Bistable Gene Expression Using CRISPR-Cas-9 Repression

Introduction

CRISPR-Cas-9 offers promise of sequence specific gene regulation with a level of control and predictability not found in protein based effectors. The nucleic acid system of Cas-9 interrogation allows it to bind to it to a given DNA site with high specificity.

Contemporary research still attempts to find the exact mechanism of the repression offered by the CRISPR-Cas-9 protein. Some interactions come from effectors attached to the protein, but there is also repression based solely on the presence of the protein itself. This "roadblocking" repression has been studied and observed in Stanly Qi's "Repurposing CRISPR as an RNA-Guided Platform for Sequence-Specific Control of Gene Expression."

Further, the phenomenon of molecular titration has been used to promote hyper-sensitive substrate response. Within the theoretically workings of Nicholas Buchler in "Molecular Titration and Ultrasensitivity in Regulatory Networks," a specific decoy molecule could interact with the gRNA substrate in order to create a non-linear equilibrium concentration of the guide RNA leading to a more ideal RNA transcription transfer function.

Using figures and mechanisms from Qi and Buchler as well as parameters from other CRISPR research, a model is created for comparisons to laboratory data and for hypothesis building. In this case, these theoretical systems are used to create a means of testing for a bistable system in the instance of two promoter sequences that repress each other.

Derivations

The model, for simplicity, assumes sgRNA guides Cas-9 binding according to the Hill model of substrate bonding. With no likely mechanisms to cause cooperativity within the binding of

sgRNA to Cas-9, a Hill coefficient of 1 is assumed. If so, at a quasi-steady state equilibrium, the amount of sgRNA-Cas-9 complex can be derived from the equilibrium state of the system.

$$K_d = \frac{[Cas9][gRNA]}{[Cas9 - gRNA]}$$

With initial concentration conditions, it is possible to find the equilibrium Cas9-gRNA complex concentration can be found as the change in concentration to equilibrium, *n*.

$$K_d = \frac{([Cas9]_0 - n)([gRNA]_0 - n)}{n}$$

In the case of multiple gRNA systems with *m* gRNAs of equal concentration, the equation is augmented as below.

$$K_d = \frac{([Cas9]_0 - mn)\left(\frac{[gRNA]_0}{m} - n\right)}{n}$$

$$\text{n} = \frac{([Cas9]_0 + [gRNA]_0 + K_d) - \sqrt{b([Cas9]_0 + [gRNA]_0 + K_d)^2 - 4[Cas9]_0[gRNA]_0}}{2m}$$

With the assumption that no initial binded Cas9 is added to the system, the movement of equilibrium is the equilibrium concentration of gRNA binded Cas 9 protein.

$$[Cas9 - gRNA] = n$$

Concentrations are for each individual gRNA complex, producing a total amount as below.

$$[Cas9 - gRNA]_{total} = mn$$

In the case of anti-gRNA, the "molecular titration" phenomenon affects the availability of gRNA at equilibrium. Following Buchler's paper[1], the amount of available gRNA at equilibrium is as below.

$$[gRNA] = \frac{([\alpha - gRNA]_0 - [gRNA]_0 - K_d) + \sqrt{b([\alpha - gRNA]_0 - [gRNA]_0 - K_d)^2 - 4K_d[gRNA]_0}}{2}$$

[1] Molecular Titration and Ultrasensitivity in Regulatory Networks. Buchler, Nicolas E.; Matthieu Louis. Journal of Molecular Biology, Volume 384, Issue 5, Pages 1106-1119. 2008.

With no likely mechanisms to cause cooperativity within the binding of sgRNA-Cas9 complex to DNA, a Hill coefficient of 1 is assumed. With standard Hill equation manipulation and the assumption of excess sgRNA-Cas9 complex, the percent amount of DNA bound to the complex is given below.

$$\%DNAOccupied = \frac{[gRNA - Cas9]}{K_d + [gRNA - Cas9]}$$

Distance was fit by Stanley Qi's experiments as linear[2], but from curve fitting the data set of the paper, exponential decay models fit published data points more effectively. Qi's paper did not publish distances, so the half-repression point could not be found. The percent repression, *R*, is as given below, where *k* is a decay constant and *x* is distance in base pairs from the start of transcription.

$$R = e^{-kx}$$

Although Qi's paper has examples of cooperative repression and non-cooperative repression, results on multiple non-overlapping binding sites appear to be multiplicative. Thus, assuming binding at isolated DNA sites is independent, the transcription rate is given as below where *m* is the number of Cas-9 specific sites. This assumes negligible effect from non-specific binding.

$$\prod_{i=1}^{m} 1 - R_i$$

Regarding Constants

Where possible, constants were found from literature, however due to a lack of quantitative data about certain aspects of Cas-9 bonding, reasonable estimates were produced. The full list of constants used in the model is below.

[2] Repurposing CRISPR as an RNA-Guided Platform for Sequence-Specific Control of Gene Expression
Qi, Lei S. et al. Cell , Volume 152 , Issue 5 , 1173 – 1183. 2013.

pdCas9-DNA Dissociation Constant[3]	0.5 /nM
RNA Decay rate[4]	0.001283 /s
anti-gRNA Dissociation Constant	1 /nM
Roadblock Decay Constant[5]	4800 bp
Cas9 Length	200 bp
Lac Repressor Hill coefficient[6]	1.84
Lac macroscopic dissociation constant[6]	0.004422
Tet Hill coefficient[6]	12.2
Tet microscopic dissociation constant[6]	15.27

Table I: Constants used in the calculation of models

Modelling gRNA Repression

Using the equations outline above, a model was constructed in Matlab (code in appendix) using between 1 and 10 gRNA. In the given simulation, the total Cas9 molarity is maintained at 1 nM. The introduction of different levels of initial total gRNA concentration is modeled in the following graph.

[3] JANAN, WHERE'S THAT PAPER
[4] "Turnover and Translation of in Vitro Synthesized Messenger RNAs in Transfected, Normal Cells". Rajagopalan, Lakshman E., and Malter, James S. *The Journal of Biological Chemistry*, Vol 271, pg 19871-19876. 1996.
[5] Derived from Qi 2013.
[6] Derived from "Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements". Lutz R and Bujard H. 1997.

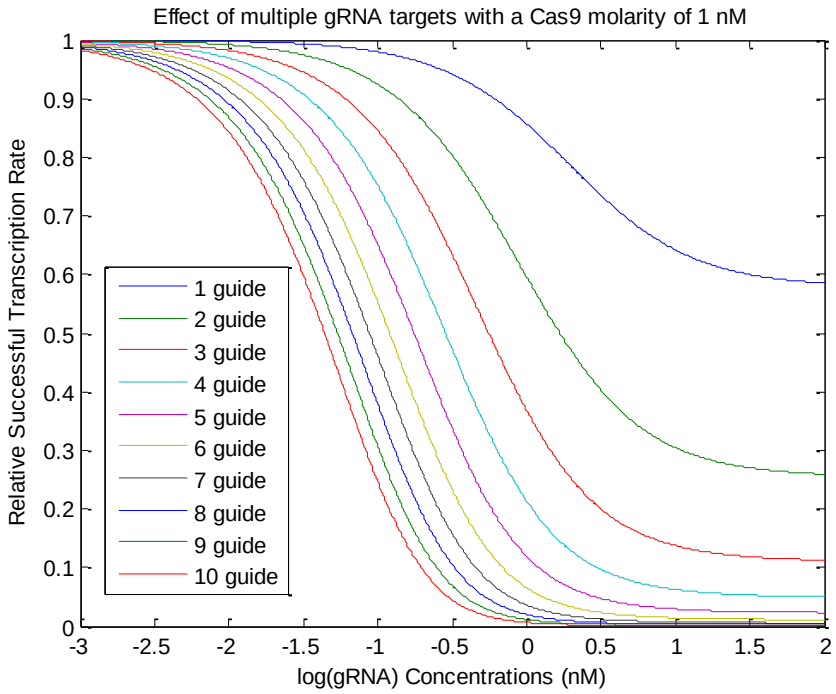

Figure 1a: Effect of various amounts of gRNA specific target sites. The modelling assumes the sites start as early as possible along the transcribed genes.

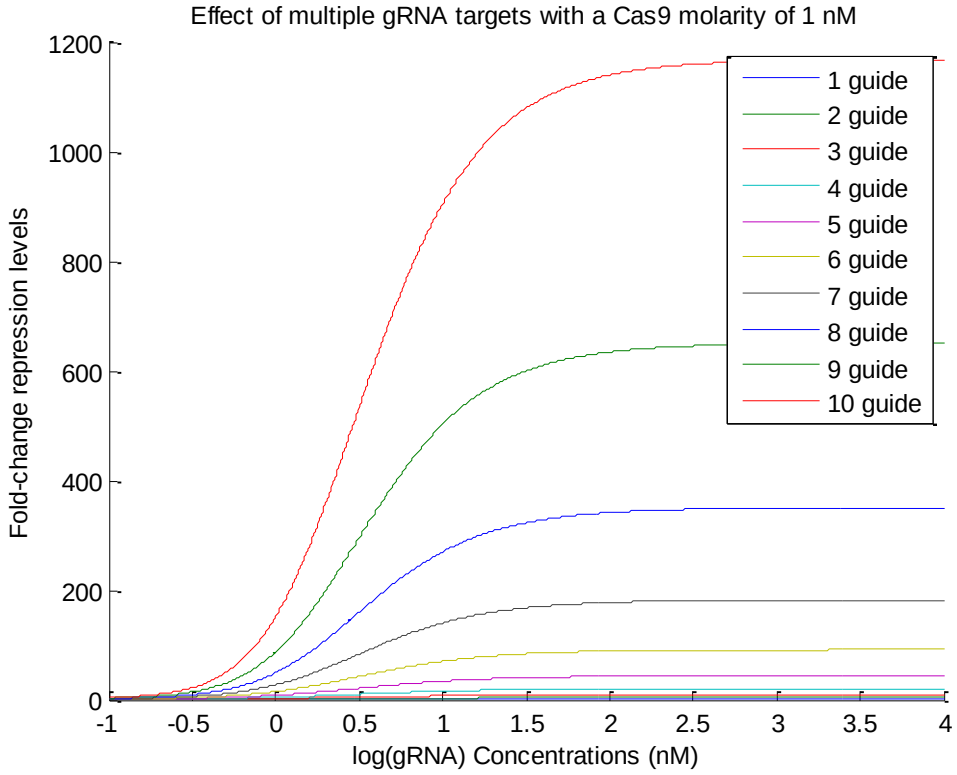

Figure 1b: When the data is changed to fold change of repression, the increase in repression with respect to number of Cas-9 binding sites appears almost exponential

The fold of repression given by the model appears to follow a roughly exponential trend with respect to number of guide RNAs. At 100 nM of total gRNA, the exponential constant is seen to be 0.5489 with a pre-exponential of 2.497. At 1 nM, the exponential constant is almost the same, 0.5631 but the pre-exponential is only 0.5489. At higher concentrations of gRNA, the graph can be described with more accuracy modelled with an exponential function, with an R^2 approaching 1.

Next, the model was used to hypothesize the effect of starting the activation sites starting at 2000 base pairs down a gene. As would be expected, the model predicts that the level of repression would be significantly lower. This effect seems to be stronger on systems with more gRNA binding sites, leading to less extreme differences between these systems.

