Chapter 11

Measuring Riboswitch Activity In Vitro and in Artificial Cells with Purified Transcription–Translation Machinery

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Abstract

We present a simple method to measure the real-time activity of riboswitches with purified components in vitro and inside of artificial cells. Typically, riboswitch activity is measured in vivo by exploiting β-galactosidase encoding constructs with a putative riboswitch sequence in the untranslated region. Additional in vitro characterization often makes use of in-line probing to explore conformational changes induced by ligand binding to the mRNA or analyses of transcript lengths in the presence and absence of ligand. However, riboswitches ultimately control protein levels and often times require accessory factors. Therefore, an in vitro system capable of monitoring protein production with fully defined components that can be supplemented with accessory factors would greatly aid riboswitch studies. Herein we present a system that is amenable to such analyses. Further, since the described system can be easily reconstituted within compartments to build artificial, cellular mimics with sensing capability, protocols are provided for building sense-response systems within water-in-oil emulsion compartments and lipid vesicles. Only standard laboratory equipment and commercially available material are exploited for the described assays, including DNA, purified transcription–translation machinery, i.e., the PURE system, and a spectrofluorometer.

Key words Riboswitch, Transcription–translation, In vitro compartmentalization, Liposome, Emulsion, Cell-free synthetic biology

1 Introduction

Riboswitches are genetically encoded control elements that respond to small molecules through direct binding. Sensing is mediated by an aptamer [1–4] sequence within the mRNA that controls the conformation of the expression platform. Usually ligand binding turns off gene expression; however, natural on-riboswitches exist [5]. The induced conformational changes either regulate transcription through terminator–anti-terminator activity, translation by modulating the accessibility of the ribosome binding
In addition to natural riboswitches, many riboswitches have been engineered by modifying previously selected aptamer sequences [7] or by mutating natural riboswitches to display new functionality [8]. Most of the characterized natural riboswitches control transcription, whereas synthetic riboswitches typically control translation.

Monitoring transcription in vitro is straightforward [9, 10] thereby allowing for the characterization of riboswitches that alter transcript length in a manner dependent upon the presence or absence of ligand. However, riboswitches that control ribosome binding site accessibility produce transcripts of the same length regardless of the presence or absence of ligand, making methods that quantify differences at the RNA level less insightful. Moreover, riboswitches ultimately control protein synthesis, regardless of the specific mechanism exploited. Therefore, more direct methods that probe the influence of riboswitch activity on protein synthesis are desirable. This is most often achieved by placing the riboswitch in question within a genetic construct that encodes β-galactosidase, a fluorescent protein [7, 11] or more recently, a protein involved in motility [12] and monitoring the activity of the reporter protein in *Escherichia coli*. In other words, the assay is carried out within the cell and absorbance or fluorescence are quantified.

The advantage of such methods is that the activity of the riboswitch within a living cell is monitored, meaning that the measured activity is not a result of imperfect in vitro approximations of in vivo conditions. However, there are several limitations of such in-cell assays. First, the influence of accessory proteins could easily be missed, since their participation in sensing or transducing chemical messages is largely uncontrolled in such experiments. Second, the putative ligand either must be capable of crossing the membrane (to allow for exogenous delivery) or easy to manipulate in terms of concentration. For example, the activity of the flavin mononucleotide (FMN) riboswitch was characterized at the transcriptional level in vitro [13, 14], but the influence of FMN on protein synthesis was not investigated, presumably due to the difficulty in quantifying and modulating intracellular FMN concentrations.

Herein we present a simple method to characterize the influence of riboswitch activity on protein synthesis in vitro. Guidelines for the design and assembly of the genetic construct, and the evaluation of in vitro riboswitch activity by monitoring the synthesis of fluorescent protein with fully defined components are described (Fig. 1). Importantly, this real-time fluorescence assay is amenable to the screening of protein accessory factors and ligands, including ligands that are metabolites, impermeable, or toxic. It should be noted that the described protein synthesis assay does not replace current methods that characterize transcriptional activity. The investigation of both transcription and translation is needed in order to fully define the mechanistic details of riboswitch activity.
We additionally describe how this riboswitch controlled in vitro transcription–translation system can be encapsulated within compartments to build cellular mimics (Fig. 1). As opposed to the majority of artificial cell studies that focus on self-replication, riboswitch sensing-based cellular mimics integrate more fully with the environment and thus could potentially serve as a platform for future technologies. The example described here uses water-in-oil (w/o) emulsion droplets [15], vesicles [16], and a previously reported theophylline riboswitch [7, 17, 18].

2 Materials

All solutions should be prepared using diethyl pyrocarbonate (DEPC) treated water. All reagents are nuclease-free, molecular biology grade. The theophylline riboswitch sequence used here is available from the Registry of Standard Biological Parts (BBa_J89000).

