PREFACE

The early years of the twenty-first century have seen an explosion of interest in the diverse capabilities of RNA. Riboswitches capture the excitement and promise of this field. They are structurally dynamic, they sense and respond to specific molecular partners, their occupancy states governs gene regulatory decisions, and they can be engineered to reprogram gene regulatory circuitry. Importantly, many of the experimental and theoretical tools that have been used to study riboswitches can also be applied to other RNAs, and tools developed for studies of other RNAs can be applied to riboswitches.

These two volumes (*Methods in Enzymology* 549 and 550) include 40 contributions that outline cutting-edge methods representing a wide spectrum of research questions and scientific themes. The first volume emphasizes natural riboswitches, from their discovery to assessment of their structures and functions. The second volume shifts the focus to applying riboswitches as tools for a variety of applications and as targets for inhibition by potential new antibacterial compounds. A third volume (*Methods in Enzymology* 553) will appear shortly after these two focusing on computational methods for predicting and evaluating dynamic RNA structures. Although the chapters are organized into discrete themes, many cut across thematic boundaries by weaving together diverse methodological solutions and several of the chapters could fit comfortably into more than one section.

VOLUME 1

Riboswitch discovery. In the early days of the riboswitch field, new riboswitches were discovered at a frenetic pace, often by comparing large sets of bacterial genomes. While that approach continues to identify new members of known riboswitch families, the pace has slowed, and new discovery methods are needed. The series begins with two chapters outlining new methods that utilize informatics approaches in combination either with RNASeq and genome-wide methods (Rosinski-Chupin) or with *in vitro* selection (Ho) to discover new natural riboswitches.

Sample preparation. Any effort to characterize purified, functional RNAs will only be as good as the corresponding sample preparations. Therefore, the next five chapters are dedicated to methods for the synthesis and

preparation of large RNAs. Three groups exploit specialty nucleic acids with functionalities of their own. The first chapter in this set describes the use of cotranscribed aptamer affinity tags that are removed by activatable self-cleavage (Di Tomasso). This is followed by methods for using catalytic deoxyribozyme ligases to assemble large RNAs from synthetic fragments, some of which carry site-specific spin labels for electron paired resonance studies (Wawrzyniak-Turek). The third chapter in this set describes the combined use of aminoacyl transferase ribozymes and chemical protection to generate charged tRNAs on a large scale (Zhang). These are followed by two chapters that integrate organic chemical methods with improved enzymology to produce photocleavable biotinylated guanosine that incorporates at the 5′ end of *in vitro* transcripts (Luo) and large quantities of selectively $^{13}C/^{15}N$-labeled RNA in previously unattainable labeling patterns for improved spectroscopic analysis (Alvarado).

Structure and function. The biochemical functions of riboswitches are inextricably linked with their three-dimensional structures. The next several chapters, therefore, provide methods for evaluating riboswitch structure and function. Updated protocols are provided for the widely utilized SHAPE method of structural probing, along with details of how to implement new software for data interpretation (Rice). It is well recognized that structural context can perturb pK_a values within RNA and DNA; hence, the next chapter details how to measure them without falling into traps of oversimplifying the underlying molecular processes (Taplyal). The next chapter provides methods for obtaining appropriate crystals for ligand–RNA complexes, with emphasis on fragment-bound TPP riboswitches (Warner). This section ends with a detailed description of experimental and analytical methods for using small-angle X-ray scattering to define RNA conformations in solution (Reyes).

Conformational dynamics. Spectroscopic methods are ideal for following riboswitch conformational dynamics in real time. The first two chapters of this section describe site-specific incorporation of spectroscopic labels and their use in addressing specific question, first with ^{19}F NMR to probe conformational exchange (Zhao) and then with spin-label probes for electron paramagnetic resonance spectroscopy of large RNAs (Esquiaqui). Single-molecule methods such as smFRET have become a staple of modern biophysical analysis. Three chapters provide detailed guidance on many facets of smFRET, from sample preparation, data acquisition, and analysis to explorations of folding landscapes (Shaw, Suddala, and Shebl). The last chapter of this section describes how to integrate surface plasmon resonance

(SPR), isothermal titration calorimetry, and circular dichroism to examine tertiary docking (Hoogstraten).

Ligand interactions. One of the most important characteristics of riboswitches is their ability to sense the presence of specific metabolites by forming bound molecular complexes. Isothermal titration calorimetry is one of the most powerful methods for evaluating the energetics of RNA–small molecule complexes (Wedekind). SPR is another powerful tool for characterizing aptamer kinetic and equilibrium binding properties and is detailed in two chapters (Chang and Schaffer). Finally, an innovative and relatively new technique known as DRaCALA is described in the last chapter of the first volume (Patel).