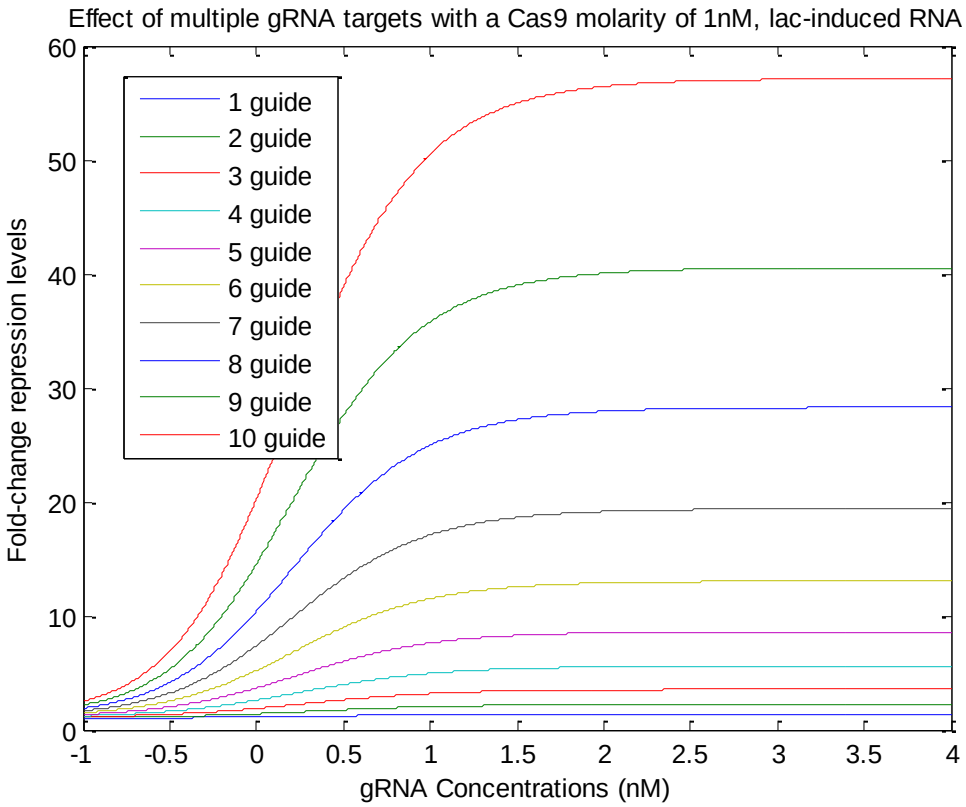

Figure 1c: Effect of various amounts of gRNA specific target sites. The modelling assumes the sites start 2000 base pairs down the transcribed genes.

The model was then used to represent a system with a higher level of Cas9 protein. In the following figure, the Cas9 molarity was kept at 10 nM in place of the previous 1 nM. The fold change increases, but it appears to increase at the same rate for all amounts of gRNA targeting sites. The only apparent difference in the repression appears to be the earlier plateau region.

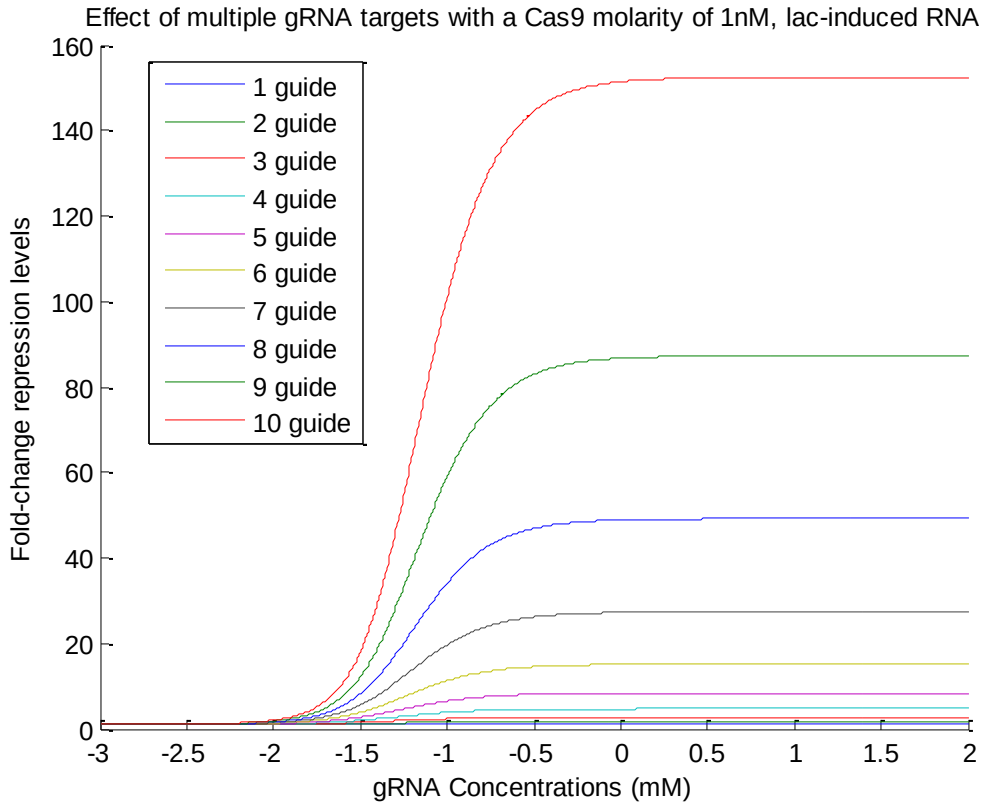

Figure 1d: Effect of various amounts of gRNA specific target sites, starting at beginning of transcription with 10 nM initial Cas-9 concentration.

When attached to the Lutz and Bujard lac promoter with a full production rate of 1 nM/s, the effect of multiple gRNA is frozen to a maximum value due to the maximum production of the lac promoter. It also makes use of the modelled cooperative effect of the lac effector to create a more binary repression response. The speed of the lac in this simulation presented a limiting factor at the current level.

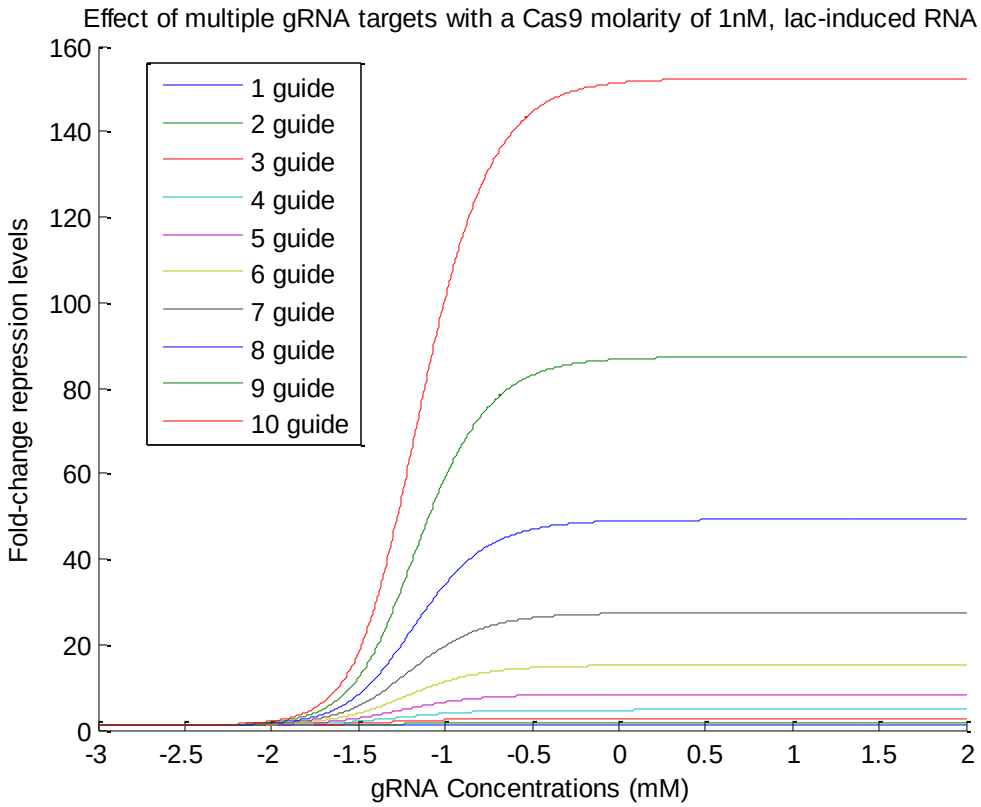

Figure 1e: The effect of IPTG productions is shown, with a maximum production of 10 nM of gRNA.

Maximum production is very flexible due to exogenous factors like copy number and promoter factors, so the exact value of this value is unknown experimentally. However, if maximum production is in excess of Cas9 production, the final protein repression will approach its maximum. Otherwise, the repression plateaus before expected maximum repression.

Modelling anti-gRNA

With the addition of anti-gRNA to the repression construct, the repression appears to become more binary in nature. This is as to be expected by the nature of molecular titration. This feature can be utilized to facilitate the creation of bi-stable systems of repression. These effects appear to hold even in the case of higher levels of Cas9 productions and molarity.

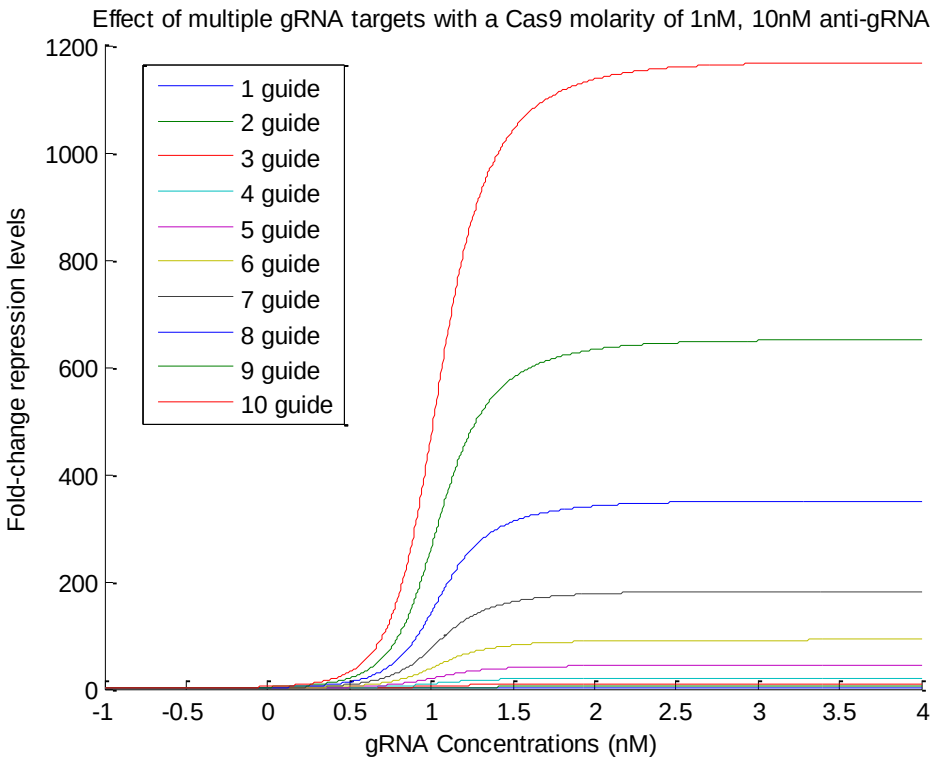

Figure 2a: Although the addition of α-gRNA appears to lower coopertivity slightly for higher numbers of gRNA, the effective K_d increases to more practical levels to change the effective cut-off point to a binary state.

