



Controlling fluxes for microbial metabolic engineering

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Controlling fluxes for microbial metabolic engineering

a dissertation presented

by

Gairik Sachdeva

to

The School of Engineering and Applied Sciences

in partial fulfillment of the requirements

for the degree of

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in the subject of

Engineering Sciences

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Controlling fluxes for microbial metabolic engineering

ABSTRACT

This thesis presents novel synthetic biology tools and design principles usable for microbial metabolic engineering. Controlling metabolic fluxes is essential for biological manufacturing of fuels, materials, and high value chemicals. Insulating the flow of metabolites is a successful natural strategy for metabolic flux regulation. Recently, approaches using scaffolds, both *in vitro* and *in vivo*, to spatially co-localize enzymes have reported significant gains in product yields. RNA is suitable for use as an *in vivo* scaffold due to its diversity of structural motifs, predictability of design, and ease of expression.

We used scaffolds made from RNA strands containing two sets of motifs. Polymerization domains bring individually expressed strands together as tiles to form a macromolecular assembly. Aptamers included in the sequence recruit RNA binding domains to these *in vivo* scaffolds. We assembled a library of eight aptamers and corresponding RNA binding domains fused to partial fragments of fluorescent proteins in *E. coli*. New scaffold designs could co-localize split green fluorescent protein fragments to produce activity as measured by cell-based fluorescence. The scaffolds consisted of either single bivalent RNAs or RNAs designed to polymerize in 1 or 2 dimensions.

The new scaffolds were used to increase metabolic output from a two-enzyme pentadecane production pathway that contains a fatty aldehyde intermediate, as well as

three and four enzymes in the succinate production pathway. Pentadecane synthesis depended on the geometry of enzymes on the scaffold, as determined through systematic reorientation of the acyl-ACP reductase fusion by rotation via addition of base pairs to its cognate RNA aptamer. Together, these data suggest that intra-cellular scaffolding of enzymatic reactions may enhance the direct channeling of a variety of chemical substrates.

In the cyanobacterium *S. elongatus* we showed that the flow of electrons can be controlled and insulated by co-expression of a heterologous hydrogenase with different interacting ferredoxin proteins. We used the system to demonstrate photo biological hydrogen production and hydrogen dependent cell growth. Finally, we expressed RNA scaffolds in *S. elongatus* using a T7 RNA polymerase system, opening doors for spatial control of metabolism in the cyanobacterium.

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FOR MY LOVING FAMILY.

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Indeed, our lack of understanding of metabolic regulation has been revealed by poor results from attempts to increase the rates of selected metabolic pathways... Our ability to interfere with an organism's genetics has far outstripped our ability to predict the effects on its metabolism

David Fell, *Understanding the Control of Metabolism*¹

1

Synthetic biology approaches for control of metabolism

SYNTHETIC BIOLOGY IS RAPIDLY ADVANCING TOWARDS ENGINEERING AT THE NANOSCALE. The ability to engineer cells with sub-cellular spatial precision is a very powerful and essential tool for synthetic biologists, who look at cells as programmable

devices². Such an approach aims to engineer biological systems to diagnose and treat diseases³, perform molecular computations⁴, and manufacture materials as parts of bio-refineries of the future⁵. The latter goal is especially urgent since biological replacements for petroleum based products can go long way in tackling the climate change problem⁶.

Efforts in metabolic engineering have benefited from the synthetic biology approach of treating genetic and cellular components as modules that can be engineered to function together in novel ways⁷. However, understanding the complexity of metabolic regulation and going beyond that to engineer metabolism for the production of chosen compounds has been a long standing challenge¹. Although there have been significant advances in both computational⁸ and empirical⁹ methods to study and design metabolism, significant challenges remain with respect to the catalytic rates of heterologously expressed enzymes within a given cell's metabolism¹⁰. One traditional approach has been to over produce the enzymes themselves; however, that is coupled with inefficiencies due to slower growth rates¹⁰. Other means to increase specific metabolic reaction yield include protein engineering¹¹, and sub-cellular co-localization of enzymes¹².

1.1 SPATIAL CONTROL FOR METABOLIC ENGINEERING

Co-localization of proteins, DNA and RNA enhances metabolic output of enzymes^{13,14}, allows novel regulation of gene expression^{15,16,17}, and can increase the specificity of therapeutics^{18,19}. This occurs primarily because co-localized macromolecules have high local concentrations, allowing their activities to be coordinated. Thus, better ability to organize proteins, RNAs, lipids etc. into synthetic supramolecular complexes should enable diverse and more complex function than can be achieved by solely engineering

individual parts.

Spatial organization of biochemical reactions is commonly observed in biological systems and allows for complex metabolic control. Natural approaches include compartmentalization, separation of reactions in different cells, and supramolecular organizations of enzymes²⁰. These strategies control the direction of metabolic pathway flux^{20,21}, prevent loss of intermediates to competing cellular reactions²², and insulate the cell itself from intermediates that may be volatile²³. Polyketide and fatty acid synthases are examples of large multi-enzyme complexes where intermediates are shuttled between enzymes to achieve end products with specific lengths and chemical groups²⁴. Similarly in bacteria, protein microcompartments for propanediol and ethanolamine utilization restrict the diffusion of toxic intermediates such as propionaldehyde and acetaldehyde respectively²⁵.

Co-localization of enzymes has been successfully employed in synthetic systems as well^{22,26}. Confinement of enzymatic reactions has been demonstrated both *in vitro*^{23,27,28,29} and *in vivo*. In bacteria, both protein^{14,30} and DNA³¹ have been used as scaffolds for heterologous enzymatic pathways with several fold increases in end product titers reported. Recently, our lab showed that RNA scaffolds can be used to modularly organize proteins in *E. coli*¹³ and increase the rate of electron transfer between enzymes. Such scaffolds, formed by tiles¹³ or repeats³² of small RNA strands, lead to higher densities of binding sites available for protein docking.

There has been debate about the mechanism by which scaffolds enable metabolic substrate channeling. The transfer of electrons between enzymes relies on physical contact and thus is limited by protein diffusion rates and competition, which are effectively addressed by scaffolding¹³. However, the role of enzyme co-localization in pathways involving diffusible intermediates is much less well understood^{33,12}. In a re-

cent study²⁸, enzymes localized in close proximity, less than 20 nanometers apart, on *in vitro* assembled DNA scaffolds exhibited enhanced rates of metabolite exchange. The transfer rates dropped precipitously with any further increase in inter-enzyme distance. Since such effects are not explicable by 3D diffusion models³³, a mechanism of metabolite substrate channeling by restricted diffusion on hydration layers across crowded protein surfaces has been proposed²⁸. RNA scaffolds, with their predictable geometry, can be used to create a range of metabolic channeling platforms and test the relative effects from these two different mechanisms.

1.2 RNA FOR *IN VIVO* SPATIAL ENGINEERING

Nucleic acids, which have important structural and catalytic roles in nature, are also enabling synthetic biology efforts for *in vivo* spatial engineering. Growing knowledge of the rules determining RNA structure and catalysis has led to the development of tools that allow one to both predict function of RNA from its sequence and rationally design RNA to assume desired motifs and interactions. Other advances have allowed RNA to be synthesized, studied, and manipulated both *in vitro* and *in vivo*. Here, we* present an account of these developments and how synthetic RNA scaffolds are advancing basic research, metabolic engineering and even therapeutics through *in vivo* spatial engineering. We offer a strong prognosis for the role of synthetic RNA devices as bioengineering tools with emerging synergies between RNA, DNA, protein nanotechnology and high-throughput sequencing.

Natural non-coding RNAs already play structural and catalytic roles in cells. A breadth of studies has established design principles that can be used to predictably

*This section forms part of a review, co-written with Cameron Myhrvold and Peng Yin, and submitted to Wiley Biotechnology Series

shape RNA secondary structures^{34,35,36,37}. Structural malleability of RNA, the ease of expressing synthetic RNA constructs in cells, their stability and advances in methods for assaying and imaging assembled structures are some of the many reasons why RNA is a useful scaffolding material. Synthetic biology efforts have demonstrated that carefully designed RNA can be used for sub-cellular targeting of probes, enzymes, and therapeutic agents.

1.2.1 STRUCTURAL ROLES OF NATURAL RNA

RNAs perform numerous biological functions as canonical gene expression agents, catalysts, gene regulation switches, and structural scaffolds. These structural and catalytic roles of RNA are due in large part to the tremendous diversity of secondary and tertiary structures assumed by natural RNA, and the fact that ribose sugars are more reactive than deoxyribose. RNA secondary structures can include intricate motifs like double helices, hairpin loops, bulges, pseudo-knots, and right-angled turns^{38,39}. Aside from the Watson-Crick base pairing, RNA has the capacity to form Hoogsteen base pairs as well as wobble base pairs. Such interactions allow motifs to be connected in higher order tertiary interactions, pre-dominantly through the non Watson-Crick base pairs^{40,41}.

RNA AS A NATURAL CATALYST

Catalytic roles of RNA during translation, like the tRNA, disrupted a simple view held by the central dogma that RNA exists merely to transfer genetic information from DNA to protein. Today we know that RNA has catalytic and regulatory roles in many other cellular processes as well. Regulatory RNA structures play a significant role in the

control of translation initiation of several bacterial genes and in bacterial immunity⁴². RNAs affect expression in *cis*, by forming secondary structures near translation start sites of the mRNA. The *cis* regions can bind to regulatory proteins or other RNAs that affect translation in *trans*⁴². Other similarly dynamic regulatory RNA regions can consist of aptamers, which are nucleic acids that selectively bind ligands⁴³. Many metabolic genes are ‘switched’ on or off, triggered by the binding of small molecule metabolites to some of these regulatory RNAs known as riboswitches⁴⁴.

RNA SCAFFOLDS IN NATURE

There are also several instances of natural RNAs that are largely structural. Some natural RNAs are known to specifically bind the coat proteins of single-strand RNA phages. Such interactions help package the RNA into viral capsids. Some RNA phages that have well-characterized RNA binding proteins include PP7⁴⁵, MS2⁴⁶ and Q β ⁴⁷. These coat proteins also act as repressors of the viral replicase translation by specifically binding RNA hairpins near the origin of replication. In the bacteriophage, ϕ 29, a short (117-174 nt) sequence of packaging RNA (pRNA) helps to pack phage DNA into pre-formed capsids⁴⁸. A DNA packaging motor is composed of a pentameric ring of pRNA, capsid proteins, ds-DNA and an ATPase⁴⁹. Studies characterizing the specificity and stoichiometries of these interactions^{45,50,51,52} have laid the foundation for RNA-tagging based applications.

RNA scaffolds are important in eukaryotic gene expression as well. Mammalian cells appear to extensively employ long noncoding RNAs. These lncRNAs are rich with secondary structure motifs^{53,54} some of which bind and coordinate proteins on scaffolds that play important roles in epigenetic regulation^{55,56} and telomere maintenance^{57,58}. Thus, natural RNA diversity offers a template of diverse structure and

function for synthetic biologists. Next, we look at how natural observations have been translated into precise engineering of the structure and dynamics of RNA for *in vivo* applications.

1.2.2 APPLICATIONS OF DESIGNED RNA SCAFFOLDS

The design principles for RNA i.e. its structural and folding properties are well understood. Dynamic properties of RNA have been engineered to create synthetic riboregulators⁵⁹, ribozymes⁶⁰, and riboswitches^{61,62}. Additionally, the self-assembly of RNA *in vitro* has also been well characterized and controlled to create precisely designed nano-structures like cubes⁶³, jigsaws⁶⁴, squares⁶⁵, and polyhedra⁶⁶. RNA sequences consisting of secondary structures and functional units designed using these empirical results and computational tools^{34,35,36,37} can be genetically expressed in cells. Such engineered RNAs have been used for tasks ranging from studying natural RNA processing in cells to metabolic engineering and therapeutic applications.

TOOLS FOR RNA RESEARCH

While mRNA has long been known to function as a template for protein translation, the spatio-temporal aspects of the various steps involved in mRNA processing remain poorly understood. Investigation of the dynamics of mRNA as it goes through translation, splicing, nuclear export in eukaryotes, localization for translation, and finally degradation requires tools to track individual RNA molecules. Aptamers and their recruitment of fluorescent proteins on engineered mRNA scaffolds have enabled such studies. Some of the earliest attempts to tag RNA *in vivo* were carried out by expressing GFP fused with bacteriophage MS2 coat protein⁶⁷ or human RNA interacting

protein domain U1A⁶⁸ along with mRNA containing the corresponding binding sites in *Saccharomyces cerevisiae*. Such tags enabled tracking of single-cell mRNA localization by microscopy. Furthermore, by incorporating tandem repeats of MS2 binding sites on reporter mRNA⁶⁹, several GFP-MS2 fusions could be localized on a single transcript, enabling tracking of individual mRNA molecules in mammalian cells. This *in vivo* tracking method was extended to other systems⁷⁰, including bacteria^{32,71}.

More recently, several efforts have addressed the longstanding question of whether or not RNA is highly localized within bacterial cells^{72,73}. A significant innovation over the above strategy came from the use of fluorescent protein complementation. In this approach, RNA aptamers are used to bring together two different protein fusion units, each with a split-fluorescent protein fused to an RNA binding domain^{74,75}. Since only the scaffolded protein units are able to reconstitute the split chromophore and fluoresce, they can be easily distinguished from the unbound ones. Such an approach hence achieves lower background signals than systems where auto-fluorescent proteins are directly tagged onto RNA. As the repertoire of aptamer-RNA binding protein pairs is being extended through several *in vitro* methods like systematic evolution of ligands by exponential enrichment (SELEX)^{76,77,78} newer combinations are being used to explore cellular function⁷⁹. The studies discussed here have led to a better understanding of RNA diffusion and localization^{32,74} in bacterial cells and measurement of transcriptional kinetics⁸⁰. These efforts also enabled localization of a diverse array of proteins (such as enzymes) on RNA scaffolds, opening up applications in therapeutics and metabolic engineering.

PACKAGING THERAPEUTICS ON RNA SCAFFOLDS

RNA-RNA interactions can be used for exciting scaffold applications *in vivo*. Packaging RNA (pRNA) from bacteriophage, $\phi 29$ has been used as a building block for bottom-up assembly of drug delivery vehicles^{18,81}. pRNA monomers consist of structural hairpin regions and dimerization/polymerization domains. Ends of the hairpin regions offer sites for tagging with drugs or targeting molecules. The polymerization domains can be engineered to favor formation of dimers, trimers, pentamers or hexamers as stable drug carriers^{18,52,81}. Heterodimers containing pRNA tagged with a CD4 aptamer, and pRNA attached to an siRNA were shown to specifically target CD4-expressing T cells, leading to cell death⁸¹. This *in vitro* study also showed stability and efficacy of the nanoscale drug delivery particles for killing cancer cells. Such systems are advantageous since the pRNA polymers are hypothesized to be stable in physiological conditions, and be less immunogenic than protein carriers⁸¹. Finally, these polymers could be made specific to many *in situ* targets by using engineered specific RNA aptamers that recognize cellular moieties.

RECOMBINANT RNA TECHNOLOGY

RNA scaffolds have also been used to serve as protective tethers for the purification of recombinant RNA⁸². In this approach, a tRNA scaffold acts as a protective secondary structure to insulate the transcript from native *E. coli* nucleases and thereby stabilize production of recombinant RNA (recRNA) *in vivo*. The characteristic clover-leaf tRNA structure formed around a recRNA is recognized by native cellular enzymes and processed as tRNA. This ensures that each single transcript is a product of specific defined length. A sephadex affinity tag was included in the expressed sequence to allow

purification of transcripts that contained RNAs of medical research interest, like the human HBV-epsilon⁸². This design thus enables collection of large amounts of purified RNA transcripts for *in vitro* structural studies and vaccine development. Recently, these efforts have been extended to expression and purification of RNA-protein complexes⁸³, providing pure samples which could be used for crystallographic studies of natural RNA-protein interactions and potential use in cell-free systems.

LOCALIZING METABOLIC ENZYMES ON RNA

As we have seen earlier, scaffolding and compartmentalization are effective strategies for optimization of metabolic pathway performance both in natural and synthetic systems^{20,26}. A few studies have used DNA structures to coordinate the assembly of enzymes and study effects of spatial co-localization *in vitro*^{27,84,29} and *in vivo*³¹. Protein scaffolds have also been used to channel metabolic substrates between co-localized enzymes in living cells^{14,30}. Scaffolding is seen as a powerful tool to specifically direct metabolic pathway flux towards enzymes of choice, prevent loss of intermediates to competing reactions, and protect the host cell from any toxic or volatile intermediates through confinement at a sub-cellular location.

A notable effort in the use of RNA scaffolds for metabolic channeling achieved a nearly fifty-fold increase in hydrogen gas production in *E. coli*¹³. This effort combined many of the techniques discussed above. Synthetic RNA strands comprising of polymerization domains and aptamers for MS2 and PP7 coat proteins were expressed in the bacteria. Dimerization and polymerization domains allowed for tiling and assembly into a supramolecular structure. The large (40-100 nanometers) intra-cellular RNA assemblies greatly enhanced flux of electrons from ferredoxin to hydrogenase when both enzymes were tethered to the scaffold with fusions to MS2 and PP7. Furthermore, sig-

nificant differences in titer were observed for scaffolding structures having different geometries, tying metabolic flux to the specific spatial positioning of the scaffold. Such an approach brings modular design and scalability⁸⁵ to metabolic engineering for bio-fuels and high value chemical synthesis, where control of intermediate metabolite flux can be critical^{86,87,88}.

1.3 IMPLEMENTING CONTROL OF METABOLIC FLUX

This thesis presents research efforts aimed at controlling metabolic fluxes in the bacteria *Escherichia coli* and *Synechococcus elongatus* to expand the synthetic biology toolkit available for metabolic engineers. *E. coli* has been a workhorse model system for studying and engineering bacterial metabolism⁸⁹. *S. elongatus* is a photoautotrophic cyanobacterium and is also genetically tractable⁹⁰, making it a desirable platform for biological production without competing with traditional feedstocks⁹¹. The central strategy discussed here attempts to increase the yield of metabolic pathways by spatially co-localizing selected enzymes to enhance metabolite transfer rates.

In the next chapter we characterize and expand the RNA scaffold toolkit for *in vivo* use. We demonstrate that the aptamers and polymerization domains of scaffold strains can be treated as modular pieces. We characterize a library of aptamers and show that their corresponding RNA binding domains (RBDs) are able to reliably recruit split GFP fragments to scaffolds inside *E. coli*. This library includes aptamer-RBD pairs with a wide range of RBD sizes and dissociation constants, while being mutually orthogonal.

In chapter 3, we use these aptamers on RNA scaffolds to bring together two cyanobacterial enzymes that convert fatty acid synthesis intermediates to alkanes in *E. coli*. We

show that scaffolding leads to increased channeling of the intermediate fatty aldehyde resulting in higher alkane yields at the expense of side products. Interestingly, we find that changing the geometric orientation of enzymes on *in vivo* scaffolds leads to changes in intermediate flux and final alkane yields. We present a molecular scale model to explain this effect. In addition, we show that the scaffolds can also be used to tether 3-4 enzymes while channeling multiple chemically diverse metabolites.