VOLUME 2

The second volume in this series takes a different perspective on riboswitches. Specifically, now that nature has shown us that RNA modules can sense metabolites and report on them, how can we take advantage of that ability to engineer new properties into cells and biochemical systems? Necessarily, this volume takes a much broader view of riboswitches than those found in nature, encompassing ligand-responsive transcriptional and translational modules, ribozymes, sensors, and modules that induce fluorescence in a fluorophore upon formation of the bound complex. It encompasses Synthetic Biology applications as tools to understand normal biological processes, and as tools to reprogram metabolite flux in workhorse organisms. Finally, it comes full circle by screening small-molecule libraries for inhibitors of natural riboswitches.

In short, this second volume details methods at the cutting edge of the translational science of riboswitches.

Artificial riboswitches. The first six chapters of the second volume provide methods for several approaches to construct and optimize artificial riboswitches. There has been substantial progress toward designing artificial riboswitches from scratch, especially when guided by experimental validation (Moerl). A contrasting approach uses *in vitro* selection/evolution to obtain ligand-responsive ligase ribozymes from highly diverse starting populations (Olea), or to reshape and reprogram the ligand-binding and expression platforms of natural riboswitches (Batey). The next chapter presents methods for optimizing signal transduction, since regulation sometimes benefits from maximizing suppression of basal expression in the OFF state and sometimes from maximizing expression in the ON state

(Goodson). The next two chapters address optimization in two very different cell-free systems, first using coupled transcription–translation to optimize a ligand-responsive self-cleaving ribozyme, or "aptazyme" (Ichihashi), and then taking advantage of a eukaryotic mechanism by which ribosomes "shunt" past certain secondary structures, which can be stabilized to increase shunting efficiency by binding to the analyte ligand (Ogawa).

Ligand-responsive fluorescent sensors. There has been longstanding interest in coupling the binding of ligands to RNA with the emission of light. One such system is that of the recently described Spinach (and Spinach2) aptamer mimics of green fluorescent protein, which are the focus of the next five chapters, each in a different system. The first chapter in this section, from the lab that discovered and first described the Spinach system, presents methods for using it to image intracellular RNA in mammalian cells (Strack). The next two chapters describe how to use these modules in bacterial cells, first as intracellular sensors of intracellular cyclic dinucleotide levels (Kellenberger) and then for simultaneous and independent monitoring of mRNA and protein levels (Pothoulakis). The next chapter takes this same question into solution and into vesicle-based artificial cells (van Nies). The fifth chapter in this section couples sensing of oligonucleotide "ligands" with Spinach2 output in real time for sequence-specific target quantitation and potential point-of-care applications (Bhadra).

Synthetic biology: Conditional control of gene expression. The third section of this volume lays out several methods for using artificial or natural riboswitches to study gene function. This has proven to be a powerful tool in organisms for which limited genetic tools are available, such as the intracellular pathogen Mycobacteria (Van Vlack), as well as in more readily manipulated, nonpathogenic bacteria such as *Streptomyces coelicolor* (Rudolph). Eukaryotes can be similarly studied. A clever variation on this approach is to make the expression of query genes to be dependent upon a regulatory protein whose expression is controlled by a natural riboswitch, as demonstrated here for the unicellular alga *Chlamydomonas reinhardtii* vitamin-repressible riboswitch (Ramundo). An alternative approach is presented in the next chapter, which describes utilization of self-cleaving aptazyme to identify sequence variants that respond to various ligands to regulate gene expression in the yeast *Saccharomyces cerevisiae* (Klauser).

Synthetic biology: Pathway optimization. The fourth section provides methods that illustrate two examples of using riboswitches as tools to optimize metabolic pathways for Synthetic Biology applications. The first chapter details a computational approach focused on kinetic folding with

experimental validation to build aptazymes that respond cotranscriptionally to the presence of metabolites, and the experimental validation of those devices (Sparkman-Yager). The second chapter describes a method for using riboswitches to impose selective growth advantages on cells that optimally channel their metabolic output into production of a desired compound (Jang).

Antiriboswitches drug screens. The final section reverses the perspective, treating riboswitches as targets for antibacterial drug development and ligand-binding specificity as the basis for identifying antibiotic candidates. The first chapter lays out a sensitive, fluorescence-based screening cascade for identifying compounds that target the T-box riboswitch antiterminator element (Liu). The second chapter describes screening platform that uses cell-free lysates to monitor translational read-through of a mammalian frameshift signal that is under the control of the preQ1-I riboswitch (Yu).

I first encountered riboswitches in a conference on RNA-Based Life in November, 2001 in separate presentations from Miranda-Ríos and Breaker. At the time of this writing (November, 2014), a PubMed search turns up 771 hits for the term "riboswitch," and it will be well over 800 by the time of the publication of these volumes. The field is moving fast and in many directions. A great number of talented people with diverse expertise have contributed to these volumes, and all of us hope that they will serve as a useful resource to advance RNA research both within the riboswitch field and beyond.

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