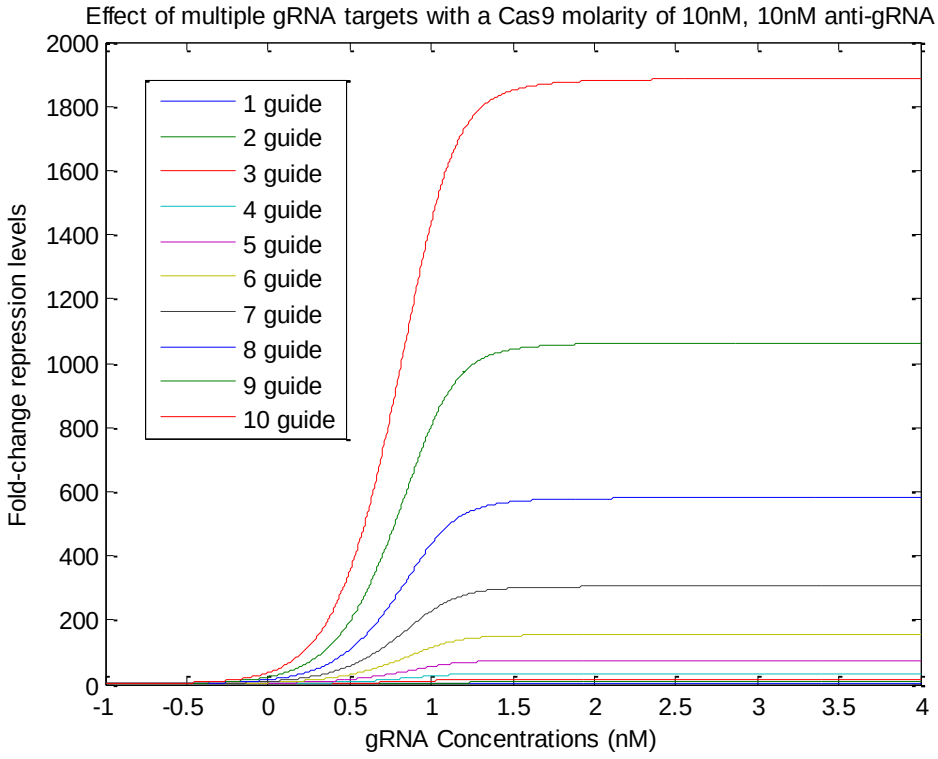

Figure 2b: A higher constituative Cas9 production increases the power of the Cas9 repression

For a better understanding of how the addition of anti-gRNA molecular titration affects the behavior of the repressive system, it is useful to compare the behavior of the system both with and without the presence of anti-gRNA. As shown below, the maximum repression does not appear to change, only the period over which the repression goes from negligible to maximum.

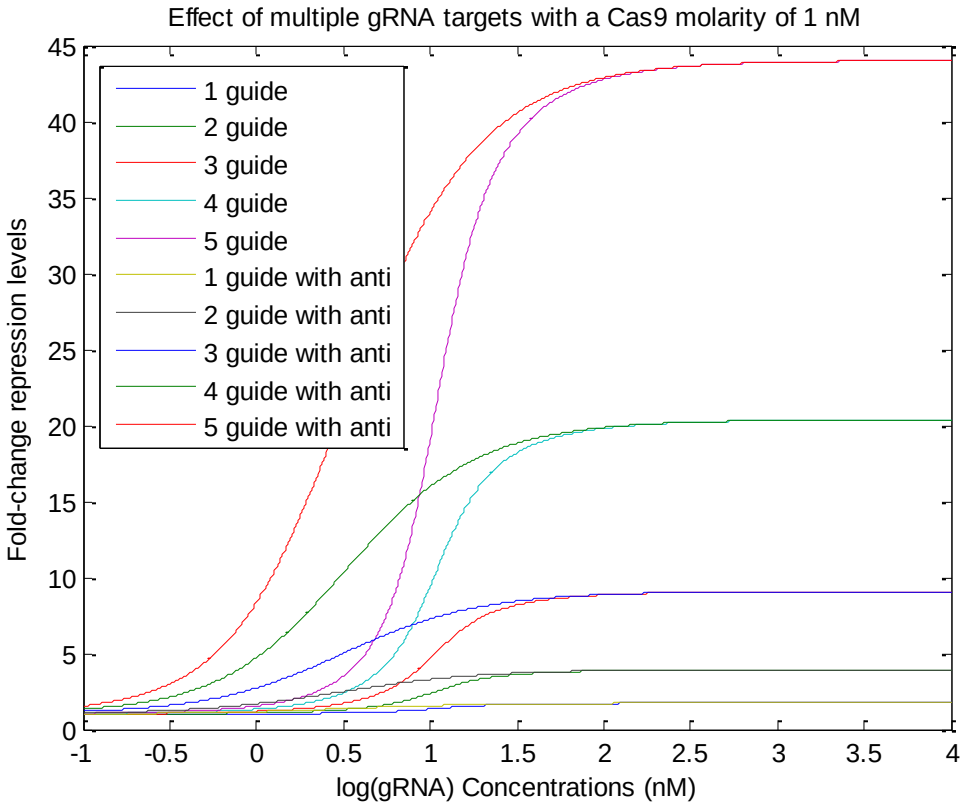

Figure 2c: Overlay of gRNA repression with and without anti-gRNA present.

This allows for a framework with which to build a bistable system by increasing the apparent Hill coefficient of the repressive system.

Bistability

Bistability is defined as the existence of two equilibrium positions given the same system conditions. In the case of a two mutual repressors, the condition with the most apparent bistability is that of high levels of repressor concentration. Therefore, in simulations, the system moves from various initial concentrations of gRNA to an equilibrium condition.

However, unlike previous simulations that feature an exogenous production of gRNA, this bistability construct acts upon the production rate of gRNA. A model cannot properly model a bistability phenomenon without also modelling how gRNA production leads to the system's development towards equilibrium. The differential equation given to describe the change in gRNA molarity is below.

$$\frac{d[gRNA]}{dt} = ProductionRate - DecayConstant * [gRNA]$$

The simulation runs with 5 nM of total Cas9 protein and with constants as listed in Table I. Code is found in Appendix A2. Equilibrium is taken as the measures at 50,000 seconds, or approximately 13.9 hours into a system's creation.

The first experiment to run as a control is a system with only one gRNA target per promoter. With this basic set up there is no instance of bistability. The equilibrium expression for both promoters appears to be monostable at around 17% of maximum transcription rates.

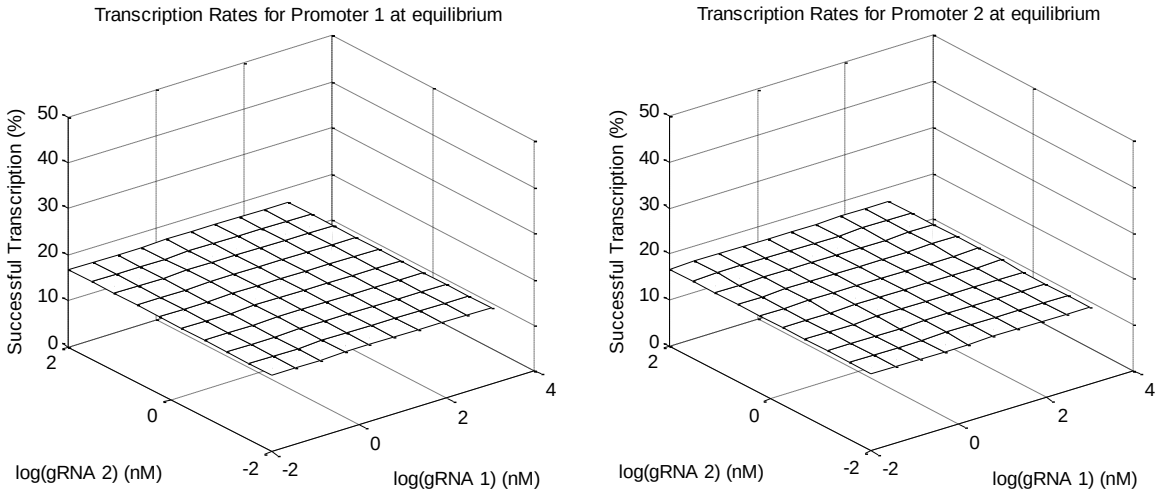

Figure 3: With 1 gRNA target and no anti-gRNA, the system is monostable for the experimental range

However, as the number of gRNA targets increases, a bistability arises and becomes more and more apparent. The first visible bistability happens with three targets and with each

target site, the amount of transcription on the favored side increases. An example of this phenomenon is below.

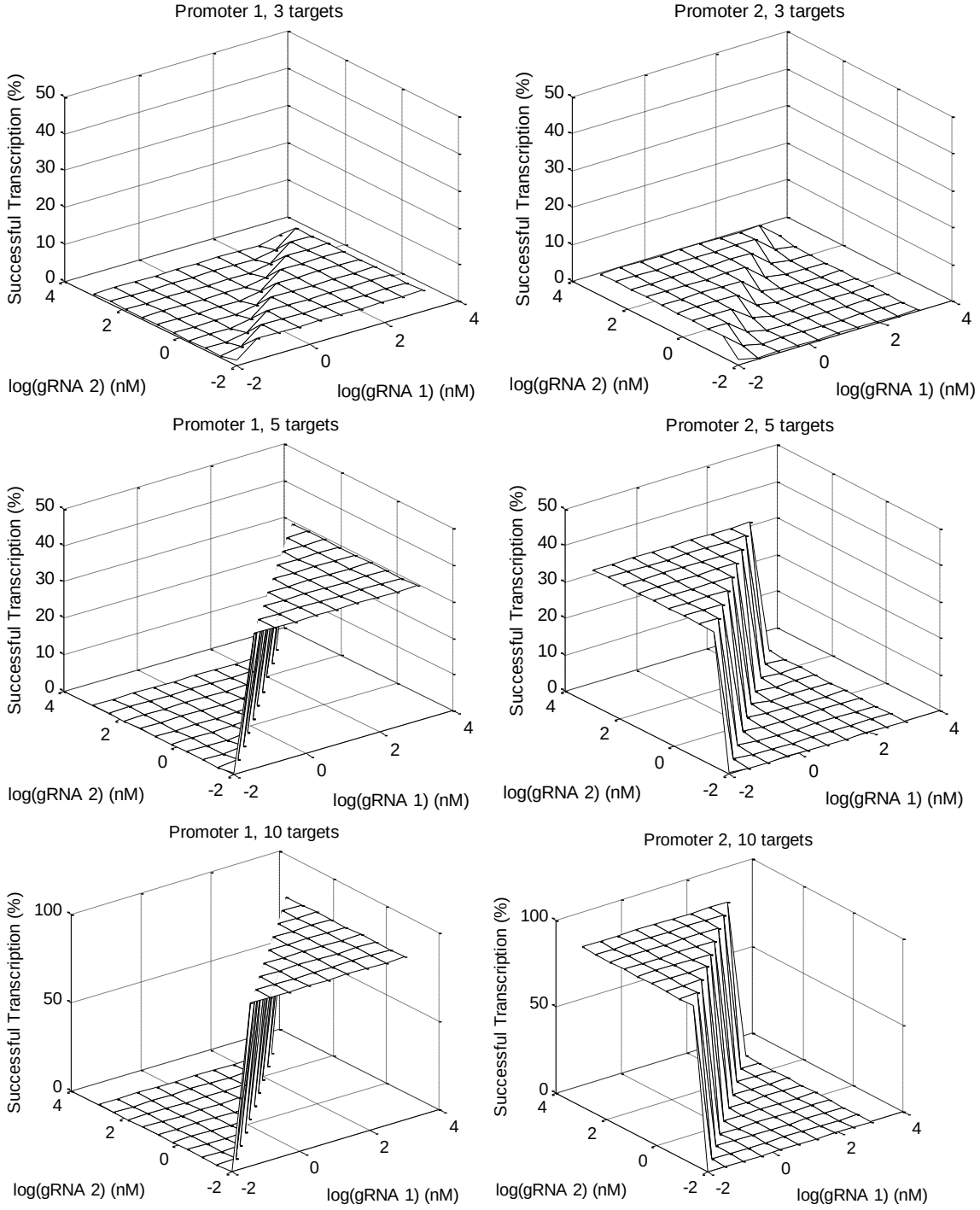

Figure 4: As the number of target sites increases, the system moves towards two clearly delineated states. As these states diverge, the favored promoter becomes more fully expressed.

From these graphs, it becomes possible to analyze the function of the maximum successful transcription of these bistable states as more gRNA target sites are added to the system.