In chapter 4, we turn to *S. elongatus*. In one approach, electronic flows in the cyanobacterium are manipulated for the biosynthesis of hydrogen gas⁸⁶, which is increasingly seen as a feasible alternative to fossil fuels⁹². Finally, we present efforts to express RNA scaffolds in *S. elongatus* and to use them as synthetic carbon fixation platforms.

Chapter 5 summarizes the results and offers a look towards the future of metabolic engineering and the role of RNA as a tool for synthetic biology.

In conclusion, we suggest that the unit in structure A is, as in structure B, two co-axial helical chains running in opposite directions.

Rosalind Franklin and Raymond Gosling, *Evidence for 2-chain helix in crystalline structure of sodium deoxyribonucleate*⁹³

2

Expanding the toolkit for *in vivo*

RNA scaffolds

THE DOUBLE HELICAL STRUCTURE⁹³ OF NUCLEIC ACID STRANDS with complementary base-paired⁹⁴ sequences allows for storage of hereditary information, translation of it into functional protein and RNA units and as noted in chapter I, also enables nu-

cleic acids to play important structural roles in nature. Motivated by the aim of using tools offered by nucleic acid nanotechnology^{95,13} for effective microbial metabolic engineering, we carried out the set of experiments presented in this and the next chapter.

Here, we* express synthetic RNA strands comprised of polymerization domains and aptamers, in bacterial cells⁸⁵. The polymerization domains allow for assembly of RNA strands into a supramolecular structure¹³. We show targeted assembly onto such structures using fluorescent protein fusions to a library of aptamer-binding domain pairs that includes small RNA binding peptides⁹⁷ as well as natural domains like MS2 and PP7. We show that modular aptamers on RNA scaffolds of a variety of polymerizing dimensions show corresponding effects in co-localizing the proteins.

2.1 EXPANDING THE REPERTOIRE OF FUNCTIONAL RNA BINDING DOMAINS

We have expanded the set of RNA binding domains that can be used to form fusion proteins that will bind to specific sites on our RNA scaffolds. The basic strategy builds on a scheme in which engineered *E. coli* express proteins fused to RNA binding domains (RBDs) that would assemble onto RNA scaffolds that include aptamers and polymerization domains. Strand design allows the aptamers to be presented on discrete (0-dimensional), 1-dimensional, or 2-dimensional scaffolds¹³ (Fig 2.1). Each protein to be docked binds to its cognate binding site on the RNA scaffold. In previous work, RNA binding domains from viral coat proteins like MS2 and PP7 were fused with fluorescent proteins or enzymes for co-localization on RNA strands^{13,32}. These viral RBDs bind tightly to their RNA aptamers^{98,45}, but are relatively large (>120 amino acids) and hence may affect folding or activity of the resulting fusion protein through steric

*The work presented in this chapter was carried out with Abhishek Garg, and has been published⁹⁶

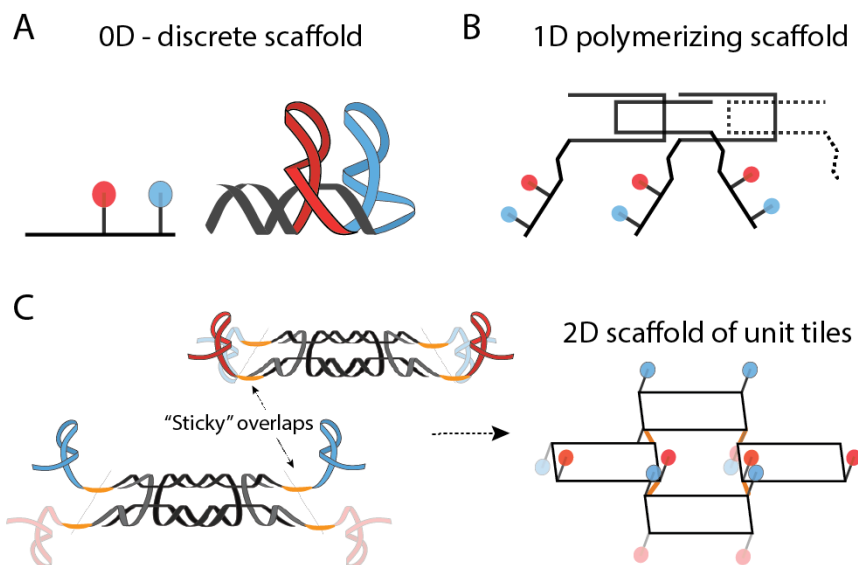


Figure 2.1: Three dimensionalities of RNA scaffolds. Our designs allow formation of three different types of RNA scaffolds¹³. (A) In the 0D case, a single RNA strand folds into a discrete unit presenting two aptamers. (B) The 1D polymerizing strands have 'sticky' ends that allow the individual units to form linear chains of aptamer sites through complementary base pairing. (C) In the case of 2D scaffolds, two different RNA oligonucleotides, A and B, come together to form unit tiles A_2B_2 , which then polymerize in two dimensions through sticky end base pairs. Each corner of the unit-tile interactions brings together two different aptamers in close proximity. Red and blue represent different aptamers and dimmed shades show aptamers in a pointing downwards from the scaffold plane.



Figure 2.2: In our assay for the formation of aptamer binding site presenting RNA scaffolds, we co-express the RNA strand with two halves of a split GFP protein⁹⁹. If the RNA scaffold folds into the designed secondary structure, the two halves are brought in close proximity through aptamer-RNA binding domain interactions and this complementation results in higher cellular fluorescence, which can be assayed by microscopy or flow cytometry.

interactions. Bayer et al⁹⁷ characterized several short arginine-rich motifs that bind RNA, along with their synthetically selected RNA aptamers. We chose 6 of these short RBD-aptamer sets (App. Fig A.1) along with MS2 and PP7, to create a library of 8 such interactions for *in vivo* applications. The 6 sets were picked so that we could obtain a set of binding domains that were both short (<25 amino acids), to enable facile tagging on enzymes, and also mutually orthogonal in binding to the corresponding aptamers⁹⁷ (App. Fig A.2).

To test *in vivo* RNA-protein interactions for the library obtained above, we made fusions of the RBDs in four pairs with inactive green fluorescent protein fragments (split GFP) A and B respectively (Fig 2.2 and Fig 2.3). The fragments were split as described by Valencia-Burton et al⁷⁴. Corresponding aptamers for each pair of RBDs were included in RNA sequences, with their secondary structures designed to fold into discrete scaffolds (Fig 2.2). These ‘oD’ scaffolds were co-expressed with split GFP-RBD fusions through plasmids in *E. coli*. In control cells, we expressed only the split GFP-RBD fusions with an empty vector so that no scaffolds for co-localizing the fragments were present. After 3 hours of induction, the cells were visualized at 100x magnification under a microscope to assay cellular fluorescence indicating co-localization of the split GFPs to create active GFP.

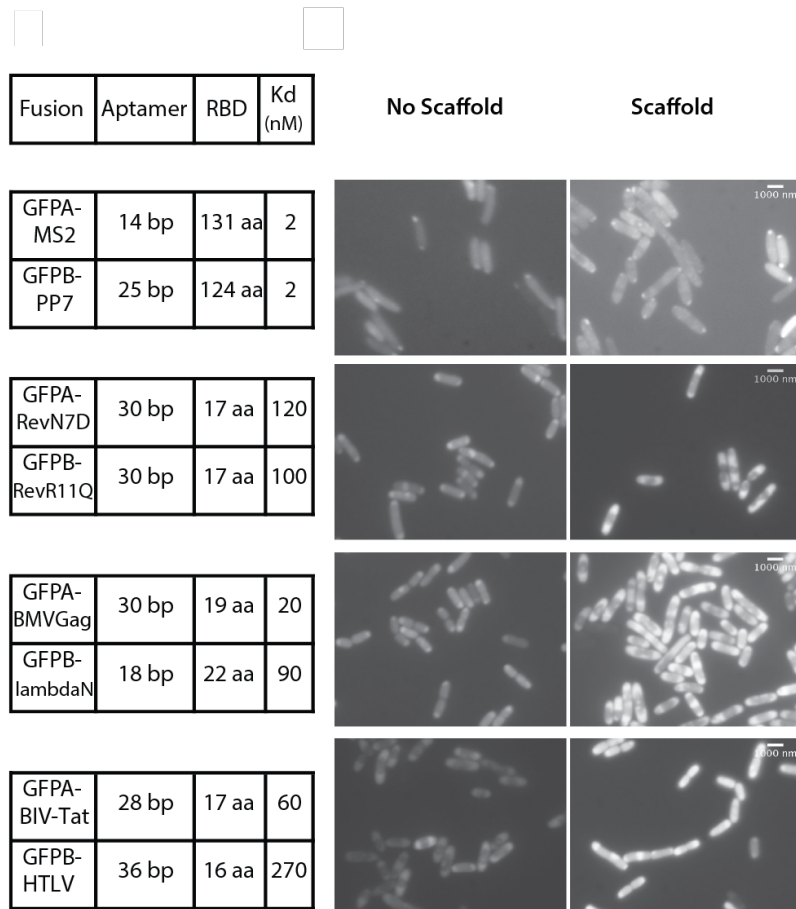


Figure 2.3: This figure shows a list of 4 different pairs with 8 unique aptamer-RNA binding sets tested. Values of dissociation constants reported previously^{97,98,45} are tabulated with domain and aptamer sizes. Also shown are FITC images revealing enhanced split GFP fluorescence *in vivo* in the presence of RNA scaffolds for all sets tested. Scale bars: 1 micrometer.

Fusion	Aptamer	RBD	Kd (nM)
--------	---------	-----	---------

GFPA-MS2	14 bp	131 aa	2
GFPB-PP7	25 bp	124 aa	2

GFPA-RevN7D	30 bp	17 aa	120
GFPB-RevR11Q	30 bp	17 aa	100

GFPA-BMVGag	30 bp	19 aa	20
GFPB-lambdaN	18 bp	22 aa	90

GFPA-BIV-Tat	28 bp	17 aa	60
GFPB-HTLV	36 bp	16 aa	270

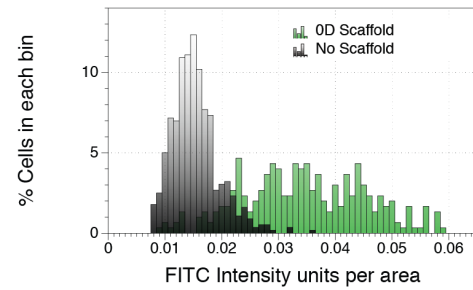
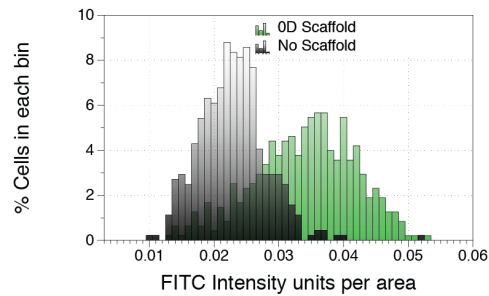
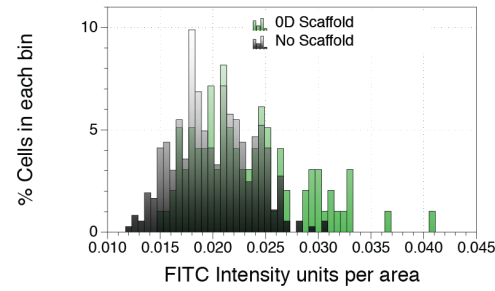
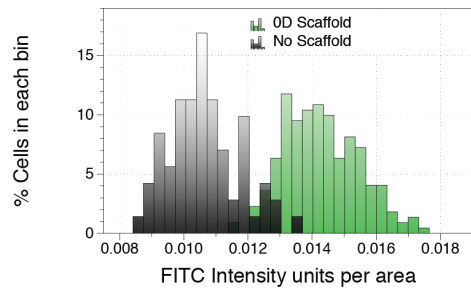


Figure 2.4: Histograms of FITC intensity profiles for 4 pairs of split GFP - RBD fusions tested on OD scaffolds in Fig 2.3. Differences in FITC intensity scales depended on the FITC exposure times chosen (50ms - 300ms) to ensure signal profiles were not saturated.

Protein-RNA binding *in vivo* was in evidence for all 8 aptamer-RBD sets tested in 4 pairs (Fig 2.3). When the split GFP fragments are expressed without scaffolds the cells are only dimly fluorescent; upon co-expression with the scaffold sequence, however, significantly brighter cells are observed, presumably because the split GFP fragments bind adjacent to each other on the RNA scaffold (Fig 2.3). Histograms showing average cellular FITC intensity from 100-400 cells for the 4 pairs are presented in Fig 2.4. For all four pairs tested, the histograms show a clearly brighter population of cells (right-shifted distribution) when the scaffold RNA was co-expressed with the split GFP-RBD fusions (Fig 2.4). We have previously shown such split GFP complementation on RNA scaffolds for the PP7 and MS2 domains' pair¹³, which served as a positive control here. We also observed enhanced GFP complementation on scaffolds from three pairs of the 6 new aptamer-RBD partners. This gives us a tool-kit of eight potential aptamer-protein interaction sets with varying sizes and binding properties.

2.2 RNA BINDING DOMAINS FUNCTION ON POLYMERIZING RNA SCAFFOLDS

We showed that the split GFP complementation is efficient on RNA scaffolds of higher dimensions (1D and 2D) with the new RBDs and aptamers as well. For this we chose two aptamer-RBD interaction sets: PP7 and BIV-Tat. We chose PP7 because of its tight binding and ability to form non-aggregating dimers on RNA aptamers⁴⁵. BIV-Tat was chosen because it was the strongest-interacting short domain for which a crystal structure of the aptamer-RBD complex is known^{97,100} (App. Fig A.2). As above, we expressed fused split GFP fragments with a plasmid expressing either no RNA or RNA strands for 0D, 1D, or 2D RNA scaffolds (Fig 2.5A). Fluorescence microscope images of the cells were analyzed using software packages MicrobeTracker¹⁰¹ and ImageJ¹⁰² to

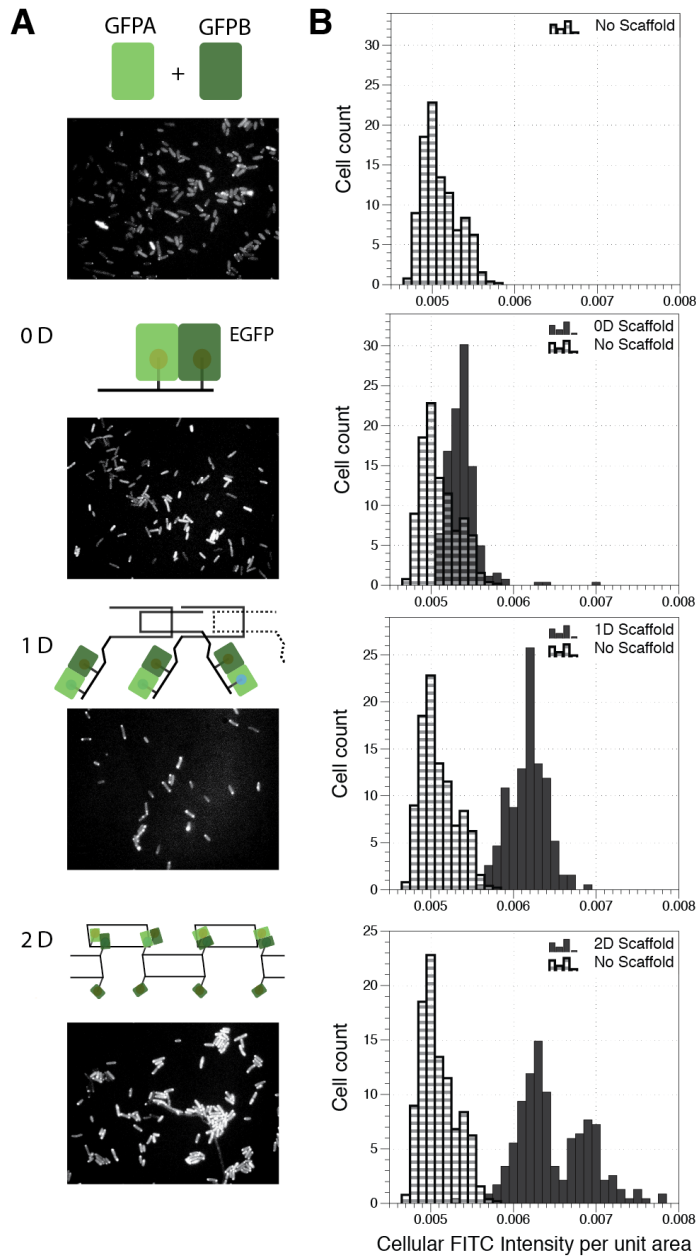


Figure 2.5: Aptamers incorporated into 0, 1, and 2 dimensional RNA scaffolds bind proteins *in vivo* (A) FITC images showing GFP fluorescence observed in cells on expression of split GFP fragments (GFP A-PP7 and GFP B-BIV-TAT) with either no scaffold, 0D, 1D or 2D scaffolds containing PP7 and BIV-TAT binding aptamers (B) Corresponding FITC quantification (histograms) for 0, 1 and 2D scaffold designs. ($p < 10^{-3}$, one-tailed t-test)

quantify average fluorescence per unit area for several images (>100 cells) of each strain. We performed statistical analyses (Fig 2.5B) to calculate the average cellular fluorescence intensity in each case. A progressive shift towards brighter cells upon expression of RNA scaffolds of the three dimensionalities was observed (Fig 2.5A,B). The automated analysis indicated two apparent populations when the GFP fragments are co-expressed with the 2D scaffold; visual inspection of the fields confirmed that there are two populations of bright cells. We hypothesize that the kinetics of RNA tiling lead to extended 2D scaffold assembly in the brightest population of cells either due to small differences in RNA expression levels or stochastic coalescing of multiple assemblies.

2.3 DISCUSSION

We show *in vivo* functionality of a library of modular RNA binding domains and aptamer scaffolds. These RBD-aptamer sets are effective in co-localizing proteins within living *E. coli*. The RBD-aptamer sets were used to reconstitute split GFP fragments on a variety of RNA scaffolds; 0, 1, and 2 dimensional RNA scaffolds, showed progressively more noticeable concentration of proteins. Split GFP fragments offer a handy tool for probing supramolecular assemblies where two disparate units needs to be spatially united. However, imaging of GFP foci often needs co-localization of tens of proteins³² and due to diffraction limits of optical imaging, precise geometric details of the assemblies are hard to capture. The modularity of our scaffolds would allow tagging with oligonucleotides that enable super-resolution imaging¹⁰³. Further, use of non-protein fluorophores¹⁰⁴ might help better understand the kinetics of scaffold assembly itself.

The library of 8 RNA binding domain-aptamer sets tested here forms a useful

toolkit for designing in-vivo protein-RNA interactions. The library of protein/RNA pairs is orthogonal and includes a range of aptamer lengths, binding domain sizes and binding dissociation constants (App. Figs. A.1, A.2). Characterization of shorter RNA binding peptides will, in principle, enable facile engineering of enzyme fusions with minimal perturbation to protein structure. We have also demonstrated the modular nature of our RNA scaffold design, by using the aptamer site library and showing assembly of functional discrete, 1D or 2D scaffolds. In the next chapter we employ these scaffolds and aptamer-RNA binding pairs to show that enzymatic pathways can be spatially co-localized *in vivo* to achieve higher metabolic fluxes in a geometrically dependent manner.