2.1 Template Preparation

1. *E. coli* DH5α or similar laboratory, cloning strain.
2. Commercial plasmid miniprep kit.
3. 25:24:1 Phenol–chloroform–isoamyl alcohol mixture (see Note 1).

2.2 In Vitro Transcription and Translation

2. Riboswitch ligand molecule (e.g., theophylline).
3. RNAse Inhibitor (RiboLock RNase Inhibitor, Fermentas).
4. Quartz ultra-micro cell cuvette (105.252-QS, Hellma).
5. QuantaMaster 40 UV–Vis Spectrofluorometer with a Peltier temperature controlled single sample holder (Photon Technology International) or a similar spectrofluorometer.

2.3 Emulsion Preparation
1. Mineral Oil.
2. Span 80.
3. Tween 80.
4. Triton X-100.
5. 9 mm Teflon stir bar.

2.4 Liposome Preparation
1. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC).
2. Cholesterol.
3. N-(carbonyl-methoxypolyethylene glycol 5000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-PEG 5000, NOF-Europe).
4. Rotary evaporator, e.g., Rotavapor R-210 with Vacuum Pump V-700 (Buchi).
5. IKA T 10 basic ULTRA-TURRAX disperser with a 5 mm diameter dispersing tool.
6. Mini-Extruder (Avanti Polar Lipids, Inc.).
7. Nucleopore Track-Etch Membrane 0.4 µm (Whatman).
8. Centrifugal evaporator, e.g., CentriVap Centrifugal Vacuum Concentrator (Labconco).
9. Tris saline buffer; 50 mM Tris–HCl, 50 mM NaCl, pH 7.4 supplemented with 10 mg/mL Proteinase K (Fermentas).

2.5 Imaging
1. Zeiss Observer Z1 microscope (Carl Zeiss S.p.A.) or similar fluorescence microscope.

3 Methods

3.1 DNA Template Preparation
The DNA template can be either a circular plasmid or a linear PCR product that contains a series of modular elements to allow for riboswitch controlled protein synthesis (Fig. 2). The sequence should contain a transcriptional promoter, a sequence encoding the riboswitch that contains a ribosome binding site (preferably the natural ribosome binding site sequence, if possible), a gene coding for a fluorescent protein to act as a reporter, and a transcriptional terminator. The transcriptional promoters for T7 and E. coli RNA polymerases are typically used. However, since the activity of a riboswitch can depend
on the RNA polymerase [19], particularly for riboswitches that use a terminator–anti-terminator mechanism, the choice of which promoter to use can significantly impact in vitro riboswitch activity. Similarly, it is advisable that the sequence chosen for the riboswitch portion of the construct contains the ribosome binding site. Riboswitches are often associated with inefficient ribosome binding sites, either due to a lack of potential base-pairing interactions with the ribosome or because of structural features of the riboswitch that obstruct ribosome binding site—ribosome interaction. Further, since riboswitches typically do not fully block protein synthesis in the off-state nor mediate robust expression in the on-state, i.e., riboswitch control is leaky and generally mediates more subtle changes in expression [7], a ribosome binding site not tuned to the activity of the riboswitch could complicate analyses. The reporter should be a protein that expresses well in vitro and is easily detectable. We find the green fluorescent proteins superfolder GFP (sfGFP) and GFPmut3b and the yellow fluorescent proteins YPet and Venus to be particularly good choices [20]. Finally, incorporating a hairpin transcriptional terminator is advisable, even if not absolutely required when using a linear PCR product as a template, because structured RNA termini increase RNA stability and thus protein yield [21].

1. The DNA template should contain from 5′ to 3′ a T7 promoter followed by two GG nucleotides to enhance transcription, a sequence encoding the riboswitch [17] and the RBS, and the gene coding for the reporter protein followed by a transcriptional terminator (Fig. 2).

2. The template should be amplified either by PCR or by transforming a typical laboratory cloning strain of *E. coli*, such as DH5α, and purified with a commercially available kit according to the manufacturer’s instructions.

3. Subsequently, the DNA is phenol–chloroform extracted [22] with an equal volume of Tris-buffered Phenol–Chloroform (*see Note 1*).

4. The DNA is ethanol precipitated [23], resuspended in sterile water, and stored at −20 °C.
3.2 In Vitro Transcription and Translation Reaction

The major advantage of working in vitro, compared with in vivo, is that the system operates only with what is provided. In other words, in vitro activity cannot depend on unidentified cellular components, because they are not present. To date, only *E. coli* [24] and *Thermus thermophilus* [25] translation machinery have been reconstituted in vitro from purified components. Of these two, only the *E. coli* system, i.e., the PURE system, is commercially available. It should be noted that in contrast to in vivo or cell-extract conditions, reactions with purified transcription–translation machinery do not contain nucleases. The lack of nucleases decreases the amount of DNA template needed. Whether protein production is more or less efficient with the PURE system in comparison with cell-extract based systems depends on the specific folding properties of the expressed protein.