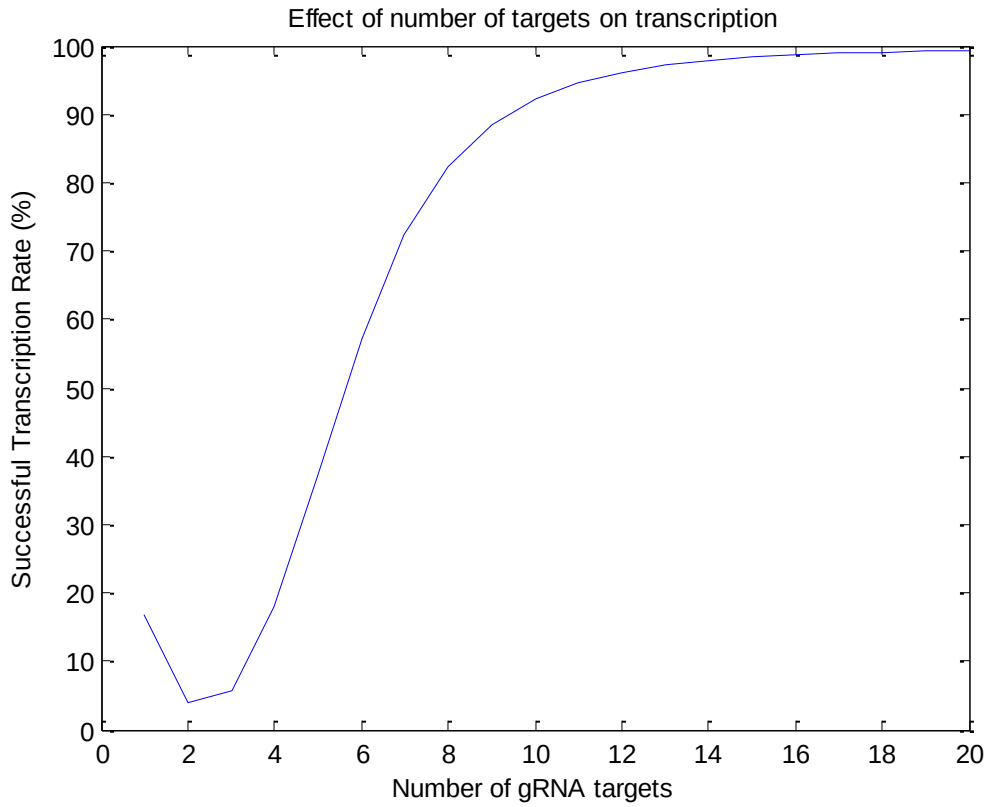

Figure 5: The maximum equilibrium transcription lulls before entering a bistable state starting at 3 targets. At that point, as more targets are added, the maximum successful transcription rate approaches 100%

Using this simulation framework, the anti-gRNA nucleic acids were added to the model. For the following simulations, the simulation constants were kept the same with the exception of a total 1 nM of anti-gRNA amongst the target sites of each gene. The effects of this phenomenon as the number of gRNA targets increase is seen below.

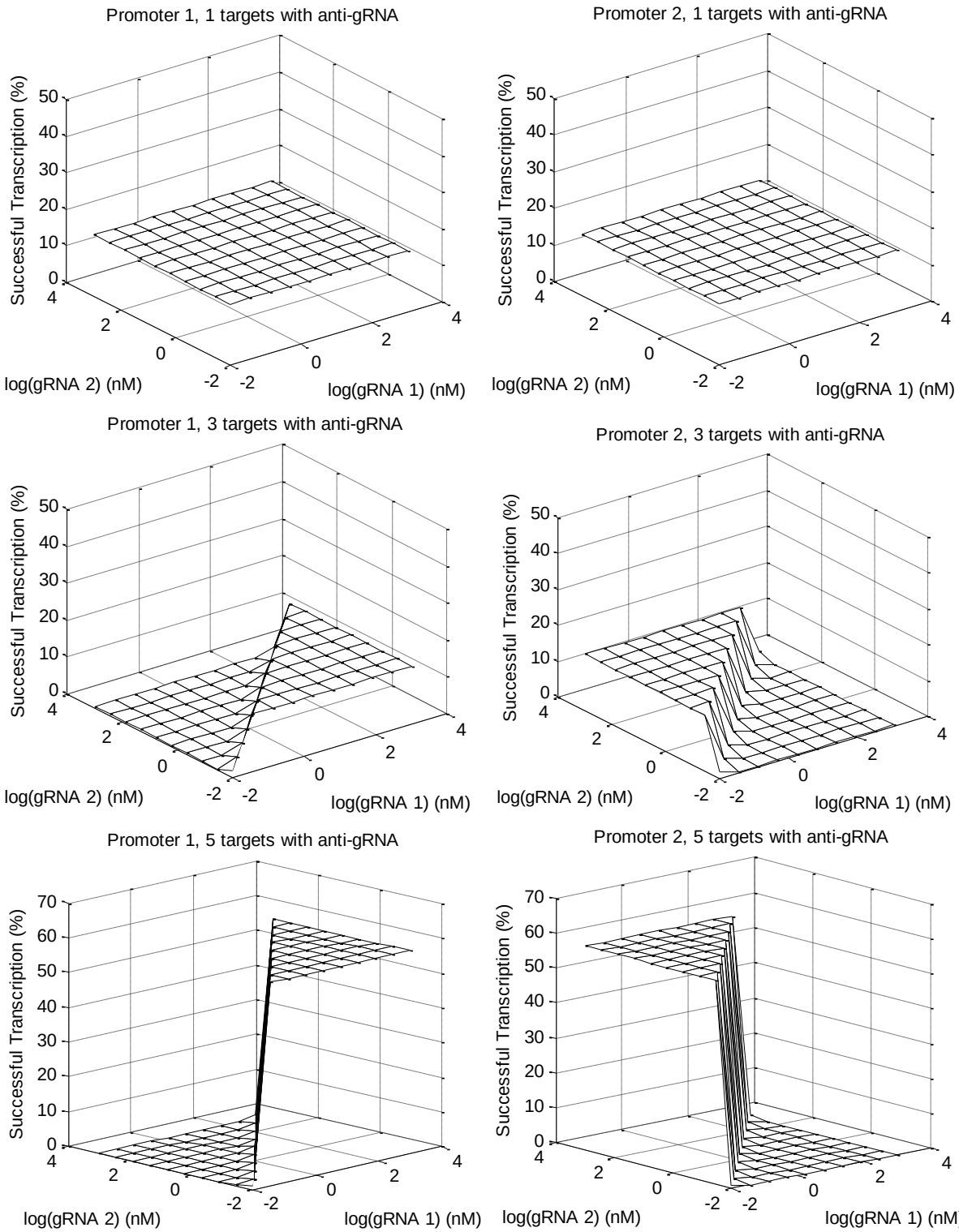

Figure 6: The system with anti-gRNA exhibits similar phenomenon to the system without anti-gRNA. The only difference appears to be the higher transcription rate of the favored Promoter.

With higher concentrations of anti-gRNA however, the amount of gRNA targets necessary to create bistability decreases. Take for example the difference in the two-gRNA-target response between systems with 1 nM of anti-gRNA and 100 nM. Not only does this higher anti-gRNA molarity create a bistability that does not exist in 1 nM, it creates a possible ambiguous state where no concentrations of gRNA are high.

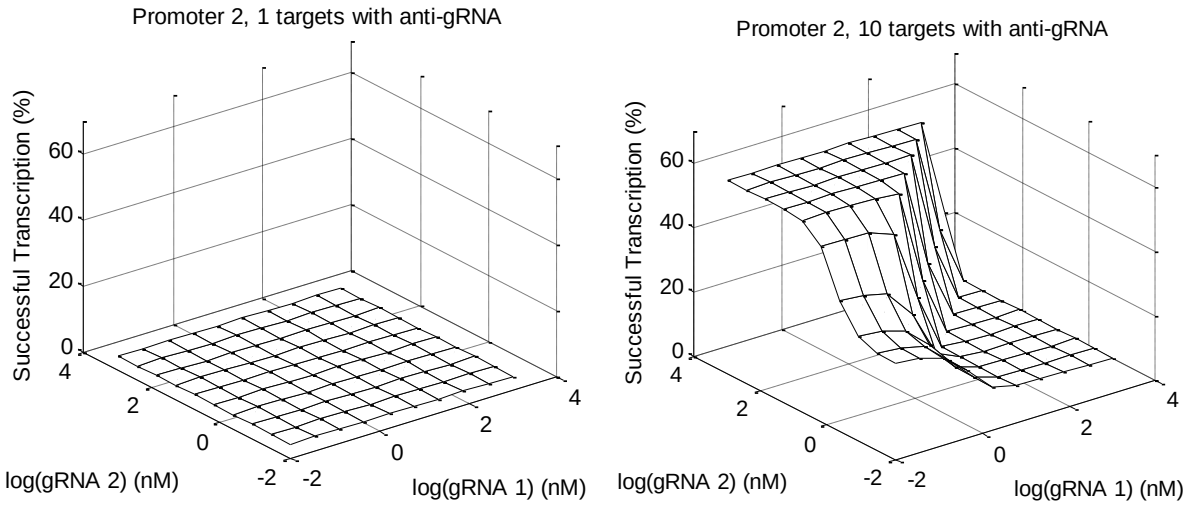

Figure 7: The behavior of the two-promoter construct is observed in the presence of 1 nM and 100 nM of anti-gRNA. At the higher level, the bistability and ambiguous regions arise.

From these graphs, it becomes apparent that system functions similarly to the system without anti-gRNA. From this observation, simulations were run that show the effect of increasing the amount of anti-gRNA in the system. This acts as a summary of the two phenomenon observed in the addition of anti-gRNA, namely the earlier bistability and higher levels of transcription associated with the higher concentrations of anti-gRNA.

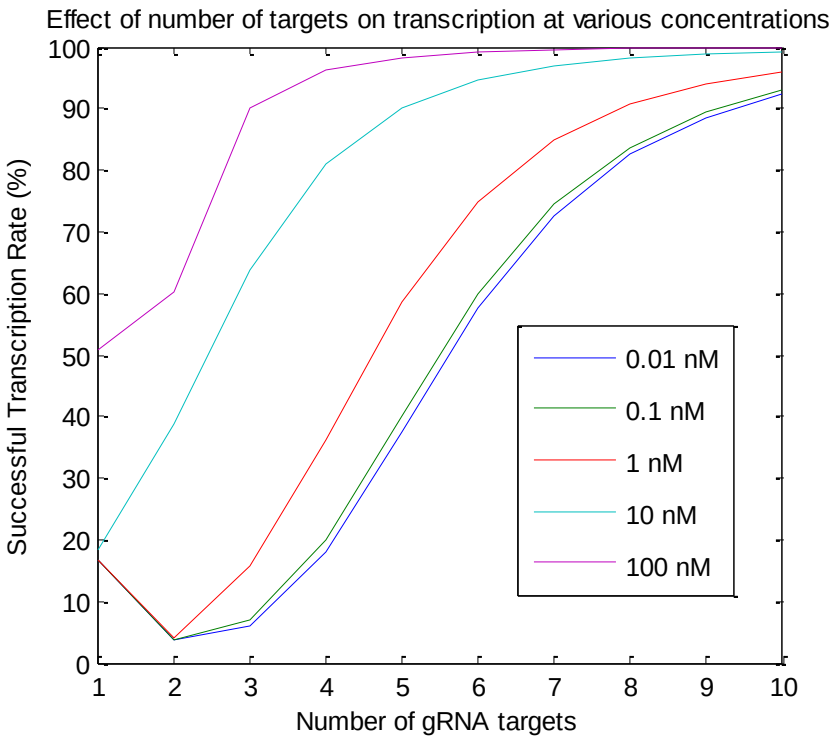

Figure 8: The Mapping of the maximum transcription rate illustrates not only the phenomenon of higher transcription rates of the favored state but also the phenomenon of bistability with fewer gRNA targets.

Another useful construct for lab is the linking of anti-gRNA to the levels of the opposing gRNA. In this construct, anti-gRNA can be thought of as a shield against the other promoter's gRNA repression. In this construct, the equilibrium bistability is reached with just one gRNA anti-gRNA pair and a maximum transcription rate of 0.1 nM/s. From there, the addition of more gRNA anti-gRNA pairs quickly approaches all-or-nothing binary. This can be seen in the equilibrium states below and the transcription rates as the number of gRNA and anti-gRNA pairs increases.

