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In vivo application of an inhibitory RNA aptamer against T7 RNA polymerase

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Abstract Synthetic biology involves the design of complex artificial biological systems with exploitable features for human benefit through an ecological and sustainable way. Recent advances in nucleic acids engineering introduced several RNA-based regulatory components for synthetic gene circuits, expanding the toolsets to engineer organisms. In this work, we designed genetic circuits implementing an RNA aptamer previously described to have the capability of binding to the T7 RNA polymerase and inhibiting its activity *in vitro*. Using *in vitro* transcription assays, we first demonstrated the utility of the RNA aptamer in combination with programmable synthetic transcription networks. As a step to quickly assess the feasibility of aptamer functions *in vivo*, a cell-free expression system was used as a breadboard to emulate the *in vivo* conditions of *E. coli*. We tested the aptamer and its three sequence variants in the cell-free expression system, verifying the aptamer functionality in the cell-free testbed. *In vivo* expression of aptamer and its variants demonstrated control over GFP expression driven by T7 RNA polymerase with different response curves, indicating its ability to serve as building blocks for both logic circuits and transcriptional cascades. This work elucidates the potential of RNA-based regulators for implementing different "biocodes" for cell programming and for improved controllability thanks to the fast production and degradation time scales of RNA molecules.

Keywords: synthetic biology, logic circuits, biological machines, molecular programming, aptamer

Introduction

Synthetic biology is a field that involves the (re)design of tools and biological systems for the development of "biological machines" that allow us to address problems in an ecological and sustainable way. To achieve this, such *de novo* biological systems need to be programmed, which is accomplished by using molecular mechanisms that work as programming codes. The design of the first synthetic gene networks as the toggle switch [1] and the repressilator [2] opened up the opportunity of programming computational tasks in living cells – ideally in such a way that we can control cell behavior in a similar manner as we do over computational softwares.

Recent advances in synthetic biology have allowed scientists to program biological organisms with functions that do not exist in nature by introducing engineered functional gene circuits into living hosts [3, 4, 5, 6, 7, 8, 9, 10, 11]. Most synthetic gene circuits are regulated by a diversity of molecular mechanisms based on protein-DNA interactions to control transcription, which results as a consequence in a big challenge to predict the behavior of synthetic circuits because all the regulatory levels involved in protein expression, including; transcription, RNA degradation, ribosome binding site recognition by ribosomes, translation, post-translational modification, protein folding, and protein degradation.

Besides, a key limitation in gene network engineering is that the circuits are based on a few characterized regulatory elements, being the size of programs limited to a small number of possible combinations, so it is of major significance the search and description of further functioning "biocodes" to program living cells.

In the recent past, RNA structures have been considered as important tools for programming gene expression by virtue of their interaction with other nucleic acids, proteins and small molecules [12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25]. Among these components, RNA aptamers posses the molecular recognition properties of antibodies for exclusive ligands that are conferred by their specific sequences, allowing these molecules to form core structures responsible for their function and binding to other components. Broadly speaking, RNA aptamers are generated and selected in vitro from large random sequence libraries, and optimized for high affinity binding to given ligands by a process known as SELEX (Systematic Evolution of Ligands by EXponential enrichment) [26, 27, 28, 29].

In a recent work, Ohuchi and co-workers [30] reported a series of RNA aptamers with the capability of binding to the T7 RNA polymerase (T7 RNAP) and inhibiting its activity *in vitro*. Although the detailed mechanism is not fully understood, it was shown that the specific sequence of the RNA molecule mediates the T7 RNAP inhibition, and modifications to it directly affect the affinity of aptamer-T7 RNAP interactions. In this study, we implemented the use of the aptamer as a NOT-gate in a regulatory circuit for in vivo applications, employing the long version of the aptamer T230 (T230-38) which in our first in vitro assays exhibited better T7 RNAP inhibition than the short version, T230-29 (data not shown). As a step to quickly assess the feasibility of aptamer function in vivo, a TX-TL cell-free system was employed as a breadboard to emulate the *in vivo* conditions of *E. coli* for circuitry testing [31, 32]. Our results clearly demonstrate that the aptamer T230-38 can be used as a NOT-gate for *in vivo* synthetic biology applications with notable T7RNAP inhibition results.

