Proceedings of the 28th IEEE
EMBS Annual International Conference
New York City, USA, Aug 30-Sept 3, 2006

INVERTING AMPLIFIER GENETIC CIRCUIT PERFORMANCE

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Abstract- **A synthetic genetic circuit has been designed whose topology and function echo those of an electronic inverting amplifier. Several variants of this circuit have been built in our laboratory. This paper reports on the testing of one of these variants and contributes to the field both in terms of evaluating the specific amplifier performance and in terms of providing a methodology for performance evaluation of analog genetic circuits. An input source was created and partially calibrated. It was then used to test the circuit through both fluorometer measurements and flow cytometry. In the discussion, consideration is given to cellular loading by the synthetic circuits and the resulting impact on circuit performance. Models developed earlier are compared with the experimental results. The circuit does indeed perform as an inverting amplifier.**

Keywords **- Genetic circuit, inverting amplifier, synthetic biology**

I. INTRODUCTION

In synthetic biology, genes and their related protein products can be connected into a variety of 'genetic circuit' configurations. Engineered genetic circuits possessing preprogrammed cellular functions enable us to analyze the behavior of systems that are comparable to natural gene networks. Synthetic biology has the potential for great benefits in the biotechnology industry and in medicine.

Synthetic genetic circuits permit installation of control logic into any cell. Gardner *et al.* report a bistable toggle switch circuit that can be flip-flopped between expression states in response to external stimuli [1]. The circuit was constructed from two sets of control elements (promoters, repressors), arranged so that the promoter of one element can be inhibited by the repressor made by the other. When one promoter was turned on, the other was turned off. Elowitz and Leibler describe a genetic oscillator circuit built from three repressible promoters connected as a closed loop [2]. Each promoter transcribed the repressor for one of the other two promoters, such that when one was turned on, the other two were turned off. The configuration produced oscillating levels of each repressor.

Neither the construction nor the testing of such circuits is as easy or as quick as that of their electronic analogs. The circuit is usually implemented as a sequence of DNA inserted into a circular DNA molecule, a plasmid, that is responsible for introducing and replicating the circuit in the cell. The circuit may also contain additional short DNA sequences due to the chosen construction approach. As well, the plasmid itself is constantly interacting with the cellular machinery. Measurements are taken against the interfering background of all the other cellular processes. This greatly complicates the evaluation of circuit performance.

An inverting amplifier ideally produces an output that decreases linearly with increasing input. In [3], we show how the genetic elements of bacteriophage λ can be used as basic building blocks for the construction of such an amplifier. We also evaluate its performance through stochastic modeling. In this paper, we assess its performance through experiment.

II. BACKGROUND

Promoters and regulatory feedback loops of gene transcription units are the fundamental elements that form naturally occurring genetic circuits. A promoter is a short DNA sequence that precedes the gene. In DNA transcription, RNA polymerase binds to the promoter and is instructed where to start synthesis of mRNA. The information on the mRNA molecule is then translated into a protein. Feedback arises when the protein is capable of interacting with the promoter that drives its own synthesis or the promoter of other genes [3]; this interaction occurs when the protein binds to a region near the promoter known as an operator.

A typical electronic circuit implementation of an inverting amplifier is shown in Fig. 1(a). There are three components in this particular implementation. Two of these components are resistors while the third is an operational amplifier. An operational amplifier is a gain element wherein the output is a very large, ideally infinite, multiple, A, of the difference between its positive and negative inputs. In this circuit, the output is $-AV_-$ as the positive input is set to zero by tying it to ground. Resistor R_i couples the circuit input, V_i, to the negative input of the operational amplifier. Resistor R_f couples the circuit output, V_o, to the negative input of the operational amplifier.

In the circuit of Fig. 1(a), the feedback of the output through R_f acts to cancel the contribution of the input. In the case where the gain element has infinite gain and R_f=R_i, equilibrium is achieved when V_o=-V_i. Overall circuit gain is defined as V_o/V_i so the gain in that case is –1. For arbitrary values of the resistors, the overall gain can be shown to be $-R_f/R_i$.

