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Application of mRNA Arrays for the Production of mCherry Reporter-Protein Arrays for Quantitative Gene Expression Analysis

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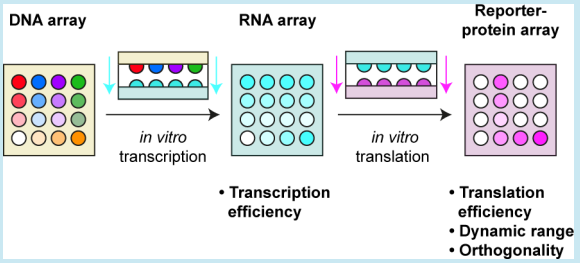
Supporting Information

ABSTRACT: The development of programmable regulators that precisely and predictably control gene expression is a major goal of synthetic biology. Consequently, rapid high-throughput biochemical methods capable of quantitatively analyzing all components of gene expression would be of value in the characterization and optimization of regulator performance. In this study we demonstrate a novel application of RNA arrays, involving the production of reporter-protein arrays, to gene expression analysis. This method enables simultaneous quantification of both the transcription and post-transcription/translation components of gene expression, and it also allows the assessment of the orthogonality of multiple regulators. We use our method to directly compare the performance of a series of previously characterized synthetic post-transcriptional riboregulators, thus demonstrating its utility in the development of synthetic regulatory modules and evaluation of gene expression regulation in general.



KEYWORDS: *gene regulation, post-transcriptional riboregulator, toehold switch, translational regulation, translation efficiency, transcriptional regulation*

A major goal of synthetic biology is to assemble versatile, programmable regulators into genetic circuits in order to achieve precise and predictable control of gene expression.[1] A logical starting point is understanding the underlying principles of gene expression that are at work in natural systems. Often, it is the complex interplay of multiple regulatory inputs that determines the final expression output. High-throughput biochemical methods capable of simultaneously evaluating these inputs/outputs would be of value to both the core gene expression and synthetic biology fields.

Regulation of gene expression occurs in all domains of life; it allows prokaryotic organisms to adapt to their environment and is critical for cell differentiation in eukaryotes. Although initially believed to be controlled primarily at the level of transcription, the weak correlation observed between mRNA and protein amounts on a global scale also highlights the importance of post-transcriptional mechanisms of regulation.[2,3] Central to both transcriptional and post-transcriptional/translational control are noncoding riboregulator RNAs (ncRNAs), which include small regulatory RNAs (sRNAs),[4] riboswitches[5] and microRNAs (miRNAs).[6] The prevalence of RNA-based regulation in nature, particularly the diverse functions performed by ncRNAs, has provided the inspiration for several synthetic biology programmes.[7−9] These include the development of artificial versions of both sRNAs[10,11] and riboswitches,[12] and completely synthetic novel riboregulators, termed "toehold switches".[13] Genetic circuits containing one or more riboregulators are typically designed *in silico* and validated *in vivo*. Commonly observed problems include incomplete repression in the "off" state, narrow dynamic range, and significant cross-talk when integrating multiple regulators in complex circuits.[13,14] In addition, the *in vivo* testing approach results in a relatively long design-build-test cycle that provides limited quantitative information about the performance of individual riboregulator components, both of which are barriers to optimization. Recently, *in vitro* transcription−translation (TX-TL) has been utilized as a rapid, quantitative method to characterize riboregulators,[15−17] demonstrating the value of controlled *in vitro* techniques in the evaluation of the performance of riboregulators, both in isolation and as part of more complex systems. Moving toward more high-throughput *in vitro* approaches would therefore be beneficial.

We recently developed a method for producing high-density functional-RNA arrays.[18] These arrays provide a platform technology for the high-throughput investigation of biologically relevant RNA-based interactions.[18] In the current work, we now demonstrate the proof-of-principle application of functional-RNA arrays to the production of reporter-protein arrays for quantifiable gene expression analysis of synthetic riboregulators *in vitro*. In contrast to combined TX-TL systems,[15−17] in our method, the transcription and translation steps are physically separated. Consequently, this allows for the independent evaluation of both the transcriptional and translational components of gene expression. We anticipate