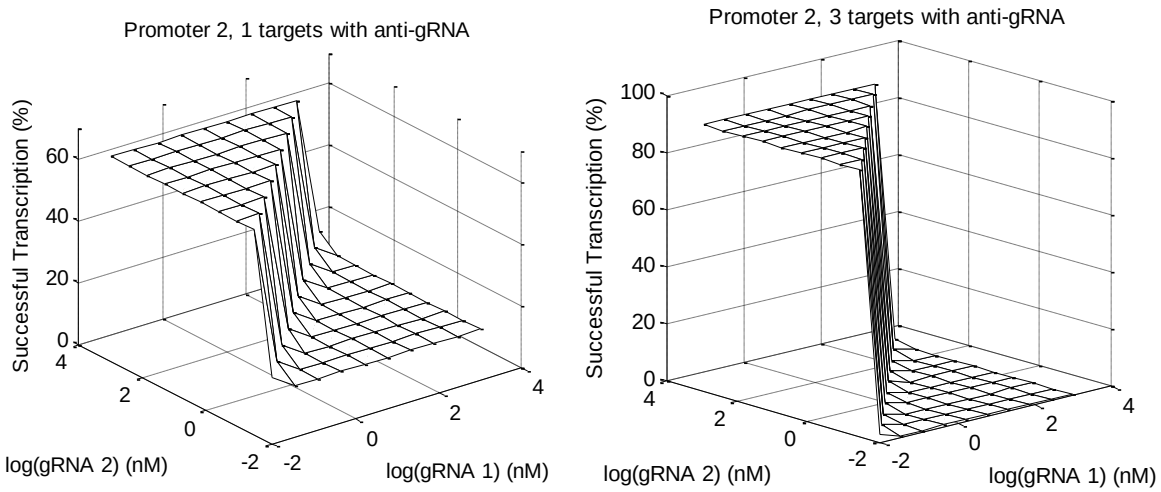

Figure 9: The effects of pairing the pairing anti-gRNA with gRNA production becomes apparent with the most binary response of the observed anti-gRNA strategies

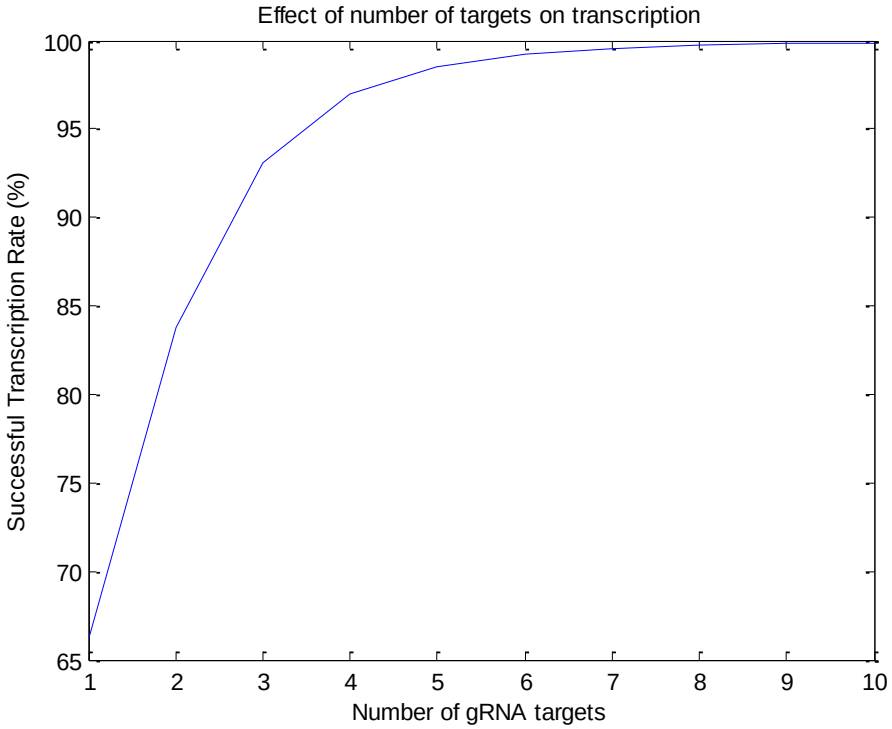

Figure 10: The number of gRNA anti-gRNA pairs increase, the residual transcription repression of the favored state appears to decay exponentially

However, in a in vivo experiment, it is unlikely that the two promoters will be perfectly balanced. For practical applications of this system, it is necessary to understand how promoter asymmetry leads to the breakdown of bistability. For the following simulations, the matched

gRNA and “defensive” anti-gRNA construct will be observed with a variety of unmatched promoter strengths in the presence of 1 nM of exogenously produced Cas9 protein. For example, an order of magnitude inconsistency can lead to a loss of bistability even with only one guide as seen below. This holds for all relative discrepancies for various transcription rates.

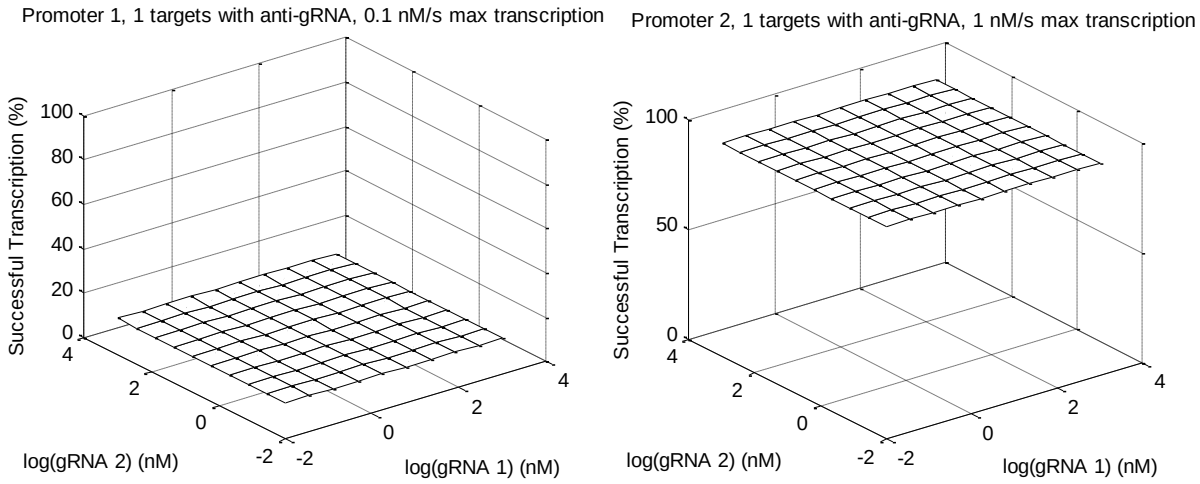

Figure 11: A pair of mismatched promoters, one with a max transcription of 0.1 nM/s and the other with a maximum transcription rate of 1 nM/s. This leads to a favoring of the faster promoter over the full range of experimentation in promoter speed and initial gRNA molarities.

However, with higher numbers of gRNA anti-gRNA pairs, the system can overcome the mismatch and still obtain bistability, although the maximum transcription rates of these two states are not balanced. For example, the above conditions can reach bistability in the presence of foue gRNA anti-gRNA pairs, regardless of absolute promoter strength. Further, with larger mismatches comes a larger number of gRNA pairs needed to create bistability. A hundred fold difference in promoter strength is not overcome until eight pairs.

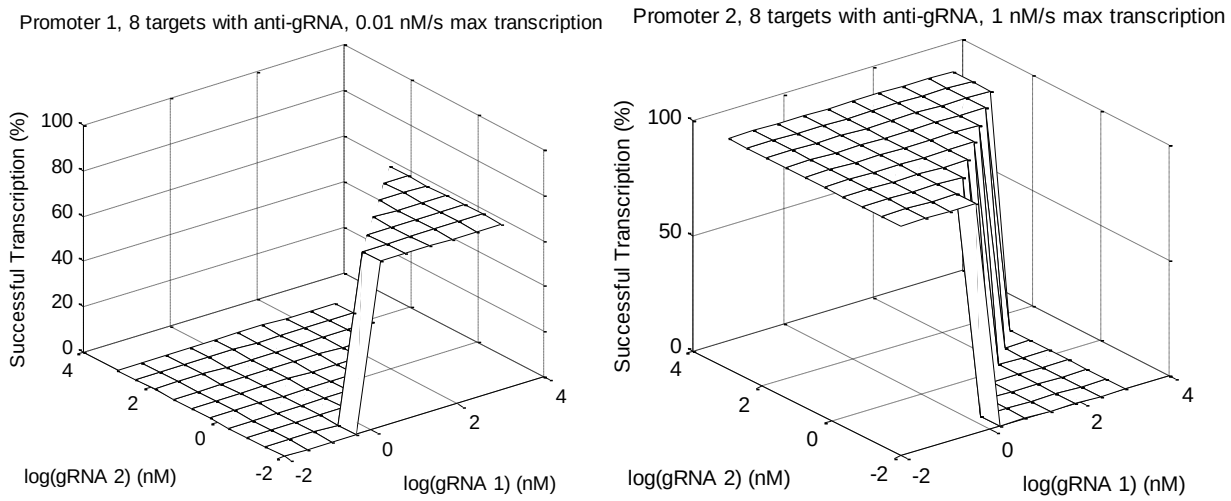

Figure 12: In the case of a hundred fold mismatch of promoter strength, the system requires eight gRNA anti-gRNA pairs to reach bistability. This bistability, however, is not symmetric in terms of either the region that approaches each equilibrium nor in the transcription success in each state.

Wet Lab Experiment Simulation

From these observations, hypothetically experiments can be modelled through the control of gRNA and anti-gRNA levels. Moving towards the construction of plausible constructs starts the transition of these bistable systems into functional synthetic gene circuitry.

However, in the laboratory setting, it is difficult to prepare and directly insert gRNA directly into the cells. The cell's own transcriptional mechanisms controlled with excitable promoters provide an easier means of creating a controlled and variable response. Consequently, the simulation utilizes lac and tet repressors controlled by IPTG and aTc respectively to model possible bistable constructions. Within this experimental framework, conditions will be kept within the plausible limits of technology and genetic design.

The scope of this paper will deal primarily with systems of up to five or six gRNA or anti-gRNA elements per promoter. Although *in vitro* plasmid design allows for the creation of longer constructs, five or six elements represents a reasonable amount that can be connected using Golden Gate cloning. With regards to promoters, even in the most controlled systems, a

mismatch could be up to a fold of magnitude apart from each other. This will act as a heuristic benchmark for testing promoter mismatch. The amount of Cas9 protein present in the simulation is moved to a higher but plausible molarity level of 5 nM.

Model System

The model used below represents two promoters transcribing mutually repressive gRNA sequences. These interact with the exogenous Cas9 protein to attach along the opposing transcription region, limiting transcription rates via road-blocking. One promoter is also controlled by the lac operon and the other is controlled by the tet promoter. Initial gRNA levels are taken as zero. This system is visualized below.

Understanding tet and lac

In simulations, the difference in character between the tet and lac promoters leads to asymmetric response in the system. In order to understand the movement of the system, the model system described about is simulated with various concentrations of IPTG and aTc and allowed to move to equilibrium. The equilibrium states of these conditions are given below. When the promoters have equal transcription rates and substrate activation, the areas of low activation favor lac whereas areas of high activation favor tet. With the addition of more gRNA anti-gRNA pairs, these two states appear nearly binary with the full transcription of one promoter and full suppression of the other.

