specify precisely those RNAs that should interact and those that should not. Third, toehold switches provide large libraries of modular RNA parts to transduce RNA hybridization into a wide dynamic range of output signals via protein production. Fourth, consolidation of RNA sensing and output modalities into the gate transcript avoids some of the signal losses typically experienced by network construction strategies employing freely diffusing intermediate signaling species.

Despite their advantages, ribocomputing devices have several limitations. Gate RNAs have translation start sites that change depending on the sensor module activated by the input RNAs. Thus, the output gene is translated with additional N-terminal residues. Although we did not observe substantial interference of these terminal residues on the function of the protein in the systems tested, they could in some cases have undesired effects on output protein folding and function. Should this become an issue, it could be avoided by incorporating cleavable peptide linkers[9] immediately upstream of the coding sequence of the output gene. Moreover, the use of input RNAs that hybridize with one another for AND and NOT operations means that these inputs must have some sequence complementarity. For instance, an AND gate that detects two endogenous cellular RNAs will require a third input RNA to assemble the endogenous inputs to activate the gate RNA. Although signal losses are reduced by co-localization within the gate RNA, the hybridization of input RNAs and gate RNAs remain a source of diffusion-mediated interactions in the circuits that can lead to signal loss via degradation by cellular RNases.

To reduce such losses, strategies for localizing biomolecular circuit components at precise locations on a surface have been proposed to enable scale-up of *in vitro* DNA computing[10-12] but have yet to be implemented experimentally. These *in vitro* localization schemes could provide a framework for further scale-up of *in vivo* computation. For instance, tethering RNA sensor modules to rigid, two-dimensional nanostructures such as RNA origami[13] could coordinate association of input strands to increase the output from AND expressions and increase processing speed due to co-localization of a greater number of components. Conversely, the successful implementation of the gate RNA co-localization strategy in a cell suggests that analogous approaches could be adopted *in vitro* for computational scale-up.

Our studies investigating the scaling of the multi-input AND and OR gates suggest that constructing ribocomputing circuits beyond more than approximately five-input AND and approximately 10-input OR expressions will likely require substantial optimization of existing network designs. AND logic computations are limited by diffusion-mediated RNA interactions and the concentration of RNAs that can be expressed by the cell while not affecting growth. These limitations can be offset by developing toehold switches with decreased leakage or higher ON state expression levels. Such modifications will ensure that even low concentrations of assembled trigger complexes yield detectable expression levels. The scaling of OR logic computations is limited by the processivity of the ribosome. Our studies indicate that

translational efficiency decreases as the ribosome encounters increasing numbers of sensor modules downstream of the translation start site. Furthermore, hairpins with stronger secondary structure are correlated with more substantial drop-offs in translation efficiency (see Extended Data Fig. 5 and Subsection S2.1.3). Increasing the number of inputs for OR computations also produces output proteins with many additional residues appended to their N-termini, which can be alleviated with cleavable peptides[9] or by employing more compact sensor RNA modules with weaker secondary structure.

Despite these limitations, the *in silico* RNA sequence design of the synthetic ribocomputing devices, facilitates the construction of diverse libraries of circuits that possess the same secondary structures yet have divergent RNA sequences within the enormous space of $>4\times10^{15}$ (or 4^{26} for the shortest input trigger length) potential sequences. This diversity of possible components suggests that ribocomputing circuits have room for aggressive scaling via parallel information processing. It is conceivable that dozens of unique gate RNAs could potentially be engineered to work within the same cell at the same time for precise regulation of multiple metabolic pathways and for constructing whole cell biosensors that simultaneously monitor dozens of analytes.

The modularity and low genetic footprint per logic operation of the integrated RNA networks also indicates that they could be incorporated into layered biological circuits to yield more complex *in vivo* information processing. However, successful use in layered circuits will require ribocomputing devices that employ RNAs as both input and output species. This functionality can be obtained by expressing RNA polymerase and transcription factors as output proteins that control the expression of ribocomputing devices in the next layer. Previous studies have demonstrated that both RNA polymerases and transcription factors can be regulated with toehold switches in layered circuits[3,14], which suggests it would be possible to integrate gate RNAs. Additional studies will be necessary to determine if the gate RNA designs described here provide sufficient dynamic range and if additional N-terminal residues interfere with transcription factor or RNA polymerase activity. If integration with layered circuits is successful, the ribocomputing device architecture can potentially be combined with advanced genetic circuit design tools such as Cello[15] to enhance overall circuit performance.

