

Fluorescence Assay for Polymerase Arrival Rates

Austin Che
austin@ai.mit.edu

November 28, 2003

Abstract

To engineer complex synthetic biological systems will require modular design, assembly, and characterization strategies. The RNA *polymerase arrival rate* (PAR) is defined to be the rate that RNA polymerases arrive at a specified location on a DNA molecule. Designing and characterizing biological modules in terms of RNA polymerase arrival rates provides for many advantages in the construction and modeling of biological systems.

PARMESAN is an *in vitro* method for measuring polymerase arrival rates using pyrrolo-dC, a fluorescent DNA base that can substitute for cytosine. Pyrrolo-dC shows increased fluorescence when in single-stranded versus double-stranded DNA. During transcription, RNA polymerase separates the two strands of DNA, leading to a change in the fluorescence of pyrrolo-dC. By incorporating pyrrolo-dC at specific locations in the DNA, fluorescence changes can be taken as a direct measurement of the polymerase arrival rate.

1 Introduction

The desire of engineers is to design and build complex systems. With the most complex systems existing in Nature, understanding, simplifying, and engineering living systems is an irresistible challenge. Both biologists and engineers have begun the process of engineering synthetic circuits using biological components [7, 9, 25, 30, 35, 37].

A key feature necessary for designing and building complex biological systems is *modularity*. Modularity

simplifies the process of engineering, facilitating reuse and abstractions. Biological components should be capable of being built as interchangeable modules.

Requirements for modularity include a module *design* strategy, an *assembly* strategy, and a *characterization* strategy. The choice of a strategy in one area influences the choices in the other areas. The primary focus here is on module characterization, with the goal of simplifying the design and assembly process. Modules will be assumed to be specified entirely as a single linear sequence of DNA. Although other types of modules are possible, DNA modules are currently the easiest to work with in practice.

1.1 PAR

Not only is there no biological equivalent of a voltmeter, there is not even a definition of a standard volt unit. As most biological networks begin with transcription, transcription is a natural boundary for describing modules. We propose using RNA polymerase arrival rates (PAR), with units of polymerase arrivals per second (PAPS), to define the boundaries between modules. Polymerase arrival rates are defined as the rate that RNA polymerase arrives at a location on the DNA.

Modular design is facilitated by abstracting both module inputs and outputs as RNA polymerase arrival rates. Transfer curves that relate the outputs as a function of the inputs can then be used to completely specify the behavior of a module. In addition, modules can be connected interchangeably as the inputs and outputs for all modules are specified in identical units.



Figure 1: The two polymerase arrival rates in this example integrate information from multiple promoters and pause sites. RNA polymerase can initiate transcription from one of the two promoters and travel from left to right, arriving at the PAR_1 and PAR_2 locations on the DNA at some rate.

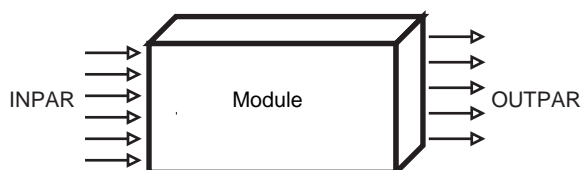


Figure 2: Modules will have some number of inputs and outputs measured in PARs. This is an example of a $\{6/5\}$ module.

The polymerase arrival rate integrates information from an entire system, taking into account polymerase binding rates, transcription initiation rates, elongation rates, and unbinding rates. For example, in Figure 1, the polymerase arrival rate, PAR_1 , depends on a variety of factors. Promoter 1 may be strong allowing polymerases to initiate transcription quickly, but the pause site may slow down those polymerases and kick some of them off the DNA. Promoter 2 may also allow some polymerases to bind and initiate. The number of polymerases arriving at PAR_1 includes the number of polymerases initiating from promoter 1 that pass the pause site and the number of polymerases initiating from promoter 2. The polymerase arrival rate PAR_2 at a location further downstream, in addition to everything that determined PAR_1 , depends on the elongation rate through the intervening sequence.

1.2 Modules

Figure 2 shows the general form for a module containing some number of input polymerase arrivals, INPARs, and some number of output polymerase arrivals, OUTPARs. A module that has i inputs and o outputs will be denoted as an $\{i/o\}$ module. The

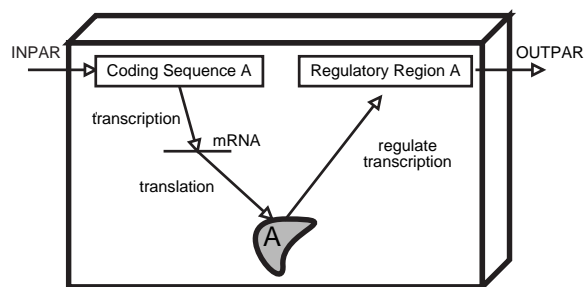


Figure 3: This simple $\{1/1\}$ module has a regulatory protein mapping the input PAR to the output PAR.

inputs and outputs of modules should be able to connect to other modules independently of each other.

1.2.1 Examples

Figure 3 shows a $\{1/1\}$ module with a single input and output. The polymerases arriving at its input lead to transcription and production of the transcriptional regulator protein A. Protein A regulates polymerase binding and transcription initiations from its regulatory region, causing polymerases to leave the module at a certain rate. The polymerases leaving the module are not necessarily the same polymerases that entered the module. The regulatory region for A does not have to be physically located immediately after the coding sequence for A.

The important **Insulator** module in Figure 4 can be considered a $\{1/0\}$ module. This module contains a transcriptional terminator that fixes the output at zero polymerase arrivals and so can be considered to not have an output. To make the module even more useful, the terminator is made bidirectional so that in both directions, the output PAR is zero. The bidirec-

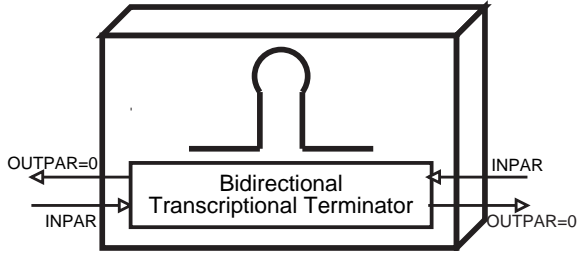


Figure 4: An example of an *Insulator* module consisting of a bidirectional transcriptional terminator. The output PAR on both sides is zero regardless of the inputs, thus insulating the modules on either side from each other.

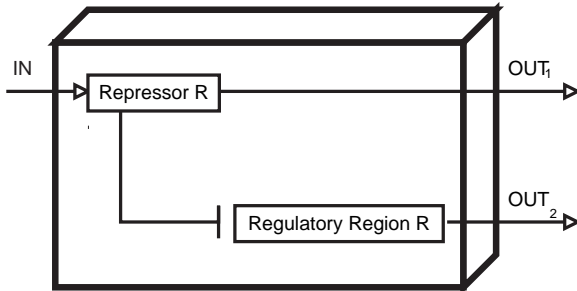


Figure 5: A $\{1/2\}$ inverting module where $OUT_1 = IN$ and $OUT_2 = NOT(IN)$.

tional transcriptional terminator could be a standard hairpin terminator with the correct bases on both sides to terminate transcription going in either direction. In effect, polymerases are blocked from going through the module in either direction.

A $\{0/1\}$ module would have no inputs and a single constant output PAR, as it is a function of no inputs. The typical $\{0/1\}$ module consists of a single constitutive promoter connected to the output. The promoter initiates transcription at a fixed rate, leading to a constant output PAR.

The $\{1/2\}$ module in Figure 5 is an inverter that also passes through the input PAR as an output. Polymerases entering from the input travel through the module and exit as OUT_1 , with the OUT_1 PAR roughly equal to the input PAR. The output PAR at OUT_2 is the logical NOT of the input.

1.3 Assumptions

Several important assumptions are made in order to effectively use PAR-modules.