To develop a genetic circuit analog to the electronic inverting amplifier, one need only find and interconnect genetic circuit elements that behave similarly to the components in Fig. 1(a). A gain element is required whose gain is negative; its output should decrease when input increases and vice versa. A repressible promoter satisfies this requirement as increasing the concentration of the repressor molecule decreases the likelihood of transcription.

Using the promoter to transcribe the repressor allows the negative feedback connection analogous to that provided by R_f to be created. Setting the input molecule to be the repressor as well creates a connection analogous to that provided by R_i. The input repressor molecules are now balanced by the feedback repressor molecules. As molecular concentrations can only be positive, to allow for negative signals, bias must be provided. This will allow an increase in input to be countered by a decrease in output and vice versa.

Our genetic circuit amplifier is designed around the P_{RM} promoter region of bacteriophage λ, a virus that infects bacteria. As seen in Fig.1(b), this promoter is overlapped by the right operator region, which contains three sites: O_R1, O_R2, and O_R3. Repressor protein (CI) dimerizes and binds to each individual operator site. When RNA polymerase binds to P_{RM}, it becomes poised to transcribe the cI gene. If, at that instance, some repressor dimer is already bound to O_R1, transcription of cI proceeds at a low level. As increasing amounts of cI are added, O_R1 and O_R2 are filled together, and O_R3 is filled only at high concentrations [4]. Repressor dimer bound to O_R2 interacts with the polymerase, enhancing its affinity for P_{RM} and increasing transcription of cI by roughly tenfold. In contrast, when repressor dimer is bound to O_R3, binding of polymerase to P_{RM} is blocked and transcription is effectively turned off. Operating this promoter at a concentration of CI above where O_R1 and O_R2 have bound dimers implies that increasing the concentration will increase the likelihood that O_R3 is bound and hence decrease the likelihood that transcription occurs. Thus this promoter provides the desired negative gain element. Further, it provides it at a positive (i.e. biased) concentration of CI thus permitting negative signal swings about this bias level.

Fig. 1(b) shows the biological design of the genetic circuit amplifier. The input to the circuit is CI monomer. Feedback of translated CI acts to balance the effect of the input. The feedback and an external source of CI monomer constitute the total input to the gain element. The output of the system is EGFP protein that is jointly transcribed with CI. This allows the amplifier output to be quantified experimentally by detecting the fluorescence of the EGFP.

III. IMPLEMENTATION

The design was implemented using two plasmids. One incorporates the source of the input CI while the other incorporates P_{RM} and feedback CI and EGFP. These are denoted as the Input Plasmid and the Amplifier Plasmid, respectively.

Plasmids replicate inside the host cells. They maintain a specific number of plasmid copies on average. This is referred to as the plasmid copy number and is different for different types of plasmids.

Copy number is a key design parameter in genetic circuits

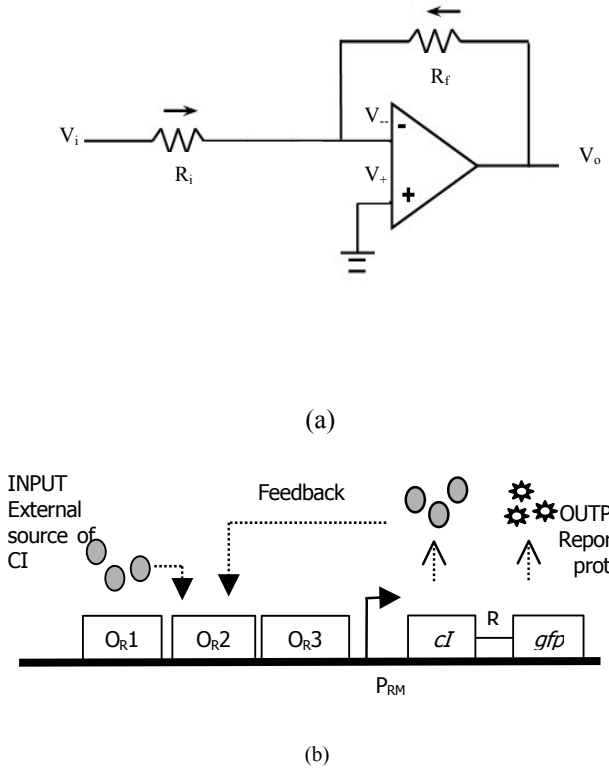
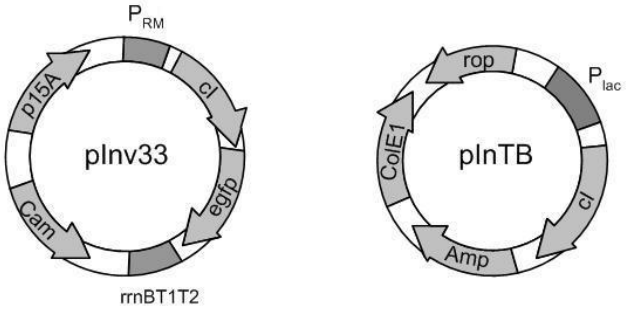