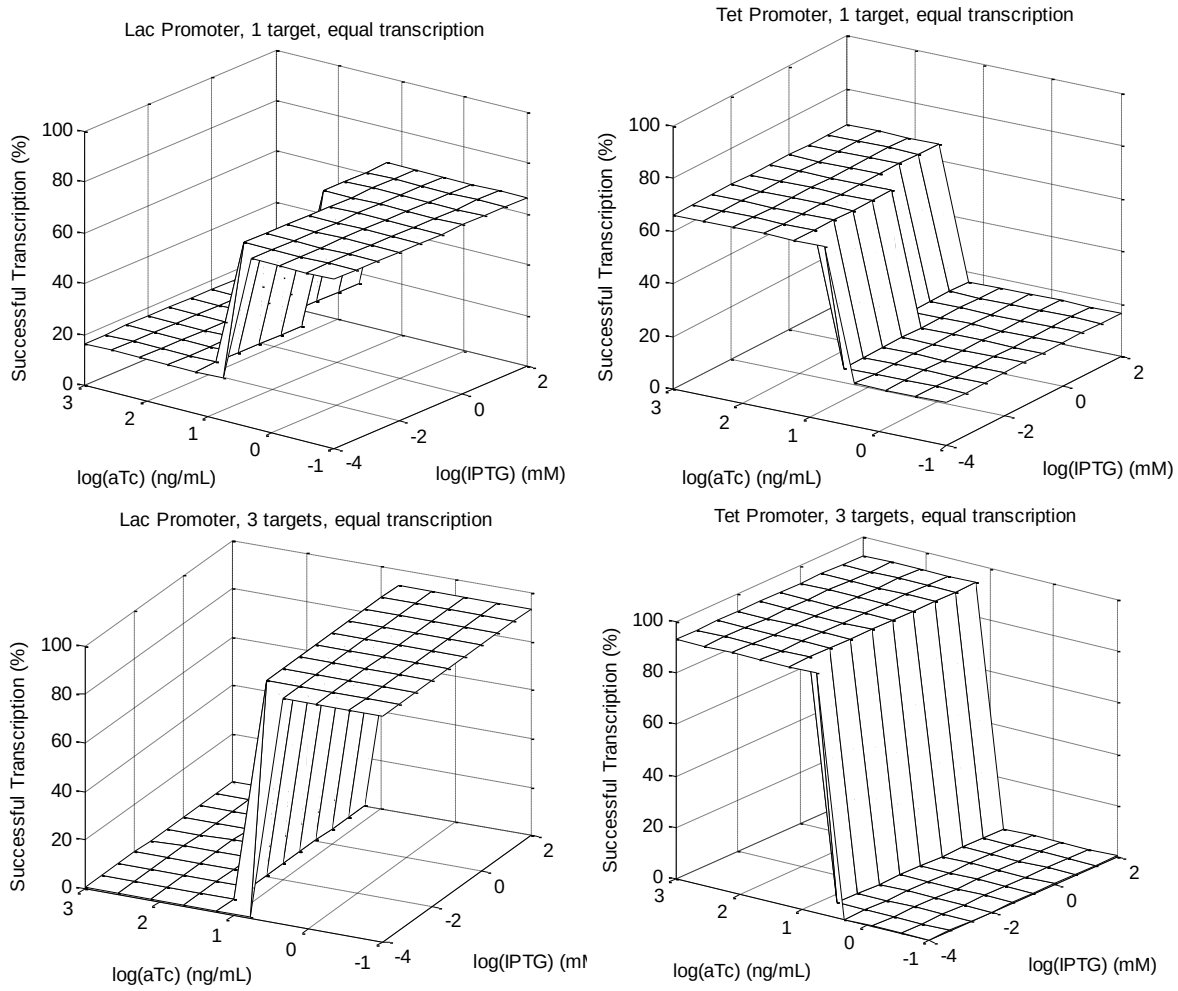

Figure 13: The equilibrium of a system of a mutually repressive tet and a lac promoter at various stages of activation.

From these preliminary simulations, aTc levels seem to affect the equilibrium state more readily than IPTG. Although this observation will not affect the means by which the system is analyzed in this paper, it is worth note for the experimental production of similar systems.

Promoter Mismatch

To understand the role of promoter mismatch on the behavior of the construct, simulations are run with a tenfold difference in max transcription rate: one with 1 nM/s and the other 0.1 nM/s. When the lac promoted sequence is more effectively transcribed, it is also

favored when high concentrations of IPTG and aTc are present, and tet maintains transcription only in cases of very low IPTG concentrations. When tet promoted sequence has a higher maximum transcription rate, the only difference is the maintenance of transcription at low levels of both IPTG and aTc.

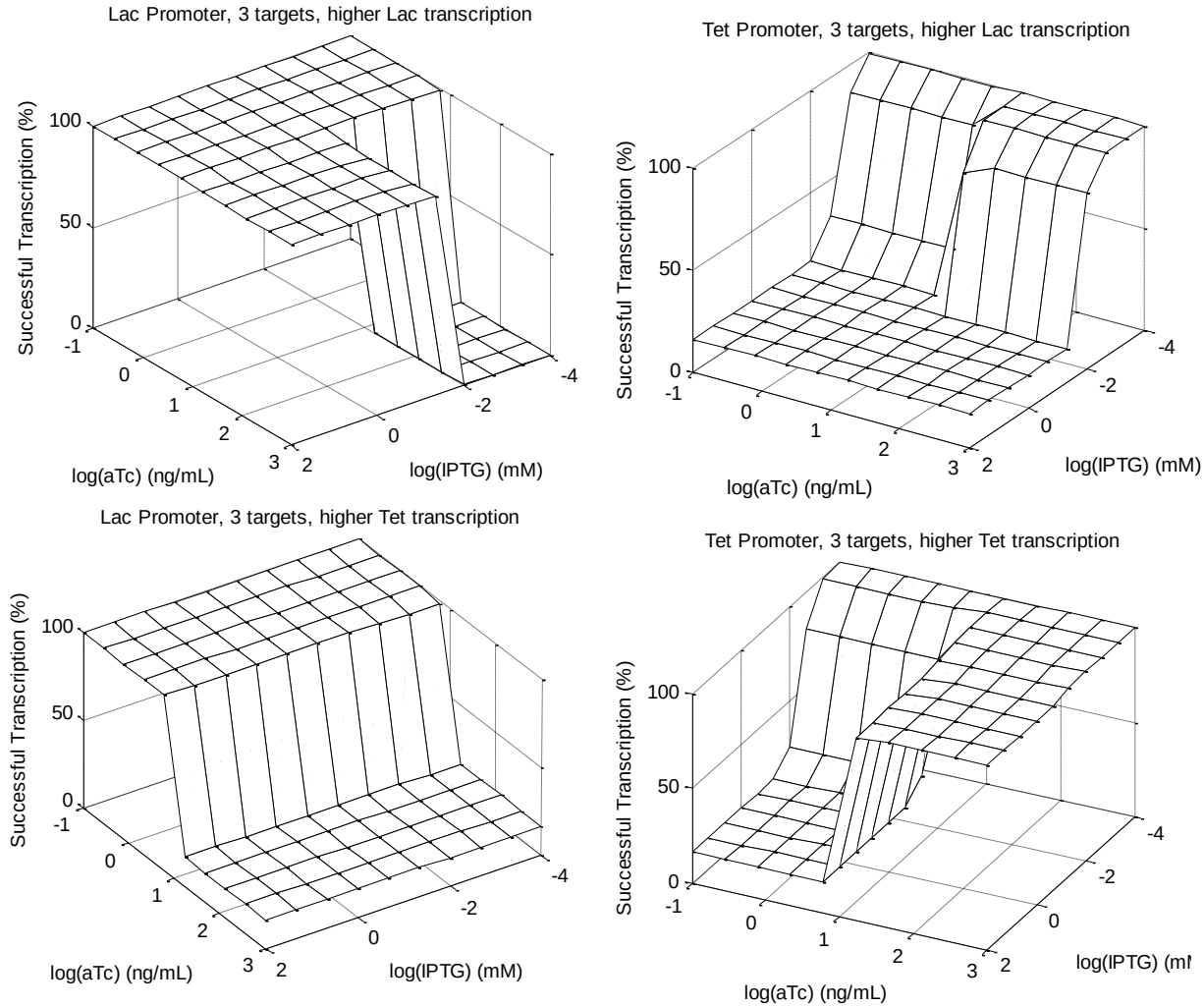

Figure 14: Mismatched promoters cause the previously indeterminate highly stimulated region to favor the more powerful promoter. The first row features higher transcription rate at the lac promoter, the second row features it at the tet promoter

Bistability Testing

These conditions are, however, not a test of bistability of itself. From this point, it is necessary to move from these conditions to a set of standard conditions. Simulations thus will

have two steps, a movement to equilibrium at various concentrations of IPTG and aTc, then to a standard concentration of the two. For purposes of the experiment, the standard condition is established as 1000 mM of IPTG and 100 ng/mL of aTc. These values represent an excess for the activation on the modelled promoters, leading to approximately full expression. Bistability is verified by the presence of two distinct phases under these new conditions.

Bistability in the absence of anti-gRNA

Using only Cas9 roadblock repression, weak bistability is predicted in the simulation with equal promoter strength. First signs of bistability appear with three gRNA, and with more gRNA targets, the promoter favored in the equilibrium becomes increasingly expressed. With six gRNA the expression of this maximum is nearly 60% of maximum transcription. Although this is enough to clearly delineate the two equilibria, it is worth comparing systems with anti-gRNA in order to increase equilibrium production towards full transcription.

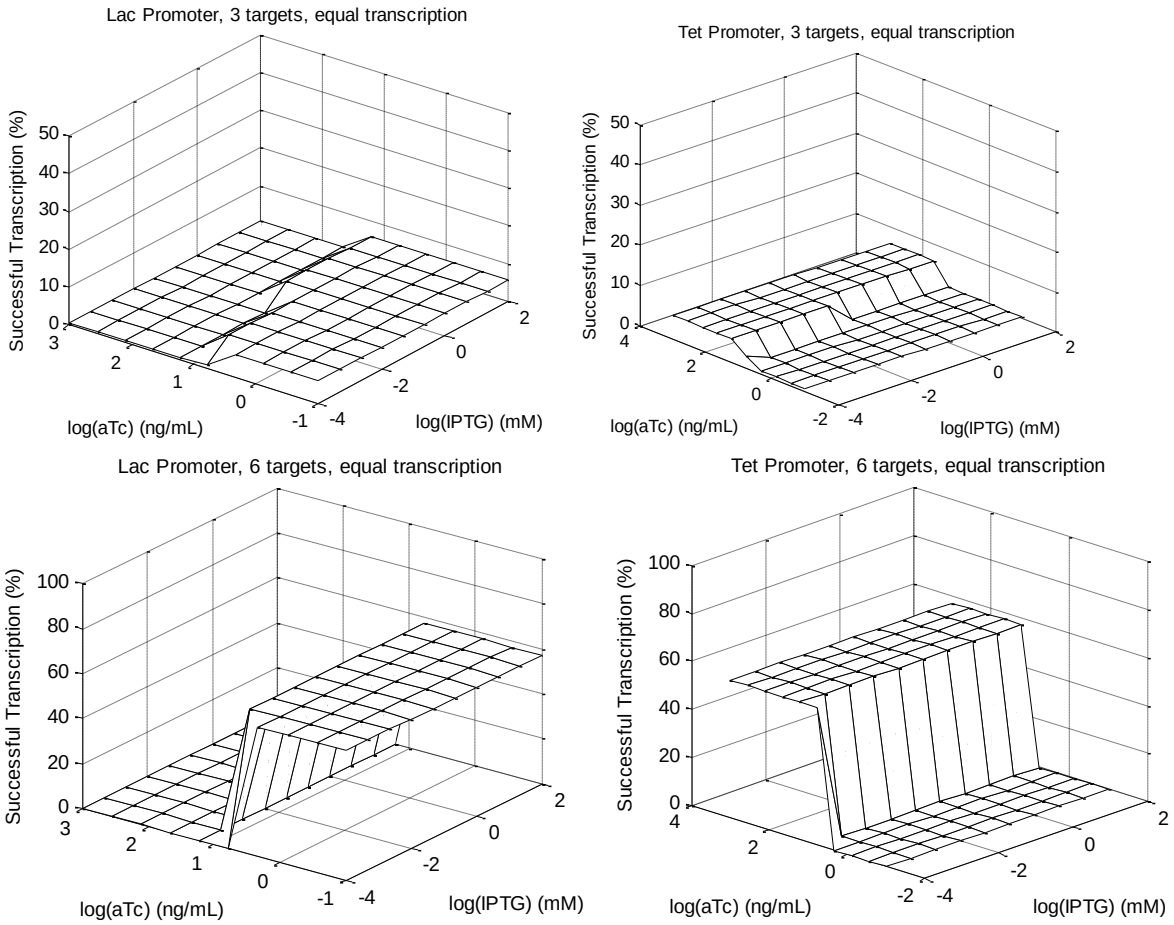

Figure 15: Without anti-gRNA, the model construct still reaches clearly defined bistability in ideal conditions with enough gRNA targets. It is slow to approach full equilibrium transcription

Bistability with matched anti-gRNA

In the case of one gRNA anti-gRNA pair and equal promoter strength, the system forms a rough bistability depending on mostly on the initial concentration of aTc with minor variation based on initial IPTG levels. With more gRNA targets, the system's two phases become very clearly defined, level and binary. However, a region of mutual repression still exists at very low initial levels of substrate activation.

