Technical Summary of PhD thesis

Scaling up Genetic Circuits in Mammalian Cells: A U1-snRNA-based Platform Enables Mammalian Cells to Compute the Bitwise Inversion of the Square Root of a Number

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Abstract

Scaling up genetic circuits in mammalian cells can lead to a new class of therapies. As result of my research as PhD candidate on exploring ways and engineering tools to scale up genetic circuits, I engineered and validated in HEK293FT cells a genetic circuit that allows those cells to compute the bitwise inversion of the square root of a number. To date, this circuit which has four inputs and two outputs is the most sophisticated genetically encoded circuit ever expressed in mammalian cells. The core processing module of the circuit is a novel miRNA-based NOT gate based on a platform that uses the U1 snRNA. We have called this platform "u.P.R.O.C.E.S.S.O.R." (U-gene-based Platform, RNAi-regulated Only, Compactly Employing Small Shuttle-miRNAs, Operates (through) RNA), which does not use any transcriptional regulators or exogenous proteins, which can cause dangerous immune responses. The design of this sophisticated logic circuit was found by executing an algorithm, which I developed, for the exhaustive search of logic circuits designs (with 4 inputs and 2 outputs). The solution tested in HEK293FT cells required just four transcriptional units and about 10kb of DNA. Furthermore, I have engineered a trans-activated gRNA (for CAS9) and a trans-activated miRNA to sense abundant nuclear RNAs by the use of the toehold-mediated strand displacement reaction. The trans-activated miRNA also dose not use any transcriptional regulators or exogenous proteins and, like the miRNA-based NOT gate, has a DNA footprint small enough to fit in a AAV virus.

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Background and Rationale One field of synthetic biology explores ways to engineer and scale up genetically encoded logic circuits. In particular, logic gates in mammalian cells have the potential to lead to completely new and more effective therapies. Previously, a genetically encoded logic circuit has been proved to distinguish, in cell culture, between Hela cancer cells and non-Hela cells (cancer classifier) [1]. More recently, an RNA-based AND gate increased the survival of mice treated with cancer immunotherapies [2]. Clearly, more sophisticated logic

circuits in mammalian cells can enable new therapies in treating cancer and other diseases as well. The Winfree lab in Caltech proved that it was possible to engineer, in cell free settings, a logic circuit that computes the square root of a number by the use of DNA strand displacement [3]. At that time, this technology appeared suitable to scale up genetically encoded circuit in mammalian cells while aiming at a small DNA footprint, for easy delivery in vivo, and without using exogenous proteins, which can cause dangerous immune responses. Because of that, at the beginning of my PhD, this work has been the basis of my efforts to scale up genetic circuits. Afterward, the focus of my research shifted toward miRNAs, which I used both with and without the strand displacement reaction.

MiRNA as tool for sensing, processing, and actuation. MiRNA is a versatile tool widely used in biotechnologies and gene therapies. On the one hand, it is used as therapeutic agent to downregulate the expression of any gene of choice. On the other hand, the extensive knowledge of miRNAs expressions in different cell lines and states allows their use as biomarkers. More importantly, the Watson and Crick base pairing, which is the mechanism that allows the RISC complex to identify the target RNA to be downregulated, provides a large design space which in turn makes miRNAs good candidates as input-output signals of complex logic circuits. Finally, there is a large body of research that is examining the use of AAV-RNAi to treat different diseases [4] (The AAV virus is considered among the safest vector in gene therapy due to its low risk profile for immunogenicity and genomic integration). As consequence, engineering miRNAs whose activities can be conditionally regulated can be valuable even outside the context of scaling up genetic circuits.

Summary of Contributions

Sensing nuclear RNAs through toehold-mediated RNA strand displacement. Toehold-mediated RNA strand displacement has been the focus of extensive academic research. A general framework, in the use of this technology, is the one where an RNA molecule changes folding state due to the interaction with an input-RNA strand. In this new folding state, the RNA molecule can then interact with an actuator, which can be an RNA, a protein or a protein complex [5]. While this framework is robust for its use in cell free settings, it has been difficult to use it in genetically encoded circuits in mammalian cells. Here, the folding of the RNA soon after transcription can lead the RNA in folding states that allow the interaction with the actuator even in case the input is absent. During my PhD, I provided design principles to reduce these side reactions. Here,

the general idea is that an RNA strand can still interact with its related actuator when the conformation of the RNA needed for this interaction is at an energy state that is far from the lowest one. As result of these design principles, I engineered genetically encoded sensors: the trans-activated miRNA and the trans-activated gRNA (for cas9). Here, nuclear RNAs are the input molecules which, through toehold-mediated strand displacement, upregulate the miRNA and Cas9 activity. Importantly, those genetically encoded devices may enable, by the use of AAV virus for instance, the conditional expression of therapeutic agents in cells that are characterized by abundant nuclear RNAs biomarkers like in the case of some viral infections.

MiRNA-based NOT gate, a building block for sophisticated information processing in mammalian cells. The trans-activated miRNA can be the sensing module of a more complex genetically encoded circuit where the processing part is implemented with miRNA-based logic circuitry. MiRNA-based logic gates might be easy to compose because input and output are molecules of the same type. As building block of miRNA-based logic gates, I engineered a miRNA whose activity can be downregulated by another miRNA (a miRNA-based NOT-gate). This was achieved without using exogenous proteins or transcriptional regulators but by employing the U1 snRNA gene, which is involved in the spliceosome and in regulating mRNA transcription [6]. Since this miRNA-based NOT gate is primarily a platform that uses the U1 snRNA, we have called it "u.P.R.O.C.E.S.S.O.R." (U-gene-based Platform, RNAi-regulated Only, Compactly Employing Small Shuttle-miRNAs, Operates (through) RNA). Although the NOT gate is the simplest logic gate (with just one input and one output), more NOT-gates can be combined to engineer more sophisticated logic circuits. Additionally, this NOT gate has a DNA footprint small enough to fit an AAV virus.

Scaling up genetic circuits. To investigate whether this technology could enable more sophisticated computation in mammalian cells without the use of exogenous proteins, I explored ways to use this NOT gate as building block to engineer the circuit computing the bitwise inversion of the square root of a number. To do that, I also developed an algorithm for the exhaustive search of logic circuits designs (with 4 inputs and 2 outputs) with some given constraints for the total number of gates, level of cascading and fan out allowed. An exhaustive search is advantageous because it enables the optimization of the circuit design according to some given parameters. One of the solution of this exhaustive search in the design space of circuits computing the bitwise inversion of the square root of a number re-

quired just 4 transcriptional units and one miRNA-based NOT gate. Importantly, those transcriptional units can be transiently transfected to mammalian cells by the use of a 10kb DNA plasmid, which is remarkably small for a circuit performing such a sophisticated information processing. Finally, the genetic circuit has been validated in mammalian cells.

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