2.4 MATERIALS AND METHODS

2.4.1 STRAIN CONSTRUCTION

E. coli strain BL21* (DE3), that carries a mutation in the *mei31* (RNaseE) gene, was used for all experiments described here. The appendix Fig A.3 lists the plasmid systems used for expression of the various components in *E. coli*. RNA and protein expression was based on Novagen (EMD Millipore) Duet vectors. pETDuet1 was used for RNA expression because of its high copy number (>40 copies/cell) while the lower copy pACYCDuet1 (10-15 copies/cell) and pCOLADuet1 (20-40 copies/cell) were used to express proteins. For co-expression, we co-transformed competent BL21* DE3 cells with the two or three relevant plasmids and selected on plates with a combination of antibiotics.

2.4.2 MICROSCOPY AND ANALYSIS

For GFP microscopy experiments, cells were grown in LB media and induced with 0.3 mM IPTG at OD 0.8. Cells were allowed to grow for 3 more hours at 37°C with shaking. They were then washed with M9 media to reduce the background fluorescence from LB media. Cells were placed on 2% clear agarose pads and analyzed using a Nikon TE-2000 microscope (100x, 1.4 numerical aperture objective, ORCA-ER charge-coupled device camera, FITC channel). The images were analyzed using the software packages ImageJ and MicrobeTracker^{101,102}. We calculated the average cellular fluorescence using MicrobeTracker and plotted the distribution as histograms using the software DataGraph 3.1.1. 2013. Visual Data Tools, Inc.

At the atomic level, we have new kinds of forces and new kinds of possibilities, new kinds of effects... I am, as I said, inspired by the biological phenomena in which chemical forces are used in a repetitious fashion to produce all kinds of weird effects.

Richard P. Feynman, *There's plenty of Room at the Bottom*¹⁰⁵

3

Increase in metabolic output from RNA scaffolds

BIOMOLECULAR ORGANIZATIONS ACHIEVE NANOSCALE PRECISION leading to the complex properties and behavior that Feynman¹⁰⁵ marveled that. Synthetic biology today enables the kind of molecular level engineering that he presciently suggested.

Continuing with the aim of using the precise molecular interactions for increasing the flux of selected metabolic pathways, this chapter details interesting novel effects at the nanoscale that facilitate such applications.

The role of enzyme co-localization in pathways that involve diffusible intermediates continues to be debated¹⁰⁶. While some analyses rely on three dimensional diffusion models to argue that the effectiveness of substrate channeling may be limited to cases where intermediate pools are dilute³³, others have suggested a more general ‘molecular crowding’ hypothesis¹². The latter view is supported by a recent study, in which the authors demonstrated increased transfer of hydrogen peroxide between enzymes scaffolded in vitro²⁸. In that study, a discontinuous increase in intermediate channeling was observed for proteins tethered less than 20 nm apart. The authors proposed a mechanism of metabolite channeling by restricted diffusion on hydration layers across crowded protein surfaces. Metabolite diffusion may be particularly restricted for hydrophobic substrates such as fatty acids and related molecules, which may associate with protein surfaces more strongly than hydrophilic substrates. In this regard, the two-step alkane synthesis pathway described by Schirmer et al.⁸⁷ is of interest because it involves an uncharged aldehyde intermediate. The two enzymes, acyl-ACP reductase and aldehyde deformylating oxygenase¹⁰⁷ can be heterologously expressed in *E. coli* to produce pentadecane. Also of interest is the channeling of cofactors and carboxylic acids, since they form intermediates in several industrially relevant metabolic products. Succinate is one such product where pathway engineering has focused on increasing supply of oxaloacetate¹⁰⁸, and NADH¹⁰⁹. Co-localization of enzymes such that the fatty aldehyde intermediate, or oxaloacetate and NADH are transferred directly from the surface of one enzyme to the other could enhance production of pentadecane and succinate respectively.

Here, as in chapter 2, we* express synthetic RNA strands comprised of polymerization domains and aptamers, in *E. coli*. We have seen that polymerization domains allow for assembly of RNA strands into a supramolecular structure upon which proteins can be effectively co-localized. In experiments described in the current chapter, fusions of RNA-binding domains with enzymes involved a two-step pentadecane pathway are co-expressed to allow assembly onto the RNA scaffolds. We show that increased production of pentadecane depends on the relative geometric orientation of enzymes. Further, we report higher metabolic output on RNA scaffolds with up to 4 enzymes co-localized from a multi-step succinate production pathway. Our results suggest that the specific orientation and placement of enzymes on the scaffold enhances pathway flux consistent with restricted substrate diffusion.

3.1 INCREASED PENTADECANE PRODUCTION ON RNA SCAFFOLDS

To use RNA scaffolds for metabolic substrate channeling, we employed a two-step pentadecane production pathway (Fig 3.1) which has recently been characterized using cyanobacterial enzymes expressed in *E. coli*⁸⁷. Two enzymes, acyl-ACP reductase (AAR), and aldehyde deformylating oxygenase (ADO), sequentially convert cellular N-carbon fatty acyl-ACPs to alkanes of length N-1. For example, AAR reduces the C16 acyl-ACP molecule to produce a fatty aldehyde, hexadecanal (C₁₅H₃₁CHO), which undergoes deformylation catalyzed by ADO¹⁰⁷ to form pentadecane. However, upon co-expression of these two enzymes in *E. coli*, the major products are alcohols and not alkanes⁸⁷. The intermediate aldehyde appears to be non-specifically reduced by aldehyde reductases in the cell¹¹⁰. We hypothesized that if hexadecanal could be selectively

*The work presented in this chapter was carried out with David Godding and Jeffrey Way, and has been published⁹⁶

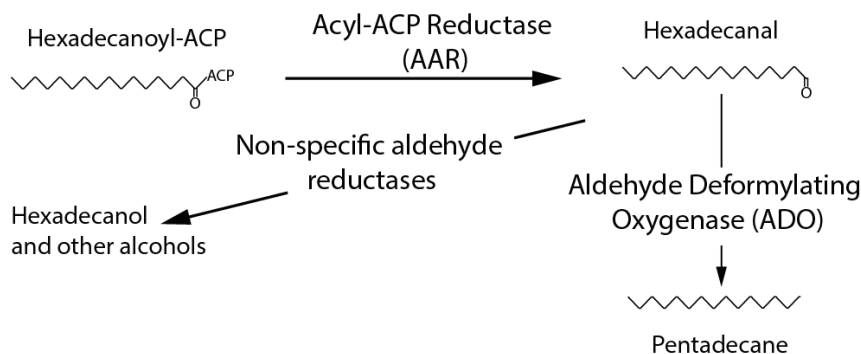


Figure 3.1: The pentadecane production pathway described by Schirmer et al⁸⁷ composed of two cyanobacterial enzymes Acyl ACP Reductase (AAR) and Aldehyde Deformylating Oxygenase (ADO). Also shown is the competing side reaction that leads to alcohol production, including hexadecanol.

channeled from AAR to ADO on scaffolds, an increase in pentadecane would occur.

Genetic fusions of AAR and ADO to RNA binding domains were constructed on the N-termini of the enzymes. AAR was tagged with BIV-Tat and ADO was fused to PP7; both fusions retained activity (Fig 3.2), although attempted fusions with the MS2 failed to show activity. *E. coli* expressing the two enzyme fusions were grown and alkane levels in the supernatant were measured (see section 3.5 - Materials and methods). Pentadecane production, which is absent in WT *E. coli*, was observed (Fig 3.2) confirming catalytic activity of the fusion proteins. We measured alkane production after 16 hours of log-phase growth in batch cultures, while earlier work has reported higher levels can be achieved from up to 40 hours of induced growth⁸⁷. In addition, we also observed other alkane products however, levels were much smaller than pentadecane, consistent with previous reports⁸⁷, and could not be accurately quantified.

When the enzyme fusions were co-expressed with 1D, or 2D RNA scaffolds containing their cognate binding sites (Fig 3.2) in *E. coli*, alkane production was enhanced by 80% compared to co-expression with an empty vector or a 0D scaffold, as assayed

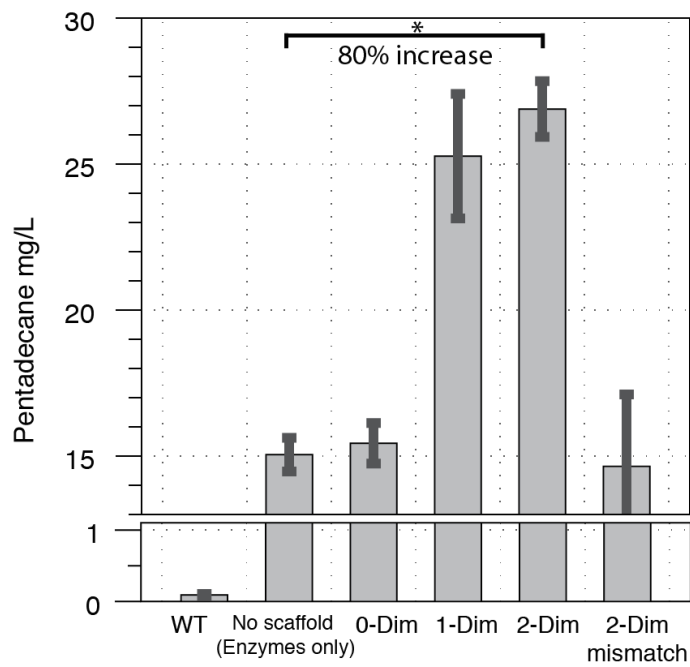
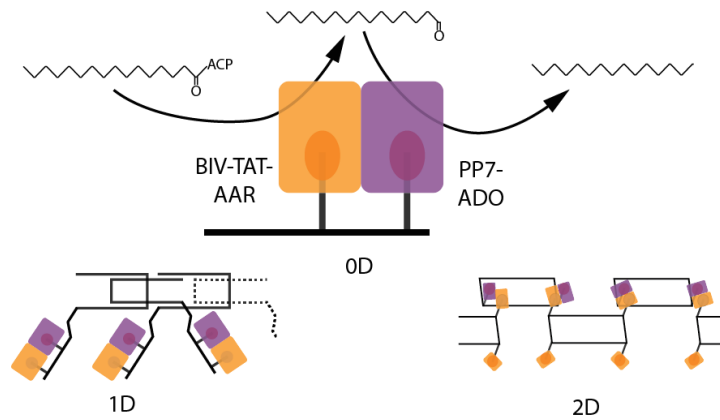


Figure 3.2: This figure shows the scaffolding design implemented to channel the hexadecyl intermediate towards pentadecane production. Fusions of AAR and ADO with BIV-TAT and PP7 respectively are co-expressed in *E. coli* with scaffolds of different dimensions carrying the corresponding binding sites. On assaying different levels of pentadecane are observed with either an empty vector (no scaffolds) or RNA scaffolds or 0,1, or 2 dimensions (with aptamers for BIV-TAT and PP7), or a 2D scaffold with mismatched aptamers (containing anti-RevR11Q and MS2). (n=3, error bars = SEM) (* indicates $p < 0.05$, one-tailed t-test)

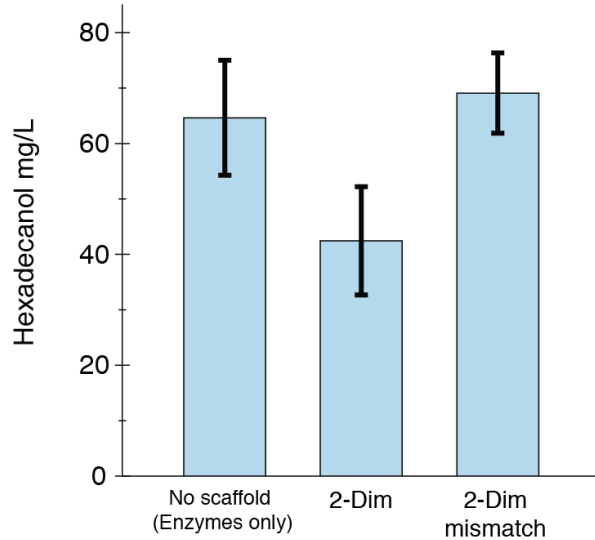


Figure 3.3: One of the pentadecane pathway side-products, hexadecanol, production measured on expression of pathway enzymes with the same empty vector, correct 2D scaffold, and mismatched 2D scaffold. (n=3, error bars = SEM)

by pentadecane levels in ethyl acetate extracts (Fig 3.2). We also measured production of hexadecanol, one of the alcohol side products reported when AAR and ADO are co-expressed⁸⁷. We observe that the hexadecanol levels are reduced when enzymes are co-localized on their cognate 2D scaffolds, compared to expression without any RNA scaffolds (Fig 3.3). As a control, expression of the enzymes with a 2D scaffold lacking the cognate aptamers showed no difference from the empty vector control (Figs 3.2, and 3.3). The mismatched scaffold carried binding sites for RevR11Q and MS2 instead of the BIV-TAT and PP7 aptamers. Lack of any effect on the mismatched scaffold shows that co-expression with the correct cognate scaffold was necessary for an effect.

We also sought direct evidence of enzyme-RNA scaffold interaction in our co-expression system. For this, we UV-crosslinked protein-RNA interactions in cells expressing the pentadecane pathway enzymes and co-purified BIV-TAT-AAR with any

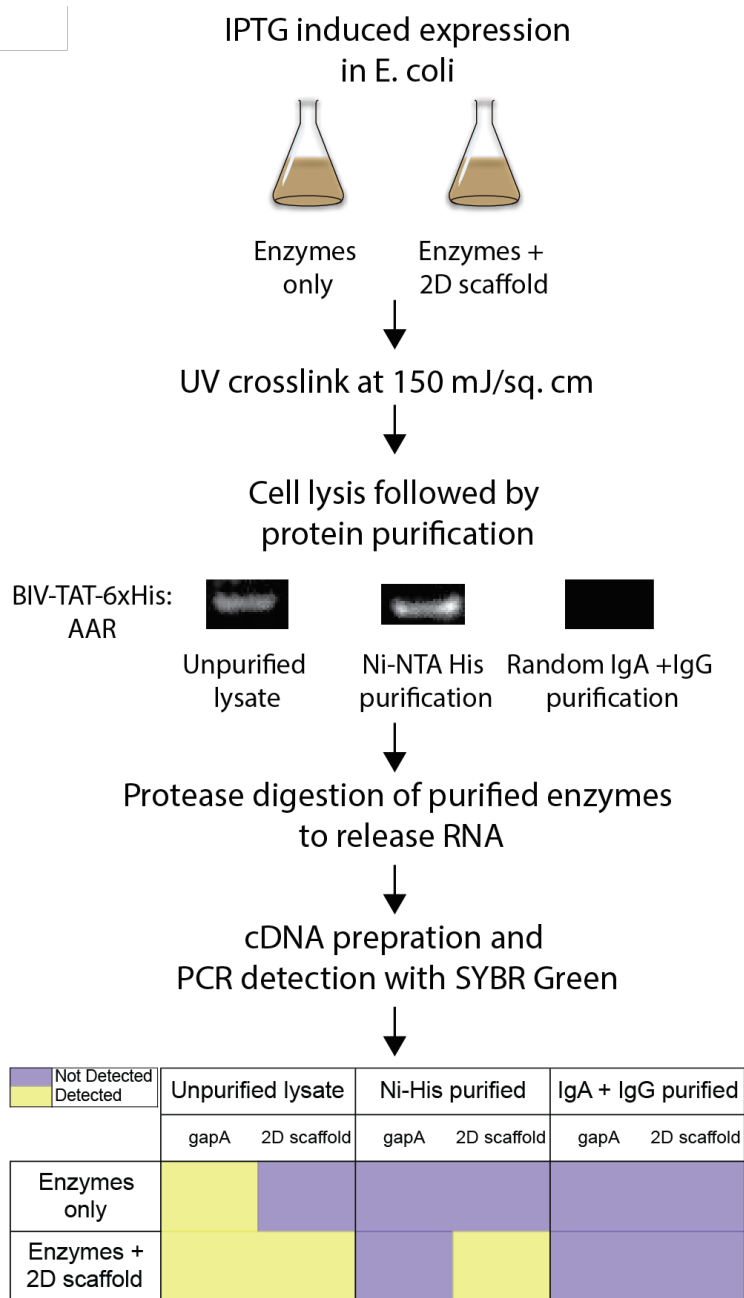


Figure 3.4: Direct evidence of enzyme-RNA interaction from experimental flow for detecting RNA associated in-vivo with 6xHis tagged BIV-TAT-AAR enzymes. Purple boxes stand for amplification detected by SYBR green and yellow boxes indicate that the RNA species was below detection threshold.

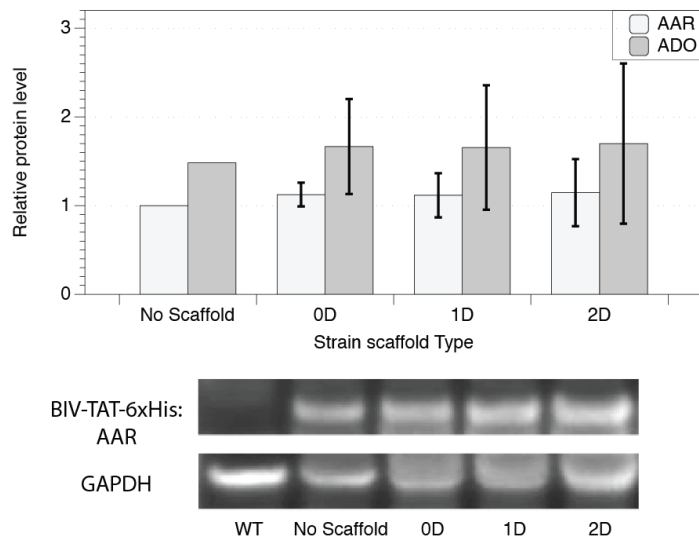


Figure 3.5: Pathway enzyme production levels measured by quantitative western blotting using 6xHis (AAR) and Strep (ADO) tags and normalized to GAPDH. Levels are shown for co-expression with an empty vector or scaffolds of different dimensionalities. (n=3, error bars = SEM)

bound RNA (Fig 3.4). BIV-TAT-AAR was tagged with 6xHistidine and could be seen only upon purification with Ni-NTA magnetic beads and not when random IgA + IgG beads were used as control (Fig 3.4). After digestion of the protein and RNA purification, we looked for presence of scaffold RNA levels by quantitative RT-PCR. Internal primers were designed to amplify scaffold RNA and as well cellular gapA mRNA. While scaffold RNA and gapA are both detected in unpurified cell lysates expressing 2D scaffolds, only the scaffold RNA is detected from the extracts co-purified with BIV-TAT-AAR. Neither gapA, nor scaffold RNA are detected from the IgA+IgG pull downs. This suggests that while a control mRNA like gapA doesn't interact with our enzymes appreciably, interactions of BIV-TAT-AAR and the co-expressed 2D scaffold are significantly higher. To ensure that the enhanced synthesis reflected in Fig 3.2 was due to scaffolding of the enzymes and not differential expression, we compared

protein levels using Strep and 6x His tags included in the fusions for AAR and ADO respectively. Quantitative Western blots were prepared on cell extracts and enzyme levels were found to be unaffected by co-expression with scaffolds (Fig 3.5). This indicates that when we expressed the two-enzyme pathway in the presence of 1 or 2 dimensional polymerizing RNA scaffolds, higher pentadecane titers were realized for the same amounts of proteins expressed.