The use of toehold switches as the sensor elements in the gate RNAs means that the synthetic ribocomputing devices could be combined with mRNA sensors to employ endogenous RNAs as circuit inputs[3] with two main requirements. First, these endogenous inputs would need to be sufficiently long to hybridize with the gate RNA (≥26 nts) and other inputs (≥34 nts for AND, ≥58 nts for NOT) to carry out their programmed functions in the circuit. Second, since input RNAs for AND logic must have sequence complementarity, incorporating endogenous RNAs into AND ribocomputing circuits would require an additional synthetic input RNA to connect pairs of native transcripts.

Supplementary References

1 Davis, J. H., Rubin, A. J. & Sauer, R. T. Design, construction and characterization of a set of insulated bacterial promoters. *Nucleic Acids Res.* **39**, 1131-1141 (2011).

2 Lutz, R. & Bujard, H. Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and AraC/I_1-I_2 regulatory elements. *Nucleic Acids Res.* **25**, 1203-1210 (1997).

3 Green, A. A., Silver, P. A., Collins, J. J. & Yin, P. Toehold Switches: De-Novo-Designed Regulators of Gene Expression. *Cell* **159**, 925-939 (2014).

4 Zadeh, J. N. *et al.* NUPACK: Analysis and design of nucleic acid systems. *J. Comput. Chem.* **32**, 170-173 (2011).

5 Mathews, D. H., Sabina, J., Zuker, M. & Turner, D. H. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J. Mol. Biol.* **288**, 911-940 (1999).

6 Zadeh, J. N., Wolfe, B. R. & Pierce, N. A. Nucleic acid sequence design via efficient ensemble defect optimization. *J. Comput. Chem.* **32**, 439-452 (2011).

7 Wolfe, B. R. & Pierce, N. A. Sequence Design for a Test Tube of Interacting Nucleic Acid Strands. *ACS Synth. Biol.* **4**, 1086-1100 (2015).

8 Owczarzy, R., Moreira, B. G., You, Y., Behlke, M. A. & Walder, J. A. Predicting Stability of DNA Duplexes in Solutions Containing Magnesium and Monovalent Cations. *Biochemistry* **47**, 5336-5353 (2008).

9 Shih, Y.-P., Wu, H.-C., Hu, S.-M., Wang, T.-F. & Wang, A. H. J. Self-cleavage of fusion protein in vivo using TEV protease to yield native protein. *Protein Science* **14**, 936-941 (2005).

10 Qian, L. & Winfree, E. Scaling Up Digital Circuit Computation with DNA Strand Displacement Cascades. *Science* **332**, 1196-1201 (2011).

11 Chandran, H., Gopalkrishnan, N., Phillips, A. & Reif, J. Localized Hybridization Circuits. *DNA Computing and Molecular Programming* **6937**, 64-83 (2011).

12 Qian, L. & Winfree, E. Parallel and Scalable Computation and Spatial Dynamics with DNA-Based Chemical Reaction Networks on a Surface. *DNA Computing and Molecular Programming* **8727**, 114-131 (2014).

13 Geary, C., Rothemund, P. W. K. & Andersen, E. S. A single-stranded architecture for cotranscriptional folding of RNA nanostructures. *Science* **345**, 799-804 (2014).

14 Pardee, K. *et al.* Paper-Based Synthetic Gene Networks. *Cell* **159**, 940-954 (2014).

15 Nielsen, A. A. K. *et al.* Genetic circuit design automation. *Science* **352** (2016).

Table S10. Comparison of Toehold Switch Design Parameters

	First-Generation Toehold Switch (Ref. 17)	Second-Generation Toehold Switch (Ref. 17)	AND-Computing Toehold Switch Type I (this work)	AND-Computing Toehold Switch Type II (this work)
Toehold length (nt)	12	15	16	15
Lower stem length (bp)	9	9	12	11
Upper stem length (bp)	6	6	6	5
Loop length (nt)	11	15	14	12
Trigger-switch binding length (nt)	30	30	28	26
Base pairs repressing RBS after trigger-switch binding	0	3	6	5