- Each molecule of RNA polymerase, at any time, is in one of a finite number of states. For example, the states may include: unbound, bound to DNA, paused, arrested, or stably elongating. One state is defined as the normal state. For our purposes, a stably elongating RNA polymerase is the normal state. Module inputs and outputs are defined in terms of the rate of polymerases in the normal state arriving at the input or output boundary of the module. We assume polymerases in other states either do not arrive at the inputs and outputs or, if they do arrive in another state, they have no effect on the perceived behavior of the module.
- To ensure modules can be arbitrarily connected together, the input and output PARs are assumed to be independent of the sequences coming before and after them. In other words, at the output locations, there should be little look-ahead by the polymerase, with the transcription rates being relatively independent of the downstream DNA sequence. At the inputs, the polymerase should behave independently of the sequence that comes before it. To ensure this, insulating sequences may need to be inserted around modules.
- The outputs should not depend on variables other than the inputs, allowing us to treat the module as a black box. Any module can, therefore, be abstracted or defined as a set of transfer curves that specify the outputs as a function of the inputs.

1.4 Motivation

PAR is defined in terms of a physically well-defined property of the system rather than based on an indirect and relative measurement of transcription. Relative definitions of transcription strength can be in arbitrary units such as amount of GFP fluorescence

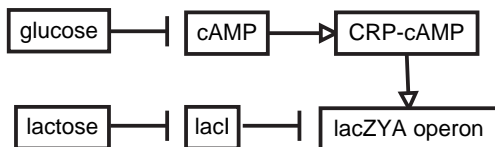


Figure 6: A conventional model representing a biological network of the lac operon. The pointed arrows represent positive regulation and the straight line heads represent negative regulation. The boxed nodes have non-interchangeable inputs and outputs.

or enzymatic activity relative to another promoter. With relative definitions, measurements of transcription only have meaning when compared with other measurements of the same type. PAR is an intrinsic feature of a biological system that is well-defined, independently of how it is measured.

1.4.1 Conventional Models

The conventional boundary for a module is at the level of translation, with the signal conveying information taken as the level of protein expressed. Although the polymerase arrival rate concept uses transcription rates to characterize modules in an unconventional way, specifying modules as PAR provides many advantages for the engineer over the standard biological models. Figure 6 shows a conventional network diagram for the well-studied *lac* operon and its regulation by glucose and lactose [24]. The boxes in the diagram represent nodes and different arrows are used to represent positive or negative regulation.

Although these models may be useful to biologists studying a fixed system, it is not at all useful for the engineer trying to build a new system. Conventional biological networks do not use a common unit for specifying connections, with inputs and outputs usually not specifically defined or defined in incompatible units, such as specific protein or RNA levels. Therefore, the nodes are not modular and cannot be re-wired or re-used easily in a meaningful way.

To allow arbitrary connections among modules, inputs and outputs need to be generic and not based on

a particular RNA sequence or protein. Thus, instead of levels, the rate that a common molecule, such as RNA polymerase, crosses between modules is used as the signal.

1.4.2 Transcription vs. Translation

Instead of polymerase arrival rates, it is possible to imagine using a similar rate for translation, the ribosome arrival rate. However, there are several reasons why the analogous approach for translation does not work as well as for transcription.

Whatever is used to define modules immediately restricts the type of modules allowed. If modules are defined at the translation level, then non-translational modules are immediately ruled out. Whereas not all genes are necessarily translated, transcription, the process of making RNA from DNA is common to the expression of all genes.

Another consideration is the type of modules that need to be built. For building interesting networks, we need many modules that can perform some type of regulatory control decision. Although translation can be regulated, most known regulatory mechanisms in existing biological systems appear to be transcriptional.

Translational regulatory networks are also much more difficult to separate into modules compared with transcriptional networks. Most regulators are proteins that themselves have to be transcribed and translated and, for these and other proteins, the desired protein sequence should be expressed. The problem with translation is that most regulation occurs around the start codon, which conflicts with the goal of controlling the exact protein sequence to be translated [4]. Transcriptional regulation also occurs around the transcriptional initiation point, but changing a 5' untranslated region of mRNA is not incompatible with expressing a desired protein sequence.

1.4.3 Non-transcriptional Interfaces

To increase the generality and usefulness of PAR-systems, it is feasible to have non-transcriptional events interact with PAR-modules. For example, al-

though module inputs and outputs are specified as PARs, it would be straightforward to generalize modules to have inputs and outputs that could be in different units in addition to PARs.

For example, a $\{0/1\}$ module could have an output that depended on the concentration of some molecule in the environment. The module would have no input PAR, but would have an input determined by the concentration of another molecule. A $\{1/0\}$ reporter module may contain only the coding sequence for a reporter protein like GFP. The input PAR is translated into some amount of GFP fluorescence. These types of modules could increase the generality of PAR-based systems by allowing modules to map between non-PAR signals, such as protein levels, and PAR signals.

1.4.4 Modularity

The most important motivating factor for using polymerase arrival rates are the benefits from modularity. Using polymerase arrival rates satisfies many desirable properties for a module characterization system:

- *Abstraction.* A non-abstract characterization method would be the DNA sequence of the module itself. Although the sequence contains all the information about the module, it lacks a useful abstraction layer needed for modular design. Using PAR effectively abstracts away the inner workings of the module, allowing a module to be specified as transfer curves mapping inputs to outputs.
- *Generality.* All modules should be characterized in a similar fashion, independent of what is inside the module. It is not general to characterize one module in terms of protein expression level and another module in terms of DNA binding strength.
- *Connectivity.* Connectivity means that an output for one module needs to match the input of another module, allowing for easy design and assembly of modules. The easiest way to ensure connectivity among modules is to have all inputs and outputs be in the same units. As all

module inputs and outputs are defined in terms of polymerase arrival rates, with the polymerase always arriving in the same state, the output of one module can be connected to the input of any other module.

- *Usefulness.* The characterization of a module must be useful in connecting and designing complex systems. An example of a non-useful characterization of a module is the %GC content, as it does not contribute to the goal of assembling systems from modules.
- *Measurable.* Requiring that modules be characterized with properties that cannot be measured is not useful. To be practical, the measurements should be easy to perform, not time consuming, accurate, and repeatable. PARMESAN, to be described later, is one proposed method to measure polymerase arrival rates.

1.5 Design and Assembly

Using PAR-modules only makes sense if it facilitates the design and assembly of systems that need to be built. Characterizing modules with polymerase arrival rates influences module design and assembly in a variety of ways.

1.5.1 Design

Modules must be designed around transcriptional events, as inputs and outputs are defined in terms of RNA polymerase arrival rates. Depending on the system that needs to be built, this may limit the inherent modularity possible. As all non-transcriptional events must be inside a module, if there are many non-transcriptional events, then modules may need to be larger, and therefore less modular, than desired.

Also, modules need to be designed carefully to limit undesirable interactions that affect the behavior of the module in an undefined manner. This may require adding insulating sequences within the module or describing the conditions under which a module can be used successfully with the specified behavior.

1.5.2 Assembly

The desired end system needs to be physically assembled into one linear piece of DNA containing all the modules connected correctly. To facilitate assembly of modules, where each module may have many inputs and outputs, the DNA for each input and output should be in its own separate tube *if possible*. Connecting the output of one module to the input of another module would then involve ligating the two corresponding pieces of DNA together using standard methods.

For some modules, it may not be possible to separate all inputs and outputs into different tubes. For example, for the module in Figure 5, OUT_1 cannot be disconnected from the input as the polymerases arriving at this output come directly from those that arrive at the input. On the other hand, OUT_2 , can be separated and placed into its own tube as it does not need to be physically near the repressor DNA for it to function correctly.

This inseparability of some inputs and outputs puts restrictions on how some modules can be connected, due to limitations in physically assembling the DNA. For example, for the module in Figure 5, OUT_1 cannot be connected back to the module's input, as this would require circularizing the DNA. Circular DNA is not practical or useful as it cannot be ligated with other pieces of DNA or cloned into a plasmid.

Once the appropriate inputs and outputs have been connected, the pieces need to be assembled into the complete final system, while preserving the expected behavior of the system. During the design process, the module designer ensures that inputs and outputs from different modules can be connected together by inserting insulating sequences within the module. The module assembler, on the other hand, needs to ensure that the behavior of the system is not changed by assembling the pieces of DNA and, so, needs to insert insulating sequences between modules. To connect the partially assembled input and output pieces together, the **Insulator** from Figure 4 or a similar module should be inserted to guarantee that polymerase arrivals from one piece do not carry over to

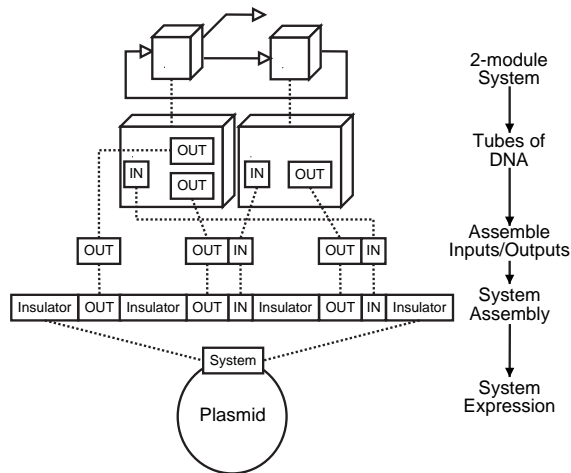


Figure 7: An example 2-module system is assembled from a schematic of how the modules should be connected. Each module has its inputs and outputs physically separated as tubes of DNA and module connections are made by ligating the corresponding input and output tubes. The pieces are assembled with additional insulating sequences.

the next, allowing the system to be assembled to the design specifications.