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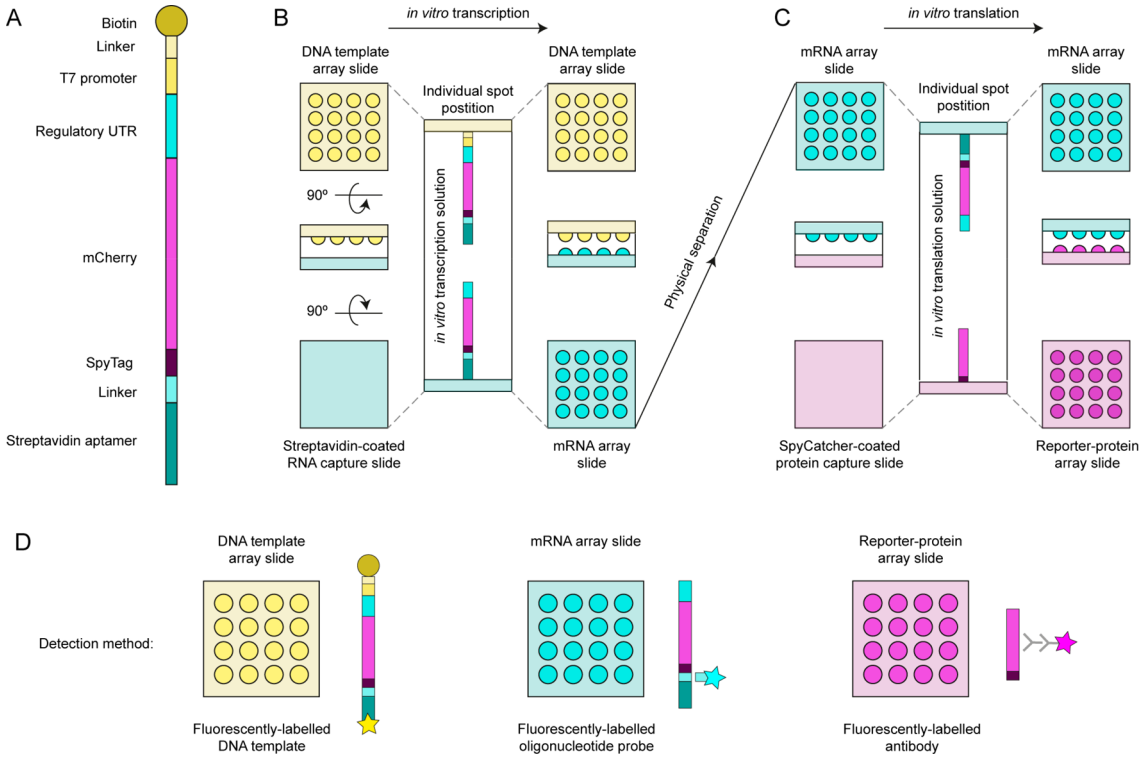

Figure 1. Application of RNA arrays to gene expression analysis. (A) Schematic design of *in vitro* transcription templates. Templates are double-stranded DNA and, from 5′ to 3′, encode a biotinylated linker, a T7 promoter, a variable regulatory UTR, an mCherry-SpyTag fusion reporter-protein, a second linker and a streptavidin RNA aptamer. (B) Schematic representation of the DNA template array slide−*in vitro* transcription solution−streptavidin-coated RNA capture slide "sandwich" used to produce mRNA arrays. (C) Schematic representation of the mRNA array slide−*in vitro* translation solution−SpyCatcher-coated protein capture slide "sandwich" used to produce reporter-protein arrays. (D) Schematic representation of the detection methods used to visualize and quantify each of the array slides. 3′ fluorescently labeled *in vitro* transcription templates allow direct visualization of DNA template array slides. mRNA array slides are probed with a Dy649-labeled DNA oligonucleotide complementary to the linker region between mCherry-SpyTag and the streptavidin aptamer. Protein arrays are probed with an anti-mCherry primary antibody followed by an Alexa647 conjugated secondary antibody.

that this technique would be utilized to characterize the properties of riboregulators, including dynamic range, orthogonality, and cross-talk.

Understanding the relative contribution that transcriptional and/or post-transcriptional/translational regulation events have on gene expression is important for understanding both the mechanism of natural gene expression and the function of synthetic biological parts. A schematic of our strategy for utilizing RNA arrays in gene expression analysis is presented in Figure 1. Underpinning the strategy is the production of both *in vitro* transcribed functional-mRNA arrays and *in vitro* translated reporter-protein arrays, generated *via* physically separated transcription and translation steps, that enables both the transcription and translation components of gene expression to be quantified. We have reported a protocol for generating functional-mRNA arrays previously.[18] Briefly, an array of double-stranded DNA *in vitro* transcription templates, all encoding an RNA of interest fused to the streptavidin-binding aptamer (Figure 1A), is positioned facing a streptavidin-coated capture surface. In this study, the RNA of interest consists of a regulatory untranslated region (UTR) fused to the coding region for an mCherry reporter coupled to a C-terminal SpyTag peptide[19] (Figure 1A and Figure S1). A solution containing only the components required for *in vitro* transcription fills the space between the DNA template array and the streptavidin-coated capture surface. As *in vitro* transcription proceeds, mRNAs are captured *via* the streptavidin-binding aptamer located at the 3′ end of the RNAs, generating an mRNA array (Figure 1B). We now present an analogous procedure for the production of reporter-protein arrays from mRNA arrays. The DNA template array-mRNA array sandwich is disassembled and the mRNA array is positioned facing a protein-capture surface that has been precoated with SpyCatcher. A solution containing only the components required for *in vitro* translation fills the space between the mRNA array and the protein-capture surface. During *in vitro* translation, the SpyTag peptide of the nascent protein binds covalently to the immobilized SpyCatcher generating a reporter-protein array (Figure 1C). The use of fluorescently labeled DNA templates allows for the direct visualization and quantification of the DNA template arrays while the mRNA arrays are visualized and quantified using a fluorescently labeled DNA oligo complementary to the linker region between mCherry and the streptavidin-binding aptamer (Figure 1D). Although we had originally intended to visualize and quantify the reporter-protein arrays using mCherry fluorescence, we were unable to detect fluorescence using our experimental setup. This was most likely due to the low amounts of mCherry protein immobilized on the reporter-protein arrays. Therefore, reporter-protein arrays were visualized and quantified using an anti-mCherry antibody (Figure 1D).

