The Spinach RNA Aptamer as a Characterization Tool for Synthetic Biology

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ABSTRACT: Characterization of genetic control elements is essential for the predictable engineering of synthetic biology systems. The current standard for in vivo characterization of control elements is through the use of fluorescent reporter proteins such as green fluorescent protein (GFP). Gene expression, however, involves not only protein production but also the production of mRNA. Here, we present the use of the Spinach aptamer sequence, an RNA mimic of GFP, as a tool to characterize mRNA expression in Escherichia coli. We show how the aptamer can be incorporated into gene expression cassettes and how co-expressing it with a red fluorescent protein (mRFP1) allows, for the first time, simultaneous measurement of mRNA and protein levels from engineered constructs. Using flow cytometry, we apply this tool here to evaluate ribosome binding site sequences and promoters and use it to highlight the differences in the temporal behavior of transcription and translation.

KEYWORDS: measurement, Spinach RNA, aptamer, promoter, ribosome binding site, fluorescence

RESULTS AND DISCUSSION

To demonstrate the utility of Spinach for measuring gene expression, we first sought to integrate the Spinach aptamer sequence into an mRNA actively translated in E. coli. Previously, Spinach has been expressed in E. coli from the pET28c-Spinach plasmid, which uses the T7 promoter to express a 171 nt tRNA scaffold containing the Spinach aptamer.7 To create a plasmid that produces a translated mRNA, we inserted the coding sequence (with RBS) for the aptamer, fluoresces when expressed in live Escherichia coli and mammalian cells in the presence of the provided fluorophore molecule, 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI). Given that the Spinach aptamer requires only a provided fluorophore and a short RNA sequence, it offers an elegant method for characterizing the transcriptional step of gene expression separately from translation. We describe here how the Spinach aptamer, when included in a construct expressing a fluorescent protein, can be used to simultaneously measure transcription and translation of mRNAs, thus providing a new tool for gene expression characterization.

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monomeric red fluorescent protein, mRFP1\textsuperscript{8} into pET28c-Spinach downstream of the T7 promoter and upstream of the Spinach aptamer. Both this new construct (pGPR01) and pET28c-Spinach were transformed into BL21(DE3) E. coli cells and grown in liquid media at 37 °C before being induced with IPTG to initiate T7 RNA polymerase-driven gene expression.
Two hours post-induction, cells were assayed for Spinach and mRFP1 expression by fluorescence microscopy (Figure 1A) and cells from the same cultures were analyzed simultaneously using two-color flow cytometry (Figure 1B and D). In the presence of the fluorophore DFHBI, green fluorescence from the Spinach aptamer can be seen from cells with both the pET28c-Spinach and pGPR01 plasmids. Red fluorescence from the protein mRFP1 is also seen from pGPR01. This verifies that the Spinach aptamer can be incorporated into a translated mRNA and measured in vivo. Spinach has previously only been measured in *E. coli* using fluorescence microscopy.7,9 The ability to measure Spinach with analytical flow cytometry allows it to be used for rapid, high-quality gene expression quantification.

Flow cytometry also helps identify multiple populations within samples. As can be seen from Figure 1B and C, not all cells in an induced culture fluoresce. This can also be seen in the microscopy images (Figure 1A, arrows). These OFF-populations (compared in Table S5, Supporting Information) consist of cells not expressing either green or red fluorescence, and so we attribute this to cells losing plasmid following cell division or acquiring mutations at the chromosomal gene encoding T7 RNA polymerase.10 The burden of high expression of recombinant DNA may be promoting both during our experiments. With flow cytometry, this population can however be readily resolved, allowing Spinach to be measured despite bimodal distributions.

