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Technical Note

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The Spinach RNA Aptamer as a Characterization Tool for Synthetic Biology

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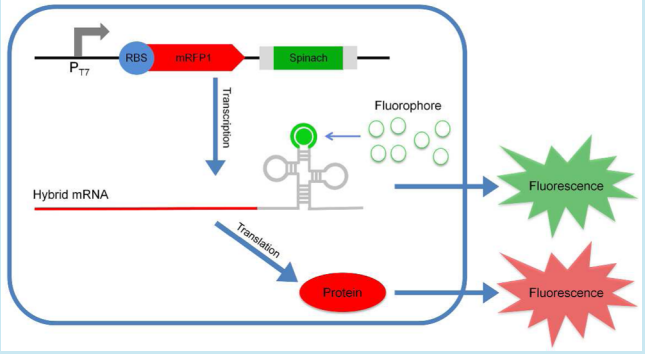
Supporting Information

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*

Accurate characterization of gene expression control elements is an important part of synthetic biology.[1] Quantitative and qualitative data informs part choice and is used to define key parameters for predictive models of gene networks and metabolic pathways.[2−4] Currently, fluorescent proteins such as GFP are the standard tool used to measure gene expression *in vivo*. Linking the expression of these proteins directly to engineered devices allows quantification of their output using high-throughput and high-sensitivity equipment such as fluorescence-enabled multiwell plate readers and flow cytometers.[5] A major limitation of fluorescent proteins, however, is that they report only on cellular protein production. Gene expression is a multi-step process where DNA is converted to mRNA and then to protein, and different control elements act at different stages within this: promoters controlling transcription, and the ribosome binding site (RBS) controlling translation initiation rates. Quantifying gene expression using fluorescent proteins measures only the final product of these stages and does not interrogate them separately. While a kinetic model of all the gene expression processes that go into GFP expression has previously been described[6] ideally data that capture transcription and translation independently would enable more accurate models.

In 2011, researchers at Cornell University described for the first time an RNA sequence that itself could mimic GFP in living cells.[7] This RNA sequence, known as the Spinach 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.

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 171nt 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

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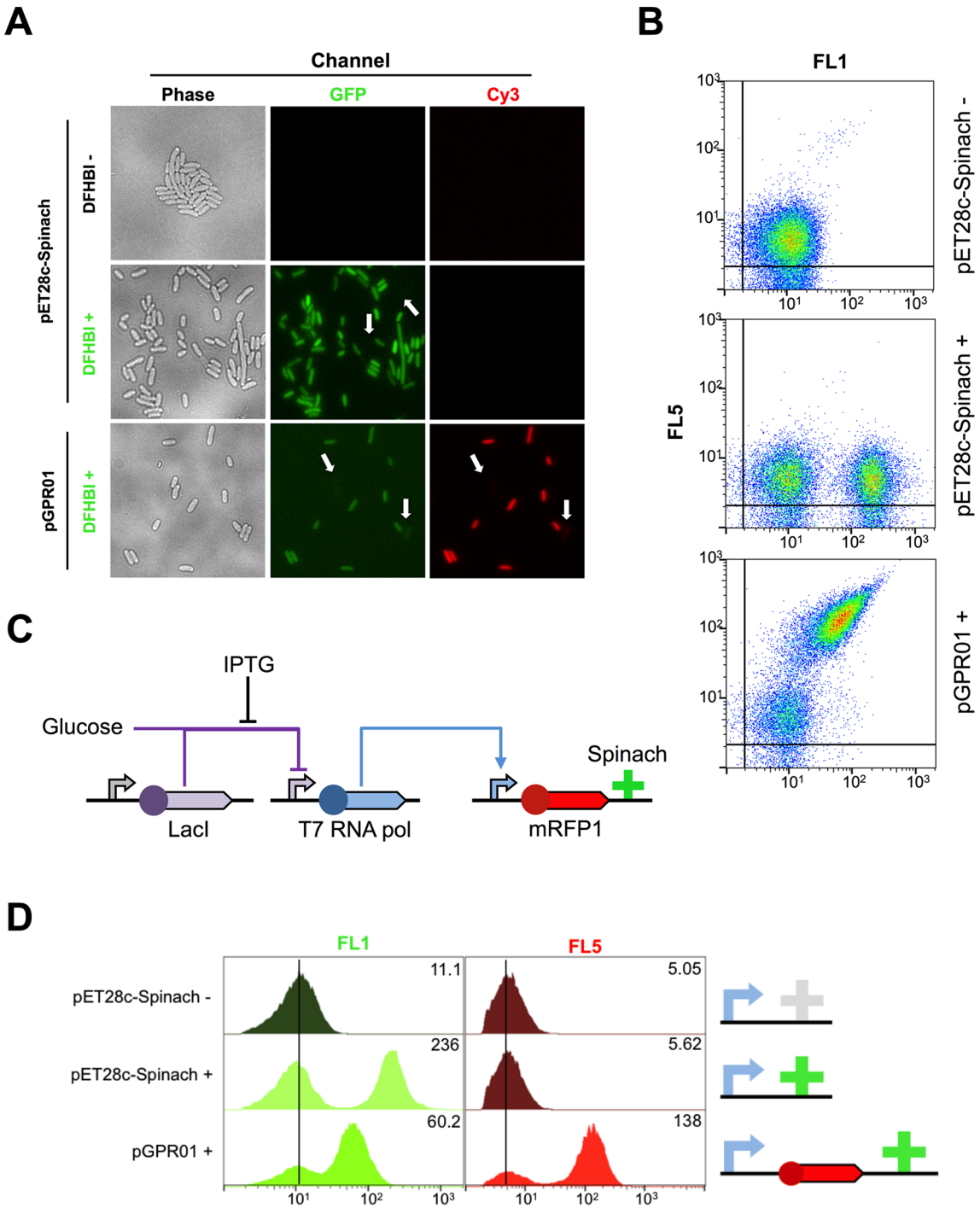