Materials and Methods

Circuit engineering and use of strains. Plasmid pAR1219 was obtained from Sigma-Aldrich, and plasmid pTara was provided by Matthew Bennetts group at Rice University. The rest of the circuits were constructed using the Gibson assembly as described in detail previously [33]. Plasmids schemes are shown throughout the text and whole sequences and full features are described in Supplementary Information S1. Plasmids were transformed into strains via chemical transformation. Escherichia coli MGZX was used for inducible expression from the TetR-regulated promoter as this strain overexpress TetR from its chromosome (it also expresses LacI, AraC, and LuxR). Plasmids pdeGFP-T230-38-4A, pdeGFP-T230-38-4G, and pdeGFP-T230-38-4Δ were derived from the plasmid pdeGFP-T230-38 via PCR amplification, employing 5′-phosphorylated oligonucleotides for subsequent ligation. Plasmids containing the aptamer T230-38 and variants have an additional XhoI site between the aptamer and the terminator sequences (the functionality of the aptamer T230-38 function was not affected by this feature). All the sequences of constructions that involved PCR were confirmed through DNA sequencing.

The sequence of all DNA molecules and expected RNA transcript sequences were chosen to minimize the occurrence of alternative secondary structures, checked by the DNA and RNA folding program NUPACK [34]. The DNA and RNA sequences used in this study are listed in Supportine Information section. (See Figure S1 for sequence domains and predicted secondary structures.) All DNA oligonucleotides and the short RNA signal iMG were purchased from Integrated DNA Technologies (USA). The T7 RNA polymerase (Cellscript, Madison, WI, USA; #C-AS2607), 10× transcription buffer and thermostable inorganic pyrophosphatase (New England Biolabs, Ipswich, MA, USA; #B9012S, #M0296S), NTP and RNase R (Epicentre, Madison, WI, USA; #RN02825, #RNR07250) were purchased. Malachite Green dye was purchased from Sigma (#M9015). Since pyrophosphatase is involved in

regulating the byproduct inorganic pyrophosphate for our transcriptional circuits and is not directly involved in the dynamics, we neglect this enzyme in our models and do not call it an "essential enzyme" for the circuit dynamics. The nominal concentrations of enzyme stocks quoted by the manufacturer were used: 10.5 μM for RNase R and 6 μM for T7 RNAP.

Media, Chemicals and Other Reagents. Strains were grown in LB liquid medium or on agar plates (1.5% agar), supplemented by antibiotics, carbenicillin (100 μg/ml) and chloramphenicol (34 μg/ml). In each experiment comprising IPTG induction, the concentration for this chemical was 10 μM. Anhydrotetracycline (aTc) experiments were conducted with different concentrations to achieve different levels of RNA aptamer induction as individually described in the figures throughout the text.

Data acquisition. For *in vitro* transcription experiments, excitation and emission for Malachite Green fluorescence were at 630 nm and 655 nm. DNA templates were annealed with 10% (v/v) 10× transcription buffer from 90°C to 20°C over 1 hour at 5 μM concentrations. Transcription reactions were prepared by combining the annealed templates, 7.5 mM each NTP, 24 mM $MgCl_2$ (to balance salt concentrations due to increased NTP concentrations), 10% (v/v) 10× transcription buffer, and 25 μM Malachite Green dye in a test tube. Transcription reactions for spectrofluorometer experiments were prepared as a total volume of 60 μL in quartz cuvettes and enzymes (RNAP and PPase) were added and mixed after the baseline fluorescence was recorded for 10 min. The fluorescence was recorded every minute using a Fluorolog-3 spectrofluorometer (Jobin Yvon, Edison, NJ, USA). Temperature was set at 29 °C.

For TX-TL experiments, samples were measured fluorescence intensity using a filter set with excitation wavelength at 488 nm and emission wavelength at 507 nm. All data were obtained using Victor X3 plate reader. Concentrations of plasmids pAR1219, pPT7-deGFP, and pTet-T230-38 were 0.8 nM for each corresponding experiment. The reaction volume was 10 μL, and the number of replicates was two for each condition.