The linear piece of DNA representing the assembled system needs to then be cloned and maintained, probably on a plasmid vector. Again, to ensure that the sequences on the plasmid do not affect the behavior of the system, insulating transcriptional terminator sequences are needed on both ends of the system. Figure 7 depicts an overview of the assembly process.

1.6 PARMESAN

For PAR-modules to be practical, a method is needed to measure polymerase arrival rates. PARMESAN, short for Polymerase Arrival Rate Measurements in (En) Standard Assay *in vitro*, is one proposed method for measuring polymerase arrival rates using a fluorescence assay [4].

The fluorescence of the normal nucleic acid bases is low, and, in proteins, only tryptophan usually has significant fluorescence. Thus, to study protein and

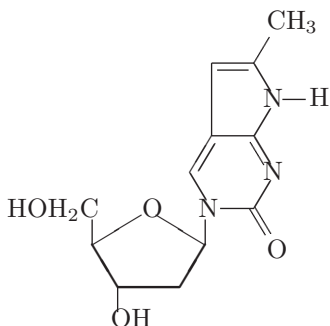


Figure 8: The chemical structure of the fluorescence base pyrrolo-dC allows it to substitute for cytosine in DNA. Pyrrolo-dC will be represented by the letter P.

DNA pairing	Fluorescence
dsDNA mismatched	High
ssDNA	Medium
dsDNA or RNA:DNA	Low

Figure 9: The relative fluorescence intensity of pyrrolo-dC in DNA varies depending on its environment.

DNA interactions, non-natural fluorescent probes are often used. PARMESAN uses the fluorescent DNA base, pyrrolo-dC, that is sensitive to the local melting of DNA.

1.6.1 Pyrrolo-dC

Pyrrolo-dC, shown in Figure 8, is a synthetic base able to form 3 hydrogen bonds with guanine and, thus, is able to substitute for cytosine in DNA [19, 20]. In addition, pyrrolo-dC has different fluorescence properties when it is in single-stranded or double-stranded DNA. The fluorescence of pyrrolo-dC in ssDNA is approximately double its fluorescence in dsDNA. The fluorescence when pyrrolo-dC is in a mismatched base pair in double-stranded DNA (e.g. paired with adenine) is even higher than in ssDNA. Also, the pyrrolo-dC fluorescence is low when in a RNA:DNA heteroduplex [20]. These fluorescence properties of pyrrolo-dC are summarized in Figure 9.

A similar fluorescent probe more commonly used is the base analogue 2-aminopurine (2-AP) that substitutes for adenine and base pairs with T only slightly weaker than the A-T pair. The fluorescence of 2-aminopurine is also sensitive to whether it is base paired.

Although 2-aminopurine has similar properties to pyrrolo-dC and been used extensively to study proteins that separate the strands of DNA, the excitation spectrum of 2-aminopurine overlaps with the intrinsic fluorescence of proteins making subtracting background measurements necessary [32]. Pyrrolo-dC has excitation maxima at 260nm and 350nm and an emission maximum at 460nm, far from the protein and nucleic acid fluorescence range [20]. The shifted spectrum of pyrrolo-dC should in theory lead to less background than 2-aminopurine.

1.6.2 Transcription Assay

By incorporating pyrrolo-dC at specific positions in a strand of DNA, fluorescence changes during transcription reactions can be used to measure the rate of RNA polymerase arrivals. In the transcription bubble, RNA polymerase separates the two strands of DNA, temporarily making single-stranded DNA from double-stranded DNA. Thus, as RNA polymerase transcribes past a location containing pyrrolo-dC, the fluorescence of the pyrrolo-dC should increase. Change in fluorescence can be used to measure polymerase arrivals at the specific location in the DNA marked with the pyrrolo-dC.

1.6.3 Measurement Issues

Pyrrolo-dC bases can be inserted on either the template or the non-template strand, potentially leading to differences in the fluorescence effect depending on the location of the pyrrolo-dC. An increase in fluorescence is only seen if a DNA region with pyrrolo-dC is separated and no RNA:DNA hybrid is formed. If the pyrrolo-dC is located on the template strand, then a RNA:DNA hybrid could quench the fluorescence, with increased fluorescence only when the pyrrolo-dC is in the transcription bubble but not paired with RNA.

The length of the RNA:DNA hybrid was determined using pyrrolo-dC for T7 RNA polymerase [20]. The transcription bubble for T7 RNA polymerase was determined to collapse close to the exiting RNA, meaning that the length of the RNA:DNA hybrid is about the same size as the transcription bubble. This means fluorescence from pyrrolo-dC bases on the template strand will almost always be quenched, either being in a DNA:DNA duplex or RNA:DNA heteroduplex.

For *E. coli*, there is disagreement about the length of the RNA:DNA pairing in the transcription bubble and the amount of unpaired DNA on the template strand [14, 15]. If the length of the RNA:DNA hybrid is small, then there would be a large number of exposed bases on the template strand in the transcription bubble, providing more time for a fluorescence signal to be seen.

Unlike the bases on the template strand, the bases on the non-template strand in the transcription bubble are oriented away from the enzyme, with the bases being susceptible to nucleases [34]. Thus, pyrrolo-dC bases should be unstacked on the non-template strand, showing an increase in fluorescence during strand separation by RNA polymerase.

1.7 Applications

Some biological applications become easier when working with modules based on polymerase arrival rates. Provided a module specified with input and output polymerase arrival rates, transfer curves can be used to completely describe the behavior of the module. It is desirable to have an easy method for measuring the transfer curves that specify a PAR-module. For any output, points along the transfer curve can be measured by fixing the inputs to known values. The entire transfer curve can be interpolated by measuring as many points as desired and used for modeling, comparisons, biological insights, or for other purposes.

Different types of natural biological phenomena can be modeled using a transcriptional rate model and by abstracting away non-transcriptional events into modules [5]. Using transcriptional modules and with reasonable measurements of transfer curves,

complex systems can be easily modeled. As the transfer curves, in theory, completely describe the behavior of modules, simulation is straightforward even with only limited points sampled from the transfer curve.

2 Methods

The experimental work has focused on applying the PARMESAN method to single promoters. The convention used here is to print DNA from left to right in the 5' to 3' direction and for the top strand to contain the promoter. Thus, the top DNA strand is the expected *non-template* strand, and the RNA polymerase will use the bottom DNA strand as the *template* strand. The RNA polymerase and systems described here all come from *E. coli*.

All promoters and other pieces of DNA to be measured are assumed to be in a standard BioBricks format [26]. Oligos containing pyrrolo-dC are synthesized that allow for the incorporation of the pyrrolo-dC into any BioBricks-conforming module. The following is the standard methodology used to measure fluorescence and transcription levels.

1. Synthesize standard oligos with pyrrolo-dC.
2. Use the standard oligos to incorporate pyrrolo-dC into promoters or other DNA regions to be measured. The two main ways of attaching a pyrrolo-dC are through ligation or through PCR.
3. Perform an *in vitro* transcription reaction by adding purified RNA polymerase.
4. Measure the fluorescence change after addition of the polymerase.
5. Interpret the data as polymerase arrivals at the location of the pyrrolo-dC.

More experimental details are provided in [4].

2.1 Oligos

Oligonucleotides were synthesized on an ABI 394 synthesizer using standard phosphoramidite chemistry.

The sequences and details for the synthesized oligos can be found in [4].

2.1.1 BB-R-P

One method of incorporating pyrrolo-dC into sequences is to run a PCR with primers containing pyrrolo-dC. The PCR primer BB-R-P, includes one pyrrolo-dC and was designed to match the BioBricks suffix. A PCR with BB-R-P and a forward primer incorporates the pyrrolo-dC on to the template strand immediately after the test promoter.