Figure 1. Parallel measurement of Spinach and mRFP1-Spinach expression by fluorescence microscopy and flow cytometry. (A) Live-cell fluorescence (GFP/Green and Cy3/Red) and phase images of BL21(DE3) *E. coli* cells carrying either the pET28c-Spinach (T7-Spinach) or pGPR01 (T7-mRFP1-Spinach) plasmid in the presence or absence of 200 μM DFHBI. Images are taken after a 2 h induction with IPTG. White arrows highlight OFF-population cells not showing any fluorescence. (B) Flow cytometry analysis showing FL1 (green fluorescence) and FL5 (red fluorescence) area plots for cells from the same samples as those imaged by microscopy. OFF-populations of cells showing no fluorescence are seen in the lower left corner. Vertical and horizontal lines show exclusion of FL1 and FL5 values below $2 \times 10^{\circ}$ A.U. (C) Diagram of gene expression control for pGPR01. T7 RNA pol (polymerase) is chromosomally encoded in BL21(DE3) cells and is inhibited in the presence of glucose and absence of IPTG. (D) Individual FL1 (green fluorescence) and FL5 (red fluorescence) histograms for the gated data shown in B. Background fluorescence for each is indicated by a line plotted through the peaks of negative control samples (i.e., pET28c-Spinach with no DFHBI). The fluorescence peak (modal value) is given for each histogram. The part composition of the constructs is shown in cartoon form: T7 promoter (blue right-angled arrow), RBS1-mRFP1 (red ball and arrow), Spinach (gray cross without DFHBI, green cross with DFHBI).

monomeric red fluorescent protein, mRFP1,[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.