1. The PURE system components should be aliquoted on ice and stored in 0.2 mL microcentrifuge tubes. Convenient volumes are 10 µL aliquots of solution A and 7.5 µL aliquots of solution B. Aliquots are stored at −80 °C.

2. Assemble the reaction components, except for the DNA template, on ice following the manufacturer’s instructions. Supplementary reagents can also be added, such as RNase inhibitor (e.g., 20 U RiboLock RNase Inhibitor) or the ribo-switch ligand (e.g., 0.5 mM theophylline).

3. The assembled reaction is transferred to a quartz cuvette and incubated at 37 °C.

4. The reaction is initiated by the addition of the DNA template and monitored by fluorescence spectroscopy for 6 h (Fig. 3) (see Note 2). The DNA template concentration should be screened. We used 250 ng of plasmid template in 25.5 µL total reaction volume. If YPet is used as the reporter protein, the excitation and emission wavelengths are 517 nm and 530 nm, respectively.

3.3 In Vitro Compartmentalization

Since cellular life is chemically distinct from the environment, efforts to mimic cells in the laboratory often times exploit w/o emulsion droplets or vesicles to approximate the compartment of the living, chemical system [26, 27]. However, even if cellular life is distinct from the environment, life cannot exist in isolation and must in some manner interface with the environment to survive [28]. Since lipid vesicles are semipermeable and more similar to the types of barriers found in biology, vesicles are better suited than w/o emulsion droplets for the construction of cellular mimics. Nevertheless, the encapsulation efficiency of w/o emulsions is nearly 100 %, whereas encapsulation efficiency in vesicles is at best 30 % [29]. It is for this reason that the screening of compartmentalized reactions is carried out with w/o emulsions. Once optimal conditions are identified, similar vesicle systems are setup.
The method described here is based on that of Davidson et al. [30] but has been scaled down to be compatible with small volume PURE system reactions. Positive displacement pipettes are used to handle oil samples.

1. The oil phase that will be used for the w/o emulsion is assembled in a 15 mL Falcon tube (see Note 3), in the following order: 474.75 µL of mineral oil, 22.5 µL of span 80, 2.5 µL of tween 80, and 0.25 µL of triton X-100.

2. The aqueous phase is first assembled in a microcentrifuge tube by mixing the PURE system components on ice as described above for the in vitro reactions. Note that the optimal DNA template concentration may be different when transcription–translation is performed in a compartment versus in vitro. We used 500 ng of plasmid DNA in 25.5 µL of total aqueous volume (see Note 4) for expression in w/o emulsion droplets. The theophylline concentration was also increased to 5 mM to compensate for partitioning into the oil phase (see Note 5).

3. The 15 mL tube containing the oil phase is placed in a 250 mL beaker filled with ice water on a magnetic stir plate. A teflon stir bar is inserted in the oil phase and the oil is mixed by stirring at maximum speed for 1 min.

4. The emulsion is formed by the drop-wise addition of the aqueous phase containing the PURE system reaction to the oil phase over 1 min with continuous stirring. The emulsion is then stirred for an additional 3 min (see Note 6).

5. Finally, the emulsion is transferred to a 2 mL microcentrifuge tube and incubated at 37 °C for 6 h. 5 µL aliquots are removed every hour for observation by fluorescence microscopy.

**Fig. 3** In vitro theophylline riboswitch activity observed by measuring the expression of the reporter protein mYPet. The presence of the ligand activates protein expression. A schematic representation of the mRNA in the ligand-bound ON state and the uncomplexed OFF state are shown. Protein production is inhibited in this case in the OFF state because the RBS is not available for base-pairing with the ribosome. The data were taken from a previous in vitro theophylline riboswitch study [18]
The freeze-dried empty liposome (FDEL) method, as described by Yomo and colleagues [16], is used with slight modification to build the vesicles that house the transcription–translation reaction. FDEL vesicles encapsulate macromolecular, hydrophilic components relatively efficiently. A variety of lipid compositions can be exploited. Here 12 μmol of 58:39:3 POPC–cholesterol–DSPE-PEG 5000 is used.

1. Each lipid is dissolved in chloroform and mixed in a 5 mL round-bottom flask.

2. The solution is subjected to rotary evaporation for 1 h. The resulting thin lipid film is then hydrated with 1 mL of DEPC-treated water and vortexed for 20 s or until a homogeneous opaque solution is formed.

3. The vesicle solution is then transferred to a 2 mL microcentrifuge tube and disrupted with an IKA T 10 basic homogenizer at high speed (level 4 setting) for 1 min (see Note 7).