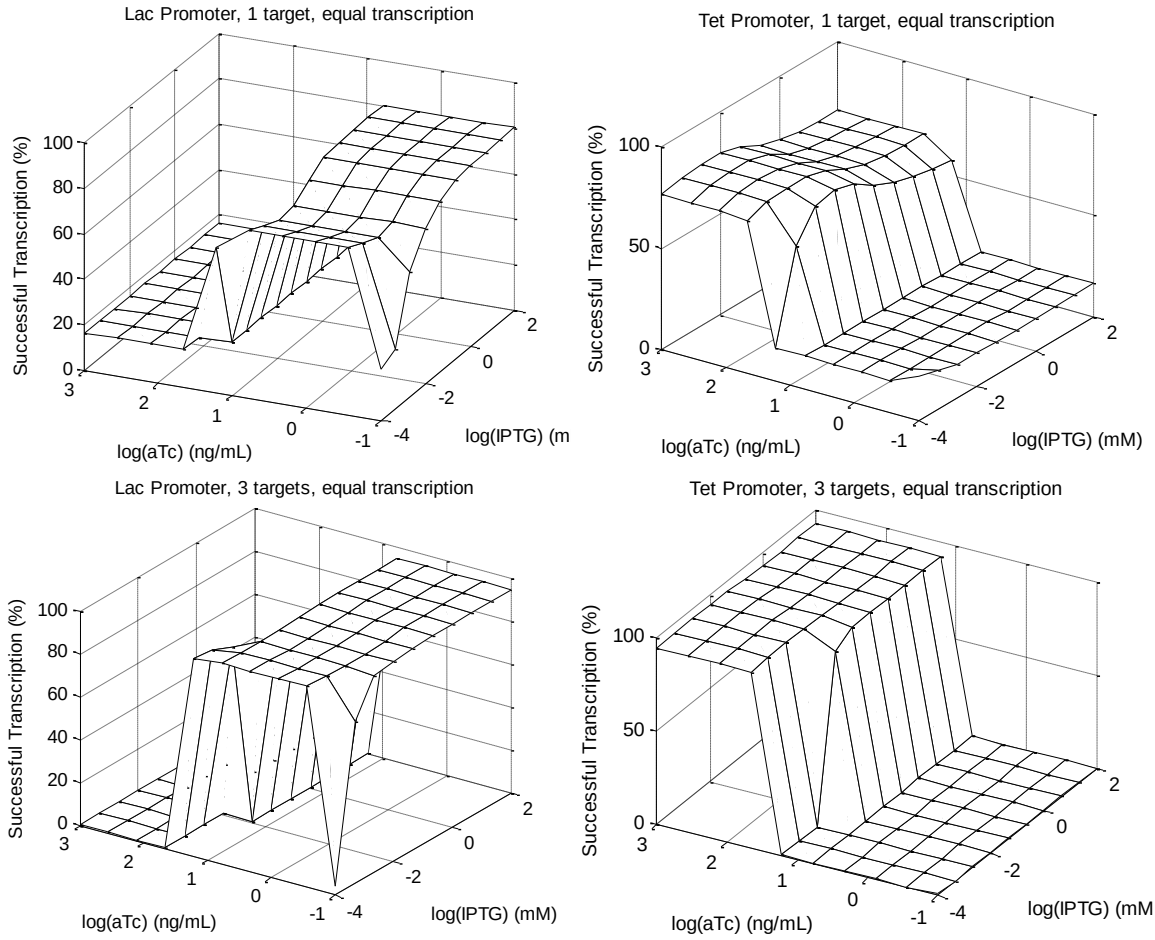

Figure 16: Equilibriums at 100 mM IPTG and 1000 ng/mL of a system primed with different levels of substrate activation. With the addition of larger numbers of gRNA anti-gRNA pairs, the system approaches binary.

When the promoters become mismatched, the bistability of the system fades very quickly. Within a tenfold difference in transcription rate as above, bistability is lost even with three gRNA and anti-gRNA pairs. Although a weak bistable state arises with 4 gRNA pairs and later goes towards strongly delineated bistability, a transcription featuring eight or more total RNA units as well as a reporter gene would be difficult to assemble in a laboratory setting.

These phenomena were present regardless of absolute differences in promotion rate. For each promoter, the margin of bistability for the 3 gRNA system is about six-fold difference although as mismatch increase the equilibrium favoring the weaker promoter exhibits lower successful transcription rate.

Bistability in a system of exogenous anti-gRNA

An alternative to the gRNA anti-gRNA pairs is to produce only gRNA along the transcription region and to have anti-gRNA expressed exogenously elsewhere. This system would allow more gRNA target sequences with the same amount of plasmid design, with the same number of components in a single gene. In this case, the anti-gRNA is held at 10 nM per promoter split evenly among the gRNA sequences. Simulations show that after reaching bistability with two gRNA sequence per promoter, it continues to approach binary response. However, this rate is slower than systems with matched anti-gRNA of a similar amount of components for simpler systems.

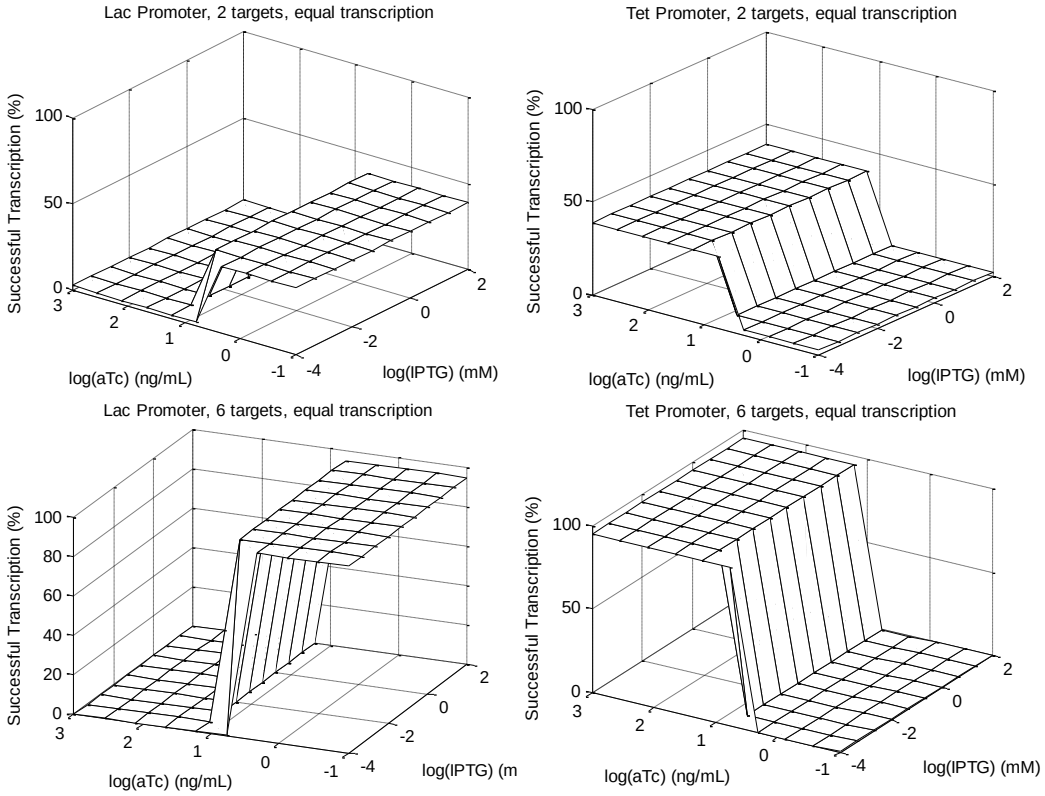

Figure 17: The behavior of the exogenous anti-gRNA bistability construct is observed to move towards binary similarly to the matched anti-gRNA construct, although lagging for simpler systems

The exogenous anti-gRNA construct also allows for bistability in more mismatched promoters. For example, promoter speeds are as above and the total anti-gRNA molarity against

each promoter's gRNAs is 10 nM. In this construct, a tenfold difference in promoter speed becomes bistable with 5 gRNA. With 6 gRNA per promoter, the system is bistable for a range of up to a thirty-fold difference in maximum transcription rate.

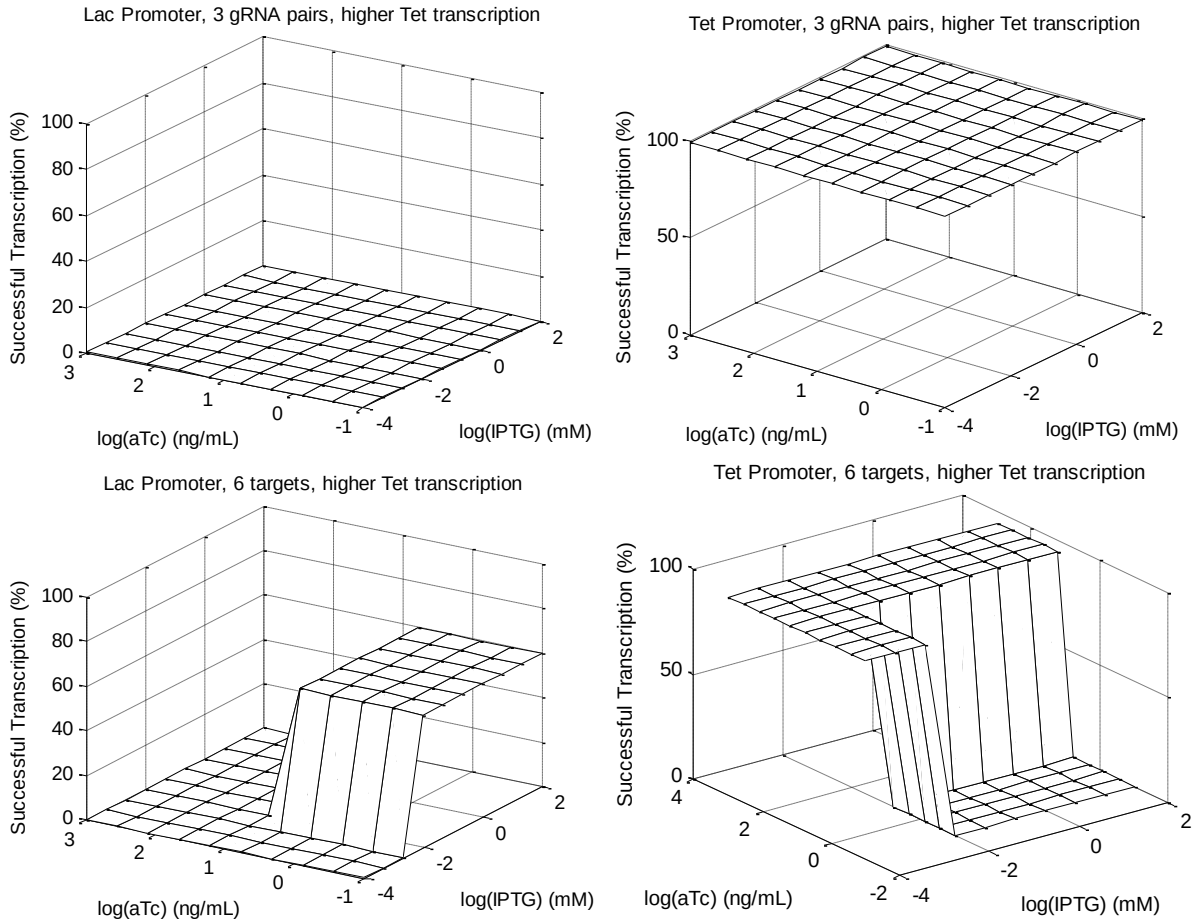

Figure 18: A comparison of equilibrium states of systems with six RNA components per promoter with a tenfold promoter mismatch.

