

Cellular Logic with Orthogonal Ribosomes

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We recently described the evolution of several orthogonal ribosome•orthogonal mRNA pairs (O-ribosome•O-mRNA pairs) in *Escherichia coli*.¹ For each orthogonal pair, the O-ribosome does not translate cellular mRNAs, and the O-mRNA is not a substrate for the endogenous ribosome. The specificity of each O-ribosome results from the incorporation of an orthogonal 16S ribosomal RNA (O-rRNA) into the ribosome. We have defined the network of molecular specificities of each O-ribosome, with respect to both cognate and noncognate orthogonal ribosome binding sites on mRNA, by considering each pairwise O-ribosome•O-mRNA interaction in isolation. Pairs of O-ribosome•O-mRNA pairs have the molecular specificities that define mutual orthogonality. For example, O-ribosome-A translates its cognate O-mRNA-A, but not the noncognate O-mRNA-C, and O-ribosome-C translates its cognate O-mRNA-C, but not the noncognate O-mRNA-A. Similarly, O-ribosome-B•O-mRNA-B and O-ribosome-C•O-mRNA-C are mutually orthogonal (Figure 1). Here we show that subnetworks of this network graph can be physically realized in a single cell and allow combinatorial cellular programming of entirely post-transcriptional Boolean logic functions.

The requirements for the realization of subnetworks are that multiple distinct ribosome•mRNA pairs can be produced in a single cell, and that these pairs function independently of other ribosome•mRNA pairs in this context. The simultaneous expression of multiple distinct mutant ribosomes in cells has not previously been demonstrated. It requires the expression and processing of two ribosomal RNAs from two compatible plasmids. It also requires that ribosomal proteins are produced from the genome in sufficient quantities to produce functional ribosomes containing wild-type ribosomal RNA as well as two functional orthogonal ribosomes, which each contain a distinct O-rRNA. This is particularly challenging since excess rRNA may be degraded *in vivo*,² leading to a depletion of both wild-type and orthogonal ribosomes. Moreover, ribosome assembly is not entirely cooperative,^{3–5} and production of mutant rRNA in excess of the cell's capacity to synthesize ribosomal proteins might lead to partially assembled, and therefore non-functional, wild-type and orthogonal ribosomes.

As a first step toward the simultaneous production of three ribosomes in the cell (the wild-type ribosome and two O-ribosomes), O-rRNAs were produced from plasmids of distinct compatibility groups, and the resulting ribosomes were assayed for function. One vector for rRNA production has a ColE1 origin of replication and an ampicillin resistance gene and is present at about 50 copies per cell. A second vector for rRNA production has an RSF origin of replication and a kanamycin resistance gene and is present at about 100 copies per cell. We have previously observed that the production of functional ribosomes incorporating plasmid-encoded rRNA can be strongly modulated by the sequences flanking the rRNA transcriptional cassette. To ascertain the effect of plasmid flanking sequences and plasmid copy number on the activity of the O-ribosomes incorporating plasmid-encoded rRNA, the translation of the chloramphenicol acetyl transferase gene (*cat*) from

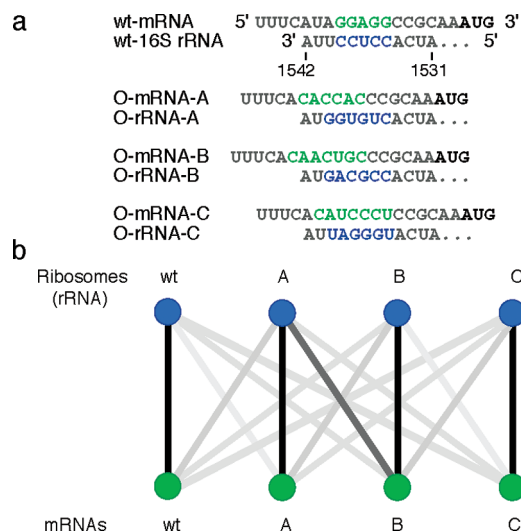


Figure 1. Orthogonal ribosome•orthogonal mRNA pairs and their network of specificities. (a) The sequence of rRNA that interacts with mRNA is shown (wt is wild-type). Mutations in O-mRNAs and O-rRNAs are shown in green and blue, respectively.⁶ (b) Pairwise ribosome•mRNA interaction strengths are indicated by grayscale intensity.