For in vivo experiments, all samples were measured fluorescence intensity using a monochromater with excitation wavelength at 485 nm and emission wavelength at 525 nm. Final fluorescence data were normalized by an OD600 value. All data were obtained using Biotek Synergy H1 plate reader. The reaction volume was 200 μL, and the number of replicates was four for each condition.

Results

Transcriptional circuit *in vitro* for aptamer functionality testing. Initially, the aptamer functionality was tested in an in vitro system implementing a transcriptional regulatory mechanism reported by Kim et al. [35, 36, 37] (Figure 1A). This mechanism consists of a promoter lacking part of the double stranded DNA, remaining as a single strand; upon addition of a DNA activator (complementary single stranded DNA) the promoter is completed except for a nick, herein, the transcription efficiency is high.

For this purpose, a transcriptional circuit was designed (Figure 1B). Given that we are dealing with a transcriptional circuit, instead of using a protein reporter, we used an aptamer (MGA) with the property of being able to bind to the chemical compound Malachite green and producing proportional fluorescence to the binding event [38].

In the circuit, T3 RNAP recognizes pT3 promoter upon addition of the DNA activator A2, and induces transcription of T230-38. Likewise, T7 RNAP recognizes pT7 promoter upon addition of the DNA activator A1, and induces transcription of MGA.

In our experiment, we first added both T3 and T7 RNA polymerases to the reaction in the minute 10th and the DNA activator A1 after 15 min of incubation, leading transcription of MGA to start. Then, the DNA activator A2 was added in the minute 20th, enabling transcription of T230-38 in order to block T7 RNAP-mediated MGA expression.

As shown in Figure 1C, aptamer-mediated T7 RNAP inhibition was observed quickly after the addition of the DNA activator A2, and even with high concentrations of T7 RNAP the aptamer inhibitory effect was quite remarkable.

Aptamer-mediated T7 RNAP inhibition in TX-TL system.

After we observed the aptamer worked in the TX system, we designed a new circuit for in vivo application assays (Figure 2A).

Creating an in vivo functioning biocircuit can be a lengthy process, involving multiple iterations of design, assembly, and testing. Similar challenges can be expected for testing novel regulatory mechanisms such as the inhibition of T7 RNAP through aptamers.

Transcription-Translation (TX-TL) cell-free expression systems are biomolecular breadboards that take advantage of shorter assembly and troubleshooting times by conducting reactions in vitro emulating *in vivo* conditions [31, 32]. Because requirements for propagating plasmids in vivo have been removed, and transformation is not needed, this results in faster design times and quicker iterations of the design, assembly, and testing process of a given circuit. It is worth mentioning that linear parts as circuit components can be also tested in the TX-TL system.

In order to emulate whether the aptamer could work in vivo, we assayed the aptamer in the TX-TL system. Plasmids shown in Figure 2B were introduced into the TX-TL system under a variety of conditions. As a nega-