Another primer, BB-R-5P-TE, also matches the BioBricks suffix but contains 5 pyrrolo-dC and the TE terminator from T7. The terminator both slows down the polymerase and terminates transcription normally rather than have the polymerase fall off the end of the DNA.

2.1.2 his-6P

A pause site was synthesized as a means of slowing down the polymerase at a specified location, allowing for easier fluorescence measurements. Two oligos, his-top-6P and his-bot, were created to allow for the insertion of the *his* pause site containing 6 pyrrolo-dC bases on to the non-template strand. The sequence included 14 bp downstream of the pause site, as this is the region determined to affect the pause strength [16]. Several mutations were introduced into the native sequence to increase the pause strength and to increase the number of pyrrolo-dC.

2.1.3 PrnB

To test directly the effect of pyrrolo-dC due to DNA separation, a promoter was synthesized containing pyrrolo-dC incorporated in the region melted during polymerase binding. The promoter used was 85 bp from the *E. coli rrnB* promoter. As the melted region in the open complex extends from -10 to $+1$ [23], several of the cytosines in this region were replaced with pyrrolo-dC.

2.2 Experiments

Promoters were first amplified via PCR to incorporate standard BioBricks' ends. Then another PCR or ligation was used to incorporate the above pyrrolo-dC oligos into the promoter DNA.

2.2.1 Promoters

Promoter sequences were taken from PromEC [10] and checked for unwanted restriction sites. All promoter sequences were the same length, 100 bp, and have at least 25 bp after the transcription start, ensuring that the initial melted region does not include the downstream pyrrolo-dC region. Promoter-specific primers were designed with BioBricks' ends and a PCR done using *E. coli* genomic DNA as the template. The result was gel purified using Qiagen spin columns.

To test termination, some promoters were attached to the TE transcriptional terminator from bacteriophage T7 via ligation and PCR.

2.2.2 PCR

After the initial amplification of the promoters, standard BioBricks primers, BB-F and BB-R, were used to PCR the promoters again. To incorporate a pyrrolo-dC after the promoter, the oligo BB-R-P was substituted for BB-R in the PCR.

2.2.3 Ligations

Although the PCR method is an extremely simple and straightforward way of incorporating pyrrolo-dC, it has the disadvantage that the pyrrolo-dC can only be incorporated on the template strand. Ligations can place the pyrrolo-dC on either the template or non-template strands. Oligos that were designed to be incorporated by ligations contained a SpeI compatible overhang and were ligated with BioBricks-compatible promoters.

2.2.4 Transcription Experiments

For transcription experiments, the temperature in the plate reader was set to 31°C and fluorescence was

measured with and without RNA polymerase. Details of the transcription reactions are in [4].

3 Results

Some of the results from PARMESAN experiments are described, many of which were unexpected and still not fully explained.

3.1 Fluorescence Detection

Fluorescence was initially measured on a Bio-Tek FL600 fluorescence plate reader using fixed wavelength filters with an excitation filter of 360nm/40nm and an emission filter of 460nm/30nm (center wavelength/bandwidth). These filters were chosen based on published data about the spectrum of pyrrolo-dC [19] and from the manufacturer (Glen Research). Fluorescence values are in arbitrary units and were usually normalized to the first fluorescence reading.

3.2 Plates

The type of plate used was found to have an enormous effect on fluorescence readings. All plates were black 96-well plates. Across different types of plates from different sources, large variations were seen in background fluorescence readings.

3.2.1 High Binding Plates

Transcription reactions done on Dynex high-binding polystyrene plates showed a curious effect. Kinetics measurements showed a noticeable bell-shape curve, with both a dramatic increase and decrease in fluorescence over time. However, the bell-shaped curve was seen even when only RNA polymerase, BSA, or other proteins were placed into wells with water. An increase in fluorescence but no decrease was seen when the oligo his-top-6P was used (Figure 10).

Protein or DNA may have been binding to the wells of the plates. However, the excitation and emission wavelengths used should have been far from any intrinsic protein or DNA fluorescence. It is therefore not known why the fluorescence kinetics appear as

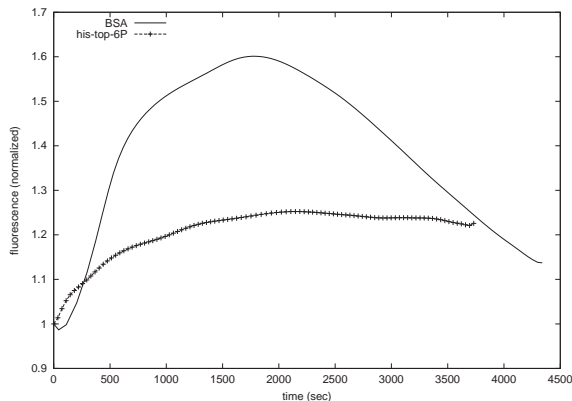


Figure 10: Fluorescence kinetics of bovine serum albumin (BSA) and his-top-6P in water on Dynex high binding plates show unexplained increases and decreases in fluorescence.

they do. By varying the measurement times and durations, the decrease in fluorescence seen in the curves do not appear to be due to photobleaching.

3.2.2 Medium Binding Plates

Another type of plates tried were Dynex clear bottom polystyrene plates, not treated to be high-binding and believed to be medium binding. Figure 11 shows the fluorescence measured for BSA in water. Although the magnitude of the increase is less than for the high-binding plates, a similar shaped curve is present on these medium-binding plates. Transcription reactions done with several promoters attached to his-6P also show upward and downward trends in fluorescence.

In Figure 12, the his-6P oligo by itself without polymerase shows a similar behavior as on the high-binding plates, with an increase but no decrease in fluorescence.

However, unlike the results on the high-binding plates, controls with only the polymerase or transcription reactions with promoters attached to a single pyrrolo-dC using BB-R-P, did not show the same bell curve but was flat (data not shown). The results indicates that the *his* pause site or the extra

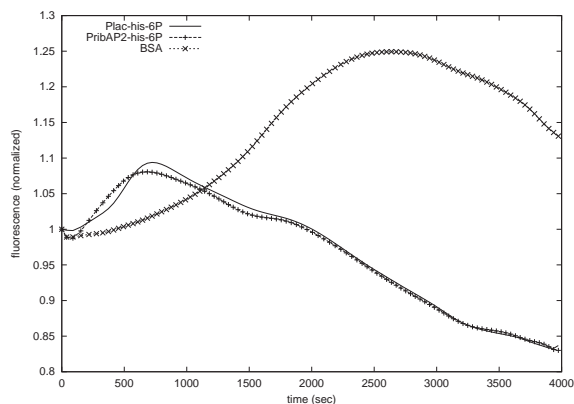


Figure 11: Measurements of BSA alone and promoters in transcription reactions on Dynex polystyrene non-high-binding plates show the same type of trend as seen on the high-binding plates.

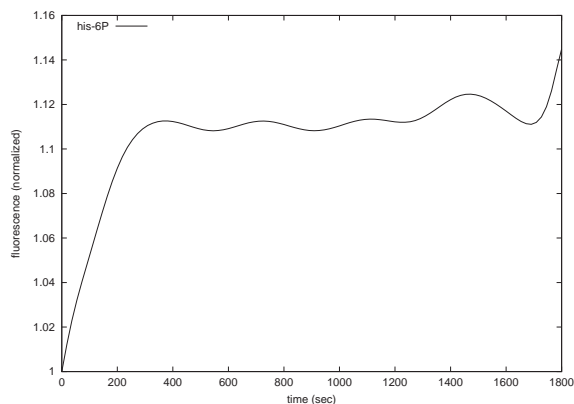


Figure 12: The kinetics of the his-6P oligo in buffer with NTPs and without RNA polymerase shows an increase but no decrease in fluorescence.