O-mRNA-Ccat (a version of *cat* with the 5' orthogonal ribosome binding site C) was measured. Cells containing RSF or ColE1 plasmids encoding rRNA-C confer resistance to chloramphenicol, with IC₅₀ values of 250 and 150 $\mu\text{g mL}^{-1}$, respectively, while *O-mRNA-Ccat* has an IC₅₀ of 10 $\mu\text{g mL}^{-1}$ in the absence of cognate ribosome. Similar results were obtained with other O-ribosome•O-mRNA pairs. These results demonstrate that highly active orthogonal ribosomes can be produced from two compatible plasmids, and that the RSF plasmid leads to a slightly greater ribosome activity than the ColE1 plasmid, as predicted based on copy number alone.

To demonstrate that multiple O-ribosomes can be produced in a single cell, and to begin to address the potential of O-ribosomes for the expression of Boolean logic, an AND gate containing O-mRNA sequences was designed. The gate is composed of two O-mRNA sequences: O-mRNA-A ω directs the synthesis of the ω fragment of β -galactosidase, while O-mRNA-C α directs the synthesis of the α fragment of β -galactosidase. Upon synthesis and assembly of both fragments into β -galactosidase⁷ ($(\alpha + \omega)_4$), cells hydrolyze fluorescein di- β -D-galactopyranoside (FDG) to fluorescein (F),⁸ which can be detected fluorimetrically (Figure 2a,b).

Cells containing a plasmid encoding both O-mRNA-C α and O-mRNA-A ω were programmed with either wild-type rRNA, rRNA-A, rRNA-C, or rRNA-C and rRNA-A together, and the conversion of FDG to fluorescein was measured. Cells programmed with wild-type rRNA produce low fluorescence, which is comparable to background. This confirms that the orthogonal ribosome binding sites A and C—developed on the *cat* gene—are portable and can confer orthogonality to a variety of genes. Cells pro-