3.1.1 ALKANE YIELD ENHANCEMENT HIGHEST IN EXPONENTIAL GROWTH PHASE

In applied and industrial settings continuous cultures are more commonly employed since they allow for faster process rates, lesser down time, and better controlled standard conditionsⁱⁱⁱ. One significant difference between batch and continuous modes of growth is the variability in growth rate. While continuous cultures maintain uniform growth rates at all times, batch cultures follow sigmoid growth curves, with the exponential phase representing a sub-period of sustained uniform growth.

We tested the 2D scaffold producing strain for pentadecane yields at different points in batch culture and compared that to the strain carrying an empty vector (Fig 3.6). We measured pentadecane production from batch cultures at three different time points 16 hours (mid exponential phase), 20 hours (early stationary phase) and 26 hours (late stationary phase) after induction. As can be seen in Fig 3.6A, in general higher titers are observed for later time points. However, when we compare the fractional increase in pentadecane production due to scaffolding, the trend is reversed. At the 16 hours time point, we observe an 80% higher titer from 2D scaffolds. However, the fractional increase became smaller at 20 hours and less significant at 26 hours after induction (Fig 3.6A). Enzyme levels were again quantified by western blots and relative protein expression levels at the three time points were compared; the average enzyme levels

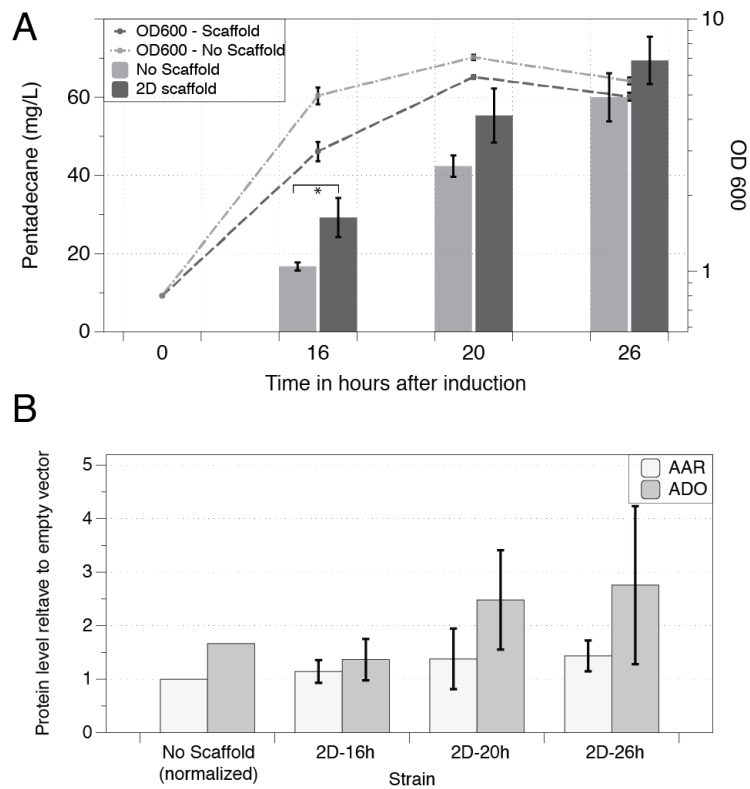


Figure 3.6: Pentadecane yield enhancement is highest in exponential growth phase (A) Time series of enhanced alkane production for 2D scaffolds at different time points in batch culture, plotted with cellular growth measured as optical density (OD) at 600 nm. (B) Enzyme levels measured by quantitative western blotting using His (AAR) and Strep (ADO) tags and normalized to GAPDH. (n=3, error bars = SEM) (* indicates $p < 0.05$, one-tailed t-test)

were not significantly different at any of the time points (Fig 3.6B). However, the later measurements carried larger error bars, showing a marked increase in variability of enzyme concentration.

3.2 FLUX ENHANCEMENT DEPENDS ON GEOMETRY OF SCAFFOLDED ENZYMES

The RNA scaffold offers structural flexibility that might be further modified to enhance the interplay of associated enzymes. Some studies have probed the effect of varying numbers of binding sites¹⁴ or different inter-enzyme distances on metabolic channeling²⁸. Here, we investigate the effect of altering relative enzyme orientation on the scaffold. The structure of two-dimensional sheets (Fig 2.1) resulting from assembly of the RNA oligonucleotides (App. Fig A.4) that make up the scaffold can be inferred based on the helicity of double-stranded RNA (11 bp/turn), the structure of Holliday junctions¹¹², and general principles of nucleic acid nanotechnology^{13,95}. Four strands (A_2B_2) come together to form a unit tile, which polymerizes in two dimensions through base pair interactions on the tile corners. The aptamers are presented on two different sides of the plane of the unit tile, as shown (Figs 2.1, and 3.7A). Each unit tile has four 6-bp sticky ends that base-pair with diagonally related units. Through these interactions, one PP7 aptamer and one BIV-Tat aptamer emerge on the same side of the scaffold plane at each corner of the unit tiles (Figs 2.1, and 3.7A).

We constructed a high-level model of this aptamer pair from PDB files of aptamer-binding protein complexes, double-stranded RNAs, and the deformylating oxygenase enzyme from a related organism^{113,114,115}. The enzymes being co-localized on the scaffold bind and form PP7-aptamer (PDB 2QUX¹¹³) and BIV-Tat-aptamer (PDB 2A9X¹¹⁴) complexes. Such complexes can be treated as rigid elements composed of PP7-linker-

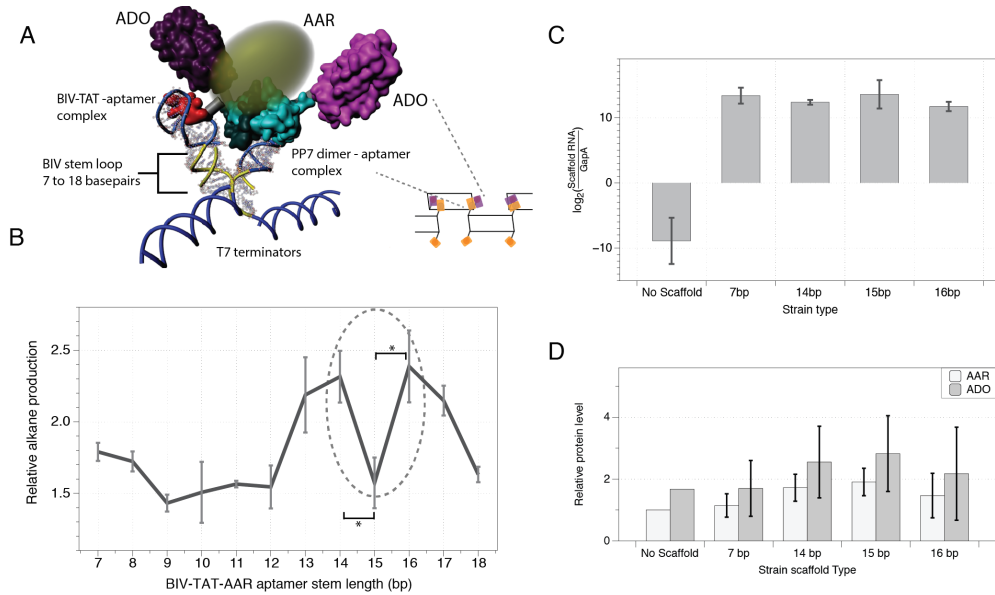


Figure 3.7: Length and orientation of aptamers affect metabolic flux increase on scaffolds. (A) Schematic showing design details of a pair of aptamers interacting on the 2D scaffold using PDB files, 2QUX¹¹³, 2A9X¹¹⁴, and 4KVQ¹¹⁵. Different base-pair lengths (7-18bp) were used for the hairpin presenting aptamer bound by BIV-Tat-AAR so that rotation of the complex may bring AAR in closer proximity to ADO on the scaffold (B) Pentadecane yields are shown for 2D scaffolds with the different BIV-Tat binding aptamer stem lengths. Concentrations were normalized to yield from enzyme expression without scaffolds and plotted as relative production levels. (C) RNA scaffold production levels, measured by qRT-PCR and normalized to endogenous gapA mRNA, for the strains showing greatest variation in yields. (Log base value $2^r \approx 1.87$) see Materials and Methods (D) Pathway enzyme production levels measured by quantitative western blotting using 6xHis (AAR) and Strep (ADO) tags. Levels are shown for co-expression of pathway enzymes with RNA scaffolds of varying BIV-TAT-aptamer lengths and are normalized to GAPDH levels. (n=3, error bars = SEM) (* indicates $p < 0.05$, one-tailed t-test)

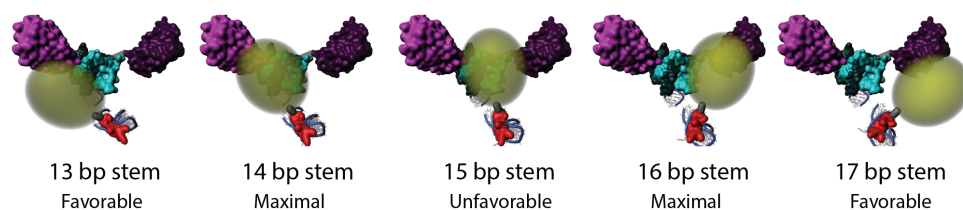


Figure 3.8: Proposed model for two maximal configurations of intermediate flux channeling. On varying the anti-BIV-TAT aptamer stem loop length, different rotational conformations of the BIV-Tat-AAR moiety are possible, relative to the PP7-ADO dimer.

ADO and BIV-Tat-linker-AAR fusion proteins which are expected to be under constrained Brownian motion as predicted by the coarse-grained molecular dynamics simulation approach of Robinson-Mosher et al¹¹⁶. In Fig 3.7A, the crystal structure of ADO is represented by *Prochlorococcus marinus* aldehyde-deformylating oxygenase (PDB 4KVQ¹¹⁵) which has 95% sequence identity to the *S. elongatus* enzyme used here. We found that systematic addition of base pairs to the stem-loop structure of anti-BIV-Tat aptamer identified scaffold variants with increased activity. To explore the effect of changing the position of the reductase, we added 0-11 base pairs to the BIV-Tat hairpin on the 2D scaffold, to go through an entire RNA helical turn (Fig 3.7A). We then measured the pentadecane synthesis from cells expressing the twelve 2D scaffolds with the BIV-Tat-AAR and PP7-ADO fusion proteins (Fig 3.7B). As before, the strains were grown aerobically, induced, and finally extracted with ethyl acetate for assaying pentadecane production. The enzymes co-expressed with an empty vector served as control. All designs showed increased pentadecane production relative to the no-scaffold case (Fig 3.7B). The increase in production ranges from 40% (9bp) to 140% (16bp) greater than co-expression without scaffolds.

A striking pattern of pentadecane synthesis was observed as a function of hairpin stem length, particularly with stems of 13-17 base pairs. Synthesis was near-maximal

with 13 and 17-bp stems, maximal with 14 and 16-bp stems, and minimal with a 15-bp stem (Fig 3.7B). These results may be interpreted in terms of a model (Fig 3.8) where the peaks in activity with stems of lengths 13, 14, 16, and 17bp may correspond to the acyl-ACP reductase being in proximity to one or the other of the deformylating oxygenases fused to the dimeric PP7 protein.

To ensure that these effects were solely due to changes in scaffold design, we measured the relative levels of scaffold RNA and enzyme production for strains with the 7, 14, 15, and 16 base pair scaffolds. We extracted total cellular RNA from cell pellets for each strain after an induction experiment and measured scaffold RNA levels by quantitative RT-PCR. Once again, primers to measure the 2D scaffold RNA level and endogenous cellular pools of gapA mRNA were used (Fig 3.7C). All measured scaffold RNAs were produced at about the same levels, with a variation of only 2 to 4-fold, corresponding to the error of qPCR. Enzyme levels were measured by quantitative western blots on cell pellet extracts from these strains (Fig 3.7D); no significant difference in enzyme concentrations between the strains and the no-scaffold controls were seen.

3.3 LOCALIZING MULTIPLE ENZYMES ON RNA SCAFFOLDS

RNA scaffolds can be used to enhance pathway flux in longer multi-step enzymatic pathways. We chose the commercially relevant succinate production pathway that has multiple rate limiting control points at the pyruvate utilization node⁸⁸ (Fig 3.9). Production of oxaloacetate through pyruvate carboxylase (PYC) enhances flux towards succinate¹⁰⁸. Further, increased succinate production is noted when a NAD⁺-dependent formate dehydrogenase (FDH) generates NADH, a co-factor used in the

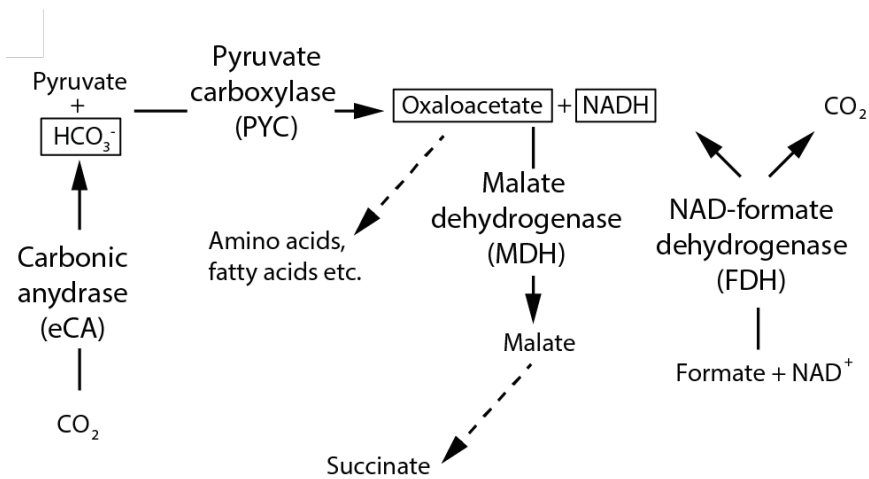


Figure 3.9: Conversion of pyruvate and oxaloacetate from significant control points for the fermentative production succinate from *E. coli*⁸⁸. Shown here are the enzymes we expressed to increase succinate production in *E. coli*. We hypothesized that the intermediate molecules shown in boxes would be preferentially channeled towards succinate production.

conversion of oxaloacetate to malate, by malate dehydrogenase (MDH)¹⁰⁹.

Thus, we wanted to test if scaffolding of PYC (from *Rhizobium etli*), MDH (from *E. coli*), and FDH (from *Candida boidinii*) on a 2D scaffold results in increased redirection of pyruvate towards succinate in *E. coli* (Fig 3.10). We also tested a 4 enzyme co-localization scheme, in which a cyanobacterial carbonic anhydrase (eCA), known to increase oxaloacetate flux towards succinate¹¹⁷ was included (Fig 3.10). We made fusions of PYC, MDH, FDH, and eCA with RNA binding domains PP7, BIV-TAT, lambdaN, and RevR11Q respectively. We observe that co-expression of PP7-PYC, BIV-TAT-MDH, and lambdaN-FDH with a 2D scaffold containing the corresponding aptamers leads to 83% higher succinate production than expression of the enzyme fusions with an empty vector (Fig 3.10). When RevR11Q-eCA is also expressed with these three enzymes, the new corresponding scaffold with 4 aptamers leads to an 88% increase (Fig 3.10). These results indicate that intermediates as diverse in their chemical composition

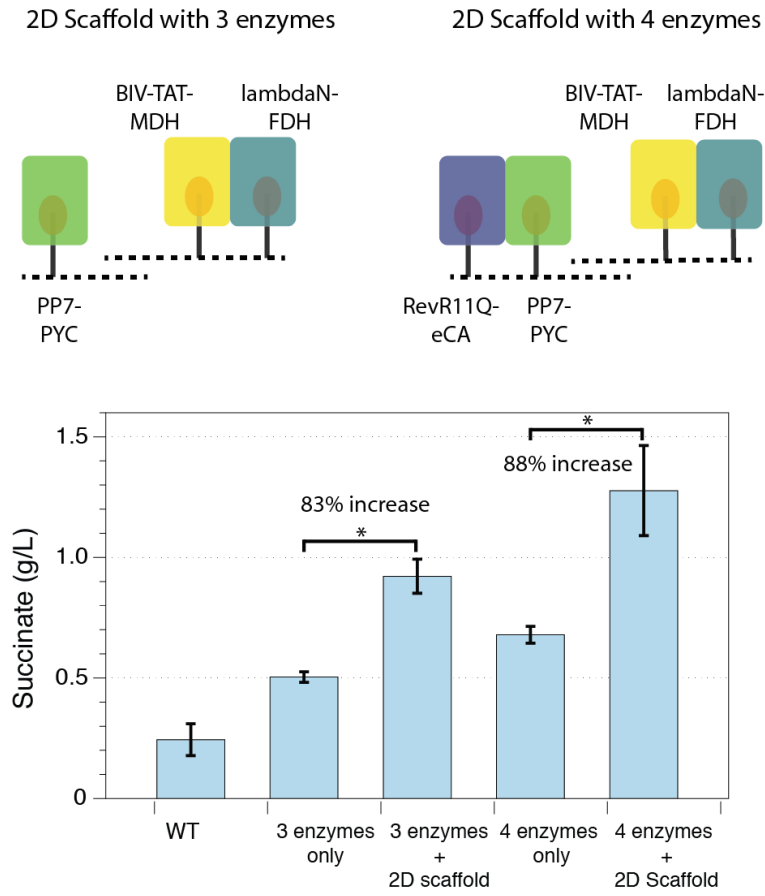


Figure 3.10: Schematics for two scaffolding approaches on 2D scaffolds to increase pathway flux towards succinate. PYC and FDH are scaffolded with MDH in the first approach to prevent loss of oxaloacetate and NADH respectively. In the scaffold with 4 aptamers, eCA is also scaffolded to provide HCO_3^- for PYC. The plot shows succinate production from *E. coli* expressing 3 enzyme fusions (PP7-PYC, BIV-TAT-MDH, lambdaN-FDH) or 4 enzymes (also RevR11Q-eCA) with and without the corresponding 2D scaffolds compared to wildtype (WT) levels. (n=3, error bars = SEM) (* indicates $p < 0.05$, one-tailed t-test)

as oxaloacetate, NADH, and HCO_3^- can likely be channeled towards desired product formation on RNA scaffolds with 2-4 different modular aptamer sites.

3.4 DISCUSSION

Compartmentalization and scaffolding of enzymes are effective strategies for the control of cellular metabolism in both natural^{20,21,22,23,24,25} and synthetic^{20,13,14,26,27,28,29,30,31} systems. In this work we established a toolkit for designing protein-RNA interactions that work inside cells and used it to enhance metabolic substrate channeling in a bio-fuel production pathway. The key features of our system are the expression in cells of RNAs that form a multidimensional nanostructure scaffold with binding sites for RNA-binding domains (aptamers) and co-expression of fusions between specific RNA-binding domains (RBDs) and enzymes defining a metabolic pathway.