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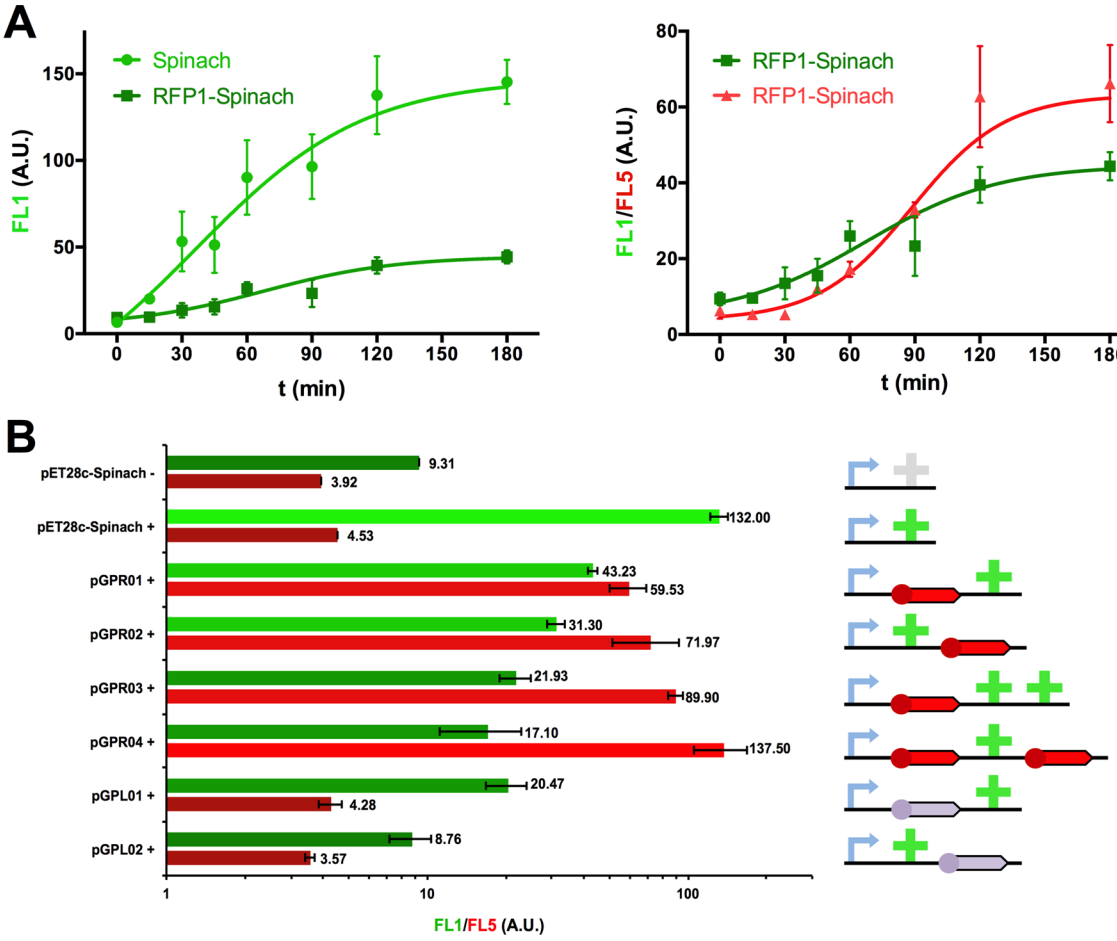

Figure 2. Temporal characteristics and optimal placement of the Spinach aptamer within a synthetic mRNA. (A) Change in green (FL1) and red (FL5) fluorescence over a 3 h induction period from *E. coli* cells expressing Spinach and mRFP1 from the pET28c-Spinach and pGPR01 plasmids. Left panel compares the green fluorescence of both constructs, and right panel shows the parallel green and red fluorescence of pGPR01. Plotted values are the modal fluorescence determined by flow cytometry, averaged across 3 repeats. (B) Green and red fluorescence of *E. coli* cells hosting either pET28c-Spinach, pGPR01, pGPL01 or variations of the pGPR01 and pGPL01 plasmids where the RBS1-mRFP1 (red ball and arrow) or RBS2-LacZ (purple ball and arrow) and Spinach (cross) parts have been rearranged in constructs expressed from the T7 promoter (blue right-angled arrow). Presence and absence of DFHBI are respectively noted by (+) and (−) and by green and gray crosses in the construct diagrams. Data show modal fluorescence as determined by flow cytometry and averaged across 3 repeats (except pGPR04, where $n = 2$).