We previously produced high-density functional-RNA arrays of both single and multiple RNA species.[18] These arrays

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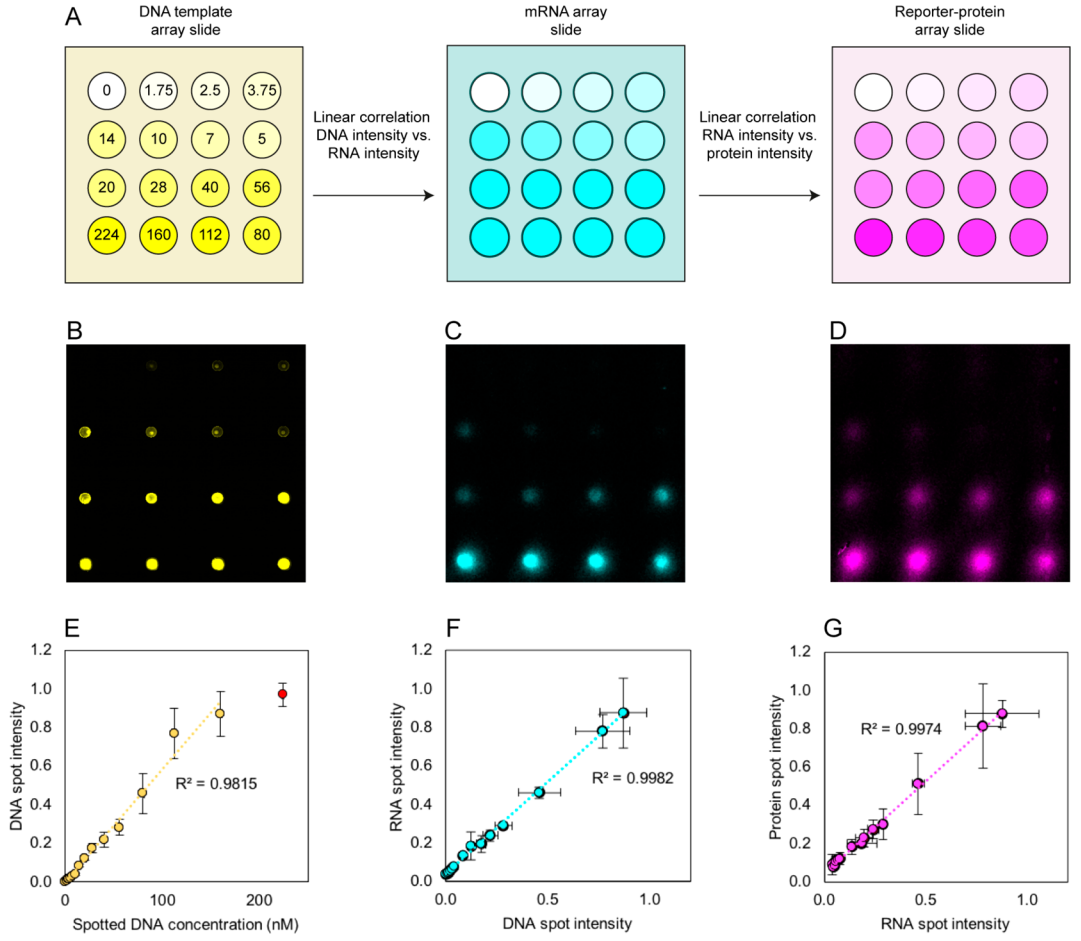

Figure 2. Gene expression analysis of mCherry-SpyTag$_{sa}$. (A) Schematics of a 4 × 4 field of an mCherry-SpyTag$_{sa}$ DNA template array and the mRNA and reporter-protein arrays expected to be subsequently generated. The concentration of the DNA template spotted at each position of the DNA template array, in nM, is indicated. The mirror image of the expected mRNA array is shown. (B) A representative 4 × 4 field of an Alexa647-labeled mCherry-SpyTag$_{sa}$ DNA template array corresponding to the schematic shown in (A). (C) The mirror image of a representative 4 × 4 field of a probed mCherry-SpyTag$_{sa}$ mRNA array, generated by *in vitro* transcription of the DNA template array in (B). (D) A representative 4 × 4 field of a probed mCherry-SpyTag reporter-protein array, generated by *in vitro* translation of the mRNA array in (C). The DNA (B), mRNA (C), and reporter-protein array (D) images have been false-colored yellow, cyan, and magenta, respectively, to aid visualization. (E) Plot of DNA spot intensity against concentration of spotted DNA. (F) Plot of RNA spot intensity against DNA spot intensity. (G) Plot of protein spot intensity against RNA spot intensity. Data in panels (E−G) were fit to a linear equation. The DNA spot intensity at a spotting concentration of 224 nM was outside the linear range (panel E, red circle). Therefore, spots corresponding to this concentration of DNA template were omitted from subsequent analysis.

mainly comprised ncRNAs and, consequently, were never subjected to expression/translation analyses. In this study, we wanted to demonstrate that we could produce quantifiable arrays of mRNAs, and that these arrays could be translated *in vitro* to produce quantifiable reporter-protein arrays as readouts of gene expression. As an initial proof-of-concept, we decided to use a DNA template array of varying levels of an mCherry-SpyTag$_{sa}$ template, encoding the mCherry-SpyTag reporter protein and streptavidin-binding RNA aptamer, to generate mCherry-SpyTag$_{sa}$ mRNA and mCherry-SpyTag reporter-protein arrays (Figure 2A−D). The template DNA was spotted at a range of concentrations between 1.75 and 224 nM in order to allow the detection limits of the technique to be ascertained (Figure 2A,B,E). The spot intensities for all three arrays were quantified. A linear correlation between the concentration of DNA template spotted and the spot intensity of the DNA array was observed for DNA template concentrations up to 160 nM (Figure 2E). Linear correlations were also observed between the DNA and RNA spot intensities (Figure 2F), between the RNA and protein spot intensities (Figure 2G), and between the DNA and protein spot intensities (Figure S2) for this DNA template concentration range. Therefore, DNA template concentrations up to 160 nM were considered to be a suitable working range for subsequent experiments.

