Chapter 6.

Conclusions

Living systems rely on the precise control of gene expression to affect appropriate phenotypic responses over different temporal and spatial scales, and therefore maintain proper cellular function. Increased understanding of natural biological systems, in addition to advances in nucleic acid and protein engineering, have inspired researchers to construct synthetic genetic systems and catalyze the emerging field of synthetic biology (1). Recent advances in the field of RNA synthetic biology led to the development of a modular platform from which ligand-responsive ribozyme-based gene-regulatory devices can be assembled (2,3). The ability to construct user-specified ribozyme devices tailored to diverse applications depends on the development and integration of several enabling technologies. Specifically, high throughput technologies that enable the efficient *de novo* generation of new sensor functions and tailoring of device regulatory properties for specific cellular applications are needed to broaden the utility of these genetic control devices.

The ribozyme device platform offers two distinct advantages in advancing our ability to generate new ligand-responsive RNA control devices with tailored regulatory properties. First, the modular composition framework of the device platform supports direct interchange of functional RNA components, including sensors, transmitters, and actuators, to program devices with user-specified regulatory activities. Second, the mechanism of action of the ribozyme component is independent of cell-specific machinery, allowing transport of the resultant ribozyme devices to *in vitro* or diverse cellular environments. These two advantages allow direct generation and optimization of the individual RNA component activities within the device platform through *in vitro* and/or *in vivo* strategies, thereby allowing the development of a scalable framework from

which ribozyme devices with tailored ligand responsiveness and regulatory properties can be efficiently generated.

We developed a high-throughput and quantitative two-color FACS-based strategy that allows rapid generation and optimization of component activities in the cellular environment. Our fluorescence-based screening strategy provides an advantage in that the device output (GFP) can be normalized by an internal standard (mCherry) across cell populations, whereas cell-based screening and selection strategies based on enzyme activities (i.e., motility, colorimetry, viability) are more prone to gene expression noise that is not normalizable (4-6). Our two-color screening approach can be extended beyond RNA device tailoring. As an example, our screening approach can be applied toward direct enzyme evolution in cellular environments. In this instance, an enzyme library is transformed into a cell host, which harbors an integrated biosensor in the form of an RNA control device responsive to the product of the enzyme-catalyzed reaction in the two-color screen construct. The RNA device can respond to the intracellular accumulation of the enzymatic product and translate this information to increased cellular fluorescence levels. As the fluorescence levels are normalized by the internal standard, enzyme variants that exhibit enhanced activities can be efficiently isolated, increasing the sensitivity of the biosensor to small changes in enzyme activity. Thus, our two-color FACS-based screen can provide a powerful strategy that allows programming of diverse biological components.

Scalable generation of biological components is currently a rate-limiting step in the process of building new genetic control devices. Generation of new component functions, such as ones that exhibit new sensing functions, requires searching large

sequence space (~10^{14}-10^{15} variants), exceeding the capacity of any existing cell-based selection or screening strategies. We proposed a solution-based *in vitro* selection strategy based on the modular ribozyme device platform. Large device libraries are generated by randomizing the sensor component within the device platform. The selection strategy links ligand binding to ribozyme cleavage activity and therefore does not require conjugation of target ligand to a solid support (i.e., column). As such, the solution-based selection process can be applied to a complex ligand pool in which mixtures of ligand targets are simultaneously presented to the RNA library to generate multiple sensing functions. We described a dual selection process, optimized by mathematical modeling, based on magnetic bead separation to facilitate partitioning of the library members. Future efforts will be directed to validating our selection strategy initially on a small control library to recover existing functional sequences and ultimately on a large library to generate new sensing functions.

The enrichment efficiencies of our *in vitro* cleavage-based selection and *in vivo* FACS-based screening strategies are closely associated with the device regulatory dynamic range. Our current platform design is based on conformational switching facilitated by a strand-displacing transmitter sequence. Previous modeling studies of riboswitch function have predicted that slow conformational switching rates may limit the device dynamic range, resulting in small changes in the output signal in response to changing concentrations of the target ligand (7). This limitation has a more pronounced impact on the enrichment efficiency of the *in vitro* selection. In contrast to the *in vivo* FACS-based screen, where library members exhibiting desired activities can be specifically sorted, the proposed *in vitro* cleavage-based selection collects all the cleaved

library members in the supernatant. As such, library members exhibiting similar cleavage rates relative to the target member will also be carried over into the next selection round, resulting in poor enrichment of the target member. To address this limitation, early efforts may be directed toward developing a transmitter design that allows for faster conformational switching. One potential strategy is to reduce the random walk step size, a measure of the number of base pairs that need to be broken or formed between two conformations. As an example, a more compact strand displacement-based transmitter sequence (with fewer nucleotides) will be designed through rational or evolutionary strategies to facilitate faster conformational switching. Alternative transmitter architectures, such as design based on a helix-slipping mechanism (8), may also be considered for the development of a next-generation device platform.

By developing enabling technologies based on the same ribozyme-based platform, we will ultimately be able to integrate our *in vitro* solution-based selection and *in vivo* two-color FACS-based screening strategies into one seamless integrated process. The *in vitro* selection strategy can be performed initially on a large device library (~10^{14}-10^{15} variants) to enrich for sequences that exhibit desired cleavage activities. The *in vitro*-enriched libraries can be directly transformed into a cell host (~10^{6}-10^{7} clones for yeast) and further enriched and optimized by the *in vivo* two-color FACS-based screen. To integrate the resulting devices into biological networks, the regulatory activities of the devices can be readily tailored by the same two-color screen to meet application-specific performance requirement. Collectively, progress in this thesis will aid in the scalable construction and programming of RNA control devices for diverse cellular engineering applications and further advance our ability to interact with and program biology.

References

1. Khalil, A.S. and Collins, J.J. (2010) Synthetic biology: applications come of age. *Nat Rev Genet*, **11**, 367-379.
2. Win, M.N., Liang, J.C. and Smolke, C.D. (2009) Frameworks for programming biological function through RNA parts and devices. *Chemistry & Biology*, **16**, 298-310.
3. Win, M.N. and Smolke, C.D. (2007) A modular and extensible RNA-based gene-regulatory platform for engineering cellular function. *Proceedings of the National Academy of Sciences*, **104**, 14283-14288.
4. Lynch, S.A., Desai, S.K., Sajja, H.K. and Gallivan, J.P. (2007) A high-throughput screen for synthetic riboswitches reveals mechanistic insights into their function. *Chemistry & Biology*, **14**, 173-184.
5. Topp, S. and Gallivan, J.P. (2008) Random walks to synthetic riboswitches--a high-throughput selection based on cell motility. *Chembiochem : A European Journal of Chemical Biology*, **9**, 210-213.
6. Nomura, Y. and Yokobayashi, Y. (2007) Dual selection of a genetic switch by a single selection marker. *Biosystems*, **90**, 115-120.
7. Beisel, C.L. and Smolke, C.D. (2009) Design principles for riboswitch function. *PLoS Comput Biol*, **5**, e1000363.
8. Soukup, G.A. and Breaker, R.R. (1999) Engineering precision RNA molecular switches. *Proc Natl Acad Sci U S A*, **96**, 3584-3589.