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

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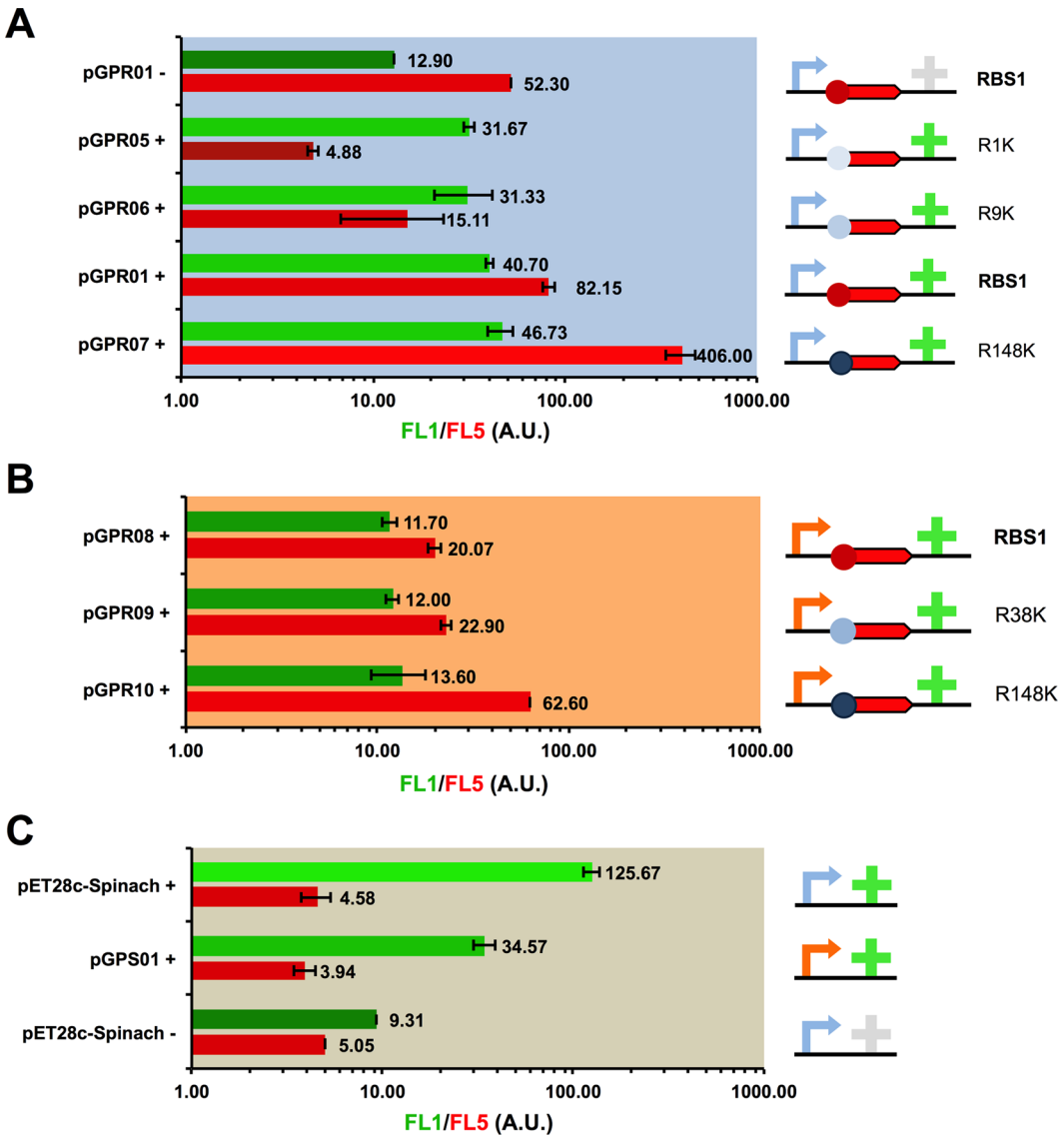

Figure 3. Characterization of designed ribosome binding site sequences and promoters. (A) Green (FL1) and red (FL5) fluorescence of *E. coli* expressing mRNA from versions of pGPR01 with designed RBS sequences (R1K, R9K, R148K) replacing RBS1. (B) Fluorescence of a version of pGPR01 where the T5 promoter replaces the T7 promoter (pGPR08) and for when RBS1 is replaced with designed RBS sequences (R38K, R148K) for this case. (C) Fluorescence of T7 promoter-driven Spinach expression (pET28c-Spinach) compared to T5 promoter-driven expression (pGPS01). Construct diagrams depict the T7 and T5 promoters as blue and orange right-angled arrows, respectively, mRFP1 as a red arrow and Spinach as a cross. RBS1 is a red ball and designed RBS sequences are shaded blue balls. Presence and absence of DFHBI are respectively noted by (+) and (−) and by green and gray crosses in the construct diagrams. Data show modal fluorescence as determined by flow cytometry and averaged across 3 repeats (except pGPR01+, where $n = 2$).

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

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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 Calculator[12,13] 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 utilizes the host RNA polymerase rather than T7 RNA polymerase,[14] allowed us to also investigate how different promoters affect expression. An attempt to detect Spinach fluorescence from the well-characterized *E. coli* AraBAD promoter[15] did not yield green fluorescence above background (data not shown), but with the T5 promoter, green fluorescence was detectable for three constructs yielding different red fluorescence (Figure 3B). A further comparison of Spinach fluorescence from the T7 and T5 promoters without mRFP1 (pET28c-Spinach and pGPS01) confirmed T5 to be approximately one-third the strength of T7 (Figure 3C).

Our work here shows that a construct consisting of mRFP1-Spinach allows for the first time simultaneous *in vivo* measurement of transcript and protein production from the same mRNA. We have demonstrated this with transcripts expressed by both T7 RNA polymerase and by the native *E. coli* transcription machinery. The Spinach aptamer is thus a valuable new tool to measure the control of gene expression in synthetic systems, providing characterization data for both steps in gene expression when paired with fluorescent protein measurement. While it is clear that there are current limitations to the use of the Spinach aptamer as a characterization tool, future improved 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-Seq[16] 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 Calculator[12,13] 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 Assembly[19] 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 OD_{600} = 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 $2 \times 10^{\circ}$ 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× CPI60 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

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