The combination of mRFP1, Spinach, and two-color flow cytometry enables temporal characteristics to be captured during gene expression. To demonstrate this, we took a time-series of green and red fluorescence measurements during a 3 h induction of Spinach and mRFP1-Spinach expression from the T7 promoter (Figure 2A). IPTG added at time zero activates expression of chromosomally encoded T7 RNA polymerase, which in turn transcribes the constructs (as illustrated in Figure 1C). Transcript expression above background can be detected 15 min following induction for Spinach alone, and at 30 min for mRFP1-Spinach. RNA fluorescence rises linearly over the first 2 h as mRNAs are generated by T7 RNA polymerase. In contrast, protein-generated fluorescence rises exponentially from 30 min onward, in line with the fact that each mRNA is translated multiple times. Beyond 2 h, as the *E. coli* enter stationary phase, fluorescence per cell no longer increases. These data confirm those observed in Figure 1 that show that transcription of the aptamer alone (pET28c-Spinach) yields ~4-fold more green
fluorescence than when transcribed in the 3′ untranslated region (3′UTR) of the mRFP1 mRNA (pGPR01). In order to investigate this effect and determine the optimum placement of Spinach within an mRNA, we next assembled a series of modified constructs placing the Spinach aptamer at different positions within mRFP1- and LacZ-encoding mRNAs and quantified their fluorescence using flow cytometry as before (Figure 2B). No alternative designs yielded greater green fluorescence per cell than pGPR01, which places Spinach in the 3′UTR. This location was also preferable when the longer LacZ coding sequence (3 kb) was used. Incorporating two copies of Spinach in tandem in the 3′UTR (pGPR03) did not increase green fluorescence and neither did placing Spinach within a bicistronic operon (pGPR04) in order to shield it from cellular ribonucleases. Immediately following fluorescence measurements for three of these constructs, we harvested cellular RNA and measured Spinach aptamer levels by RT-PCR (Figure S1, Supporting Information). This verified that Spinach gives a reliable measurement of mRNA transcript levels in vivo. Taken together, the results of Figure 2 and Figure S1, Supporting Information show that the 3′UTR is the optimal placement of Spinach within an mRNA but this leads to decreased expression compared to expressing Spinach alone, with the decrease magnified when longer transcripts are used. Decreases in Spinach expression and fluorescence in different contexts within mRNAs may be being promoted by the presence of translation machinery enzymes with helicase activities, notably the ribosome.11 Mis-folding of mRNA may also lead to changes in mRNA degradation rates and to stalled transcripts; however, it is worth noting that the aptamer used here is maintained...
within a stable tRNA scaffold so should be shielded from most mis-folding and RNA degradation.

Crucially, Spinach also allows control elements such as the promoter and RBS to be assessed separately using the same construct. To demonstrate this, we designed three RBS sequences (R1K, R9K, and R148K) of different predicted strengths using the Salis Lab RBS Calculator12,13 and incorporated these within T7-expressed pGPR01. Measured green fluorescence from these constructs (i.e., mRNA levels) remained equivalent to that of the parent construct (pGPR01), but red fluorescence (i.e., mRFP1 expression) varied in accordance to the predicted RBS strength (Figure 3A).

Replacing the T7 promoter with the T5 promoter, which itself imposes a cost to the cell.17 Perhaps the potential benefits of Spinach and future in vitro designs will likely resolve issues of sensitivity and mis-folding. Interrogation of the RNA folding within Spinach-containing mRNAs using new methods such as SHAPE-Seq5 may shed light on design strategies that ensure that the aptamer correctly folds and is efficiently insulated from local sequences, ribosomes, and ribonucleases. The use of Spinach and future variants as measurement tools in synthetic biology offers great potential benefits. The order-of-magnitude faster turnover of RNA compared to protein in cells makes sensitivity more challenging but offers more accurate temporal measurements and a new tool to investigate mRNA degradation. As we demonstrate here, mRFP1-Spinach constructs can be used to evaluate designed RBS sequences and Spinach alone can also be used to measure promoter strength without requiring translation, which itself imposes a cost to the cell.17 Perhaps the most interesting insights to be gained using Spinach will come from simultaneously inspecting the individual effects on transcription and translation caused by changes in cell state, such as growth phase, or by design features other than the promoter and RBS that can lead to changes in gene expression, such as codon usage.