Having shown (in chapter 2) assembly of 0, 1, and 2 dimensional RNA scaffolds, which were progressively more effective in sub-cellular co-localization of proteins, we next used these tools to demonstrate metabolic channeling on RNA scaffolds inside cells. A two-enzyme pentadecane synthesis pathway with a labile aldehyde intermediate was localized on the scaffolds. Scaffolds with 1- and 2-dimensional assemblies significantly increased pathway yields. We showed direct evidence of enzyme-RNA interaction and systematically reoriented one of the enzymes on the scaffold. We found that, in the best case, a 2D RNA scaffold led to 2.4 fold higher pentadecane production levels than observed without scaffolding. We could also demonstrate the utility of RNA scaffolds to enhance flux through 3-4 steps of a succinate production pathway with diverse intermediates.

Our system offers a convenient, modular design to optimize metabolic channeling

by changing one design element at a time. The enzymes AAR and ADO are associated with aptamers emerging on the same side of the unit tile plane, where they are well positioned to interact with each other. At each corner junction between unit tiles, 6-bp sticky ends bring together two different stem-loop aptamer segments, followed by RNA tails corresponding to the T7 terminator sequence at the 3' end of the transcripts (App. Fig A.4). Because six bases correspond to half of a turn of an RNA helix, these are likely positioned on the same side of the plane of the scaffold, and electrostatic repulsion between the aptamers, the T7 terminator RNA, and the scaffold itself will roughly determine the placement of the two interacting aptamers. In the solved structure of the PP7 protein-RNA complex (PDB 2QUX¹¹³) the two PP7-ADO units are at an angle of 120°. The BIV-Tat-aptamer interaction is a one-to-one interaction (PDB 1MNB¹⁰⁰). Our data suggest that for some conformations of the BIV-Tat-AAR moiety that are separated by about 120°, the AAR interacts favorably with the one or the other of the PP7-ADO dimers, while other rotational conformations of the aptamer make transfer of the metabolic intermediate less favorable (Fig 3.8).

The geometric dependence of RNA scaffold performance that we report here points to a mechanism where phenomena other than just 3D spatial diffusion of metabolites³³ are in play. Our results are consistent with direct, surface-limited metabolite transfer between aligned enzyme active sites²⁸. The range of flux enhancement on 2D RNA scaffolds reported here illustrates the importance of getting the precise relative orientation right when co-localizing enzymes. In the future, mathematical modeling of protein surface diffusion phenomena will shed insights into how our results and those of other groups^{28,12} can be mechanistically explained. Experimentally investigating the behavior of substrates with different sizes and hydrophobicity could also help develop such a model.

Our RNA scaffolds have consistently shown better efficacy with higher dimensionalities of polymerization¹³. To understand why a similar number of aptamer-pairs result in significantly different metabolite transfer rates¹³ we need a mechanism that involves transfer of intermediates not just to the nearest neighbors on a 1D or 2D scaffold, but also to downstream enzymes with more than 1 or 2 degrees of separation on the same scaffold structure. The localization of substrates to a crowded scaffold assembly would provide such a mechanism. Since scaffolds of 1 and 2 dimensions have more such neighboring pairs progressively, we would expect stronger channeling on those structures.

The economics of biofuel synthesis are particularly challenging because biofuels compete with petroleum products that derive from carbon fixed over millions of years¹¹⁸. Our results indicate that rational scaffolding of enzymes can be useful in improving yields of pathways with intermediates of a variety of different chemical properties. The 2.4-fold increase in pentadecane synthesis observed here could be synergistically combined with other genetic techniques for biofuel yield enhancement⁸⁹, leading to higher overall synthesis rates.

3.5 MATERIALS AND METHODS

3.5.1 STRAIN CONSTRUCTION

E. coli strain BL21* (DE3), that carries a mutation in the *mei31* (RNaseE) gene, was used for all experiments described here. The appendix Fig A.3 lists the plasmid systems used for expression of the various components in *E. coli*. RNA and protein expression was based on Novagen (EMD Millipore) Duet vectors. pETDuet1 was used for RNA expression because of its high copy number (>40 copies/cell) while the lower

copy pACYCDuet1 (10-15 copies/cell) and pCOLADuet1 (20-40 copies/cell) were used to express proteins. For co-expression, we co-transformed competent BL21* DE3 cells with the two or three relevant plasmids and selected on plates with a combination of antibiotics. For all metabolic production experiments, triplicates correspond to three independent transformant colonies.

3.5.2 METABOLIC PRODUCTION CULTURE CONDITIONS

For alkane production experiments, *E. coli* cells were grown in M9 media with 3% glucose, added minerals and 0.1% Triton X-1000 as described in Supplemental information for Schirmer et al⁸⁷. Cultures were inoculated at OD 0.8 with 1mM IPTG. 5mL cultures were grown in 20 mL glass test tubes at 37°C with shaking. Alkane yields were measured 16 hours after induction unless otherwise stated. For succinate production experiments, *E. coli* cells were grown anaerobically in LB media, with 2% glucose, 2% bis-tris, 100mM formate and induction with 2mM IPTG. For this, overnight LB cultures, still in exponential phase, were centrifuged and resuspended in the above media at a starting OD of 5.0 and the batch cultures were sparged to create a headspace of 16% CO₂ and balance Nitrogen. Succinic acid levels were measured 7 hours after anaerobic growth at 37°C.

3.5.3 MEASURING PRODUCTION LEVELS

Following induction and incubation, cell cultures were centrifuged, and the supernatant decanted. In a typical pentadecane assay, 3mL of supernatant vigorously mixed with 1mL of 1M NaCl and 1mL pure HPLC grade ethyl acetate (Sigma Aldrich). After 1 hour of shaking, the ethyl acetate extract was separated by centrifugation (10 min

at 4000g) and obtaining the top layer. Extracts were analyzed on an Agilent GC-MS 5975/7890 (Agilent Technologies) with the HP-5MS column (30 m length, 0.50 mm diameter). Following a 1-3 μ l split-less injection, the inlet temperature was held at 100°C for 3 minutes and ramped up to 320°C at 30°C per minute. For quantification, standard curves of pentadecane and hexadecanol were generated, by using the pure compounds (Sigma Aldrich). Succinic acid production assays were similarly performed on decanted supernatant. Roche (r-biopharm) enzymatic succinic acid assay kits (CN: 10176281035) were used to measure production levels using a Victor3V 1420 Multilabel plate reader (Perkin Elmer).

3.5.4 MEASURING ENZYME EXPRESSION

For enzyme level quantification, we collected centrifuged cell pellets from cultures that were grown in media condition described for alkane production. Cultures grown for 16 hours after induction were used. The cell pellets were lysed with a bacterial protein extraction reagent (B-PER II, Thermo Scientific), boiled for 5 minutes at 100°C, and a protease inhibitor cocktail (Roche) was immediately added. We used 4-20% Tris-Glycine gels (Novex) to separate the proteins by mass, transferred them to a nitrocellulose membrane (Novex iBlot) and blotted with antibodies. We used Abcam antibodies for endogenous GAPDH (ab85760) and 6x His Tag (ab1187), and Novagen antibody for Strep Tag II (#71591). The software ImageJ¹⁰² was used to analyze and quantify western blot images.

3.5.5 PURIFICATION OF RNA-BOUND PROTEINS

For co-purification of RNA interacting with enzymes, we performed UV crosslinking with 150 mJ/cm² of *E. coli* cultures in 6 well plates. This was followed by gentle cell lysis using B-PER I (Thermo Scientific) containing protease inhibitors, RNaseOUT (Life Technologies) and DNase I (Sigma Aldrich). The solution was subjected to 2 freeze thaw cycles (-20°C/4°C) to further ensure cell lysis. Then the lysate was centrifuged at 12000g for 10 minutes and the supernatant was processed through the HisPur Ni-NTA magnetic beads protocol (Thermo Scientific) to purify 6xHis-tagged AAR fusion protein. IgA and IgG magnetic beads (Thermo Scientific) were used as controls to pull down random proteins. The purification was verified by Western blots and then subjected to protease K (Thermo Scientific) treatment, to release any co-purified RNA.

3.5.6 MEASURING CELLULAR RNA LEVELS

Intracellular RNA was either collected from cultures grown for 16 hours after induction or from protease digestion of the purified UV crosslinked enzymes. RNA directly from cell pellets was extracted using Trizol and a Purelink RNA Mini Kit (Ambion, Life Technologies). RNA from a protease treated purified enzyme extract was extracted through acid-phenol-chloroform extraction and overnight ethanol precipitation at -20°C. From this, cDNA was prepared using SuperScript III First-Strand cDNA synthesis system (Life Technologies). We used random hexamers to reverse transcribe the entire cellular RNA pool. 500 ng of total RNA was elongated at 25°C for 10 min, followed by 50°C for 50 minutes and termination at 85°C for 5 min. RT-PCR was performed using 7.5 µl of SYBR Green Supermix (Ambion), 4.5 µl of 10x diluted cDNA and 3µl of 2nM primer solution. Fig A.5 in the appendix lists the primers that

were used for amplification. The gapA primers served to enable measurement of endogenous cellular gapA mRNA pools, which were used as internal controls. The 2D primers shown here amplified scaffold sequences accurately as tested by a standard curve using 4 fold dilutions of a single test sample. Quantitative PCR was performed on an Eppendorf Mastercycler ep realplex thermal cycler (upto 55 cycles) and the qPCR software Biogazelle Plus was used for calculating scaffold RNA concentration relative to gapA mRNA. Quantitative PCR efficiency was estimated to be 1.87/cycle for scaffold RNA and 2.03/cycle for gapA using a dilution series. Hence, relative amplification, r , for scaffold/gapA per cycle is $r \approx 0.92$.

3.5.7 SCAFFOLD STRUCTURE REPRESENTATION

The Make-NA tool (James Stroud 2004, 2011), available on the University of Southern California servers, was used for constructing PDB representations of our stem and T7 terminator hairpins. The software Autodesk Maya (student version 2014) was used to render the 3D representation in Figs 3.7 and 3.8, along with a Molecular Maya toolkit (Digizyme).

Cyanobacteria are some of the simplest microbes capable of harnessing energy from sunlight and are among the most evolutionarily ancient organisms. Indeed, cyanobacterial biochemistry, acting over millions of years, radically altered the atmosphere and enabled life as we know it on Earth today. It seems therefore fitting that cyanobacteria could be an important component to help us address some of today's urgent energy and atmospheric problems.

Danny Ducat, Michigan State University

4

Controlling metabolic fluxes in cyanobacteria

CYANOBACTERIA ARE UBIQUITOUS PHOTOAUTOTROPHIC BACTERIA that convert solar energy into fixed electrons at 3-12 times higher efficiencies than land plants^{119,91} while also being relatively easy to engineer⁹⁰. This makes them very exciting candidates

as platforms for the renewable production of both a variety of high value chemicals⁹¹ and feedstocks usable as substrates for bioreactors¹²⁰. To that end, in this chapter I present research efforts aimed at understanding the control of electronic and metabolic fluxes in the cyanobacterium *Synechococcus elongatus*.

In section 4.1 I present work where we^{*} elucidated the flow of reducing equivalents in engineered strains of *S. elongatus* and showed that electron transport reactions in cyanobacteria can be employed for renewable hydrogen production. In section 4.2, I present a design strategy we[†] implemented with the goal of expressing 2D RNA scaffolds (discussed in Chapters 2, and 3) in *S. elongatus* and using them to carry out the carbon fixation reaction naturally occurring within protein shells called carboxysomes.

4.1 REWIRING HYDROGENASE-DEPENDENT REDOX CIRCUITS IN CYANOBACTERIA

It has been shown that photosynthetic organisms are capable of hosting functional hydrogenase enzymes, carrying out the reversible reaction, $2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2$ ¹²¹. There are two classes of hydrogenase enzymes found naturally, nickel-iron [NiFe] and iron-iron [FeFe], classified based on the nature of the reactive metallocluster at their active sites¹²¹. The two kinds of hydrogenases are phylogenetically and structurally unrelated. [NiFe] hydrogenases are found in a variety of organisms, however [FeFe] hydrogenases are typically prokaryotic, or found in some algal species. [NiFe]-hydrogenases are typically exchange electrons with NAD(P)H, (reducing potential ≈ 320 mV). [FeFe] hydrogenases interact with ferredoxins, which are electron bearing proteins and have

^{*}The research presented in section 4.1 was carried out with lead author Daniel C. Ducat and the material has been published⁸⁶

[†]Work presented in section 4.2 was carried out with Simon Alexander Thomas Kretschmer

significantly lower redox potentials, which brings the potential energy of their electrons closer to the redox potential of the H_2/H^+ pair (≈ 420 mV)¹²². Thus [FeFe]-hydrogenases thermodynamically favor production of H_2 compared to [NiFe] hydrogenases, which are predominantly carry out H_2 uptake¹²³.

Hydrogenases, because of their metallocluster active sites are usually oxygen sensitive, and thus it can be a challenge to employ them for biological hydrogen production in photosynthetic organisms¹²⁴. Several efforts to this end have used various strategies to temporally insulate the hydrogenase reactions from the water splitting (hence O_2 producing) activity of the photosystem II (PSII). This involves growing photosynthetic cultures normally to allow generation of internal stores of reducing equivalents, then changing the cellular environment of one that is both anaerobic and inhibits PSII activity¹²⁵. Both, anaerobic light fermentation¹²⁶, and dark fermentation¹²⁷ have been shown to result in biological hydrogenase activity leading hydrogen production.

Here, we expressed a *Clostridial* [FeFe] hydrogenase HydA¹²⁸ in *Synechococcus elongatus* to achieve hydrogen production in the cyanobacterium. *S. elongatus* is obligately aerobic and photoautotrophic. It also does not contain any nitrogenases or nitrogen fixing capability¹²⁹. Nitrogenases can sometimes carry hydrogenase activity, but any hydrogen production in our system can be solely attributed to the expressed HydA enzyme. We show that HydA can be functionally integrated into the electron transport machinery of the cell and is capable of hydrogen production. We also show that the channeling of electrons to and from HydA can be rationally designed to achieve cell growth based on hydrogen consumption and also conversely, the use of dark fermentation reactions, converting cellular carbohydrate pools into hydrogen gas.

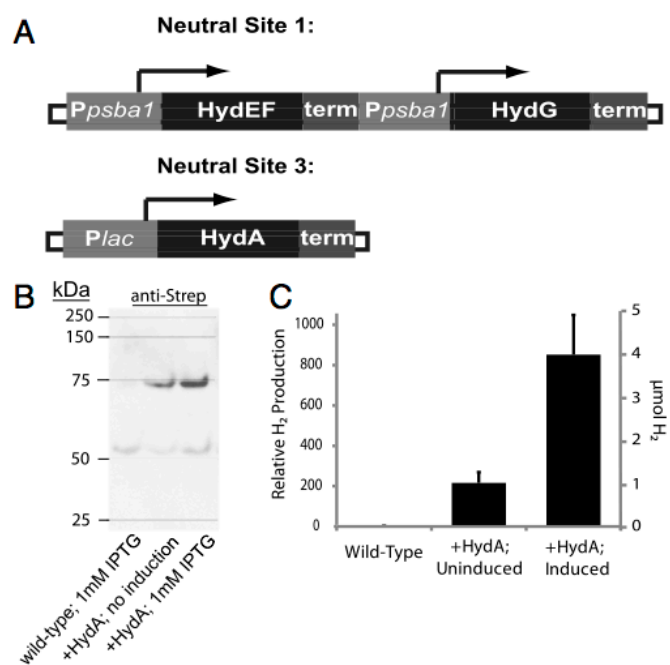


Figure 4.1: Expression of Hydrogenase A in *Synechococcus elongatus* sp. 7942. (A) A schematic showing the genes integrated into the genome. Constitutive promoter *psba1* are used to express maturation factors (HydEF,G) and HydA is placed under IPTG-inducible *PLac* control (B) A western blot showing IPTG induction of HydA in transformed strains (expected molecular mass : 65.6 kDa) (C) Evolution of hydrogen gas from WT and HydA expressing strains of *Synechococcus* lysates after 60 minutes anaerobic methyl viologen treatment

4.1.1 EXPRESSION AND *IN VITRO* ACTIVITY OF HYDA

We expressed the [FeFe] hydrogenase (HydA) from *Clostridium acetobutylicum* in *Synechococcus elongatus* sp. 7942. In previous work at our lab with synthetic hydrogen-producing circuits in *Escherichia coli*, the HydA from *C. acetobutylicum* was shown to have the highest level of activity among several hydrogenases tested¹³⁰.

We also expressed hydrogenase maturation factors HydEF and HydG¹³¹, by cloning them downstream of the *S. elongatus* *psba1* promoter. The *psba1* promoter controls the expression of D1 subunit of photosystem II (PSII), leading to constitutive expression. This cloned unit was integrated into the cyanobacterial genome using the neutral site

1⁹⁰ which is a genomic location that has been shown to allow genetic integration without any noticeable phenotypic effects (Fig 4.1A). HydA was similarly inserted at the neutral site 3¹³² (Fig 4.1A). HydA was however placed until the IPTG inducible PLac promoter. Expression of a StrepII-tagged HydA could be confirmed by Western blot analysis and the protein expression could be induced (approximately 3-4 fold) by addition of IPTG (Fig 4.1B). Next, we used a standard methyl viologen assay¹²⁸ to confirm the catalytic activity of the expressed enzyme.

Methyl viologen is a promiscuous electron donor that can reduce both [FeFe] and [NiFe] hydrogenases. Thus, if cell lysates are mixed with an excess of methyl viologen, the hydrogen gas evolved is an accurate measure of the hydrogenase activity¹²⁸. We incubated cell lysates with methyl viologen in an anaerobic environment for 45 minutes and observed >500-fold increase in the amount of hydrogen gas detected in the headspace (Fig 4.1C) with HydA expressing cultures. Under similar conditions, wild-type (WT) cyanobacterial cultures yielded no H₂ gas.

4.1.2 *IN VIVO*, LIGHT DEPENDENT HYDROGEN EVOLUTION

To achieve functional hydrogenase activity *in vivo*, the enzymes must interact with the endogenous redox machinery and in order to be able to accept reducing equivalents. However, due to the extreme oxygen sensitivity of HydA, and most hydrogenases in general, it is necessary to spatiotemporally separate the water-splitting reaction of PSII from hydrogenase expression. We used 5 μM diuron [3-(3,4-dichlorophenyl)-1,1-dimethyl-urea (DCMU)], to inhibit PSII activity after cells had been grown to a healthy density (OD₇₅₀ ≈ 0.5-1). At this point we also sparged the cyanobacterial cultures' headspace with 2.5% CO₂ (N₂ balance) to create an anaerobic environment. As before, expression of hydrogenase was induced with IPTG. We then incubated the cul-

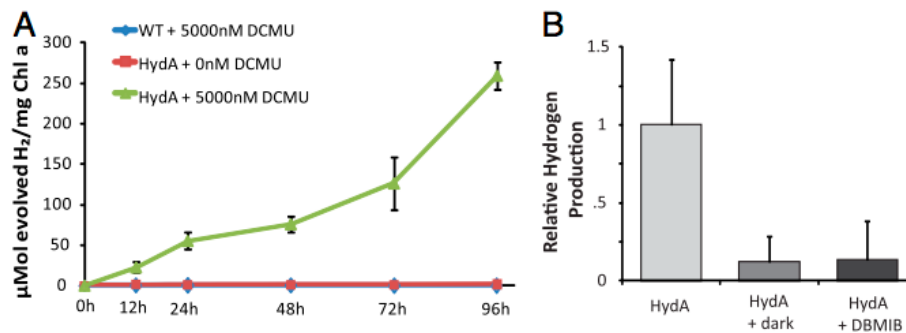


Figure 4.2: *In vivo* hydrogen production from HydA expressing *Synechococcus elongatus*. (A) Hydrogen production under anaerobic conditions with dichlorophenyl dimethyl urea (DCMU) added to media to prevent oxygen evolution from photosystem II (B) *In vivo* hydrogen production depends on light and electron transport from plastoquinone, which can be inhibited by dibromothymoquinone (DBMIB).

tures with or without light and assayed hydrogen evolution into the headspace. No hydrogenase activity was detected in the parent *S. elongatus* strain, while significant levels of hydrogen gas were produced by HydA-expressing cell cultures. Production was only observed when O_2 generation was suppressed (Fig 4.2A). The average measured hydrogenase activity of HydA-expressing strains over the first 96 hours was $2.8 \mu\text{mol H}_2 \text{ h}^{-1} \cdot \text{mg Chl-a}^{-1}$ (Fig 4.2A), where Chl-a is chlorophyll a, measured by methanol extraction¹³³.