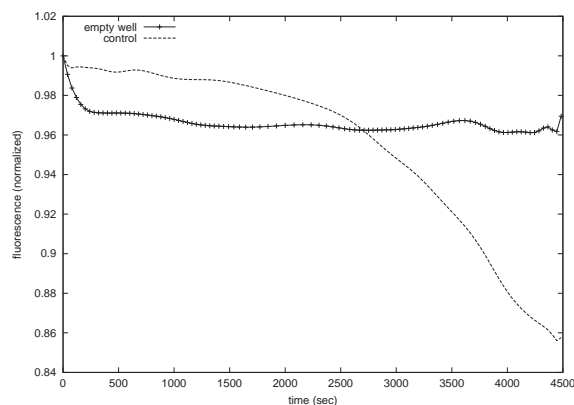


Figure 13: A fluorescence control without DNA on a polypropylene plate shows relatively flat kinetics followed by a decrease. The control well contained buffer, NTPs, and RNA polymerase.

pyrrolo-dC signals on the his-6P oligo may have been producing a measurable fluorescence effect, although probably not the expected effect due to transcription.

3.2.3 Polypropylene Plates

To test potential problems with protein binding to wells, polypropylene plates were tried, as the amount of binding on polypropylene was expected to be less than polystyrene. As seen in Figure 13, a control well on a Costar clear bottom plate containing RNA polymerase and no DNA shows relatively flat kinetics for a long time before a decrease in fluorescence, presumably due to bleaching.

Transcription reactions with Plac-his-6P and one that includes the TE terminator, Plac-TE-his-6P, were done simultaneously with the previous control. As can be seen in Figure 14, the bell-shaped curve is present again but can no longer be attributed to protein binding. Similar results were obtained with another promoter, PribAP2-his-6P and PribAP2-TE-his-6P, as seen in Figure 15.

Different curves were seen depending on the promoter and whether there was an attached transcriptional terminator. The increase in fluorescence was faster for the PribAP2 curves compared with the Plac

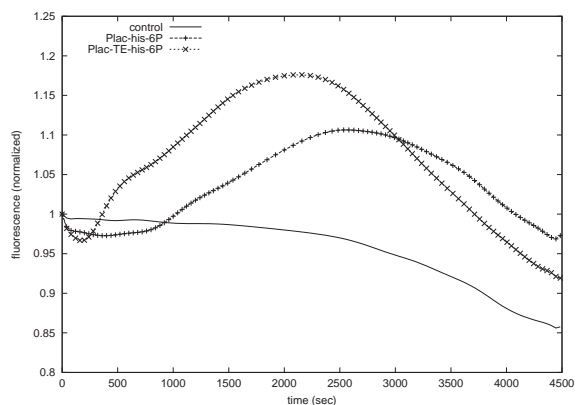


Figure 14: The kinetics of transcription reactions with the *Plac* promoter with and without a terminator show bell-shaped curves not present in the control. The control well is the same as in Figure 13.

curves. In addition, the terminator for both promoters shifted the peak to the left.

3.2.4 Corning NBS Plates

To reduce potential binding further, Corning NBS (non-binding surface) treated polystyrene, clear bottom, plates were used. In Figure 16, the fluorescence kinetics of his-top-6P and his-bot mixed together clearly show a decrease in fluorescence over time, indicating a quenching of fluorescence as the strands anneal.

Unlike all of the previous plates, transcription reactions and BSA controls on these NBS plates do not show a bell-shaped fluorescence curve and are essentially flat. As all measured fluorescence curves were flat, not many useful results could be determined using these plates.

3.2.5 Greiner Fluotrac 200 Plates

Another type of plates tried were Greiner Fluotrac 200, polystyrene, medium binding plates. At the same sensitivity setting, the background fluorescence of an empty well from a Greiner plate was about half that of a well from a Corning NBS plate.

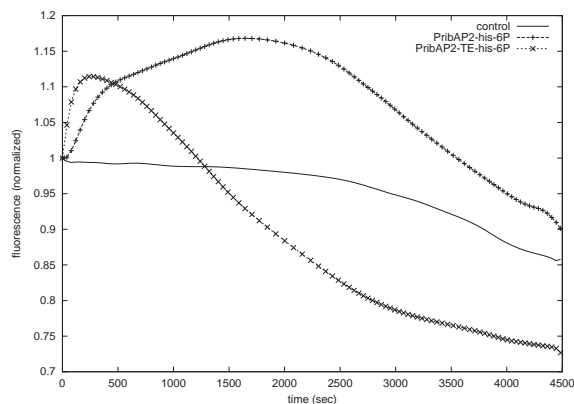


Figure 15: Transcription reactions with the *PribAP2* promoter with and without a terminator show similar results to the reactions with *Plac* above. The control well is the same as in Figure 13.

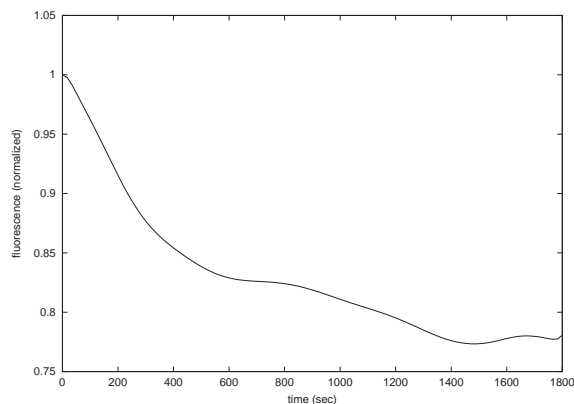


Figure 16: Fluorescence of his-top-6P annealing with his-bot shows a gradual decrease, due to quenching of the pyrrolo-dC fluorescence in double-stranded DNA.

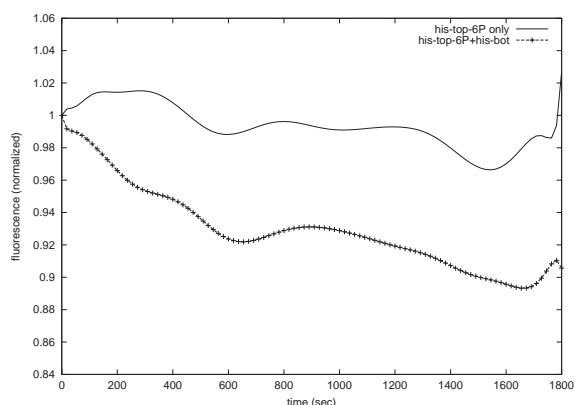


Figure 17: The kinetics of *his-top-6P* annealing with *his-bot* on Greiner plates shows a decrease in fluorescence as would be expected. No change in fluorescence is seen with only *his-top-6P*.

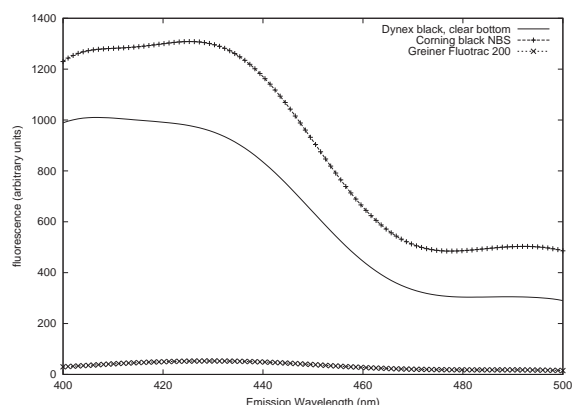


Figure 18: The emission spectrum measured from empty wells of different types of plates show that the Greiner plates have a much lower background fluorescence. Excitation was set at 360nm/12nm.

The fluorescence kinetics for BSA measured on these Greiner plates showed a similar bell curve as on the other plates. After measuring BSA, the well was emptied and rinsed with water, fresh BSA was put into the well, and the fluorescence measured again. The second measurements were extremely flat, not showing the same curve.

Thus, the bell-shaped curves seen on the plates are probably due to binding of protein to the well. After the protein saturates the well, it blocks further protein from binding. Thus, for all further experiments, these Greiner plates were used, and, before use, the wells were pre-soaked with BSA to protect the well from protein binding during the experiment.

The same annealing experiments were done as in Figure 16 on the Greiner plates. As seen in Figure 17, the fluorescence curve for the annealing condition shows a decrease over time, whereas the control reaction containing only the top oligo does not show an appreciable decrease in fluorescence.

3.3 Machine

Other plate readers were tried to compare their sensitivity and to obtain a fluorescence spectrum, something that was not possible on the Bio-Tek FL600

machine. The most useful results came from a Tecan Safire plate reader.

3.3.1 Plate Background

Using the Safire, the background fluorescence from empty wells of all the previous plates was measured. Excitation was fixed at 360nm/12nm and the emission scanned between 400nm and 500nm. All the plates had extraordinarily high fluorescence compared with the Greiner plates. Figure 18 shows the data for a couple of the plates. In addition to a high background, there is an unexplained sharp drop off at 450nm for most of these plates.