Having successfully demonstrated the ability to quantifiably analyze gene expression of an array of identical DNA templates at both the transcriptional and post-transcriptional/translational levels, we next wanted to analyze the expression of a more complex array. We selected a series of previously characterized synthetic post-transcriptional riboregulators.[13] These riboregulators, termed toehold switches, are structured RNA elements that are designed to span the translation initiation region of mRNAs such that gene expression is activated post-transcriptionally in the presence of a *trans*-acting trigger RNA[13] (Figure S3). We prepared a DNA array, in quadruplicate, that consisted of four toehold switch-mCherry-SpyTag$_{sa}$ templates (TS1, TS3, TS8, and TS9) and mCherry-

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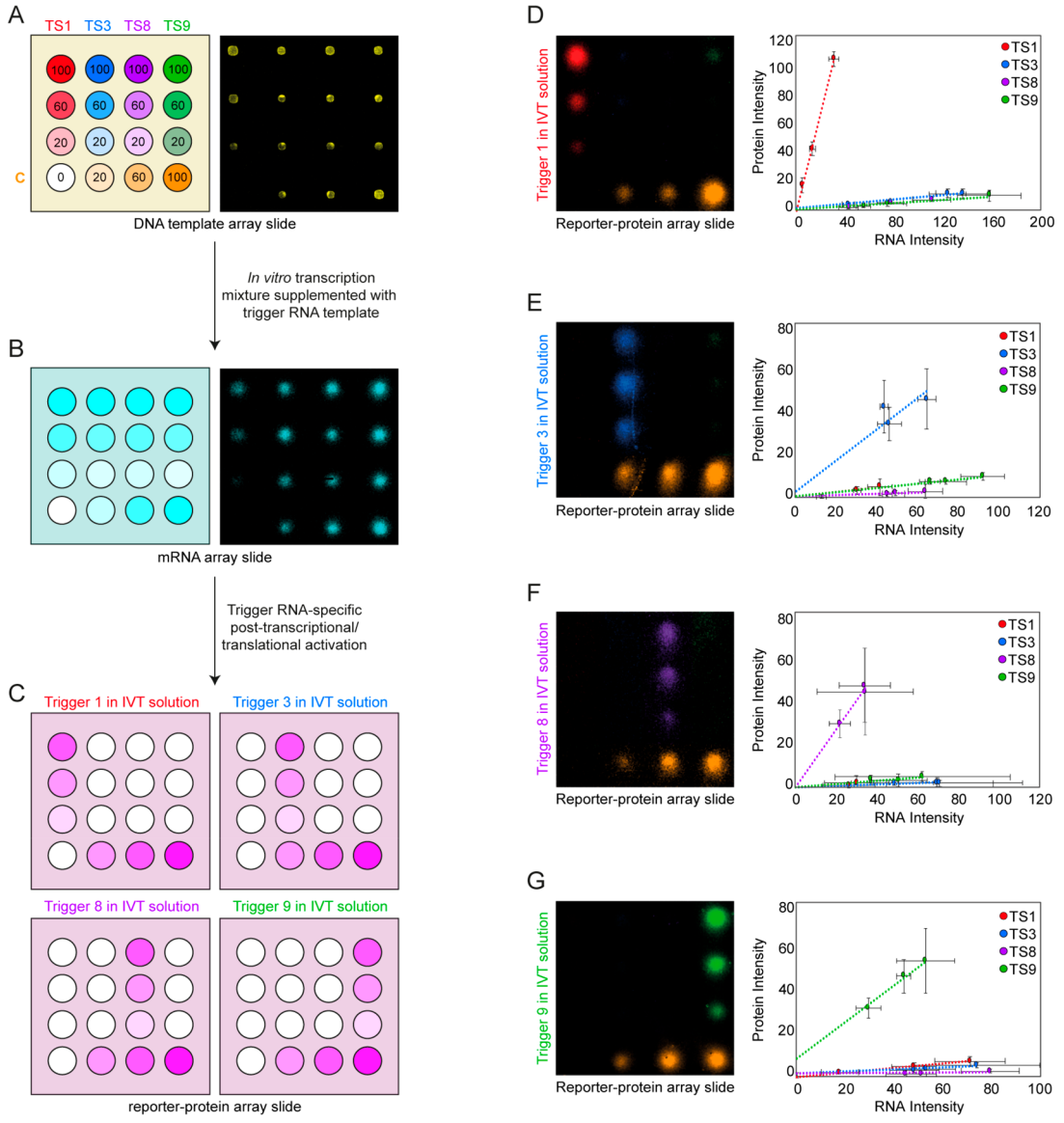