Cyanobacteria and alga can anaerobically metabolize carbohydrates to generate reducing equivalents that may be used by hydrogenases through either light-dependent or dark reactions^{134,135}. In light-dependant reactions, the electrons from carbohydrate metabolism are transferred to the plastoquinone pool, and reenergized through Photosystem I (PSI) before interacting with hydrogenases through petF- class plant-type ferredoxins¹³⁴. During dark fermentation however, reducing equivalents from the breakdown of glycogen can either be directly donated to the hydrogenase, or may be transferred through electron carriers like NAD(P)H or ferredoxin^{136,137}. We observe

that HydA-expressing *Synechococcus*, hydrogen production is greatly reduced in the absence of light or in the presence of the plastoquinone inhibitor dibromothymoquinone (DBMIB) (Fig 4.2B). Therefore, we can conclude that the electron transfer to HydA is largely dependent cyanobacterial electron transport chain function.

4.1.3 HYDA ACTIVITY SUPPORTS LIMITED GROWTH WITH H₂ AS SOLE ELECTRON SOURCE

In several algal species, native hydrogenases function by recovering electrons from hydrogen, that may have been produced as a side product from the promiscuity of nitrogen fixation reactions. For efficient uptake of reducing equivalents, the hydrogenases need to interact with cellular machinery in the opposite direction from the previous sub-section. They need to deposit the hydrogen-derived electrons towards the rest of cellular metabolism. It has been shown that a native bidirectional [NiFe] hydrogenase of *S. elongatus* can uptake H₂ and reduce NAD⁺. This activity has been examined in cell extracts and was measured to be very low (85 nmol h⁻¹ · mg protein⁻¹)¹³⁸. Such a rate is likely insufficient to support autochemolithic growth using H₂ as the only source of reducing equivalents, and H₂-dependent cell division has not been observed in cyanobacteria^{138,139} (Fig 4.3A, WT curves).

Hence, we evaluated the the *in vivo* activity of HydA with regard to its capacity to consume hydrogen gas as an alternative source of reducing equivalents within *S. elongatus*. We tested this under conditions where PSII water-splitting reactions were inhibited by addition of DCMU. Additionally, cells were incubated in anaerobic sealed cultures with 5% CO₂ and 5% H₂ (balance N₂), and growth was assayed by OD750 (Fig 4.3A). We notice that HydA-expressing strains can support limited cell growth using hydrogen under DCMU inhibition when incubated anaerobically under illumination.

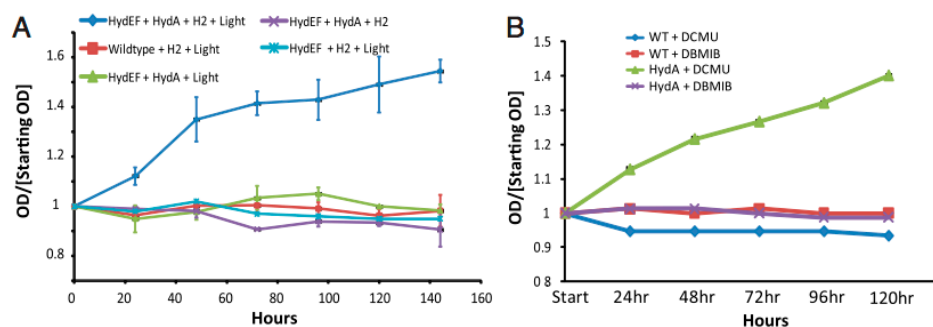


Figure 4.3: Hydrogenase A dependent chemoautotrophic growth of *Synechococcus*. (A) HydA activity supports growth in anaerobic conditions with hydrogen gas and co-expression of maturation factors HydEF (B) Using dibromothymoquinone (DBMIB) to inhibit plastoquinone electron transfer prevents hydrogenase mediated growth

Most of the growth was observed in the first 3-4 days following the switch to a hydrogen containing atmosphere, and HydA-supported growth depended on the presence of H₂, the absence of O₂, HydA maturation factors, and illumination (Fig 4.3A).

Carbon fixation through the Calvin cycle requires supplies of both ATP and reducing equivalents in order to increase cell mass. Under illuminated conditions, photosynthetic cells carry an excess of reducing equivalents relative to ATP, and electrons from ferredoxin can reenter the ETC through the plastoquinone pool in an essential process termed cyclic electron transport¹⁴⁰. Although cyclic electron transport increases the proton gradient across thylakoid membranes, hence, stimulating ATP production, light absorption at PSI is essential to keep ferredoxin pools reduced, for supplying electrons to Calvin cycle. Oxidation of hydrogen, however, can only provide a source of electrons. Based on the observation that HydA-dependent growth required light as well, we hypothesized that hydrogenase-derived electrons were being channeled through the ETC and undergoing cyclic electron transport to create proton gradients and generate ATP. To test this, we attempted to block electron flow, using the plastoquinone inhibitor DBMIB. We noticed that HydA-dependent growth was disrupted

when electron flow from plastoquinone was blocked by the addition of DBMIB (Fig 4.3B). We also observed that HydA-expressing strains were more viable after extended incubations under the above conditions (DCMU/hydrogen), as evident by culture bleaching and colony forming unit (CFU) counts when plated on agar plates. Taken together, these results show that heterologously expressed *Clostridial* HydA is capable of both accepting and donating reducing equivalents from/to the endogenous redox circuitry of *S. elongatus*.

4.1.4 HYDA ACTIVITY DEPENDS ON THE CO-EXPRESSED FERREDOXINS

As mentioned earlier, it is likely that HydA is interacting with endogenous *S. elongatus* petF plant-type ferredoxins, leading to H₂ production that is dependent on both light and ETC function (Fig 4.2B). However, it is also possible that the endogenous plant-type ferredoxins may be only weakly interacting with the heterologous HydA. In a previous project in the lab, employing a synthetic circuit in *E. coli* to test interactions of HydA with a several ferredoxin proteins, it was found that some ferredoxin-hydrogenase affinity pairs could influence levels of hydrogen output from the circuit¹³⁰. We tested these pairs to facilitate enhanced electron transfer to HydA and increase hydrogen production from *S. elongatus*. The strongest ferredoxin pairs, one plant type and one bacterial type (from *Spinacia oleracea* and *C. acetobutylicum*, respectively)¹³⁰, were integrated into the genome of the HydA-expressing strain, and were also cloned downstream of an IPTG-inducible promoter⁹⁰. We found that co-expression of the *C. acetobutylicum* (CACo303) ferredoxin¹⁴¹, the strongest pairing from previously reported synthetic circuits¹³⁰, increased the rate of H₂ production by nearly twofold (Fig 4.4A). This suggests that expression of the *Clostridial* ferredoxin helped to redirect cellular reducing equivalents toward HydA more efficiently. We found that co-expression

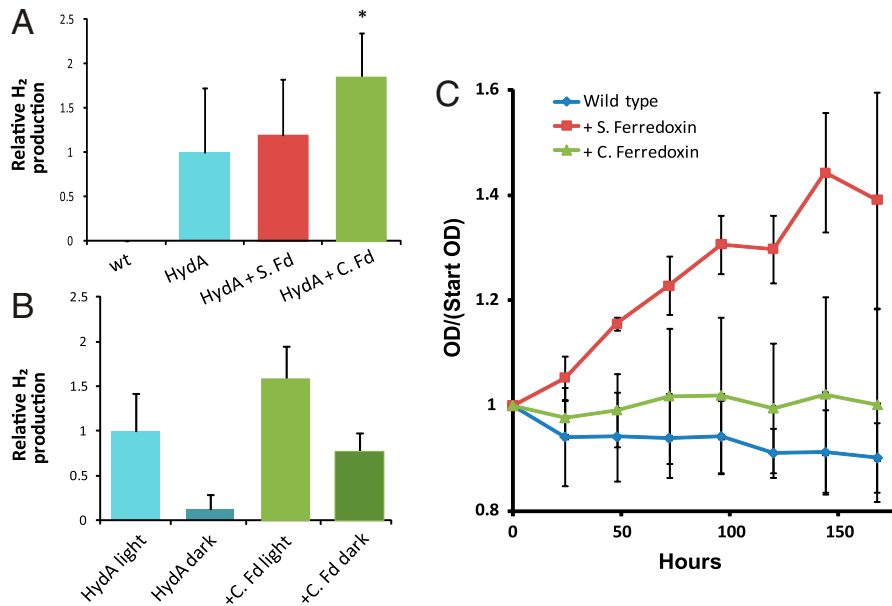


Figure 4.4: Coupling with ferredoxin affects HydA electron flow. (A) Effect of co-expressing ferredoxins from *Spinacea oleracea* (S. Fd.) and *Clostridium acetobutylicum* on hydrogen production through HydA (B) C. Fd. enables hydrogen gas production from dark fermentation (C) Strains expressing C. Fd. are unable to support HydA dependent growth in an anaerobic hydrogen environment.

of the plant-type ferredoxin I from *S. oleracea* did not significantly enhance hydrogen production.

Although the ferredoxin from *C. acetobutylicum* has previously shown a strong functional interaction with HydA in a synthetic circuit¹³⁰, it is a bacterial-type ferredoxin and is unlikely to be capable of direct interactions with PSI in a manner similar to the endogenous *S. elongatus* plant-type ferredoxins. Therefore, we asked if the observed increase in hydrogen production in strains expressing both HydA and *C. acetobutylicum* ferredoxin was the result of utilization of reducing equivalents from a distinct source. Indeed, *C. acetobutylicum* ferredoxin-bearing strains were capable of producing hydrogen in the dark and/or in the presence of DBMIB, whereas these conditions largely abolished hydrogen production when expressing HydA alone (Fig

4.4B).

Since the heterologous expression of a ferredoxin enhanced the ability of HydA to produce hydrogen (Fig 4.4A), we examined if new ferredoxin pairings would affect the ability of HydA to support hydrogen dependent anaerobic growth as well (Fig 4.3A). We found that strains co-expressing *S. oleracea* ferredoxin showed HydA-supported growth in the presence of hydrogen as before (Fig 4.4C). However, remarkably, the presence of *C. acetobutylicum* ferredoxin attenuated H₂-dependent growth (Fig 4.4C).

Together, these experiments imply that co-expression of exogenous ferredoxins can both increase the flux of electrons toward HydA, and “rewire” the redox pathway, depending on the specific ferredoxin-HydA links being optimized. Our hypothesis is that the *C. acetobutylicum* ferredoxin provides an alternative electron circuit that bypasses the need for light reactions and anaerobic fermentation through PSI for the reduction of HydA. However, since this exogenous ferredoxin, which is presumably a preferred interacting partner of HydA, appears less integrated with the cyanobacterial redox pathways, it may “short circuit” the HydA interactions with native machinery that were responsible for H₂-supported growth (Fig 4.5).

4.1.5 HYDA EXPRESSION LIKELY CREATES NEW ELECTRONIC FLOWS IN *SYNECHOCOCCUS ELONGATUS*

In this work⁸⁶, we demonstrated the functional expression of an exogenous [FeFe] hydrogenase (HydA) in cyanobacteria *in vivo*. In particular, we showed activity within the context of the endogenous redox machinery and the electron transport chain. We show significantly enhanced hydrogen evolution rates relative to those of wild-type *S. elongatus*, which rely on native [NiFe] hydrogenases (Figs 4.1C and 4.2A). It is relevant to note that in an earlier study attempting to use a [FeFe] hydrogenase from *Clostrid-*

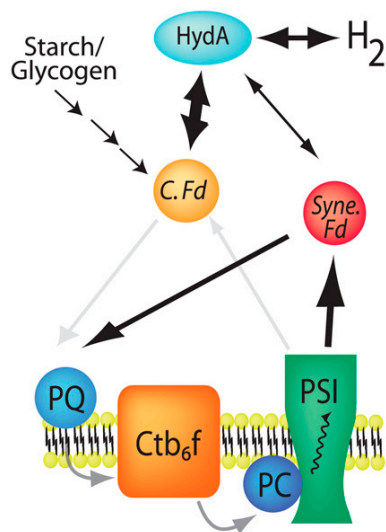


Figure 4.5: Model of ferredoxin-mediated HydA electron flows in *Synechococcus elongatus*. We hypothesize that cyanobacterial endogenous ferredoxins (Syne. Fd) mediate electron transfer from photosystem I to hydA, enabling both light dependent hydrogen production, and hydrogen dependent growth. C. Fd can interact more strongly with HydA and also likely picks up electrons from dark reactions. However, C. Fd interactions with plastoquinone are inefficient (faded arrows), preventing hydrogenase dependent growth.

ium pasteurianum within cyanobacteria¹⁴², the hydrogenase exhibited only limited (nearly two-fold) increase in activity over the native hydrogenases. Here, we show that an HydA is capable of interacting with endogenous redox circuits to evolve hydrogen from cellular sources of reducing equivalents at $2.8 \mu\text{mol H}_2 \text{ h}^{-1} \cdot \text{mg Chl-a}^{-1}$ (Fig 4.2A). We also show that HydA can support chemoautotrophic growth of *S. elongatus* by uptake of hydrogen gas as the sole source of reducing equivalents when photosystem II is chemically inhibited.

Our lab has previously shown that *C. acetobutylicum* HydA can functionally interact with a variety of ferredoxin proteins from diverse organisms¹³⁰, and here we show that electron carriers within *S. elongatus* can also donate and/or receive reducing equivalents to/from HydA. Additionally, our results show that selected ferredoxin-

hydrogenase combinations may optimize hydrogen production and change input/output sources for the reaction, such as allowing for fermentation of internal reducing equivalents without the assistance of light (Figs 4.4B and 4.5). Although the presence of distinct (i.e., light-dependent and light-independent) pathways for electron transfer to hydrogenases has been documented^{137,143}, to our knowledge this is the first demonstration of genetic means to enhance the rate of one pathway relative to the other. Rewiring the flow of electrons by such a strategy may open up the possibility of implementing parallel redox pathways that are insulated from host redox machinery (Fig 4.5). Our results suggest that characterization and optimization of the key residues forming the ferredoxin-hydrogenase interface could be a big step towards deterministic control over novel electron pathway design.

Employing cyanobacteria for the biological synthesis of hydrogen gas and other high value chemicals is an environmentally friendly approach to meet future energy and material needs⁹¹. In this work, strains expressing [FeFe] hydrogenase showed a >500-fold increase in hydrogen production *in vivo* relative to unmodified strains of *S. elongatus*¹³⁹. A successful hydrogen producing organism would likely need to be both photoautotrophic and need minimal additional care in the form of bioreactors. In this regard, discovery or engineering of hydrogenases with increased oxygen tolerance would enable direct synthesis of hydrogen gas, bypassing inefficiencies associated with energy transfer and storage through carbohydrate intermediates. The ability of HydA to oxidize hydrogen to support limited growth in *S. elongatus* also offers a possible method for directed evolution of HydA variants with desirable traits, particularly greater oxygen tolerance. To our knowledge, there have been no previous reports of cyanobacteria capable of chemoautotrophic division through the utilization of hydrogen. Given the relative ease of genetically modifying *S. elongatus*, it may be possible to make further

improvements to this strain similar to those shown in other cyanobacteria or algae that enhance starch accumulation¹⁴⁴, reduce cyclic electron transport¹²⁶, modify PSII activity¹⁴⁵, or reduce phycobilisome size¹⁴⁶ to allow for increased hydrogen output. Finally, optimal culture conditions and cell immobilization may further enhance the yield of photobiologically produced hydrogen^{147,148}.

4.1.6 MATERIALS AND METHODS

STRAINS, PLASMIDS, AND CULTURE CONDITIONS

Wild-type *S. elongatus* was obtained from the American Type Culture Collection. Cultures and in vivo assays utilized BG11 medium with light illumination of 4000 lux at 30°C and a 12/12 h day/night cycle. All constructs were cloned in BioBrick format in *E. coli* (Assembly Standard 21)¹⁴⁹. *S. elongatus* cultures in log phase (OD₇₅₀ ≈ 0.4) were transformed with 100 ng plasmid DNA overnight and plated on BG11 plates with antibiotics. Neutral site vectors for genomic integration were obtained from their respective lab of origins [NS1/pAM2314; NS2/pAM1579^{90,150}; NS3/ pHN1-LacUV5¹³²]. Hydrogenase (HydA) from *Clostridium acetobutylicum*, *Clostridium acetobutylicum* ferredoxin CACo303, ferredoxin I from *Spinacia oleracea*, and hydrogenase maturation factors (HydEF, HydG) from *Chlamydomonas reinhardtii* were cloned and synthesized as previously described¹³⁰.

HYDROGEN PRODUCTION AND UPTAKE ASSAYS

S. elongatus was grown under a standard atmosphere with appropriate antibiotics, diluted to OD₇₅₀ ≈ 0.1 at volumes 10-50 mL within 25-240 mL clear glass vials and sealed with rubber septa. Where applicable, the following chemicals were supplemented in

the media: 5 μ M Diuron (DCMU), 20 μ M DBMIB, 0.1-1.0 mM IPTG, 20 mM HEPES pH 8.0. Cultures were sparged with 2.5% CO₂, nitrogen balance (Airgas) and placed in 30°C incubators with light as above (or dark, where appropriate). For in vitro methyl viologen assays, we adapted the method from King et al¹²⁸, lysing cyanobacterial sealed cultures with bacterial protein extraction reagents (Pierce) in the presence of 1 mM methyl viologen (Sigma) and 5mM sodium dithionite for 60 min (Fisher). Headspace was measured by gas chromatography (Shimadzu GC-14A). For hydrogen-dependent growth assays, headspace was sparged with 5% H₂, 5% CO₂, nitrogen balance.

4.2 TOWARDS A SYNTHETIC CO₂ FIXATION MODULE BASED ON RNA SCAFFOLDS

Ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) is the most abundant protein on the planet. It catalyses a key step in the fixation of carbon dioxide into carbohydrates as a part of the Calvin cycle, hence feeding nearly all life on earth. It is widely held that Rubisco's slow catalytic rate of 3-10 molecules of CO₂ per second, which likely explains its abundance, "is a relic of a bygone age"¹⁵¹ and that the 'inefficiencies' of the enzyme are due the primitive environmental gas compositions at the time of its origin. One of the key inefficiencies, is a competing oxygenation reaction where O₂ can react with the 5 carbon substrate at rubisco's active site, causing losses of upto 25% of fixed carbon in some species¹⁵² through a process called photorespiration. Photosynthetic organisms have evolved several adaptations to counter this loss by protecting Rubisco through carbon concentrating mechanisms like C₄ photosynthesis¹⁵² in arid land plants and carboxysomes in cyanobacteria¹⁵³.