3.3.2 Pyrrolo-dC Fluorescence

In Figure 19, the emission and excitation spectrum of pyrrolo-dC was measured using a two nucleotide test oligo, PG. The excitation spectrum used an emission filter at 440nm and was similar to published data. The emission spectrum was measured using an excitation wavelength of 350nm/7.5nm, similar to the wavelength in a previously published report [19]. Surprisingly, the emission spectrum shows a peak around 440nm and a *minima* around 460nm. The shape of

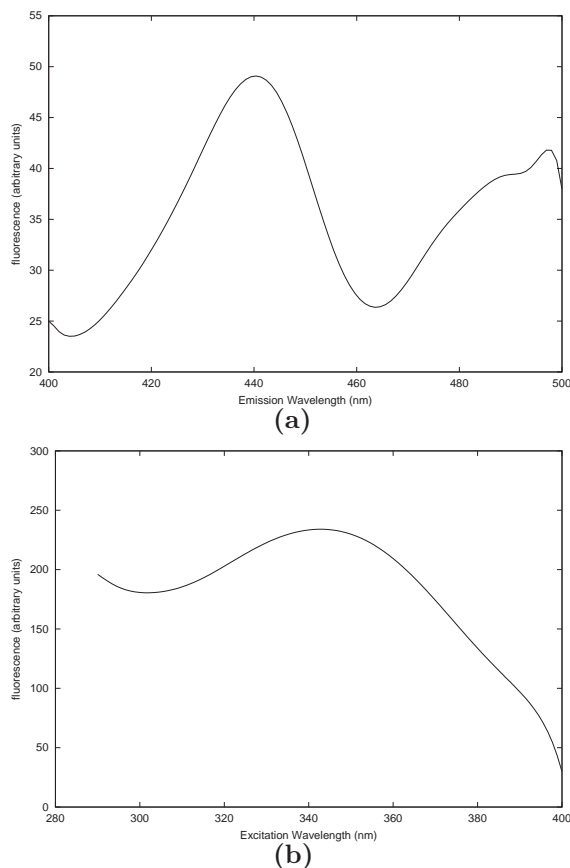


Figure 19: (a) The emission spectrum of pyrrolo-dC was measured with the oligo PG with excitation at 350nm/7.5nm. (b) The excitation spectrum of pyrrolo-dC was measured with the oligo PG with emission measured at 440nm/7.5nm.

the measured spectrum curve was quite different from the published emission spectrum, which was the data used as the original basis for choosing a 460nm emission filter.

Using a Greiner plate, the emission spectrum of a sample containing 4 μ M of the two nucleotide test oligo, PG, was measured both on the Safire reader and the Bio-Tek plate reader. The signal-to-noise ratio on the Bio-Tek was low, about 1.5, compared with 7 on the Safire. The relative insensitivity of the Bio-Tek fluorescence readings may partially explain some lack of results, especially on the original plates with high background. In addition, the emission filters being used may have been suboptimal. The highest sensitivity with the Bio-Tek machine using available filters was found with the excitation at 360nm and emission measured at 516nm/20nm.

3.4 Transcription Experiments

The following measurements were made on the Bio-Tek machine using Greiner plates with excitation at 360nm and emission at 516nm.

3.4.1 PrnB

To test that a change in fluorescence is measurable due to strand separation by RNA polymerase, the *rrnB* promoter was synthesized with 5 pyrrolo-dC bases in the initial melting region. After addition of RNA polymerase without NTPs to the promoter, the fluorescence increased (136%) but also decreased after some time in a manner that could not be attributed to bleaching. Under the same conditions, a control containing the RNA polymerase in water showed no change in fluorescence over time.

3.4.2 BB-R-P

Figure 20 shows an example of measuring fluorescence during a transcription experiment. Two promoters, Plac and PribAP2, had pyrrolo-dC incorporated on to the template strand via PCR with BB-R-P or BB-R-5P-TE. Controls were also done with BB-R to leave the promoter unlabeled. All PCRs were verified on an agarose gel.

Fluorescence measured immediately after the addition of RNA polymerase, without mixing, showed little change in fluorescence across the conditions. Fluorescence was measured again after mixing the wells, and the fluorescence for the control promoters went down while all other samples remained relatively constant.

The ratio of the fluorescence with RNA polymerase to without was calculated. For the controls, the fluorescence with polymerase goes down, giving ratios less than 1. However, the ratios are higher for the promoters with either a single or 5 pyrrolo-dC attached to the end, indicating a potential increase in fluorescence due to the RNA polymerase melting the region around the pyrrolo-dC.

It is unclear why fluorescence decreases for the promoter samples without pyrrolo-dC and does not decrease for the samples with pyrrolo-dC. In addition, the lack of a significant *increase* in fluorescence for the pyrrolo-dC samples is puzzling. Similar results were obtained in independently replicated experiments.

4 Discussion

We have presented the motivation for using polymerase arrival rates as the standard unit to describe modules. PAR depends on many things including the strength of RNA polymerase binding, percent of abortive transcripts, promoter clearance rate, elongation rate, dissociation rate, and re-transcription rates. The purpose of many biochemical studies is to separate out many of these effects to obtain individual kinetic rates, but this is not needed nor desired for the application of polymerase arrival rates.

Although PAR-modules provide a useful abstraction layer for modeling and manipulating biological systems, a method for measuring PAR is also critical. The goal of the PARMESAN method is to characterize PAR using a fluorescent assay. In theory, the PARMESAN method is a relatively easy and inexpensive way to assay transcription rates. In practice, after many failed experiments, only some decent results were obtained, leaving many open questions. We discuss several problems and possibilities for additional experiments.

4.1 Future Work

4.1.1 Method Soundness

The first set of experiments should be to confirm the soundness of using pyrrolo-dC fluorescence as a measure of transcription rates. Controls with pyrrolo-dC located in other places, for example, before a promoter, can be used to test that fluorescence changes are due to the location of the pyrrolo-dC. Fluorescence changes across promoters compared with known promoter strengths to determine if there is a relationship between fluorescence changes and promoter strength.

4.1.2 Template DNA

The linear DNA templates used may have problems with polymerases binding to the ends [21, 33]. There may also be issues involving runoff transcription. At the end of a DNA template, the polymerase presumably just falls off, but this is a boundary condition that may be handled differently than normal transcription. Forcing the polymerase to terminate transcription normally may be a more accurate indicator of its behavior in a real system.

4.1.3 Fluorescence Measurements

The major experimental problems have been with the fluorescence measurements, either with the plates or with the plate reader. Although the issues with the plates may be resolved, unresolved issues with the plate reader may still exist. It is not clear how important the sensitivity of the plate reader is to obtain useful results. Boosting the signal may be necessary by increasing the number of fluorescent bases used.

4.1.4 Reaction Conditions

Previous *in vitro* studies of transcription have shown a strong and often complex dependence on reaction conditions. Variables such as source of RNAP, temperature, and position of fluorescent bases can affect transcription [6]. Even the measured *relative* strength of promoters has been shown to vary depending on

	(a)	(b)	(c)	(d)	
	Empty	-RNAP	+RNAP	+RNAP	ratio
			(-mix)	(+mix)	(d/b)
Plac	809	821	812	671	0.82
Plac-P	845	757	742	720	0.95
Plac-5P-TE	821	775	739	772	1.00
PribAP2	845	949	943	699	0.74
PribAP2-P	842	821	748	748	0.91
PribAP2-5P-TE	851	742	739	726	0.98
Empty	861	912	964	949	1.04

Figure 20: Fluorescence of several constructs with the promoters *Plac* and *PribAP2* before and after addition of RNA polymerase shows a possible effect due to transcribing polymerase. The -TE samples contained the TE transcriptional terminator from T7. Column (a) gives the fluorescence of the empty well without the sample. Column (b) shows the fluorescence after adding the sample. Column (c) and (d) show the fluorescence after adding polymerase, with (c) measured immediately after adding polymerase and (d) measured after thorough mixing of the wells. The final column gives the ratio of column (d) to (b). The last row of the table shows a well that was left empty for all measurements.

temperature, salt concentration, and enzyme to DNA ratio [3, 12, 13, 17, 22].