**METHODS**

The pET28c-Spinach plasmid was a kind gift of Samie Jaffrey (Cornell University) and contains the Spinach aptamer inside a tRNA scaffold.18 All other plasmid constructs in this study were derived from this. The mRFP1 gene for the pGPR plasmids was amplified by PCR from part BBa_E1010 from the Registry of Standard Biological Parts (http://partsregistry.org), with the forward primer encoding an RBS sequence (RBS1). In all constructs random, synthetic 30 nucleotide spacer sequences were added between the promoter, the RBS-mRFP1 region, and the scaffold-held Spinach aptamer, in order to insulate parts from local folding and ribosome binding. The R1K, R9K, R38K, and R148K RBS sequences were designed using the Salis Lab RBS Calculator12,13 and have predicted strengths with mRFP1 of 938, 9166, 38394, and 148124 A.U., respectively. All constructs were assembled scarlessly in vitro from PCR-amplified parts using Gibson Assembly19 before transformation into BL21(DE3) E. coli cells. Details of all constructs and sequences of all parts are provided in the Supporting Information.

For the Spinach and mRFP1 measurements, cells were grown in shaking liquid culture (LB+kanamycin media supplemented with 1% glucose) at 30 °C overnight. Following this, 150 μL of culture was diluted in 3 mL of LB+kanamycin media and grown at 37 °C until at OD600 = 0.4. IPTG was then added to 1 mM final concentration. Incubation with shaking was continued at 37 °C for 2 h (except for the time course). To measure fluorescence, 10 μL of culture was diluted into 100 μL LB and DFHBI (Lucerna Technologies) was added to 200 μM final concentration. Samples were incubated at 37 °C with shaking for 10 min, placed on ice for 10 min, and then analyzed.

Flow cytometry analysis used a modified two-color Becton Dickinson FACScan flow cytometer measuring 2 μL of each sample in 1 mL of water for 45 s (approximately 80 000 cells). A 488 nm laser was used for excitation of green fluorescence detecting through a 530 nm band-pass filter (FL1) with gain 890. Red fluorescence was excited with a 561 nm laser and 610 nm filter (FL5) with gain 850. Data were analyzed using FlowJo (Tree Star), gating samples for forward scatter and side scatter and excluding FL1 and FL5 values below 2 × 104 A.U. Graphs were prepared using GraphPad Prism, with the average fluorescence and standard deviation of replica experiments calculated using the FL1 and FL5 modal values as determined by FlowJo using the Mode statistic.

Fluorescent images were taken through a 60× CFP60 objective mounted on a Nikon Eclipse Ti inverted microscope, with live cells imaged on a 1% M9-agar pad slide. Excitation, emission filters, and exposures were respectively 480 nm, 535 nm, 4000 ms for the GFP channel (Spinach) and 532 nm, 590 nm, 1000 ms for Cy3 channel (mRFP1). NIS-Elements Microscope Imaging Software (Nikon) was used for capture and ImageJ (National Institutes of Health) was used for image presentation.

**ASSOCIATED CONTENT**

* Supporting Information

Figure S1 (Comparison of Spinach and RT-PCR), Table S1 (Constructs), Table S2 (RBS Sequences), Table S3 (Spacer Sequences), Table S4 (RT-PCT primers), Table S5 (OFF-populations), Part DNA sequences. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.
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ABBREVIATIONS

DFHBI: 5-difluoro-4-hydroxybenzylidene imidazolinone; IPTG: Isopropyl β-D-1-thiogalactopyranoside; RBS: Ribosome Binding Site; PCR: Polymerase Chain Reaction; RT-PCR: Real-Time Polymerase Chain Reaction; LB: Luria–Bertani medium; A.U.: Arbitrary Units

REFERENCES