Carboxysomes are protein micro compartments in cyanobacteria²¹ that co-localize

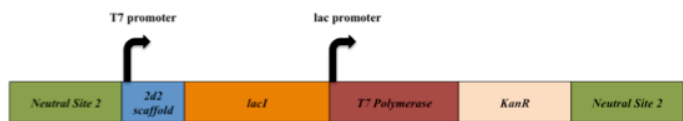


Figure 4.6: Schematic for inserting 2D RNA scaffold expression module into *Synechococcus elongatus*. This module includes the T7 RNA polymerase needed for RNA scaffold strand expression and is designed to be targeted to the neutral site 2 region⁹⁰ of *S. elongatus*

carbonic anhydrase and rubisco¹⁵⁴. Carbonic anhydrase is a part of the carbon concentrating mechanism in cyanobacteria and catalyses the conversion of dissolved (and concentrated) bicarbonate into gaseous CO₂, thus providing a high local concentration of CO₂ relative to oxygen inside the carboxysomes. Carboxysomes are complex compartments - their biogenesis is only starting to be elucidated^{155,156}, and attempts have been made to express them in other hosts, like *E. coli*¹⁵⁷.

There is some debate about the necessity¹⁵⁸ and mechanisms²⁵ of selective permeability of the carboxysomes for HCO₃⁻, CO₂, and O₂. We decided to examine whether the carbon concentrating effect of carboxysomes can be mimicked on the *in vivo* RNA scaffolds¹³ described in Chapters 2 and 3. For this, we expressed the RNA scaffolds in cyanobacteria alongside with fusion proteins of rubisco and carbonic anhydrase with aptamer binding domains - to co-localize the enzymes, and then tested the function of a proposed assembly in strain where natural carboxysomes are not functional.

4.2.1 EXPRESSING RNA SCAFFOLDS IN *S. ELONGATUS*

The first step was to achieve expression of RNA scaffold strands in *S. elongatus*. The Fig 4.6 shows the gene construct used to achieve this. In the figure, the green regions labeled *Neutral Site 2* were targeting regions to achieve genomic integration of the construct⁹⁰. The scaffold expression system consists the 2D RNA scaffold strands with

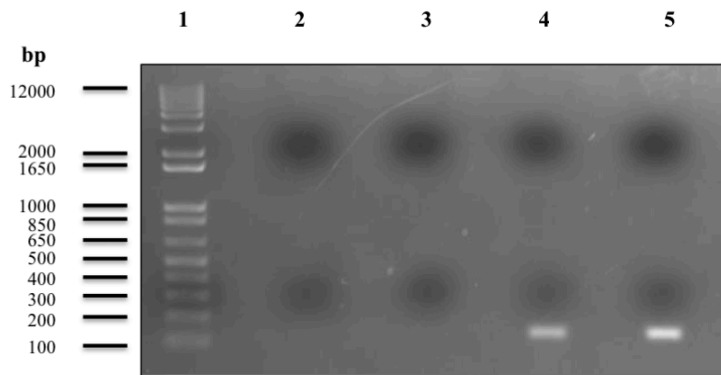


Figure 4.7: Expression of scaffold RNA in *Synechococcus elongatus*. The different columns are (1) 1kb+ DNA ladder, (2) WT *S. elongatus* with no IPTG, (3) WT *S. elongatus* with 1 mM IPTG, (4) *S. elongatus* transformed with 2D scaffold - no IPTG, and (5) *S. elongatus* transformed with 2D scaffold - 1 mM IPTG.

MS2 and PP7 aptamers¹³ under the control of a PLac-T7 promoter⁸⁵. The T7 RNA polymerase - promoter system has a short GGG pre-mRNA transcription start sequence¹⁵⁹ that allows for better control over the expressed scaffold sequence folding. To allow use of this, we included the T7 polymerase under the control of an IPTG inducible PLac system. Since both promoter cloned in were controlled by the lac operator, we included a constitutive LacI expression sequence into this construct. Finally, Kanamycin antibiotic resistance was included to be able to select for the transformed colonies.

We tested expression of the RNA scaffolds after integration of the construct into *S. elongatus* by reverse transcriptase PCR and agarose gel analysis of the cDNA. We used random hexamers for cDNA preparation and the primers (Fig A.5) for 2D scaffold, also used in Chapter 3, for amplification of the cDNA of interest (Fig 4.7). We can note from the columns 4 and 5 of Fig 4.7, that the RNA scaffold strands could be inducible expressed in *S. elongatus* using the designed expression system (Fig 4.6).

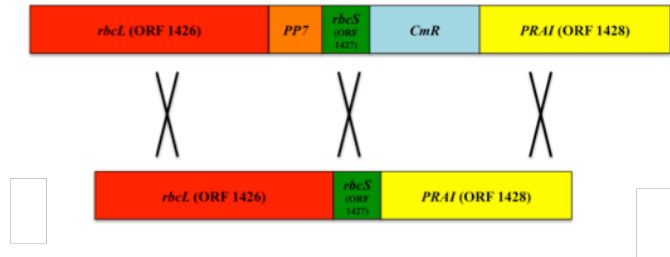


Figure 4.8: Schematic for inserting a PP7-rbcS (rubisco small subunit) fusion in *Synechococcus elongatus* sp. 7942 at the rbcS native gene operon location

4.2.2 INSERTING RNA BINDING DOMAIN FUSIONS INTO THE GENOME OF *S. ELONGATUS*

We decided to make fusions of rubisco with PP7 and carbonic anhydrase with MS2, for co-expression and localization with the 2D scaffold. In order to mimic natural function, we decided to express these fusions by inserting the constructs into the genomic locations for rubisco and carbonic anhydrase respectively, with minimal perturbation of the genomic operons barring the fusion protein insertions and selection markers.

RUBISCO FUSION WITH PP7

We designed and cloned to construct shown in Fig 4.8 to express a fusion of rubisco with the PP7 domain in order to localize it to the 2D RNA scaffold. The functional rubisco holoenzyme is a hexadecamer composed of eight large (rbcL) and eight small (rbcS) subunits^{160,161}. We studied the rubisco holoenzyme crystal structure (PDB:1GK8¹⁶¹) to decide on making a N-terminal fusion of the rbcS subunit with PP7. This decision was supported by a report where an rbcS N-terminal fusion with GFP was shown to assemble and retain catalytic function as a part of the rubisco holoenzyme and within active carboxysomes¹⁶². To enable the homologous recombination mediated genomic

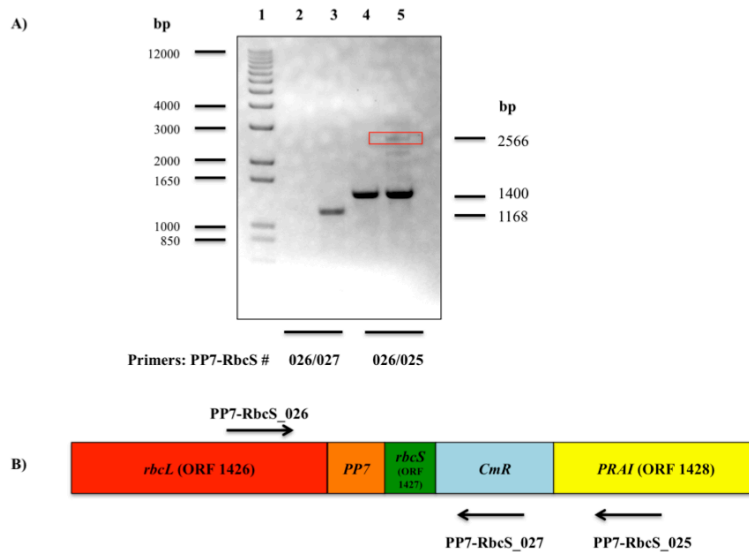


Figure 4.9: Verifying the insertion of Rubisco-RNA binding domain fusion in *Synechococcus elongatus* sp. 7942. This gel shows products from a genomic PCR of the WT (columns 2 and 4) and transformed chloramphenicol resistant (columns 3 and 5) *S. elongatus* strains. Primers 026 and 027 are only expected to yield a band from the inserted fusion constructed that includes a chloramphenicol resistance marker. Primers 026 and 028, however would yield a product of length ≈ 1500 b.p. from the WT genome, and one of length 2566 b.p. from the transformed strain.

integration of this construct in a way that replaces endogenous gene with the fusion (genomic knock in - Fig 4.8), we included the upstream and downstream genomic sequences, *rbcL* and *PRAI* respectively. Chloramphenicol resistance was used as a selection marker.

In cyanobacteria, often most of the population consists of cells with 3-6 copies of the genome⁷. It is common to verify that colonies produced after a transformation are complete genomic knock-ins, i.e. all the genomic copies have been altered. When we carried out such a test on the *rbcS*-PP7 fusion insertion into the genome (Fig 4.9) we found the despite the fact the colonies and cultures were all chloramphenicol resistance, only a small fraction of the genomes took up our PP7 fusion insertion. In Fig 4.9, this can be seen from the PCR products on the gel. In column 5, the PP7 fusion



Figure 4.10: Schematic for inserting chloramphenicol resistance marker gene into *Synechococcus elongatus* to generate a strain which could control for growth rate changes due to presence of an antibiotic and a marker gene.

would have resulted in a product of length 2566 bp using the primer locations shown, while the WT genomic copy would yield a band of length nearly 1500 bp. We see that while the PP7 insertion is present in the transformed cells (Fig 4.9A - column 3), the overwhelming majority if the locations are still WT (Fig 4.9A - column 3). We hypothesized that this reflects a severe fitness disadvantage on creating a rbc-PP7 fusion, which might not function at optimal rates in the carboxysome. We maintained this stain with chloramphenicol, and wanted to test if including a 2D scaffold and localization with carbonic anhydrase (CA) might help rescue the fitness for PP7-rbcS fusions.

However, we also wanted to test whether the current strain (a mix of WT and PP7-rbcS genomes) were already showing a fitness disadvantage. To test this, we cloned a control construct, which expresses only the chloramphenicol resistance gene at the neutral site 3 (Fig 4.10) and compared the its transformants with those tested in Fig 4.9. The growth curves in Fig 4.11 show that no growth rate difference was noticeable and that the current mixed strain maintained close to WT growth rates.

CARBONIC ANHYDRASE FUSION WITH MS₂

The genome of *S. elongatus* PCC 7942 carries three ORFs labeled with putative carbonic anhydrase activity. ORF 1388 codes for ecaA, an α -CA that localizes to the periplasm and is believed to play a role in the regulation of CO₂/HCO₃⁻ as a part of the carbon concentrating mechanism¹⁶³. On the other hand, ORF 1447 is the thought to code for the major carbonic anhydrase ccaA associated with cyanobacterial carboxysomes¹⁵⁴.

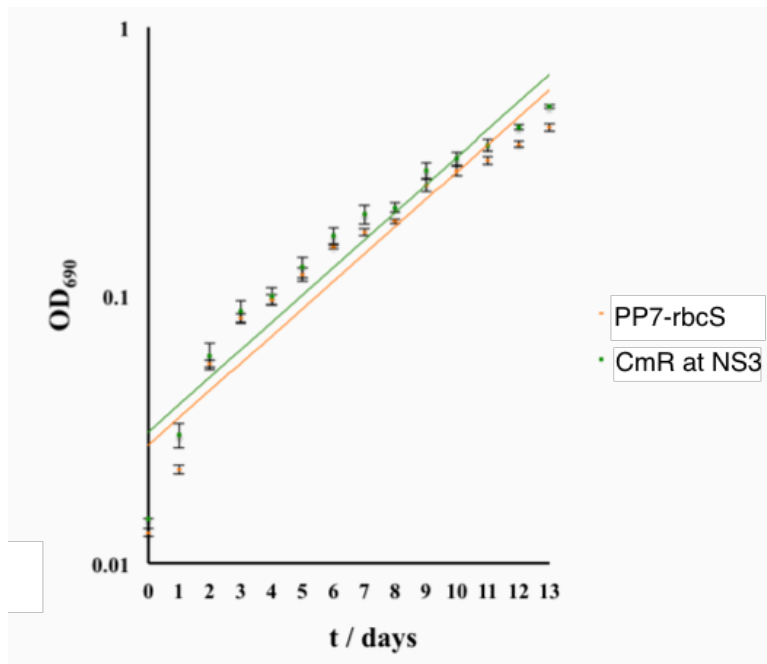


Figure 4.11: Comparing growth rates for transformed strains of *Synechococcus elongatus* - the green fit shows estimated log phase growth for a strain carrying just the chloramphenicol resistance marker, and the orange fit is growth of the mixed PP7-rbcS/WT genome carrying chloramphenicol resistant strain. The slopes of the two are the same - revealing comparable growth rates.

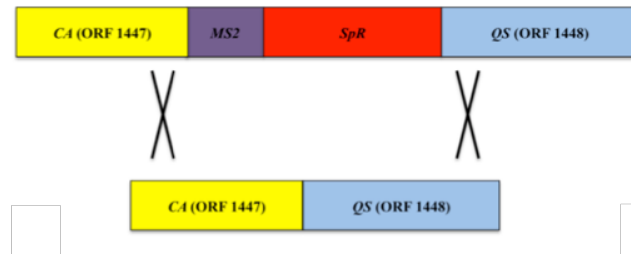


Figure 4.12: Schematic for inserting *ccaA*-MS2 fusion into *Synechococcus elongatus* at the ORF 1447, i.e. the native genomic location for the *ccaA* gene.

Finally, the protein *ccmM*, known to be a carboxysomal shell component, also bears homology to archaeon γ -CA at its N-terminus, though no direct evidence of its catalytic activity exists¹⁶⁴.

Thus, we picked the protein *ccaA* (encoded by ORF 1447) to make a C-terminal fusion with the MS2 aptamer binding domain (Fig 4.12). As before, the upstream and downstream sequences (ORFs 1447 and 1448) were flanking the MS2 fusion region and a spectinomycin resistance marker, to allow genomic integrations (Fig 4.12). Unlike with the *rubisco* fusion, this time we were able to achieve complete integration of the fusion into all copies of the genome. Once again, the strains carrying these fusions of *ccaA*-MS2 did not show any growth defect compared to WT.

4.2.3 TESTING CARBON FIXATION ON SCAFFOLDS IN A CARBOXYSONE-AFFECTED STRAIN

Next, we needed to create a strain with dysfunctional carboxysomes, so that we could show that localizing of *rubisco* and carbonic anhydrase on 2D RNA scaffolds could rescue their growth phenotype. The dysfunction of central components of the carbon concentrating mechanism and the carboxysomal shell proteins results in a high carbon

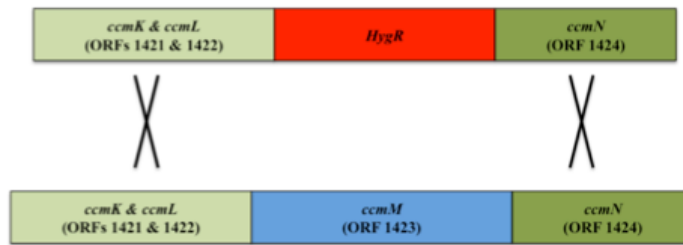


Figure 4.13: Scheme to knock out carboxysomal shell protein *ccmM* from *Synechococcus elongatus* by inserting a selectable hygromycin resistance marker in its genomic location

requiring (*hcr*) phenotype. The *hcr* phenotype is characterized by the need for elevated levels of CO₂ in media (or environment) to support growth¹⁶⁰. It has been shown that specifically, the deletion of a protein discussed above, *ccmM* leads to a the high carbon requiring phenotype since *ccmM* is essential for functional carboxysomal assembly¹⁶⁵.

We designed the construct shown in Fig 4.13 to knock out the *ccmM* expression (ORF 1423) and replace it with a selectable hygromycin antibiotic resistance marker. We could show that this strain had a high carbon requiring phenotype, as seen in Fig 4.14. As can be seen in Fig 4.14A that the growth rate for the hygromycin resistant strain with *ccmM* knocked out has a growth rate in air that is significantly lower than the WT growth rate. However, the strain's growth rate is comparable to WT levels under high carbon (2% CO₂) conditions (Fig 4.14B).

With all the parts in place, we decided to test whether co-expression of the PP7-*rbcS* and *ccaA*-MS2 fusions with the 2D scaffold strands carrying PP7 and MS2 aptamers will be able to rescue the high carbon requiring phenotype of a *ccmM* knockout strain. For this, a strain was created that contained all of the mutations described above and was tested for growth rate, with controls, both in air and high CO₂ conditions (Fig 4.15). As expected, all strains showed healthy growth rates in 2% carbon dioxide envi-

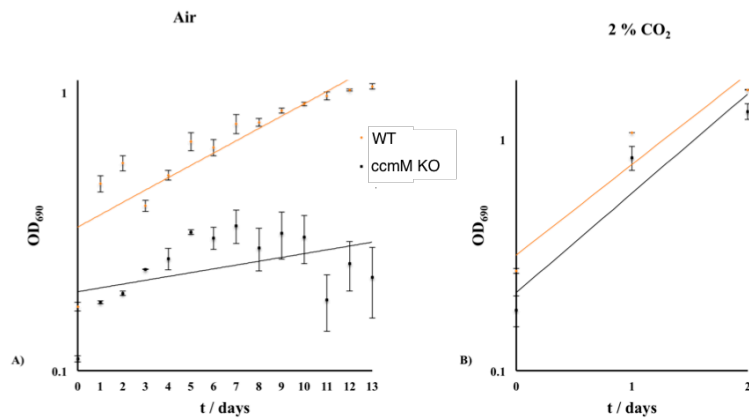


Figure 4.14: High carbon dioxide dependent growth in *Synechococcus elongatus* lacking carboxysomal shell protein. The orange curves are fits for WT growth and the black curves fit growth of the ccmM knock out strain. (A) Growth measured in batch culture in flasks grown in air, (B) growth measured from batch culture flasks in a 2% CO₂ incubator

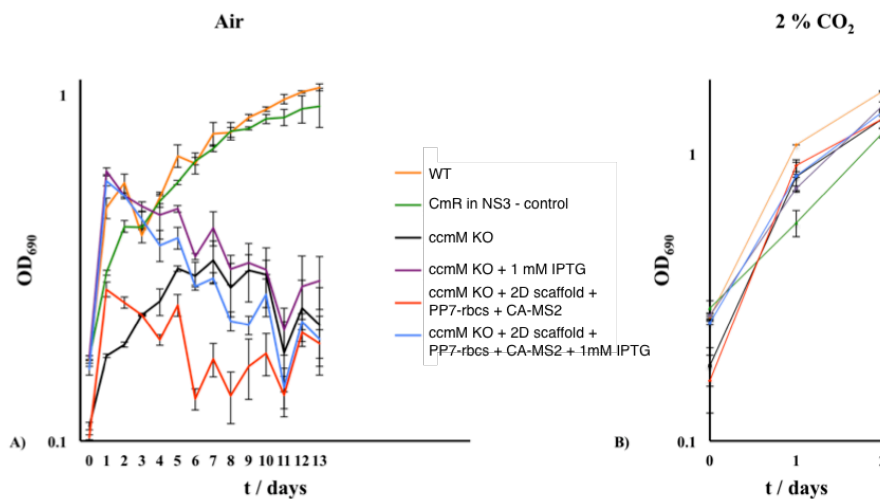


Figure 4.15: Testing localization of carbon fixation on RNA scaffolds in *Synechococcus elongatus* lacking carboxysomal shell protein. Growth rates for the various positive control (WT, and CmR), negative control (ccmM KO) and tested (ccmM KO + 2D scaffold + PP7-rbcS + ccaA-MS2) strains are shown from batch cultures in (A) air, and (B) 2% CO₂

ronment (Fig 4.15B). WT and the chloramphenicol resistant strains showed normal growth rates in air (Fig 4.15A). The *ccmM* knock out strain, expected to be the negative control here, did not grow in air. However, the *ccmM* knock out strain carrying PP7-*rbcS*, *ccaA*-MS2 fusions, and with the scaffolding system induced with 1mM IPTG, did not show growth either (Fig 4.15A). Hence, in its first pass, the system was unable to mimic carboxysomal function and rescue the *hcr* phenotype of a *ccmM* knockout strain. We believe that even there can be several ways to design this system more effectively and there are also likely to be some conditions that are less stringent than air, where such a system may show an effect. Regarding the former, since the rubisco hexadecamer is sterically larger than most holoenzymes (at nearly 8 nm across), is likely that the current 2D scaffold design, with just a 6bp-linker (see Fig 3.7) connecting the two aptamers, might be too crowded to see effective distribution of rubisco with carbonic anhydrase. Even if the 2D scaffold is able to channel some of the CO₂ locally to rubisco holoenzymes associated with the 2D scaffold, the increase in local concentration may not be high enough for rubisco to preferentially interact with CO₂ instead of O₂. Testing a new set of designs at intermediate CO₂ concentrations (between 0.04% and 2%) may be important to find a regime where function is revealed.