Fig. 1. (a) Schematic representation of the genetic circuit amplifier and (b) its analogous electronic circuit.

as it defines the number of available operator sites, etc. Simulation studies revealed that best amplifier performance was expected to occur for small copy numbers (ie less than 50). Further, separating the input circuit onto a different plasmid allowed changing the input range through changing the type of plasmid.

Fig. 2 details the two plasmids. The Amplifier Plasmid, pInv33, is based on pBAD33 and thus has a copy number near 10. The Input Plasmid, pInTB, is based on pTrueBlue-rop and has a copy number of 15-20. This plasmid incorporates the promoter P_{lac} which allows transcription to increase with increasing concentration of the inducer IPTG. In addition another plasmid was constructed that was essentially pInTB with the gene cI replaced by egfp. This was to allow calibration of the input.

The contents of the plasmids were verified using PCR and restriction digests. A PCR reaction was performed with EGFP primers and using the input calibration plasmid as a template. The PCR confirmed the presence of an egfp gene. The plasmid DNA was also extracted from a cell culture and linearized using a restriction digest. The extracted DNA was of the correct length. The contents of the input plasmid were also verified using a restriction digest, and the contents of the amplifier plasmid were verified using PCR screening.



(a) (b)

Fig. 2. (a) Schematic representation of the Input Plasmid, pInTB, and (b) the Amplifier Plasmid, pInv33.

IV. PERFORMANCE TESTING

Experiments were conducted to calibrate the input plasmid, measure the inverting amplifier's average response through fluorometer studies and obtain statistics on single cells through cytometer measurements.

For all the experiments reported here E. coli strain DH5α-z1 cells were used. This strain has been modified to produce large quantities of the LacR repressor thus ensuring that P_{lac} is repressed in the absence of the inducer. Cells from single colonies transformed with the indicated plasmids were grown overnight in liquid LB media and applicable antibiotics. They were then diluted 50:1 into fresh media with antibiotics and the appropriate inducer concentration was added. The cells were then grown for an additional period as will be indicated. The cells were then spun down, washed in PBS and resuspended at a 2:1 dilution in PBS.

For fluorescence and absorbance measurements, the cells were then loaded onto a Fluostar Optima microplate reader. The values reported here are normalized fluorescences obtained by subtracting PBS fluorescence and absorbance values from the sample measurements then taking the ratio of the so adjusted fluorescence to the adjusted absorbance.

For cytometer measurements the resuspended cells were loaded directly onto a Partec CyFlow cytometer. To facilitate comparison of data between different samples, the cytometer histogram 'counts' have been normalized by the total number counts.

a) Input Calibration

The input plasmid calibration is shown in Fig. 3. Error bars represent ± 1 standard deviation. Ampicillin was the selective antibiotic. Here the cells were grown for 4-5 hours before measurement. Four replicates were used. In Figure 3, two distinct regions are visible: 1) where the output increases linearly with the logarithm of IPTG concentration and 2) a saturation region. This is consistent with a simple reaction model of the inducer, repressor and promoter activity. The solid line in Fig 3 is the result of fitting a simple model to the data. Note that even at zero IPTG there is EGFP production, a phenomenon referred to as promoter leakage.