Figure 3. Gene expression analysis of toehold switch-mCherry-SpyTag$_{sa}$ templates with cognate trigger RNA templates in solution. (A) A schematic (left) and a representative (right) 4 × 4 field of a Dy549-labeled toehold switch-mCherry-SpyTag$_{sa}$ DNA template array. TS1 (red), TS3 (blue), TS8 (purple), and TS9 (green) indicate toehold switches 1, 3, 8, and 9, from Green *et al.*, 2014,[13] respectively, inserted upstream of mCherry-SpyTag$_{sa}$. The mCherry-SpyTag$_{sa}$ control is indicated by a letter C (orange). The concentration of the DNA template spotted at each position of the DNA template array, in nM, is indicated. (B) A schematic (left) and a representative (right) 4 × 4 field of a probed mRNA array, generated by *in vitro* transcription of the DNA template array in (A) using *in vitro* transcription solution supplemented with template for trigger RNA 1, 3, 8, or 9. Both the schematic and the data image are shown as mirror images. (C) Schematics of a 4 × 4 field of the reporter-protein arrays expected to be generated from the mRNA array in (B). (D) A representative 4 × 4 field of a probed mCherry-SpyTag reporter-protein array, generated by *in vitro* translation of an mRNA array that had been transcribed using *in vitro* transcription solution supplemented with template for trigger RNA 1 (left). Plot of protein spot intensity against RNA spot intensity (right). Data were fit to a linear equation. (E–G) As in (D) but generated by *in vitro* translation of mRNA arrays that had been transcribed using *in vitro* transcription solution supplemented with template for trigger RNA 3, 8, or 9, respectively. The data images in panels A, B, and D–G have been false-colored to aid visualization.

SpyTag$_{sa}$ template as a control (Figure 3A). Toehold switches TS1, TS3, TS8, and TS9 correspond to the forward-engineered, second-generation toehold switches 1, 3, 8, and 9, described in Green *et al.*, 2014[13] (see Table S1 for sequences), respectively. Each DNA template was spotted at three concentrations (20, 60, and 100 nM), selected to be within the established linear range for the mCherry-SpyTag$_{sa}$ control (Figure 2E). The DNA spot intensity of the toehold-mCherry-SpyTag$_{sa}$ templates displayed a similar linear correlation with the spotted DNA concentration (Figure S4). An mRNA array was generated from each of the DNA template arrays using *in vitro* transcription mixture supplemented with DNA template encoding either the cognate trigger RNA for toehold switch TS1, TS3, TS8, or TS9 (Figure 3B). We made the assumption that the trigger RNA would be transcribed in parallel with the mRNAs and would therefore be available to specifically activate its cognate mRNA. All of the mRNAs were produced in the presence of each of the trigger RNAs and all displayed a linear relationship between the DNA and RNA spot intensities (Figure S5). Finally, each of the mRNA arrays were used to generate a reporter-protein array (Figure 3C–G). Significant amounts of protein were only

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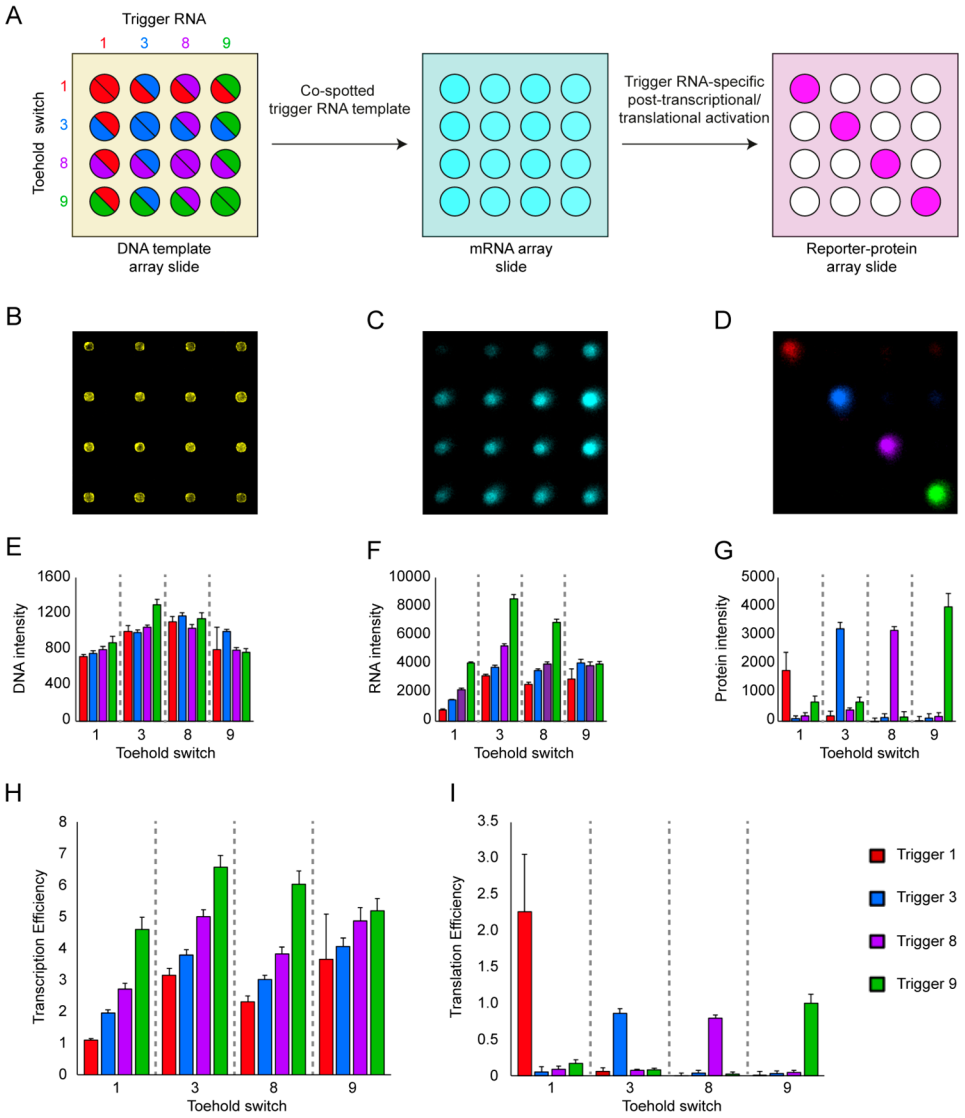

