SYSTEMATIC PROMOTER TUNING IN NEGATIVE AUTOREGULATORY TRANSCRIPTION NETWORKS

Pavel Zach*, Hynek Kasl*, Václav Babuška+ and Daniel Georgiev*
*Department of Cybernetics, Faculty of Applied Sciences, University of West Bohemia
+Faculty of Medicine in Pilsen, Charles University in Prague

Introduction

Precise and predictable gene expression is fundamental for creating new synthetic circuits inside cells. Since gene expression is mainly controlled by the promoter sequences, being able to quickly design promoters with minimal experimental workload is desired. Existing promoter libraries provide a design starting point but the final promoter sequence will vary with experimental conditions, additional binding sites, and other design elements. Here, we present a simple algorithm for tuning the negative autoregulatory transcription network motif using differential in vivo RNA- based computations. The algorithm comprises two stages: sensitivity to perturbations is minimized first and the steady state level of the gene product is set second.

Tuning is achieved by adjusting spacings between the consensus sequences in the -10/-35 and transcription factor binding site promoter regions. Differential RNA- based computations are proposed to minimize measurement sensitivity to external noise. Computations are realized by hybridization reactions between modified 3'- UTRs and 5'-UTRs of mRNA transcribed from different promoter designs. Convergence of the algorithm to the desired design irrespective of the binding kinetics is shown analytically. Accuracy of the method in measuring differences in gene expression from different promoter sequences is shown in silico. The feasibility of the approach and the underlying sequential tuning protocol are demonstrated in vivo.

Algorithm

The previously derived algorithm holds in silico for general promoter functions and hence is compatible with a wide range of repressor proteins. Minimal prior information regarding the design-to-parameter relationship is required.

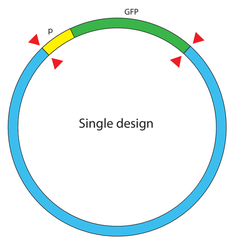
The algorithm comprises two stages. In each stage, the steady state expression levels from competing promoter designs are measured and subtracted. The sign of the difference and the location of the promoter alteration then determines the promoter design that is retained. Mutations in the operator regions are treated separately from mutations in the -10/-35 promoter region. Changes in consensus sequences as well as changes in the consensus sequence spacings are possible. Herein, in order to perform sensitivity analysis of the proposed experimental methods, tuning is performed by adjusting spacings between the consensus sequences in the -10/-35 and transcription factor binding site regions.

Step 1: Design alteration

~ 2 hours

Site-directed mutagenesis is performed on plasmids with single parts (promoter with fluorescent or repressor protein).

These part are equipped with overlaps for Gibson assembly and blunt restriction sites, which allow easy removal of each part using restriction enzymes.



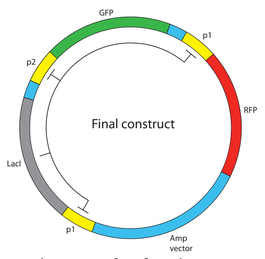
Example of a single design

Step 2: Assembly

~ 1 hour

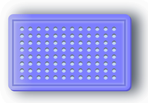
Parts with appropriate overlaps from *Step 1* are assembled together using the Gibson assembly method.

Final plasmids with ampicillin resistance are then transformed into cells.



Scheme of a final construct

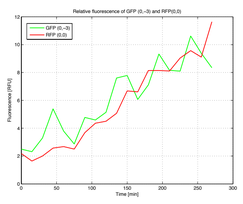
Step 3: Measurements



~ 3-5 hours

Fluorescence of cultures from *Step 2* is measured in parallel over time.

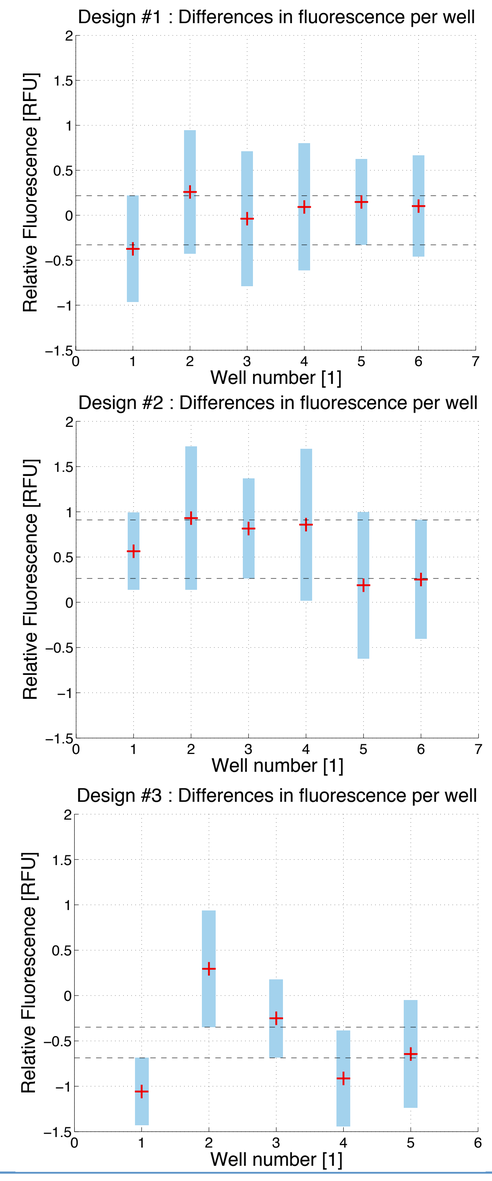
Differences between wavelengths of the used fluorescent proteins (GFP and RFP) allows for parallel measurement of protein levels from two competing designs.