Fig. 3 may be used to infer that the concentration of cI produced by pInTB will increase linearly with the logarithm of IPTG concentration. EGFP has a 24 hour halflife and cI concentration is usually assumed to decrease only through cell division. Thus over the time scales reported here, the concentration of either is essentially just the integral of their production. Assuming the production rate is dominated by the transcription step then we can infer that cI concentration as a function of IPTG will have a similar form as seen in Fig. 3.

b) Amplifier Response (Fluorometer)

The amplifier response to cI produced by the input plasmid under induction by IPTG is shown in Fig. 4. To select for cells containing both plasmids, the antibiotics ampicillin and chloramphenicol were used. Cells were grown for 3.5 hours after application of IPTG.

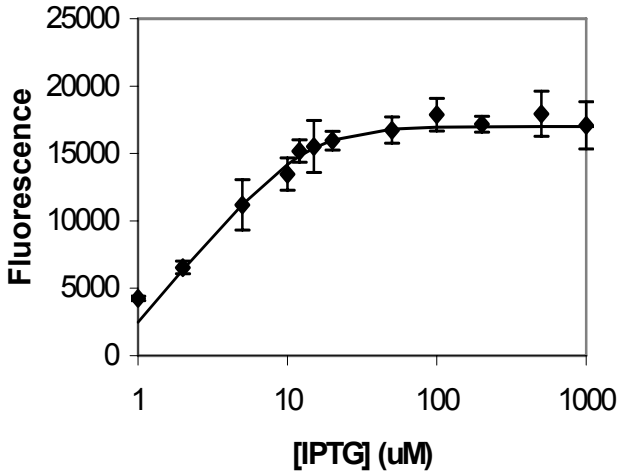


Fig. 3. Input plasmid transcription as a function of inducer concentration as measured using a EGFP reporter. The fluorescence observed for 0 IPTG is arbitrarily plotted on the y-axis (ie at 1 IPTG).

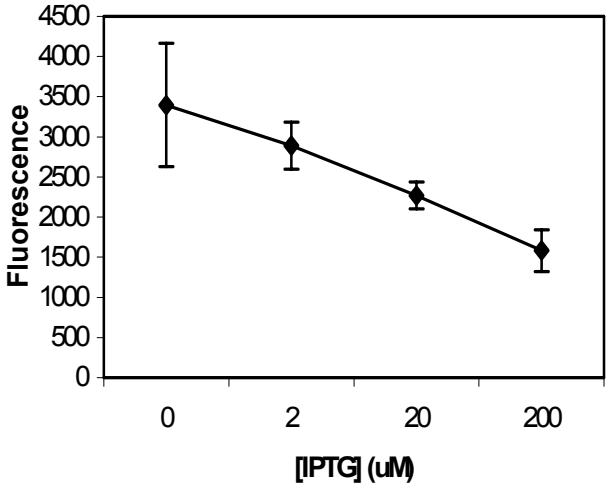


Fig. 4. Amplifier response

Six replicates were used. As may be seen in Fig 4, the output decreased with increasing IPTG concentration. Importantly, the output decreased linearly with the logarithm of IPTG concentration. This what we would expect as from Fig. 3 we expect cI to be increasing with the logarithm of IPTG concentration and the amplifier output to be linearly decreasing with cI concentration. Fig. 4 indicates that the circuit is working as an inverting amplifier.

c) Amplifier Response (Cytometer)

To further explore the amplifier behavior cytometer measurements were obtained. Whereas the flurometer provides average measurements incorporating billions of cells, the cytometer measures one cell at a time to form a histogram of the number of cells in different fluorescence intensity ranges.