To increase the applicability of the PARMESAN method, the buffer and reaction conditions need to be optimized to provide the most useful results. As the goal is to have standard conditions for measuring transcription, it may be ideal to have conditions as similar to intracellular conditions as possible. For example, in a real system, there will be other template DNA floating around, competing for the RNA polymerase. To make more realistic measurements, extra DNA such as fragments of the *E. coli* genome could be added to compete for the RNA polymerase.

4.1.5 Strand Dependence

Although different results are expected depending on whether the pyrrolo-dC is on the template or non-template strand, the current experiments have not been able to provide decisive evidence to show that one strand should be preferred over the other. The non-template strand is a safer choice for pyrrolo-dC incorporation, as the fluorescence of pyrrolo-dC on the non-template strand is not quenched during transcription. However, it is easier to incorporate pyrrolo-dC on the template strand via a PCR reaction, and

if the template strand is a workable alternative, then it may be the preferred strategy.

4.1.6 Transcription Regulators

This method could also be used to characterize various transcriptional signals. For example, the difference in PAR of a promoter followed by a terminator and the promoter alone is a measure of the terminator efficiency. Other transcriptional signals that could be characterized include pause sites and elongation rates.

In addition to the signals located on the DNA template, many proteins influence transcription. To measure the impact of these transcriptional regulators on polymerase arrival rates, these proteins can be added to *in vitro* transcription reactions. For example, by adding a varying amount of repressor protein and measuring PAR, the effect of the repressor on transcription can be determined. For more complex modules, a cell-free translation system can be added in addition to the transcription machinery [31]. An *in vitro* transcription and translation system should still be a more controlled environment, leading to more reproducible results, than in a free-living cell.

4.1.7 Real-Time Kinetics

One benefit of using a non-destructive fluorescent assay with pyrrolo-dC is the potential to obtain real-time kinetic information. Kinetic measurements should be possible, having been done before in other applications with 2-aminopurine [2, 6, 11].

4.1.8 Pyrrolo-dC Characterization

Most previous experiments using a fluorescent DNA base in a manner similar to the PARMESAN method have been done with 2-aminopurine (e.g. [1, 8, 27, 28, 29, 32, 36]). However, not much is known about pyrrolo-dC and few experiments have been done with it. For use as a measurement tool, pyrrolo-dC needs to be characterized and understood better. The amount of disturbance an artificial base like pyrrolo-dC has on the natural transcription process is not known. Although pyrrolo-dC can pair with guanine, it is possible the artificial base induces changes in the DNA structure that affect the transcription process.

4.1.9 Polymerase States

For modular assembly to function correctly, the polymerase arrival rates should be in identical units, allowing them to be interchangeably connected together. To do this, all polymerase arrivals were assumed to be measured at a point when the polymerase is in a fixed normal state.

The PARMESAN measurement method cannot distinguish, for example, between polymerases stalled at the pyrrolo-dC and polymerases actively transcribing through the pyrrolo-dC site. Although this method does not necessarily have to distinguish among polymerase states, a separate method may be required to guarantee that the assumptions are valid for the module.

Another possible problem is the non-directionality of polymerase measurement using the pyrrolo-dC technique. The polymerase could potentially be arriving and moving in the opposite direction from expected. The measurement may be directional if a fluorescence difference exists depending on whether the pyrrolo-dC is on the template or non-template

strands. But separating the effects from the two directions may be difficult.

4.2 Method Comparison

A summary of some advantages and disadvantages of the PARMESAN method are presented below.

4.2.1 Advantages

- Measurements can be done *in vitro*. With *in vitro* experiments, it is not necessary to worry about toxicity to cells due to strong promoters putting an unbearable burden on cells.
- The conditions of transcription can be well-defined and controlled. Inside a cell, many additional reactions affect transcription, many of which cannot be accounted for in a defined manner. For example, DNA polymerase during replication interacts with the transcribing RNA polymerase [18]. Undoubtedly, these are important to real systems, but for measurement and characterization purposes, these are side effects that should be eliminated.
- No radioactive labeling is needed, simplifying experiments and eliminating radioactive waste.
- It is not required to synthesize new labeled oligos for every piece of DNA to be tested, as the oligos are designed to be general enough to work with existing and future modules.
- The method is not limited to *E. coli* RNA polymerase and any purified polymerase could be substituted.
- Measuring fluorescence of pyrrolo-dC is non-destructive, allowing for repeated and continuous measurements.
- The data collection is fast, with the fluorescence change being immediately detectable, providing the potential to look closely at the kinetics of polymerase arrivals.

- The BioBricks assembly scheme is used here, but PARMESAN is relatively independent of the module assembly strategy. Different pyrrolo-dC oligos could be designed for another assembly method.
- There is no need to worry about different RNA half-life times.
- There is minimal disturbance placed on the natural transcriptional system. By not using a modified enzyme or non-natural drugs like rifampicin, most of the transcription process should proceed as normal. If the artificial base does have an effect, it likely only has an effect on the region around the site of incorporation. As we are measuring the rate the polymerase arrives at that point and do not care what happens after it passes, the disturbance on the system being measured is probably minimal.
- Only a single fluorescence probe needs to be used. The method is not limited to pyrrolo-dC. Other fluorescent bases, such as 2-aminopurine, could be substituted for pyrrolo-dC. Multiple fluorescence probes can be used simultaneously, as long as the excitation and emission wavelengths do not overlap significantly. For example, a real-time *in vitro* system has been previously developed using three different fluorescent probes to measure three properties simultaneously: protein/DNA complex formation, transcription bubble formation, and RNA production [6].
- The method is theoretically simple and straightforward to do with materials common in molecular biology labs.

4.2.2 Disadvantages

- Results have not yet shown convincingly that the method as described here is a reasonable assay for polymerase arrival rates.
- Differences in promoter strengths have been found between *in vitro* and *in vivo* experiments, undoubtedly due to many elements in living systems we do not yet understand. The *in vitro* measurements may not accurately reflect *in vivo* behavior, with the cellular “noise” an important determinant of the behavior of the system.
- Measurements need to be done *in vitro*. Using a fluorescent base is not directly adaptable to measurements in live cells.
- A source of purified RNA polymerase is required.
- It may be difficult to measure complex systems *in vitro*.

4.3 Conclusion

Several key ideas have been proposed:

- Modularity is the ultimate goal.
- All modules are defined by a fixed set of inputs and outputs measured in units of polymerase arrival rates (PAR).
- The fluorescent DNA nucleotide, pyrrolo-dC, shows a fluorescence change in single vs. double stranded DNA.
- PAR is measured by the fluorescence change of pyrrolo-dC as the RNA polymerase locally melts the DNA region during transcription.
- By standardizing the input and output units, modules can be connected and modeled easily.

To design and build biological systems that have reasonable chances of functioning correctly requires fully characterized components. A quantitative characterization of the behavior of all biological components from ribosome binding sites to promoters to protein sequences is essential. PARMESAN is one potential method for standardly quantifying the strength of promoters and general modules by measuring the ability to promote transcription activity. With modules designed around polymerase arrival rates and the possibility of characterizing them using the PARMESAN method, engineering more complex synthetic biological systems may become a less daunting task.