4.2.4 DIRECTIONS

In this section we showed that RNA scaffolds can be expressed in *S. elongatus* and attempted to use them for demonstration of carbon dioxide channeling. The T7 RNA polymerase based RNA scaffold system can be generally used for many other applications in cyanobacteria. RNA based tools for sub-cellular organization can help us probe unanswered questions in basic biology¹⁶⁶, customize metabolism in synthetic compartments¹⁶⁷, or even help us be able transfer efficient bacterial mechanisms like

carbon concentrating, into plants to increase their carbon fixation efficiencies¹⁶⁸. As we have seen in Chapter 3, RNA scaffolds offer customizable control over precise location of, and flux through co-localized enzymes, which when combined with a photosynthetic chassis¹²⁰ opens doors for cheap, renewable, production of biochemical products directly in oceanic or marine environments.

There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one: and that, whilst this planet has gone circling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved.

Charles Darwin, *On the origin of species by means of natural selection*¹⁶⁹

5

Conclusion

Today, synthetic biology has brought us to the brink of creating life itself¹⁷⁰. Evolution through natural selection¹⁶⁹ provides us with many diverse templates revealing how biological systems achieve complexity¹⁷¹, robustness¹⁷², and adaptive increases in efficiency¹⁵². Here we have demonstrated means of engineering bacterial systems with biologically inspired design to achieve increased metabolic outputs.

We were able to expand the toolkit for expressing *in vivo* RNA scaffolds for pro-

teins. Complementation of split GFP fragments served as a reliable assay for characterizing function of these new scaffolds in bacteria. We then went on to localize metabolic enzymes from two different enzymatic pathways inside cells. Our scaffolds were able to tether 2-4 enzymes, and channel intermediate molecules as diverse as fatty aldehydes and NADH co-factors. The design flexibility of RNA offered us an opportunity to study the effect of changing enzyme orientations on pathway yield. We presented a model that explains our experimental results wherein we see geometrically dependent metabolite channeling on *in vivo* RNA scaffolds.

We also showed that electronic flow in cyanobacteria can be rewired for biological H₂ production. We could demonstrate both light dependent hydrogen synthesis and hydrogen dependent cell growth. The latter can be accessed for directed evolution of new enzymatic properties and pathways. We end with showing that RNA scaffolds can also be expressed in cyanobacteria, enabling the combination of spatial metabolic control with renewable, photo-biological production.

Metabolic engineering is entering a new era propelled by cheaper gene-synthesis technologies¹⁷³, a better understanding of metabolic regulation¹⁷⁴, tools for spatial control¹⁷⁵, and an advanced ability to design enzyme catalysis¹⁷⁶. Additionally, *in silico* metabolic models are more rigorous than ever⁸. Thus, biorefineries, which are already affecting substantial global impact¹⁷⁶ can be expected to play a more significant role across manufacturing industries in the future^{177,178}.

RNA is a powerful tool to synthetic biologists. RNA scaffolds can be composed of many structural, dynamic and functional regions. As we have shown, the structure of RNA can be designed predictably, and there are several assays being developed for proper assembly. In addition, recent advances in DNA construction^{173,179} have made *in vivo* testing of designs faster and more reliable.

Synthetic biologists are constantly seeking to increase the complexity of their devices. RNA synthetic biology is offering tools to enable such control¹⁸⁰. One such goal is the construction of synthetic ribosomes¹⁸¹, capable of orthogonally incorporating non-natural amino acids wherein altered tRNA-protein interactions enable an expanded genetic code¹⁸². RNA scaffolds are also being employed to devise more precise genome editing tools¹⁸³. Therapeutic applications of *in vivo* RNA scaffolds include functionalizing natural RNA scaffolds to enable drug delivery or isolation of pure samples. Similar developments in the fields of DNA packaging and origami for drug delivery^{184,28} could offer strong synergistic opportunities for clinically applicable technologies to be implemented. More generally, the ability to simulate and predict the dynamics of structure-receptor binding interactions should enhance the design of such therapeutics¹¹⁶.

Moving forward, innovations in high throughput design, synthesis and assaying functions for RNA structures will enable a greater range of applications to be developed. Developments in chip-based synthesis^{179,185}, new structure assembly assays such as SHAPE-seq¹⁸⁶, and improved genetically encodable electron microscopy tags^{187,188} are enabling unprecedented scales of RNA synthesis, characterization and testing. Thus, the discovery of a variety of natural RNA structures and functions, an ever increasing understanding of how such features can be designed and an ability to rapidly implement and test ideas are indicators of a significant role for RNA scaffolds in future synthetic biology applications.

A

Supplementary information for *in vivo* RNA scaffold use

This appendix contains supplemental information* for experiments and designs described in Chapters 2 and 3. It presents detailed sequence information about the new

*The material in this section also forms the supporting online material for the published work

RBD	Amino acid Sequence	Aptamer RNA sequence
RevN7D	TRQARRDRRRRWREERQR	GACAAGUUGGUCCGCACAGUUGCGAGGUGU
RevR11	TRQARRNRRRQWRERQR	CGCUUAUGGUCAUUGAGUAUCUCCUGCCGA
BMV	KMTRAQRRAAARRNRWTAR	GGCGGUGGGUUUGGAAAACGGUAACGGGCA
Lambda	MDAQTRRRERRAEKQAQWK	GGGCCUGAAGAAGGGCCC
BIV-Tat	SGPRPRGTRGKGRIRIR	GGCUCGUGUAGCUCAUUAGCUCGAGCC
HTLV-1 Rex	MPKTRRRPRRSQRKRP	GCUCAGGUCGAGGTACGCAAGTACCUCCUUG GAGC

Figure A.1: Sequence information for new aptamer-RNA binding domain pairs

aptamers tested and used on RNA scaffolds, sequence information about the RNA scaffold strands themselves, a list of plasmids that they were characterized, and information about the development of models shown in Figs. 3.7 and 3.8.

RNA BINDING DOMAIN-APTAMER SETS

The sequences for arginine-rich, short RNA binding peptide domains and their corresponding RNA ligands⁹⁷ added to our toolkit of aptamers for use on *in vivo* RNA scaffolds are shown in Fig A.1.

The new RNA binding domain-aptamer sets are mutually orthogonal, as seen from the table of K_d values in A.2 of RNA binding domains (RBDs) tested *in vitro* and corresponding target aptamers (as reported in Bayer et al⁹⁷).

We picked BIV-Tat from this list since it has an X-ray crystal structure reported¹⁰⁰ and showed high affinity to its aptamer in the above reports.

CLONING AND EXPRESSION STRATEGY

The following table lists the plasmid systems used for expression of the various components in *E. coli*.

	RevN7D	RevR11Q	BMV Gag	Lambda N	BIV Tat	HTLV-1 Rex
anti-RevN7D	120	>1024	>1024	N.T.	>1024	>1024
anti-RevR11Q	>1024	100	>1024	N.T.	>1024	>1024
Anti-BMV	>1024	>1024	20	N.T.	>1024	>1024
Lambda box	>1024	>1024	>1024	90	>1024	>1024
BIV TAR	>1024	>1024	>1024	>1024	60	>1024
HTLV XBE	>1024	>1024	>1024	>1024	>1024	270

Figure A.2: Table showing K_d values for new RNA binding domain-aptamer sets. First row contains the names of RNA binding domains (RBD). The first column is the list of the corresponding aptamers. Each entry in the table represents the tested dissociation constants (K_d) in nM, as tested and reported in Bayer et al ⁹⁷. N.T.: not tested.

The following tables list DNA sequences used for expression of various RNA scaffolds and

The Do, and D₁ correspond to sequences that form discrete scaffolds, and one-dimensional polymerizing scaffolds respectively, as described in the main text and by Delebecque et al ^{13,85}. The two-dimensional polymerizing scaffolds are formed by two different strands, 2D_A and 2D_B, tiling together in the pattern shown in Fig 2.1. The geometric variants for the stem loop presenting the anti-BIV-Tat aptamer were created as shown with the 1-11 base-pairs added to the original 2D_B design stem loop.

MODEL DEVELOPMENT FOR FLUX TRANSFER ON SCAFFOLDS

The scaffold for co-localization of AAR and DCB used in this work is based on the 2D design of Delebecque et al. ¹³, with the difference that the BIV-Tat aptamer now replaces the MS2 aptamer used in that work.[†] This change is not expected to affect the overall scaffold structure, as it lies outside the sequences that mediate scaffold

[†]The model developed in this section was worked out and explained with help from Jeffrey Way.

Plasmid Name	Vector Backbone (resistance)	Expressed sequences
pPSGS088	pACYCDuet1 (Cm)	GFP _A _(Gly ₄ SerGly ₄)_ RevN7D
pPSGS089	pACYCDuet1 (Cm)	GFP _A _(Gly ₄ SerGly ₄)_ RevR11Q
pPSGS090	pACYCDuet1 (Cm)	GFP _A _(Gly ₄ SerGly ₄)_ BMV Gag
pPSGS091	pACYCDuet1 (Cm)	GFP _A _(Gly ₄ SerGly ₄)_ Lambda N
pPSGS092	pACYCDuet1 (Cm)	GFP _A _(Gly ₄ SerGly ₄)_ BIV-Tat
pPSGS093	pACYCDuet1 (Cm)	GFP _A _(Gly ₄ SerGly ₄)_ HTLV-1 Rex
pPSGS094	pCOLADuet1 (Kan)	GFP _B _(Gly ₄ SerGly ₄)_ RevN7D
pPSGS095	pCOLADuet1 (Kan)	GFP _B _(Gly ₄ SerGly ₄)_ RevR11Q
pPSGS096	pCOLADuet1 (Kan)	GFP _B _(Gly ₄ SerGly ₄)_ BMV Gag
pPSGS097	pCOLADuet1 (Kan)	GFP _B _(Gly ₄ SerGly ₄)_ Lambda N
pPSGS098	pCOLADuet1 (Kan)	GFP _B _(Gly ₄ SerGly ₄)_ BIV-Tat
pPSGS099	pCOLADuet1 (Kan)	GFP _B _(Gly ₄ SerGly ₄)_ HTLV-1 Rex
pPSGS100	pACYCDuet1 (Cm)	BIVTAT_(6xHis)_SerGlySer_AAR (6xHis)_SerGlySer_PP7_(Gly ₄ SerGly ₄)_ADO
pPSGS139 (Fig S1)	pACYCDuet1 (Cm)	BIVTAT_(6xHis)_SerGlySer_AAR (Strep-Tag)_SerGlySer_PP7_(Gly ₄ SerGly ₄)_ADO
pPSGS083 pPSGS087, pPSGS118 pPSGS125-135	pETDuet1 (Amp)	Please see Scaffold Expression table for details of relevant DNA sequences expressed (2D scaffold vector shown in Fig S2)
pPSGS153	pETDuet1 (Amp)	2D scaffold with anti-RevR11Q and MS2
pPSGS144	pACYCDuet1 (Cm)	PP7-PYC, mdh-BIV-TAT, lambda-FDH
pPSGS146	pETDuet1 (Amp)	2D scaffold with PP7, anti-BIV-TAT, and anti-lambdaN
pPSGS151	pACYCDuet1 (Cm)	RevR11Q-eCA, PP7-PYC, mdh-BIV-TAT, lambda-FDH
pPSGS152	pETDuet1 (Amp)	2D scaffold with annti-RevR11Q, PP7, anti-BIV-TAT, and anti-lambdaN

Figure A.3: List of plasmids used for Chapters 2 and 3.

Scaffold Expression	DNA sequence
D0 (BIV-Tat and PP7) pPSGS083	GGAGGACTCGGCTCGTGTAGCTCATTAGCTCCGAGCC GAGTCCTCGAATACGAGCTGGGCACAGAAGATATGGCTTCGTGCC CAGGAAGTGTTCGCACTTCTCTCGTATTCGATTCCC_<T7TERM>
D1 (BIV-Tat and PP7) pPSGS087	GGGTAGGCGCCTAGCCTAATGTACATTAAGTTATTTTTCCGGATGAATAG AATATAATTCTAATAACGCAGGACTC GGCTCGTGTAGCTCATTAGCTCCGAGCC GAGTCCTCGAATACGAGCTGGGCACAGAAGATATGGCTTCGTGCC CAGGAAGTGTTCGCACTTCTCTCGTATTCGATTGCG_<T7TERM>
2D_A (PP7) pPSGS118	GGGTCAGGAATCCTCCTGATAGCTATTTGGACAATTACGTACGTAGTTGA TGACAACTACATGAAAATAAGGGCACAGAAGATATGGCTTCGTGCC CTCTAGA_<T7TERM>
2D_B (BIV-Tat) pPSGS118	GGGTAGTTGTTATGGATTCTGATTATGGGACCCT GGCTCGTGTAGCTCATTAGCTCCGAGCC AGGGTCC_ ACTAGT_<T7TERM>
2D_BIV_variants (2D_B) pPSGS125-135 (Default 7bp stem)	GGGTAGTTGTTATGGATTCTGATTATGG ACCCT_<Rota-Variant> _GGCTCGTGTAGCTCATTAGCTCCGAGCC_<Rota-Var- ReverseComplement>_AGGGTCC_ ACTAGT_<T7TERM>
Rota-Variant-8bp stem	G
Rota-Variant-9bp stem	TA
Rota-Variant-10bp stem	GGA
Rota-Variant-11bp stem	UGGC
Rota-Variant-12bp stem	AGGGA
Rota-Variant-13bp stem	GAGACC
Rota-Variant-14bp stem	AGUGAUG
Rota-Variant-15bp stem	GUUCCACG
Rota-Variant-16bp stem	AGUUCGUGA
Rota-Variant-17bp stem	GCAGUUCAAA
Rota-Variant-18bp stem	UGGUCAUCAA
PP7 aptamer	GGCACAGAAGATATGGCTTCGTGCC
MS2 aptamer	CCACAGTCACTGGG
T7Term	CTAGCATAACCCCTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTG

Figure A.4: DNA sequences used for expression of various RNA scaffolds

RT-PCR primer	Primer sequence
2D_F	GGGTCAGGAATCCTCCTGATAGC
2D_R	GGTTATGCTAGTCTAGAGGGCACG
GAPA_F	ACTGACTGGTATGGGGTTCC
GAPA_R	AGGTTTAACGGCAGCTTTGA

Figure A.5: Primers used to measure scaffold RNA levels

assembly. The RNA structure is expected to form a unit tile of the form A_2B_2 . Six-base pair single-stranded sticky-end extensions emerge from the corners of the unit tile that allow connection to other tiles. The overall structure of assembled unit tiles is planar¹³. The RNA aptamers and T7 terminator element emerge from the 3' ends of the 6-bp extension and will be forced out of the plane of the unit tiles (Fig 2.1). Polymerization of the unit tiles via the 6-bp extensions is expected to force the PP7 and BIV-TAT aptamers that emerge from a given base-paired extension to be on the same side of the scaffold plane. This is because six base pairs correspond to about $\frac{1}{2}$ of a turn of an RNA double helix; the aptamers are attached to opposite strands. Thus, at the corners of the unit tiles, the two different aptamers will be in close proximity. (Figs 2.1, 3.7A) In principle, long-range interactions across the unit tile are also possible. For example, the assembly pattern in Fig 2.1 places diagonally related aptamers on one face of the scaffold plane and others on the opposite face. This configuration assumes a planar configuration around the Holliday junctions in the central RNA segments, and is based on: (1) an axis of 180 rotational symmetry around a y-axis drawn through the center of the structure, and (2) the shorter RNA, with the sequence UAGUUGUUAUGGAUCCUGAUUUAUG-BIVTAT, has 26 bases in the segment within the scaffold, corresponding to about $2\frac{1}{2}$ turns of a helix; so that the aptamers at the beginning and the end of such a segment would be expected to be on opposite faces of the scaffold plane. Alternative configurations are possible; for example, the RNA Holliday junctions could be tetrahedral rather than planar, and the RNA segments passing through the Holliday junctions could have additional base-pairs per turn, changing the relative emergence sites of aptamers in different parts of the scaffold.

The representations shown in Fig 3.7 and 3.8 represent the above established arrangement using PDB files, 2QUX¹¹³, 2A9X¹¹⁴, and 4KVQ¹¹⁵ from the NCBI protein

structure database. We have also used the Make-NA tool (James Stroud 2004, 2011) available on the University of Southern California servers for constructing the PDB representations of our stem and T7 terminator hairpins. The software Autodesk Maya (student version 2014) was used to render the 3D representation, along with a Molecular Maya toolkit (Digizyme).

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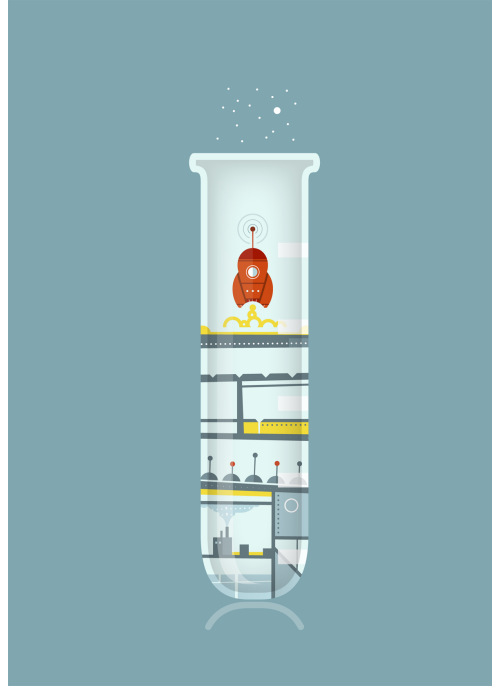
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