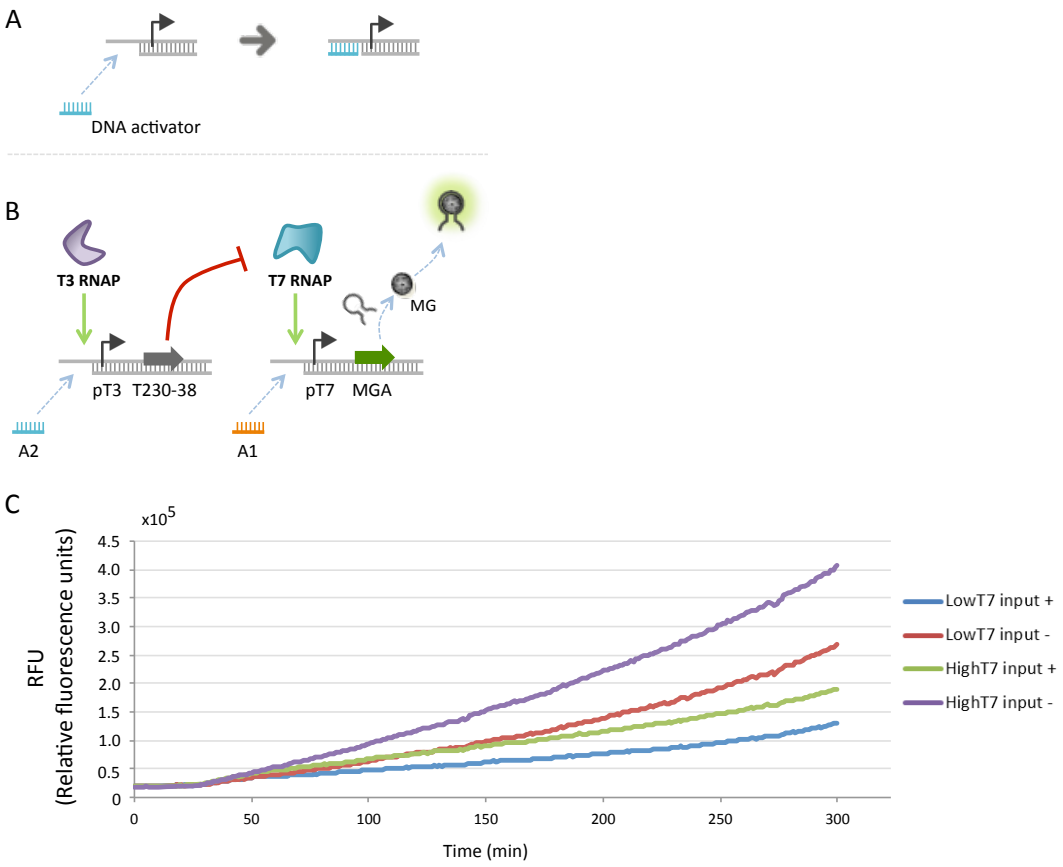

Figure 1: *In vitro* transcriptional circuit for Aptamer functionality testing. (**A**) Inducible transcription system in which a promoter lacks part of the double stranded sequence. After addition of a single stranded DNA activator the promoter is complete except for a nick, and thus, the transcription efficiency is high. (**B**) Transcriptional circuit for characterization of T7 RNAP aptamer-mediated inhibition. Promoters pT3 and pT7 are able to be induced by T3 RNAP and T7 RNAP, respectively, upon addition of the corresponding DNA activator (A2 and A1). When the aptamer MGapt is expressed, it can binds to the chemical compound Malachite green, producing fluorescence that is proportional to its concentration. (**C**) Transcriptional assay of the circuit (B). T3 RNAP and T7 RNAP were added to the reaction in the 10th minute. DNA activator A1 was introduced in the reaction after 15 min incubation. DNA activator A2 was added 20 min later. LowT7 and HighT7 refer to low and high T7 RNAP concentration, and input refers specifically to A2.

tive control, we introduced to the TX-TL system a reaction with the plasmids pAR1219 and pPT7-deGFP in the absence of IPTG. Another reaction containing the same plasmids in the presence of IPTG was implemented as a positive control, and a third reaction to test the aptamer functionality containing the plasmids pAR1219, pPT7-deGFP and pTet-T230-38 in the presence of IPTG. In this case, the aptamer was being expressed constitutively since TetR repressor was not incorporated among the plasmids used.

As shown in the Figure 2C, 10-fold reduction of deGFP output was obtained in the presence of the plasmid containing the aptamer, while on its absence deGFP was notably expressed, being this better than our first result obtained in the TX system.

In vivo **experiments and aptamer structure disruption.** Since the results obtained in the TX-TL experiment were very promising, we continued with the in vivo experiments. deGFP and Aptamer cassettes contained in the plasmids pPT7-deGFP and pTet-T230-38 (including from promoter to terminator in each case), were joined together using the plasmid pTara (Figure 3A) as a backbone to give rise to the plasmid pdeGFP-T230-38 (Figure 3B).

The purpose of using pTara as a backbone was to obtain a different replication origin and antibiotic resistance than the plasmid pAR1219 intending to be able to perform the double transformation of *E. coli* MGZX, which contains a constitutively expressed TetR repressor in its genome, allowing us to complete the whole circuit as shown in the Figure 2A.