References

- [1] Rajiv P. Bandwar, Yiping Jia, Natalie M. Stano, and Smita S. Patel. Kinetic and thermodynamic basis of promoter strength: Multiple steps of transcription initiation by T7 RNA polymerase are modulated by the promoter sequence. *Biochemistry*, 41(11):3586–3595, March 2002. [4.1.8](#)
- [2] Rajiv P. Bandwar and Smita S. Patel. Peculiar 2-aminopurine fluorescence monitors the dynamics of open complex formation by bacteriophage T7 RNA polymerase. *Journal of Biological Chemistry*, 276(17):14075–14082, April 2001. [4.1.7](#)
- [3] Melanie M. Barker, Tamas Gaal, Cathleen A. Josaitis, and Richard L. Gourse. Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation *in vivo* and *in vitro*. *Journal of Molecular Biology*, 305(4):673–688, January 2001. [4.1.4](#)
- [4] Austin Che. Fluorescence assay for polymerase arrival rates. Master’s thesis, MIT, 2003. [1.4.2](#), [1.6](#), [2](#), [2.1](#), [2.2.4](#)
- [5] Austin Che and Bogdan Fedeles. The modular modeling of biological networks using transcription rates. http://austin.che.name/docs/modular_modeling.pdf. [1.7](#)
- [6] Kevin S. Dunkak, Michael R. Otto, and Joseph M. Beechem. Real-time fluorescence assay system for gene transcription: Simultaneous observation of protein/DNA binding, localized DNA melting, and mRNA production. *Analytical Biochemistry*, 243(2):234–244, 1996. [4.1.4](#), [4.1.7](#), [4.2.1](#)
- [7] Michael B. Elowitz and Stanislas Leibler. A synthetic oscillatory network of transcriptional regulators. *Nature*, 403(6767):335–338, January 2000. [1](#)
- [8] Andrew M. Fedoriw, Huaying Liu, Vernon E. Anderson, and Pieter L. deHaseth. Equilibrium and kinetic parameters of the sequence-specific interaction of *Escherichia coli* RNA polymerase with nontemplate strand oligodeoxyribonucleotides. *Biochemistry*, 37(34):11971–11979, August 1998. [4.1.8](#)
- [9] Timothy S. Gardner, Charles R. Cantor, and James J. Collins. Construction of a genetic toggle switch in *Escherichia coli*. *Nature*, 403(6767):339–342, January 2000. [1](#)
- [10] Ruti Hershberg, Gill Bejerano, Alberto Santos-Zavaleta, and Hanah Margalit. PromEC: An updated database of *Escherichia coli* mRNA promoters with experimentally identified transcriptional start sites. *Nucleic Acids Research*, 29(1):277, 2001. <http://bioinfo.md.huji.ac.il/marg/promec/>. [2.2.1](#)
- [11] Yiping Jia and Smita S. Patel. Kinetic mechanism of GTP binding and RNA synthesis during transcription initiation by bacteriophage T7 RNA polymerase. *Journal of Biological Chemistry*, 272(48):30147–30153, November 1997. [4.1.7](#)
- [12] Thomas R. Kadesch, Steven Rosenberg, and Michael J. Chamberlin. Binding of *Escherichia coli* RNA polymerase holoenzyme to bacteriophage T7 DNA: Measurements of binding at bacteriophage T7 promoter A₁ using a template competition assay. *Journal of Molecular Biology*, 155:1–29, 1982. [4.1.4](#)
- [13] Masayuki Kajitani and Akira Ishihama. Determination of the promoter strength in the mixed transcription system. II. Promoters of ribosomal RNA, ribosomal protein S1, and *recA* protein operons from *Escherichia coli*. *Nucleic Acids Research*, 11(12):3873–3888, 1983. [4.1.4](#)
- [14] Natalia Komissarova and Mikhail Kashlev. Functional topography of nascent RNA in elongation intermediates of RNA polymerase. *Proc. Natl. Acad. Sci. USA*, 95:14699–14704, December 1998. [1.6.3](#)
- [15] Nataliya Korzheva, Arkady Mustaev, Maxim Kozlov, Arun Malhotra, Vadim Nikiforov, Alex Goldfarb, and Seth A. Darst. A structural model of transcription elongation. *Science*, 289(5479):619–625, July 2000. [1.6.3](#)
- [16] Donna N. Lee, Le Phung, Judith Stewart, and Robert Landick. Transcription pausing by *Escherichia coli* RNA polymerase is modulated by downstream DNA sequences. *Journal of Biological Chemistry*, 265(25):15145–15153, September 1990. [2.1.2](#)
- [17] S. Leirmo and M. Thomas Record, Jr. Structural, thermodynamic and kinetic studies of the interaction of E σ^{70} RNA polymerase with promoter DNA. In Fritz Eckstein and David M.J. Lilley, editors, *Nucleic Acids and Molecular Biology*, volume 4, pages 123–151. Springer-Verlag, 1990. [4.1.4](#)

- [18] Bin Liu, Mei Lie Wong, Rachel L. Tinker, E. Peter Geiduschek, and Bruce M. Alberts. The DNA replication fork can pass RNA polymerase without displacing the nascent transcript. *Nature*, 366:33–39, November 1993. [4.2.1](#)
- [19] Cuihua Liu and Craig T. Martin. Fluorescence characterization of the transcription bubble in elongation complexes of T7 RNA polymerase. *Journal of Molecular Biology*, 308(3):465–475, May 2001. [1.6.1](#), [3.1](#), [3.3.2](#)
- [20] Cuihua Liu and Craig T. Martin. Promoter clearance by T7 RNA polymerase: Initial bubble collapse and transcript dissociation monitored by base analog fluorescence. *Journal of Biological Chemistry*, 277(4):2725–2731, January 2002. [1.6.1](#), [1.6.1](#), [1.6.3](#)
- [21] Paul Melançon, Richard R. Burgess, and M. Thomas Record, Jr. Direct evidence for the preferential binding of *Escherichia coli* RNA polymerase holoenzyme to the ends of deoxyribonucleic acid restriction fragments. *Biochemistry*, 22(22):5169–5176, 1983. [4.1.2](#)
- [22] Jacqueline S. Miller and Richard R. Burgess. Selectivity of RNA chain initiation *in vitro*. 3. Variables affecting initiation of transcription. *Biochemistry*, 17(11):2064–2069, May 1978. [4.1.4](#)
- [23] Rachel Anne Mooney, Irina Artsimovitch, and Robert Landick. Information processing by RNA polymerase: Recognition of regulatory signals during RNA chain elongation. *Journal of Bacteriology*, 180(13):3265–3275, 1998. [2.1.3](#)
- [24] Benno Müller-Hill. *The lac operon: a short history of a genetic paradigm*. Walter de Gruyter, 1996. [1.4.1](#)
- [25] Vincent Noireaux, Roy Bar-Ziv, and Albert Libchaber. Principles of cell-free genetic circuit assembly. *Proc. Natl. Acad. Sci. USA*, 100(22):12672–12677, October 2003. [1](#)
- [26] Registry of Standard Biological Prats. <http://parts.syntheticbiology.org/>. [2](#)
- [27] Edward L. Rachofsky, Roman Osman, and J. B. Alexander Ross. Probing structure and dynamics of DNA with 2-aminopurine: Effects of local environment on fluorescence. *Biochemistry*, 40:946–956, 2001. [4.1.8](#)
- [28] Kevin D. Raney, Lawrence C. Sowers, David P. Millar, and Stephen J. Benkovic. A fluorescence-based assay for monitoring helicase activity. *Proc. Natl. Acad. Sci. USA*, 91(14):6644–6648, July 1994. [4.1.8](#)
- [29] Jennifer J. Sullivan, Keith P. Bjornson, Lawrence C. Sowers, and Pieter L. deHaseth. Spectroscopic determination of open complex formation at promoters for *Escherichia coli* RNA polymerase. *Biochemistry*, 36:8005–8012, 1997. [4.1.8](#)
- [30] Synthetic Biology. <http://www.syntheticbiology.org/>. [1](#)
- [31] Martin J. Tymms, editor. *In Vitro Transcription and Translation Protocols*, volume 37 of *Methods in Molecular Biology*. Humana Press, 1995. [4.1.6](#)
- [32] Andrea Újvári and Craig T. Martin. Thermodynamic and kinetic measurements of promoter binding by T7 RNA polymerase. *Biochemistry*, 35:14574–14582, 1996. [1.6.1](#), [4.1.8](#)
- [33] Peter H. von Hippel, David G. Bear, William D. Morgan, and James A. McSwiggen. Protein-nucleic acid interactions in transcription: A molecular analysis. *Annual Review of Biochemistry*, 53:389–446, 1984. [4.1.2](#)
- [34] Daguang Wang and Robert Landick. Nuclease cleavage of the upstream half of the nontemplate strand DNA in an *Escherichia coli* transcription elongation complex causes upstream translocation and transcriptional arrest. *Journal of Biological Chemistry*, 272(9):5989–5994, February 1997. [1.6.3](#)
- [35] Ron Weiss. *Cellular Computation and Communications using Engineered Genetic Regulatory Networks*. PhD thesis, MIT, 2001. [1](#)
- [36] Daguang Xu, Kervin O. Evans, and Thomas M. Nordlund. Melting and premelting transitions of an oligomer measured by DNA base fluorescence and absorption. *Biochemistry*, 33(32):9592–9599, 1994. [4.1.8](#)
- [37] Yohei Yokobayashi, Ron Weiss, and Frances H. Arnold. Directed evolution of a genetic circuit. *Proc. Natl. Acad. Sci. USA*, 99(26):16587–16591, December 2002. [1](#)