Step 4: Data analysis

Differences in GFP and RFP levels is compared for three designs:
Design 1 - GFP and RFP expressed from identical promoters. This design is used to calibrate the other measurements.
Design 2 - GFP is expressed from a mutated promoter. This promoter is derived from the RFP promoter by deleting 3bp separating the palindromic consensus sequences of the LacI operator. The derived promoter is expected to be less efficient. Hence, higher relative GFP levels than RFP levels are expected. The data shows a small but significant difference in agreement with the expected results.
Design 3 - GFP is expressed from a mutated promoter. This promoter is derived from the RFP promoter by inserting 3bp separating the palindromic consensus sequences of the LacI operator. The derived promoter is expected to be less efficient. Hence, higher relative GFP levels than RFP levels are expected. The data is inconclusive in this case, suggesting a negligible difference in operator efficiencies.

At this stage of the algorithm, since the alterations were made in the operator region, the design with the lowest expression levels would be retained. Hence, Design 1 or Design 3 would be retained and the algorithm would proceed to the second phase where the concentration of the protein levels would be set.



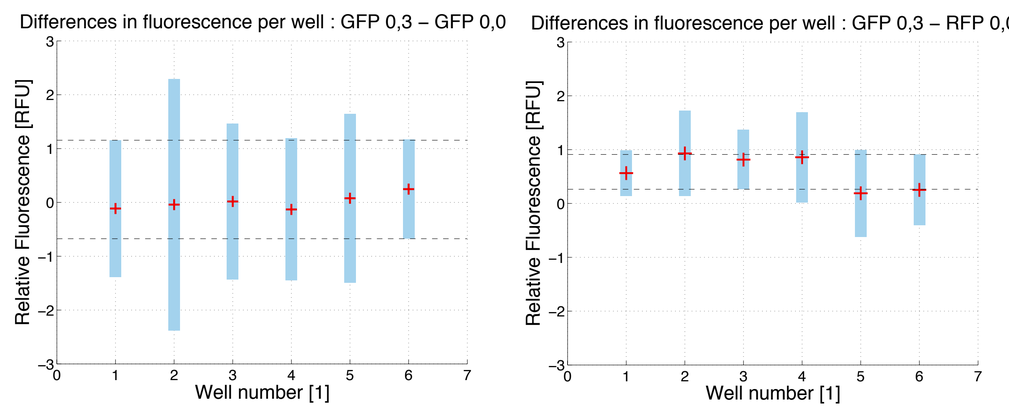
Comparison with non-parallel measurements

Advantages of proposed parallel measurements:

- *higher sensitivity to changes in design*
- *lower sensitivity to disturbances*
- *lower experimental complexity*

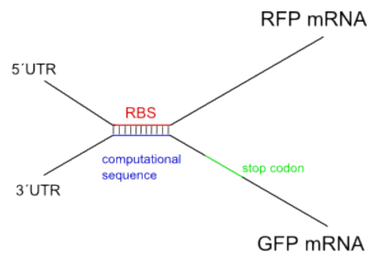
In comparison to non-parallel measurements which use only GFP under control of different promoters, our proposed method offers higher sensitivity in the detection of changes.

This can be seen in the neighboring plots, where differences between fluorescence levels of GFP with default promoter and GFP with altered promoter (with the same change as above – i.e. 3bp deleted from the operator) are shown. Using the traditional approach, no difference between competing designs is detectable.



RNA interference

Here we propose another step of improving our genetic network tuning algorithm - the "computation" of difference between the expression of GFP and RFP is done inside the cell through intercellular mechanisms instead of outside through data analysis. By introducing RNA interference into the tuned genetic network we hope to achieve more sensitive measurements of differences in protein expressions. In our construct (see above), we modified the 3'UTR of the GFP gene by inserting the reverse complement of the region surrounding the RBS of the RFP gene. Transcribed GFP mRNAs would therefore block the RBS of RFP mRNAs preventing their translation. This would mean we could directly measure the difference in protein expression by measuring the fluorescence of RFP. Furthermore, we hope to show that increased complementarity between the two mRNAs will increase the accuracy of the measurements by decreasing leaky expression - the error in cell's computation program. Further increase in accuracy may be possible using RT qPCR - instead of inhibiting translation, one of the mRNAs will be designed to block the others reverse transcription.



References

Rosenfeld, N., Elowitz, M. B. a Alon U, 2002. Negative Autoregulation Speeds the Response Times of Transcription Networks. Journal of Molecular Biology.
Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., & Smith, H. O. ”Enzymatic assembly of DNA molecules up to several hundred kilobases.”*Nature methods* 6.5 (2009): 343-345.
R. Egbert , “Tuning Gene Networks with Simple Sequence Repeats.” Presented at q-bio, 2011 Santa Fe, New Mexico.

This work (article, contribution, study etc.) was supported by the European Regional Development Fund (ERDF), project "NTIS – New Technologies for the Information Society", European Centre of Excellence, CZ.1.05/1.1.00/02.0090