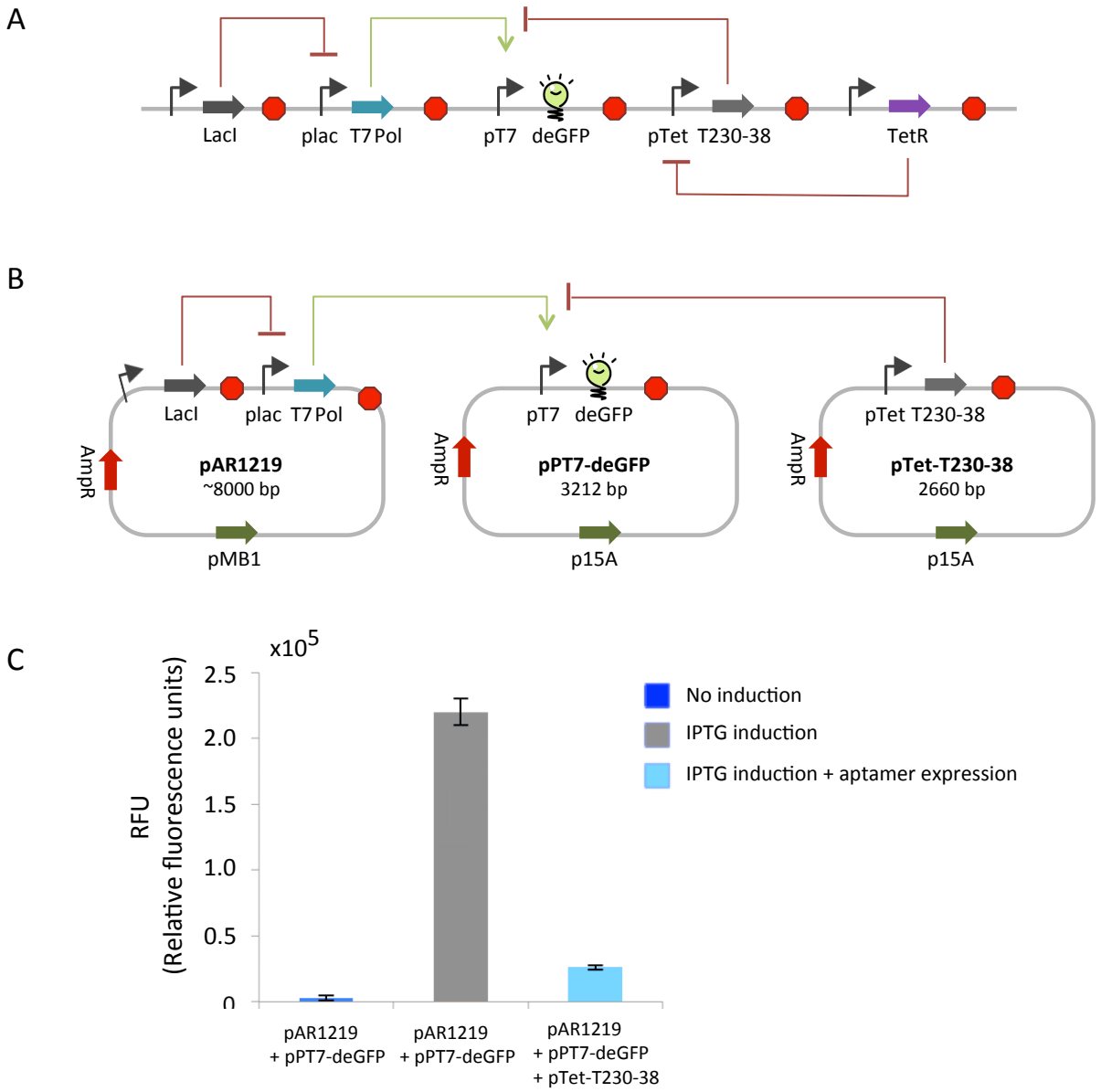

Figure 2: Genetic circuits and aptamer-mediated T7 RNAP inhibition in the TX-TL system. (**A**) Designed circuit for in vivo experiments. (**B**) Circuits for aptamer-mediated T7 RNAP inhibition in the TX-TL system. (**C**) Aptamer-mediated T7-RNAP inhibition TX-TL results showing that deGFP expression was approximately 10-fold decreased in the presence of the aptamer.

Afterwards, mutant variants of the T230-38 aptamer were constructed through site-directed mutagenesis in order to disrupt the aptamer structure supposed to be responsible for T7 RNAP inhibition. The purpose of this was to demonstrate that T7 RNAP inhibition was not mediated by the cellular stress caused by aptamer overproduction or inducer (aTc) addition to the media.