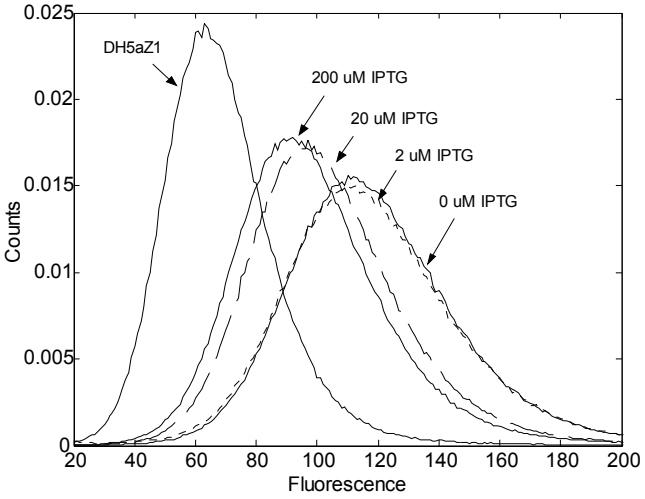
The conditions and procedures were as for the fluorometer measurements with two exceptions. The growth time after inducer application was 4 hours. Also the samples were diluted into a greater volume of PBS (2ml) to match cytometer requirements.

The results are shown in Fig. 5(a). Here we see the probability of lower fluorescence values increasing with increasing IPTG and hence increasing cI. Also shown is the histogram for untransformed DH5α-z1 cells. It is known that cell have some natural level of background fluorescence; clearly, the transformed cells are producing additional fluorescence.

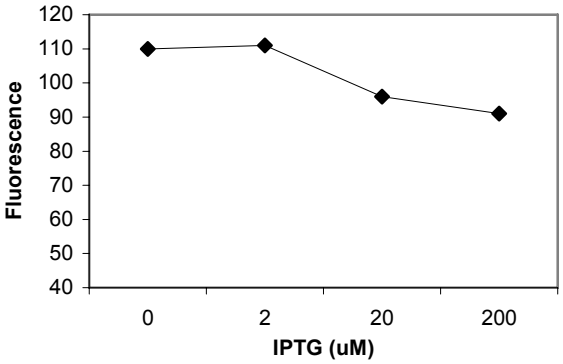
While inversion is evident in Fig. 5(a), the 0 and 2uM IPTG data are very similar, much more than we would have expected from the fluorometer results. To facilitate comparison of these different data types, a summary measurement was formed from the histogram data. We attempted to estimate the peak location of the cell fluorescence probability density function (pdf). Each histogram was treated as a function. All points above half the peak histogram value were least squares fitted to a cubic function. The location of the peak of the cubic function was taken as the cell fluorescence pdf. This is plotted in Fig. 5(b). While the results decrease monotonically with IPTG, they do not display the linearity with the logarithm of IPTG as seen in Fig. 4. The range also does not appear to be as large as was seen in Fig. 4. Further work is underway to investigate these differences.

V. Conclusion

A genetic circuit inverting amplifier has been constructed and found to function as expected. This finding required the interpretation that the input circuit produced a signal proportional to the logarithm of the inducer. Signal inversion is consistent across experiments. The amplifier provides a new method for setting a negative linear relationship between two cellular constituents.



(a)



(b)

Fig. 5. Amplifier response observed with a cytometer (a) normalized histograms and (b) estimated maxima locations.

References

[1] T. Gardner, C. Cantor, and J.J. Collins, "Construction of a genetic toggle switch in *Escherichia coli*", Nature, Vol. 403, pp.339-342, 2000.

[2] M.B. Elowitz and S. Leibler, "A synthetic oscillatory network of transcriptional regulators", Nature, Vol. 403, pp.335-338, 2000.

[3] G. DeRubertis, S.W. Davies, "A genetic circuit amplifier: design and simulation", IEEE Trans. Nanobioscience, Vol. 2, No. 4, pp.239-246, Dec., 2003.

[4] M. Ptashne, et. al., "How the λ repressor and Cro work", Cell, Vol. 19, pp.1-11, Jan. 1980.