Figure 4. Gene expression analysis of toehold switch-mCherry-SpyTag$_{sa}$ templates with cognate trigger RNA templates cospotted on the DNA template array. (A) Schematics of a 4 × 4 field of a cospotted toehold switch-mCherry-SpyTag$_{sa}$/trigger DNA template array and the mRNA and reporter-protein arrays expected to be subsequently generated. The mirror image of the expected mRNA array is shown. (B) A representative 4 × 4 field of a cospotted Alexa647-labeled toehold switch-mCherry-SpyTag$_{sa}$/trigger DNA template array. Toehold switch-mCherry-SpyTag$_{sa}$ and trigger RNA templates were spotted at a concentration of 50 nM. (C) The mirror image of a representative 4 × 4 field of a probed mRNA array, generated by *in vitro* transcription of the DNA template array in (B). (D) A representative 4 × 4 field of a probed mCherry-SpyTag reporter-protein array, generated by *in vitro* translation of the mRNA array in (C). The data images in panels (B−D) have been false-colored to aid visualization. (E) Spot intensities for the DNA template array. (F) Spot intensities for the mRNA array. (G) Spot intensities for the reporter-protein array. (H) Transcription efficiencies for each of the toehold switch/trigger RNA combinations. (I) Translation efficiencies for each of the toehold switch/trigger RNA combinations.

detected for the control mCherry-SpyTag$_{sa}$ mRNA and the toehold switch-mCherry-SpyTag$_{sa}$ mRNAs for which the cognate trigger RNA template had been present in the *in vitro* transcription solution, demonstrating the orthogonality of the individual toehold switch-trigger RNA pairs (Figure 3D−G). Where protein was produced, there was a linear correlation between the RNA and protein spot intensities (Figure 3D−G).

An experiment such as that described above may be useful for evaluating the behavior of a single, or low number of, regulator(s) of gene expression. However, if a large number of regulators need to be directly compared on a single array in order to evaluate orthogonality and cross-talk, a higher throughput method would be required. In order to achieve this, we decided to cospot the DNA templates for both the toehold switch-mCherry-SpyTag$_{sa}$ reporter and the trigger RNAs (Figure 4A), rather than adding the latter in solution to the *in vitro* transcription mixture. In theory, the number of pairwise regulatory interactions that could be investigated on a single array is limited only by the available array size and density. A DNA array was prepared that consisted of templates encoding the same four toehold switch-mCherry-SpyTag$_{sa}$ mRNAs used above, cospotted with templates for either their cognate trigger RNA or a noncognate trigger (Figure 4A,B). This was used to generate an mRNA array (Figure 4C) that was then used to produce a reporter-protein array (Figure 4D). As reported above, significant amounts of reporter-protein were only obtained for cognate toehold switch-trigger RNA pairs, demonstrating the expected orthogonality (Figure 4D).

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However, when quantification of the spot intensities was performed for all three arrays (Figure 4E−G) this revealed that detectable levels of reporter-protein were also reproducibly produced for toehold switches 1 and 3 with noncognate trigger RNAs, indicating the presence of low-levels of cross-talk (Figure 4G). Interestingly, since our technique allows both the mRNA and reporter-protein amounts to be quantified, we were also able to determine the transcription and translation efficiency of each of the toehold switch-mCherry-SpyTag$_{sa}$/trigger RNA combinations (Figure 4H,I). Although the maximum protein expression was achieved with toehold switch 9/cognate trigger RNA 9 (Figure 4G and Table 1), transcription of toehold switch 3/noncognate trigger 9 was the most efficient (Figure 4H) and toehold switch 1/cognate trigger 1 was translated most efficiently (Figure 4I and Table 1).

Table 1. Comparison of Toehold Switch Performance *In vivo* and *In vitro* with Cognate Trigger RNAs

toehold switch	*in vivo* on/off ratio[a]	*in vitro* protein spot intensity (arbitrary units)[b]	*in vitro* translation efficiency (protein spot intensity/RNA spot intensity)[b]
TS1	665 ± 135	1767 ± 617	2.26 ± 0.80
TS3	557 ± 67	3206 ± 229	0.86 ± 0.07
TS8	393 ± 41	3145 ± 138	0.79 ± 0.05
TS9	381 ± 32	3976 ± 459	1.00 ± 0.12

[a]Data extracted from Green *et al.*, 2014.[13] [b]Data extracted from Figure 4.

Since the toehold switches utilized here were originally characterized *in vivo*,[13] we next decided to compare our *in vitro* results to the published *in vivo* results. Table 1 presents the on/off ratios for each toehold switch/cognate trigger RNA that were determined by Green *et al.* using a GFP reporter and flow cytometry[13] along with the raw protein levels obtained *in vitro* (Figure 4G) and the translation efficiency determined *in vitro* (Figure 4I) for comparison. We were unable to accurately determine on/off ratios using our technique due to the extremely low levels of protein produced in the absence of cognate trigger RNA, and our raw protein levels do not correlate with the *in vivo* on/off ratios (Table 1). However, when the raw protein levels are normalized to the RNA levels to give translation efficiencies, the relative translation efficiencies for toehold switches 1, 8, and 9 are consistent with the previously reported *in vivo* results (Table 1). Only the relative translation efficiency of toehold switch 3 differs from what would have been expected based on the *in vivo* data (Table 1). A possible explanation for this discrepancy could be the use of a GFP reporter-protein for the *in vivo* studies[13] compared to the mCherry reporter-protein used *in vitro* here. It has been noted that toehold switch performance can be affected by the downstream coding sequence, although the reasons for this have not been explored.[13,20] Nevertheless, the agreement between the *in vivo* and *in vitro* data for three out of four toehold switch/cognate trigger RNA combinations suggests that our technique is suitable for predicting *in vivo* behavior.