The selected target for site-directed mutagenesis was a conserved region found among all the versions reported by Ohuchi et al. [30], which includes the nucleotides 28–31 of the T230-38 sequence. These four nucleotides were replaced either by AAAA, GGGG, or deleted, obtaining three resultant plasmids, each with a different mutant variant; pdeGFP-T230-38-4A, pdeGFP-T230-38-4G, and pdeGFP-T230-38-4Δ, respectively (Figure 4A).

Before proceeding to any vivo experiments, we introduce pAR1219 together with each variant of the plasmid pdeGFP-T230-38 into the TX-TL system. Given that

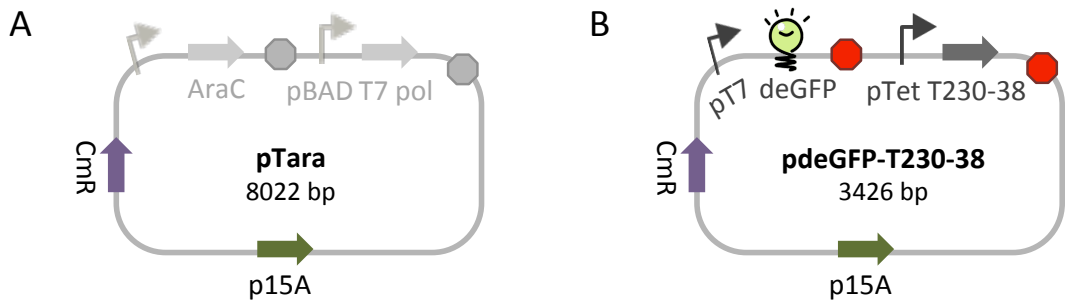

Figure 3: Assembly of deGFP and aptamer cassettes into a single plasmid. (**A**) Plasmid pTara was used as a backbone to join deGFP and aptamer cassettes. The shadowed parts in pTara plasmid were discarded in the process. (**B**) Plasmid pdeGFP-T230-38 for in vivo experiments.

TetR was planned to be expressed from E. coli MGZX, for negative and positive controls in the TX-TL system we replace pdeGFP-T230-38 (and variants) with pPT7-deGFP. The results obtained after 6 h incubation in the TX-TL system were downright as expected, the aptamer T230-38 showed 10-fold inhibition of the T7RNAP as in previous experiments, whereas the mutant versions showed decreased inhibition capability, thought to be doubt to the disruption of the core structure of the aptamer, result of the modification of the conserved region.

After the success obtained in the TX-TL system we carried out experiments in vivo, where the results obtained clearly showed aptamer-mediated T7 RNAP inhibition (Figure 5A-B). When T230-38 aptamer expression was induced with high concentration of aTc (1 μg/mL), 5-fold reduction in the deGFP output was obtained. In contrast, the mutant variants when induced by the same concentration of aTc showed half or less effect on the deGFP output (Figure 5C-F), where the inhibition achieved by the mutant T230-38-4Δ was practically null, assenting the assumption that T7 RNAP inhibition is mediated by the T230-38 aptamer, and not by some kind of cellular stress, nor other unspecific RNA molecules.

Something unexpected was the result obtained for the negative control (cells with no chemical induction), which displayed high deGFP output in all cases (T230-30 and mutant variants). This could be due to a weak repression of T7 RNAP expression or a leakage, considering that even a small amount of T7 RNAP is sufficient to direct high-level transcription from a T7 promoter. Nevertheless, when cells were induced with IPTG, the deGFP output obtained is higher, and repression is obtained when added aTc, evidencing that the aptamer effectively works in a noteworthy manner.

Additionally, something we considered is that in our TX-TL experiment the concentration for the plasmids pAR1219, pPT7-deGFP, and pTet-T230-38 was 0.8 nM for each one, meanwhile pMB1 and p15A are 15–20 and 10–12 copy number replication origins, respectively, explaining in part the little variation observed in the aptamer-mediated T7 RNAP repression among the in vitro and in vivo results (≈10-fold against ≈5-fold repression achieved, respectively), but yet giving us an idea of the high effective aptamer-mediated T7 RNAP inhibition can be in vivo for further constructs if considering replication origins usage, as well as regulation mechanisms selection, which in this case was LacI from the predesigned commercial plasmid pAR1219 for T7 RNAP, and TetR from the*E. coli* strain MGZX for the aptamer T230-38.