4. Samples are extruded through 400 nm polycarbonate filters 11 times with an Avanti mini-extruder. 40 μL aliquots of the vesicles are placed in 1.5 mL microcentrifuge tubes, frozen in liquid nitrogen (see Note 8), and lyophilized with a centrifugal evaporator overnight at 30 °C. A thin opaque lipid layer can be observed at the bottom of the microcentrifuge tube after lyophilization. At this stage the samples can be stored at −20 °C.

5. A PURE system reaction is assembled on ice in a total volume of 22.1 μL, including 500 ng of the template plasmid (see Note 4). 20 U of RiboLock RNase inhibitor is added to the solution.

6. 10 μL of the assembled PURE system reaction is added to an aliquot of FDEL vesicles on ice and incubated without agitation for 2.5 h (see Note 9). The unused portion of the PURE system reaction can be stored at −80 °C.

7. The hydrated liposomes are then diluted 20-fold in Tris saline buffer supplemented with proteinase K in a 0.2 mL microcentrifuge tube and incubated at 37 °C. The inclusion of proteinase K is to degrade extravesicular proteins.

8. At this point the ligand to be sensed, e.g., 5 mM theophylline (see Note 5) is added. Theophylline is capable of diffusing across the membrane, binding directly to the mRNA, and activating translation thereby resulting in fluorescence. Control reactions in the absence of ligand should result in no or significantly reduced fluorescence.

9. The reactions are incubated at 37 °C for 6 h. 5 μL aliquots are removed from the reaction every 1.5 h and visualized by fluorescence microscopy.
Detecting activity inside of vesicles by fluorescence microscopy is more difficult than in vitro measurements with a spectrofluorometer. First, encapsulation efficiency is low, particularly when over 80 different components need to be encapsulated within one vesicle in order for protein synthesis to proceed [31] (see Notes 4 and 9). Second, riboswitches typically have weak ribosome binding sites and thus produce less protein than constructs typically exploited for recombinant expression. Finally, fluorescent proteins photobleach and require time to mature. We use a monomeric version of the yellow fluorescent protein YPet as a reporter because the characteristics of YPet are more amenable to investigation by microscopy. YPet is one of the brightest fluorescent proteins and is more photostable than the majority of available fluorescent proteins [32]. The expression of monomeric YPet with the PURE system requires approximately 2 h to reach half maximal fluorescence [20].

1. 5 µL aliquots are removed from the reaction and spotted on a clean glass slide. A cover slip is added.
2. The slide is then left for 2 min to rest on the bench. This step helps decrease the number of rapidly moving vesicles.
3. The sample is then observed by bright field and epifluorescence with 100× magnification (Fig. 4). Care should be taken to decrease photobleaching by decreasing exposure time.

### Notes

1. It is preferable to avoid Phenol–Chloroform solutions that contain ethylenediaminetetraacetic acid (EDTA), since the chelation of metals by EDTA can interfere with enzyme activity.
2. A plate reader or a real-time PCR machine can be used in place of a spectrofluorometer.
3. We also made w/o emulsions in 13 mL Sarstedt tubes with stirring with x-shaped spinplus stir bars, as described by Davison et al. [30]. The resulting emulsions were more homogeneous and more stable than the emulsions we obtained with Falcon tubes and linear stir bars. However, we found the Davidson et al. emulsion droplets to be smaller and thus more difficult to observe by microscopy.
4. The PURE system instruction manual suggests screening between 25 to 250 ng of template DNA for 25 µL reactions. For the in vitro characterization of the theophylline riboswitch, 250 ng of plasmid template was found to be optimal. However, better results were obtained with 500 ng DNA template for compartmentalized reactions.
5. Since the oil–water partition coefficient of theophylline is low [33], much more theophylline is required to activate the riboswitch in the presence of oil than in aqueous solution.

6. Stirring is an important parameter to consider when generating an emulsion. A constant stir force should be used and the stir bar must be compatible with the tube holding the aqueous-oil mixture. For example, the conical shape of a Falcon tube is not compatible with x-shaped stir bars. The efficiency of mixing can be qualitatively assessed by eye by including in the aqueous phase 1 mM HPTS (8-hydroxypyrene-1,3,6-trisulfonic acid) and observing the distribution of green color throughout the tube during stirring.

7. We also tested sonication as a possible disruption method. Sonication at 70 A of amplitude for 5 min resulted in higher dispersion. However, the number of liposomes observed by
microscopy was lower than that observed with the IKA T homogenizer.

8. Freezing can also be performed with dry ice without any appreciable difference in liposomes formation.

9. The slow vesicle formation process mediated by the natural swelling method described herein results in fewer, but larger vesicles that are easier to observe by microscopy than by other methods that exploit vortexing. Additionally, compartment size impacts protein synthesis efficiency with larger vesicles being more compatible with protein synthesis [34].

References