Normalizing the protein levels to the RNA levels, to allow calculation of translation efficiency, is critical for obtaining this agreement between *in vivo* and *in vitro* data due to the variability in transcription efficiency. We observed both trigger-dependent variability for a specific toehold switch (*e.g.*, compare toehold switch 1 and all four trigger RNAs in Figure 4H) and toehold switch-dependent variability in the transcription efficiency for a specific trigger RNA (*e.g.*, compare all four toehold switches with trigger 8 in Figure 4H). It is possible that the variability in transcription efficiency, and the subsequent lack of correlation to protein levels, is an artifact of the physical separation of the transcription and translation steps and/or the simplified *in vitro* transcription and translation mixtures employed here. *In vivo*, factors such as coupling of transcription and translation[21] and macromolecular crowding[22] are known to affect gene expression. Transcription efficiency can also be affected by promoter sequence and transcript secondary structure. However, given that all of the toehold switches contain identical T7 promoter sequences and were designed to adopt equivalent secondary structures,[13] this observed apparent difference in transcriptional efficiencies was a little surprising. One possible explanation could be due to the use of a minimal T7 promoter sequence. Minimal T7 promoter sequences, which span from position −17 to position +3, relative to the transcriptional start site, are used because they ensure efficient transcription while appending the minimum number of exogenous nucleotides to the 5′ end of the resultant transcript. However, the full T7 promoter consensus sequence spans from position −17 to position +6,[23] with nucleotides in positions +4 to +6 known to affect transcription efficiency.[24] Since the +4 to +6 sequence is different for each of the toehold switches tested here, this could account for the observed variability in transcription efficiency. The trigger RNA-dependent effect on transcription efficiency most likely stems from similar variability in the sequences and transcription efficiencies of the trigger RNA templates resulting in differences in the relative competition for transcriptional resources between the toehold switch and trigger RNA templates. Interestingly, it has been shown that optimal activation of toehold switches is sensitive to the concentration of trigger RNA template, with high template concentrations negatively affecting activation due to sequestration of transcription reagents.[17]

The abundance and diversity of riboregulators in natural biological systems have provided a foundation for the development of synthetic RNA-based components for integration into synthetic genetic circuits.[7−9,12] However, recurring limitations of synthetic riboregulators have been low dynamic range and cross-talk.[13,14] Progress has been made, by engineering systems that utilize both transcriptional and translational control mechanisms.[25,26] Consequently, the ability afforded by our technique to independently evaluate the contribution of the transcription and translation components to the overall gene expression process is anticipated to be a valuable tool for characterizing such synthetic regulatory modules, and for understanding natural gene expression mechanisms. While well-established high-throughput techniques exist for monitoring transcriptional regulation (*e.g.*, DNA microarrays and RNA sequencing (RNAseq)), there are fewer options available for independently and/or simultaneously investigating post-transcriptional/translational control, particularly by the growing number of synthetic and natural noncoding riboregulators. To our knowledge, only one other study has directly investigated the post-transcriptional/translational component of synthetic riboregulator performance.[17] This was achieved through the use of an mRNA, rather than DNA, template in an *in vitro* transcription−translation system.[17] It is our RNA and reporter-

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protein arrays' capacity to independently and/or simultaneously investigate post-transcriptional/translational control that we envisage will be its greatest utility.

We have previously demonstrated the utility of RNA arrays in screening RNA−RNA and RNA−small molecule interactions.[18] We now anticipate that the transcriptional and post-transcriptional/translational assays developed here could be applied to the high-throughput screening of transcriptional and/or post-transcriptional/translational inhibitors. Furthermore, since the final readout of our assay involves the generation of a reporter-protein array, the technique could be applied to the production of heterotypic functional protein arrays. There are competing technologies available for the production of functional protein arrays, including PISA (protein *in situ* array),[27] NAPPA (nucleic acid programmable protein microarray),[28] and DAPA (DNA array to protein array).[29] However, each of these technologies involves a single combined transcription/translation step. In circumstances where optimal transcription and translation steps are required, *e.g.*, poorly expressed proteins, our separated transcription and translation steps may provide an advantage.

In summary, we have demonstrated the proof-of-concept application of RNA and reporter-protein array technology to the analysis of the dynamic range, orthogonality, and cross-talk of riboregulators of gene expression. The spatial and temporal separation of the transcription and translation components allows for the independent evaluation of their relative contributions to overall gene expression. We anticipate that this will be a powerful tool for the synthetic biology, gene expression, and drug discovery fields.

METHODS

Preparation of DNA Template Arrays and Generation of mRNA Arrays. Methods for the preparation of DNA template arrays and the subsequent generation of mRNA arrays were modified from those described previously[18] (see Supporting Methods in Supporting Information).