Discussion

Synthetic gene circuitry and cell programming has opened up new opportunities to deal with several worldwide problems through an ecological and sustainable way. RNA molecules have previously been shown to function in living organisms as regulatory elements. The incorporation and demonstrated function of specific-sequence nucleic acid molecules to regulate cell functions in vivo indicates an exciting option to program living cells in a synthetic biology context as an alternative to regular protein-based regulation mechanisms. Here we show that the aptamer T230-38 has the capability of inhibiting the T7 RNAP in vivo and can serve a NOT-gate. The sequence-specific properties of the aptamer seen in our results provide worthy evidence to think about how much remains to be done with this kind of alternatives to predesigned biological tools found in nature.

As can be seen in the present work, synthetic well-characterized biological components are fundamental for engineering biological devices and yield accessibility to undertake projects in an increasingly custom approach, and TX-TL cell-free systems offer a practical breadboard to quickly test their functionality. Further work need to be done to obtain improved mechanisms that let us think about obtaining robustness as in electronics, however the

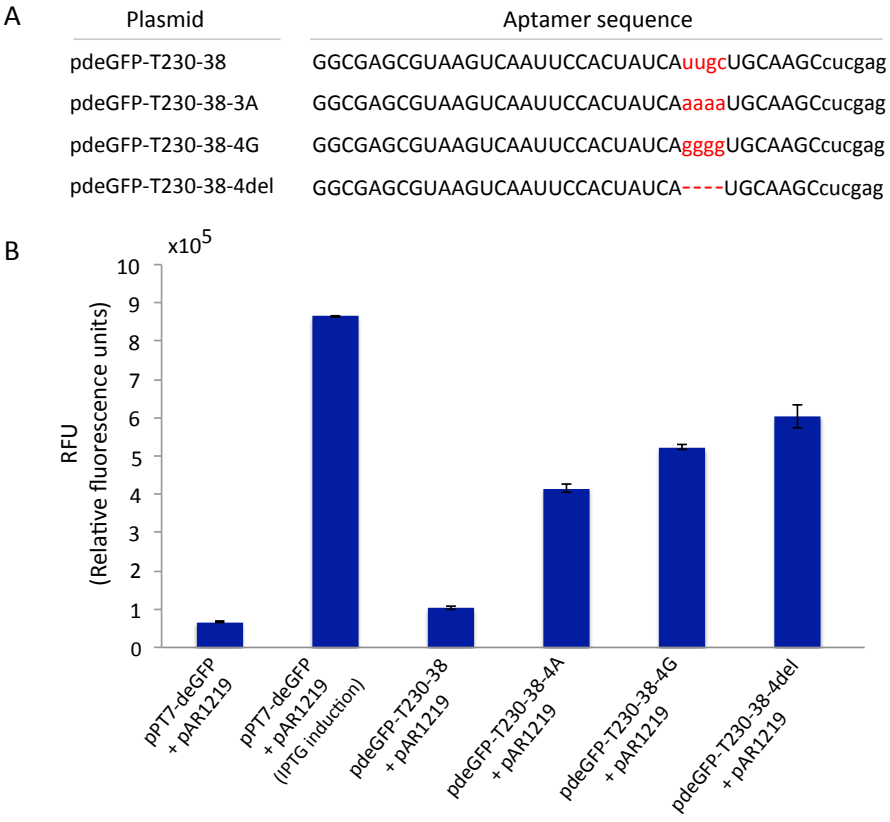

Figure 4: Characterization of the aptamer mutant variants in the TX-TL system. (**A**) Representation of the aptamer sequence contained in each variant of the plasmid pdeGFP-T230-38. The conserved domain from which we developed the mutant versions is displayed in red color. (**B**) Results obtained in the TX-TL system after 6 h at 29 °C.

current picture show us big promising facts.

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Conflict of interest statement. None declared.