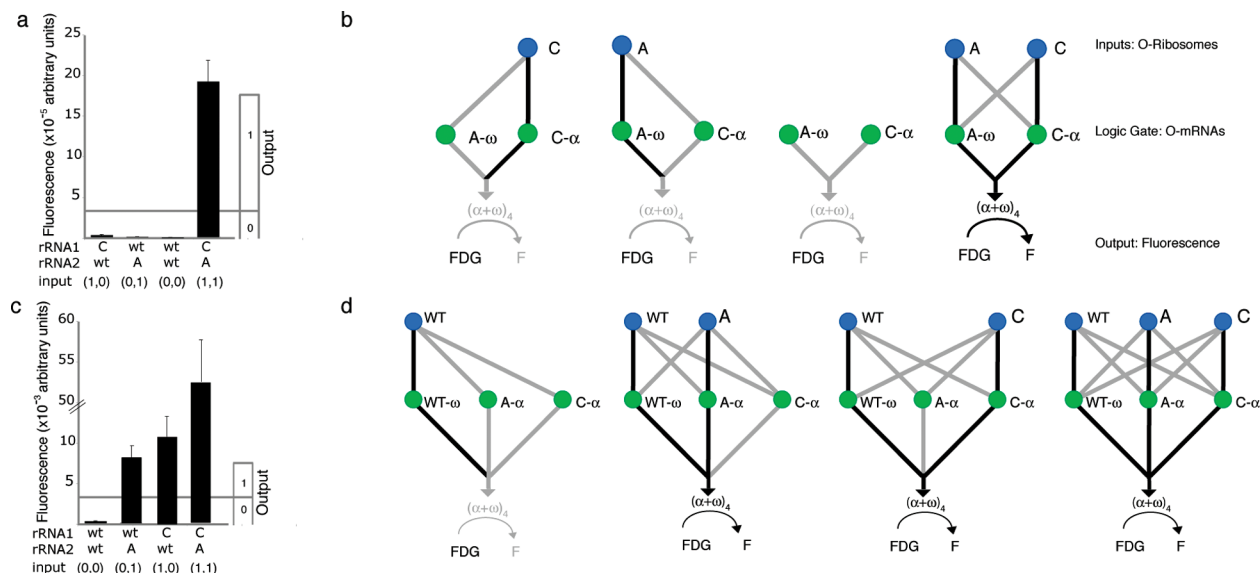


Figure 2. Combinatorial logic with orthogonal ribosomes. (a) The fluorescence generated as a function of ribosome inputs for the AND gate. Fluorescence is normalized for cell density and time of incubation, as detailed in the Supporting Information. Error bars represent the standard error of at least three independent trials. (b) Each state of the AND gate. Black lines indicate functional connections, while gray lines indicate components that are insulated from each other. (c and d) As for (a) and (b), but for the OR gate.

grammed with rRNA-A also produce low fluorescence, as do cells programmed with rRNA-C. However, cells programmed with both rRNA-A and rRNA-C give a fluorescent signal 20-fold greater than that of other rRNA combinations. These data demonstrate that multiple mutually orthogonal ribosomes can be functionally expressed in a single cell. Moreover, they show that rRNA-A and rRNA-C can be used as inputs in a post-transcriptional AND function. Similar AND functions were also obtained with cells containing other mutually orthogonal ribosomes and their cognate O-mRNA α s and O-mRNA ω s.

Next, we attempted to create a Boolean OR gate. The OR gate is composed of two O-mRNAs (O-mRNA-A α and O-mRNA-C α), each of which directs the synthesis of the α fragment of β -galactosidase (Figure 2c,d). In this system, the ω fragment is constitutively produced from a wild-type ribosome binding site. Cells programmed with wild-type ribosomes produce a fluorescence comparable to that observed in the absence of plasmid-encoded α fragment. Cells programmed with rRNA-A produce a fluorescence signal 10-fold above background, while cells programmed with rRNA-C produce a level of fluorescence 15-fold above background. Cells programmed with both rRNA-C and rRNA-A give a fluorescent signal more than 50-fold above background. The increase in fluorescent signal indicates that in this system the ω fragment is present in excess of the α fragment. When wild-type ribosome binding sites are used to replace the orthogonal ribosome binding sites on the mRNA, a similar result is observed. This suggests that the mismatch in cellular concentration of the ω fragment and α fragment results from a deficiency in either the transcription or lifetime of the α fragment mRNA, or degradation of the α fragment peptide. Overall, these results demonstrate that rRNA-A and rRNA-C can be used as inputs in a Boolean OR function. The OR function can also be created using other mutually orthogonal rRNAs and cognate O-mRNAs.

In conclusion, we have demonstrated that O-ribosomes and O-mRNAs can be used to create entirely post-transcriptional combinatorial logic in living cells. The Boolean gates described require multiple distinct orthogonal ribosomes as inputs and could not be assembled using the wild-type ribosome since its removal from the cell is lethal, precluding a value of zero for its input. Our results begin to demonstrate how unnatural, orthogonal, modular components and a knowledge of the noncovalent interactions between components⁹ may be used to synthesize unnatural network architectures and logical functions in living matter.¹⁰ Extensions of our approach may ultimately allow the synthesis of cellular computers in which signals are carried and specified not by electrical wires, but rather by molecules with unnatural specificities.¹¹

Supporting Information Available: Experimental procedures and details of materials. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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