Generation of Reporter-Protein Arrays. The reporter-protein capture surface was prepared as described for the DNA and mRNA capture surfaces (Supporting Information) except that 2 mg/mL SpyCatcher (see Supporting Information for preparation) was used in place of streptavidin. An mRNA array and SpyCatcher-coated protein capture slide were mounted onto the slide separator device described in Figure S6 and assembled in a sandwich format with 12 μL of protein expression mixture (5.5 μL PremixPlus, 4.5 μL T7 Extract, and 2 μL nuclease-free H_2O; S30 T7 High-Yield Protein Expression System (Promega)) as the aqueous filling. This was incubated in a humidity chamber at 37 °C for 10 min. The slides were separated in Milli-Q H_2O and the newly generated reporter-protein array was washed and dried using a standard wash and dry step, performed as follows: a wash with 50 mL PBST (phosphate buffered saline pH 7.4 (PBS) containing 0.02% (v/v) Tween 20) at room temperature, with rocking, for 5 min followed by a wash with 50 mL Milli-Q H_2O at room temperature, with rocking, for 30 s and a final rinse with Milli-Q H_2O. The slides were placed in a 50 mL Falcon tube and dried by centrifugation at 180*g*, at room temperature for 30 s.

mRNA Array Probing. Experiments were performed in pairs to allow two mRNA array slides to be assembled in a sandwich format with 25 μL of 28 nM Dy649-labeled single-stranded DNA probe (5′-Dy649-GTG TGT GTG TGT GTG TGT GT-3′) in hybridization buffer (2× saline-sodium citrate (SSC) containing 0.1% (w/v) SDS) as the aqueous filling. The assembly was incubated in a humidified chamber at room temperature for 30 min. The mRNA array slides were separated in Milli-Q H_2O and washed and dried using the standard wash and dry step.

Reporter-Protein Array Probing. Experiments were performed in pairs to allow two reporter-protein array slides to be assembled in a sandwich format with 25 μL of 16 ng/μL rabbit polyclonal anti-mCherry antibody (ab167456 (Abcam)) in 1× PBS containing 1% (w/v) BSA as the filling. The assembly was incubated in a humidified chamber at room temperature for 30 min. The reporter-protein array slides were separated in Milli-Q H_2O. The reporter-protein array was washed and dried using the standard wash and dry steps. The slide sandwich was reassembled with 25 μL of 32 ng/μL goat antirabbit IgG Alexa647 conjugated antibody (A-21245 (Invitrogen)) in 1× PBS containing 1% (w/v) BSA as the filling. The assembly was incubated in a humidified chamber at room temperature for 30 min. The reporter-protein array slides were separated in Milli-Q H_2O and washed and dried using the standard wash and dry step.

Visualization of the Arrays. Fluorescently labeled DNA template arrays and probed mRNA and reporter-protein arrays were visualized using a GenePix 4300A microarray slide scanner (Molecular Devices). Excitation wavelengths of 635 or 532 nm and Standard Red or Standard Green filters were used for the Alexa647/Dy649 or Dy549 fluorophores, respectively.

Quantification of the Arrays. For each experiment, the DNA, RNA, and/or protein spot intensities were measured from four identically prepared fields. Each spot intensity was background-subtracted using the intensity from a null-concentration spot. Thereafter, between quadruplicate fields, spot intensities were normalized to that with greatest brilliance within the same field. For the toehold switch-trigger RNA experiments, transcription and translation efficiencies were calculated as the relative intensity of RNA and DNA or protein and RNA spots, respectively, following background-subtraction and normalization.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00266.

Supporting methods, references, tables, and figures (PDF)

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Author Contributions

This authorship contributions statement has been prepared by the corresponding authors using evidence-based information provided by the individual authors in regard to their individual contributions to this manuscript. The chosen format and wording are not intended to either diminish or enhance the contribution of any particular author or authors. The overall strategy for the generation of mRNA arrays from DNA

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template arrays and the subsequent generation of reporter-protein arrays from mRNA arrays, particularly with regard to the slide "sandwich" arrangement, was conceived by A.J.C. Strategies for the practical implementation of specific aspects of the method were conceived by M.N. (RNA detection, protein detection) and A.R.P. (protein-capture). M.N. proposed the use of toehold switches for demonstrating the capabilities of the method, following-on from discussions with A.J.C., L.E.B. and H.A.V. regarding post-transcriptional riboregulators/riboswitches. Application of mRNA and reporter-protein arrays to gene expression analysis, particularly within the synthetic biology field, was an idea collectively developed by M.N., H.A.V., L.E.B. and A.J.C. Protocols were developed by M.N. (modified version of mRNA array production, mRNA array detection, reporter-protein array production and reporter-protein array detection), L.E.B. (preliminary quantification) and A.R.P. (extensive quantification of DNA, mRNA and reporter-protein arrays), with support from L.E.B. and A.J.C. All authors were involved in the experimental design. All experimental work was performed by M.N. A.R.P. quantified the data. All authors were involved in interpretation of the data. All authors were involved in the preparation of the text of the original draft (outline: M.N., L.E.B., H.A.V.; abstract: H.A.V.; introduction: H.A.V. and L.E.B.; results: H.A.V. and A.J.C.; discussion: H.A.V.; methods (except for quantification): M.N., H.A.V. and L.E.B.; methods (quantification): A.R.P. All authors contributed to the design of the figures. The final versions of Figures 1−4, S2, S4 and S5 were prepared by H.A.V., using graphs prepared by A.R.P., and the final versions of Figures S1, S3 and S6 were prepared by M.N. Tables were prepared by M.N. and H.A.V. All authors edited and revised the manuscript. All authors approved the final draft. Funding for the project was acquired by A.J.C.

Notes

The authors declare the following competing financial interest(s): Some of the authors of this paper are also named inventors on patents and/or patent applications that relate to aspects of the work reported here. University of Portsmouth applied for a patent (US patent number: 9777268 by A.J.C. and European patent application number: 12721901.2 by A.J.C.).

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