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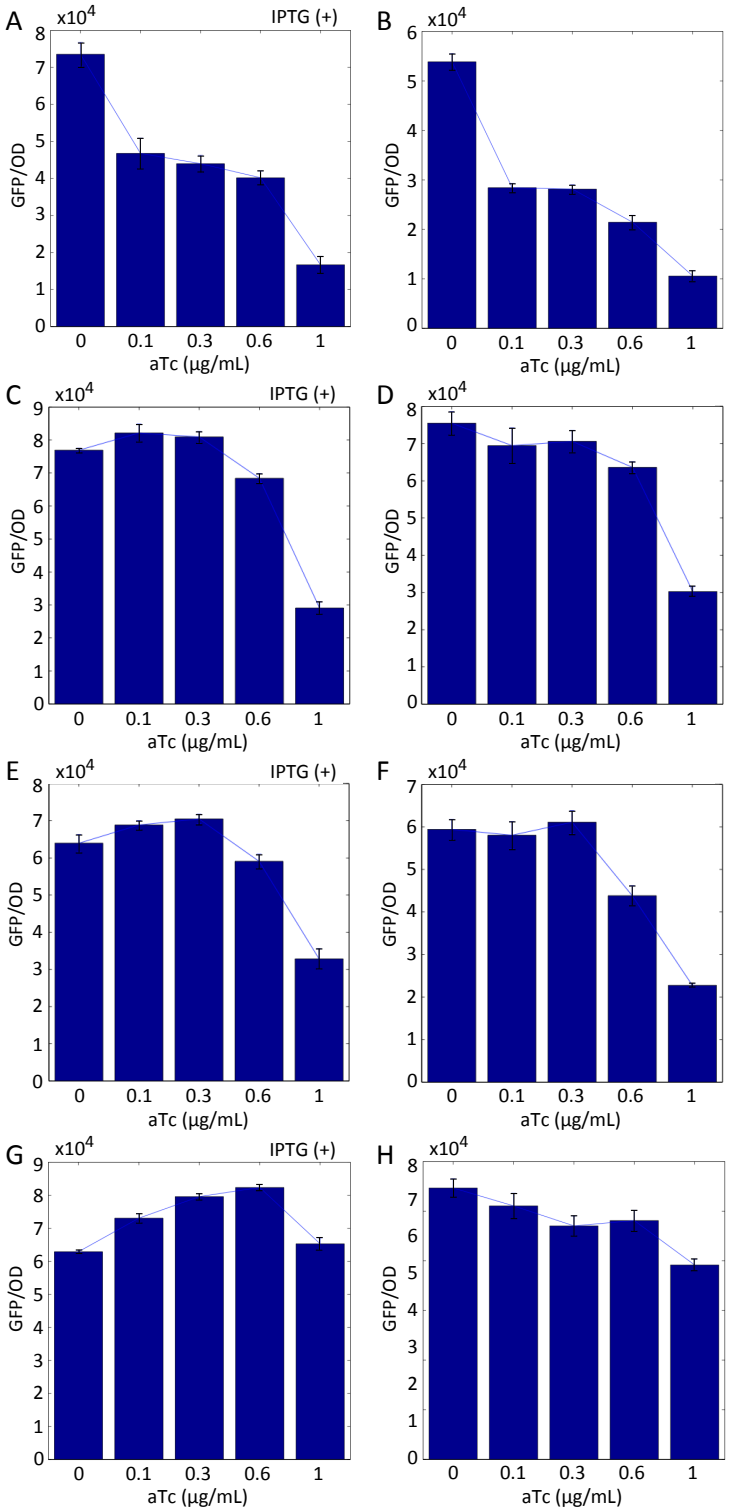

Figure 5: Aptamer-mediated T7 RNAP inhibition *in vivo* results. *E. coli* MGZX containing a constitutively expressed TetR allowed us to complete the circuit shown in the Figure 2A and carry out the *in vivo* experiments after we performed double transformations indicated as follows. (**A, B**) pAR1219 + pdeGFP-T230-38. (**C, D**) pAR1219 + pdeGFP-T230-38-4A. (**E, F**) pAR1219 + pdeGFP-T230-38-4G. (**G, H**) pAR1219 + pdeGFP-T230-38-4Δ. The results shown were obtained after eight hours of incubation.