Conclusions

Within this theoretical framework, it is possible create a bistable on-off switch using Cas9 roadblock repression. This can be done using the combined repression of multiple gRNA target sites along the promoted genes. With six gRNA targets, even without anti-gRNA present, the predicted fold change of transcription is over 3600. When anti-gRNA elements are present exogenously, the fold change for six gRNA targets is over 6000.

Although the promoter transcribed anti-gRNA increases fold change difference even further, it does not create a fold change comparable to the amount of elements in the transcription region. With three gRNA targets and three anti-gRNA sequences, the fold change is only predicted 190.

However, the two systems of anti-gRNA control maintain different benefits beyond fold change that effect their usability. Although the fold change of the matched anti-gRNA system does not scale to complexity, the amount transcribed is higher. This holds true especially for simple systems such as one gRNA target with anti-gRNA or two gRNA. In these cases, the amount transcribed is nearly twice as much in the system of matched anti-gRNA. The matched anti-gRNA construct is also fully self-contained in two promoter sequences rather than needing an engineering exogenous source of anti-gRNA.

The exogenous anti-gRNA system allows for more robust bistability. If the promoter strengths cannot be matched with certainty, there is more room for error in the fixed anti-gRNA system than in the matched. In situations where multiple binary switches depend on each other, this added margin of error may be worth the lower transcription rates. In cases where high transcription rates are necessary, with larger amounts of gRNA targets the exogenous anti-gRNA system still approaches full production.

However, within the model there are multiple sources of error and incompleteness. Many of the constants used are not fully known and were retrofit from data points and from other circumstances. Further, the lac and tet promoters are not exactly given by the Hill equation, and thus could be improved. Differences in gRNA and anti-gRNA concentrations based on the location of coding sequence within the transcription region are ignored.

Another major issue of the model is the lack of research performed on the interactions between multiple gRNA target sites. Observations of interactions between sites show that the cumulative repression is very temperamental and hard to predict. If the factors guiding the interaction of gRNA sites are better understood a more complete and representative model can be developed to predict the repression and bistability of Cas9 roadblock based systems.

Finally, the model is based on equilibria and averages and thus ignores stochastic variations within the system. It may be worth expanding the model to include these factors in order to create a more robust model of bistability and the interactions between the two equilibria.

Appendix A: Code

A1: Repression simulation used for Figures 1a through 2c, featuring one repressor system

```
clear
clf

%% Constants
%k1=### %Cas9-gRNA Kon rate
%k2=### %Cas9-gRNA Koff rate
FullLacPromotion=1; %(nM/s) This is made up
KpdCas9RNA=5; % Kd of Cas9-RNA binding (1/nM), close to other similar
              % protein interactions, Ryder and Recht, 2010
Kd=0.5; %Kd of specific binding of Cas9 to DNA (nM),  Sternberg 2014
KdTitration=1; %(nM) UNSUPPORTED, assumed similar to RNA-DNA
RoadblockDecay=4800; %Derived from Qi 2013, decay constant
Cas9Length=200; %UNSUPPORTED, NEED SOURCE

%% Code
hold on
figure(1)
%clf

%% Basic Code without Anti-gRNA
for n=1:10 %Number of gRNA
   for m=1:1000


%       %LacPromoterStuff
%       IPTG=10^(m/200-3); %(mM)
%
%       y(2)=FullLacPromotion.*IPTG.^1.84./(0.004422+IPTG.^1.84); %Derived from Lutz
and Bujard, 1997
%        %y(2) is total amount of gRNA (nM)
%        %Note: If you want parallel production, y(2) of each gRNA:
%                 %y(2)=y(2)*n

      gRNA=10^(m/200-3);
      antigRNA=0;
      y(2)=0.5*(gRNA-antigRNA-KdTitration+((gRNA-antigRNA-KdTitration)^2+...
           4*gRNA*KdTitration)^0.5);


      y(1)= 1; %Cas9 (nM) (Changed to 10 in Figure 1d)
      %y(2)= 1; %gRNA total (nM)
      y(3)=0; %Cas9-RNA Construct (nM)

      Cas9RNA=(y(1)+y(2)+KpdCas9RNA- ...
          ((y(1)+y(2)+KpdCas9RNA)^2-4*y(1)*y(2))^0.5)/2*n; %Derived from
                                    ... KDCas9RNA=[Cas9][Individual gRNA]
                                    ...           /[Individual Cas9-gRNA]

      PercentOccupied=Cas9RNA/(Kd+Cas9RNA); %Hill equation, coeff=1
          %Per site

      TrialProteinProduction=1; %Assume 100% success
      for k=(1:n)+0 %Constant simulates distance downstream
          TrialProteinProduction=TrialProteinProduction*...
              (1-PercentOccupied*exp(-(k+10)*Cas9Length/RoadblockDecay));
          %May or may not be linear, unfounded

      end
```

```
      SuccessfulTranscription(n,m)=TrialProteinProduction;
      FoldChange(n,m)=1/TrialProteinProduction;


      CasPlot(m)=gRNA;
      CasLog(m)=log10(gRNA);

   end
end

figure(1)
plot(CasLog,SuccessfulTranscription(1,:),CasLog,SuccessfulTranscription(2,:)...
   CasLog,SuccessfulTranscription(3,:),CasLog,SuccessfulTranscription(4,:),...
   CasLog,SuccessfulTranscription(5,:),CasLog,SuccessfulTranscription(6,:),...
   CasLog,SuccessfulTranscription(7,:),CasLog,SuccessfulTranscription(8,:),...
   CasLog,SuccessfulTranscription(9,:),CasLog,SuccessfulTranscription(10,:))

title('Effect of multiple gRNA targets with a Cas9 molarity of 1nM, lac-induced RNA')
xlabel('gRNA Concentrations (mM)')
ylabel('Relative Successful Transcription Rate')
legend('1 guide', '2 guide', '3 guide', '4 guide', '5 guide', ...
    '6 guide', '7 guide', '8 guide', '9 guide', '10 guide')

figure(2)
plot(CasLog,FoldChange(1,:),CasLog,FoldChange(2,:),...
    CasLog,FoldChange(3,:),CasLog,FoldChange(4,:),...
    CasLog,FoldChange(5,:),CasLog,FoldChange(6,:),...
    CasLog,FoldChange(7,:),CasLog,FoldChange(8,:),...
    CasLog,FoldChange(9,:),CasLog,FoldChange(10,:))

title('Effect of multiple gRNA targets with a Cas9 molarity of 1nM, lac-induced RNA')
xlabel('gRNA Concentrations (mM)')
ylabel('Fold-change repression levels')
legend('1 guide', '2 guide', '3 guide', '4 guide', '5 guide', ...
    '6 guide', '7 guide', '8 guide', '9 guide', '10 guide')
```

A2: Bistability Simulation Code

```
clear

%% Constants
%k1=### %Cas9-gRNA Kon rate
%k2=### %Cas9-gRNA Koff rate
FullPromotionRate=0.1; %(nM/s) This is made up
KDCas9RNA=0.5; % Kd of Cas9-RNA binding (1/nM)
Kd=0.5; %Kd of specific binding of Cas9 (nM),  Janan has paper
RoadblockDecay=4800; %Derived from Qi 2013, decay constant
Cas9Length=400; %(bp) UNSUPPORTED, NEED SOURCE
KdTitration=1; %(nM) UNSUPPORTED, NEED SOURCE
RNADecay=0.001283; %(1/s) Rajagopalan 1996, Turnover and Translation ...

for a=1:20

Time=50; %s (Iteration time)
Number=a; %Number of gRNA targets
Duration=1000; %Number of iterations

%% Define sizes

[StartingGRNA1, StartingGRNA2] = meshgrid(logspace(-2,3,10), logspace(-2,3,10));
gRNA1=zeros(1,Duration+1);
```

```
antigRNA1=zeros(1,Duration+1);
gRNA2=zeros(1,Duration+1);
antigRNA2=zeros(1,Duration+1);
EquilibriumArray1=zeros(length(StartingGRNA1));
EquilibriumArray2=zeros(length(StartingGRNA1));
SuccessfulTranscription1=zeros(1,Duration);
SuccessfulTranscription2=zeros(1,Duration);
SuccessfulTranscriptionArray1=zeros(1,length(StartingGRNA1));
SuccessfulTranscriptionArray2=zeros(1,length(StartingGRNA1));

%% Movement to 2 mM IPTG and 80 ng/mL aTc

for n=1:length(StartingGRNA1)
for m=1:length(StartingGRNA1)

gRNA1(1)=StartingGRNA1(n,m); %(nM)
gRNA2(1)=StartingGRNA2(n,m); %(nM)
antigRNA1(1)=0; %(nM)
antigRNA2(1)=0; %(nM)

for t=1:Duration

       Cas9= 5; %Cas9 (nM)
       FreegRNA1=0.5*(gRNA1(t)-antigRNA1(t)-KdTitration+...
           ((gRNA1(t)-antigRNA1(t)-KdTitration)^2+...
           4*gRNA1(t)*KdTitration)^0.5);
       FreegRNA2=0.5*(gRNA2(t)-antigRNA2(t)-KdTitration+...
           ((gRNA2(t)-antigRNA2(t)-KdTitration)^2+...
           4*gRNA2(t)*KdTitration)^0.5);


       Cas9gRNA1=(Cas9+FreegRNA1+KDCas9RNA- ...
          ((Cas9+FreegRNA1+KDCas9RNA)^2-4*Cas9*FreegRNA1)^0.5)/2*Number;
                           %Derived from KDCas9RNA=[Cas9][Individual gRNA]
                           %                      /[Individual Cas9-gRNA]
      Cas9gRNA2=(Cas9+FreegRNA2+KDCas9RNA- ...
          ((Cas9+FreegRNA2+KDCas9RNA)^2-4*Cas9*FreegRNA2)^0.5)/2*Number;

      PercentOccupied1=Cas9gRNA2/(Kd+Cas9gRNA2); %Hill equation, coeff=1
      PercentOccupied2=Cas9gRNA1/(Kd+Cas9gRNA1); %Hill equation, coeff=1

      TrialProteinProduction1=1; %Assume 100% success
      for k=1:Number
          TrialProteinProduction1=TrialProteinProduction1*...
              (1-PercentOccupied1*exp(-k*Cas9Length/RoadblockDecay));
          %May or may not be linear, unfounded
      end
      SuccessfulTranscription1(t)=TrialProteinProduction1;
     gRNA1(t+1)=gRNA1(t)+(-gRNA1(t).*RNADecay...
         +SuccessfulTranscription1(t).*FullPromotionRate).*Time;
     antigRNA2(t+1)=0;

      TrialProteinProduction2=1; %Assume 100% success
      for k=1:Number
          TrialProteinProduction2=TrialProteinProduction2*...
              (1-PercentOccupied2*exp(-k*Cas9Length/RoadblockDecay));
          %May or may not be linear, unfounded

      end
      SuccessfulTranscription2(t)=TrialProteinProduction2;
      gRNA2(t+1)=gRNA2(t)+(-gRNA2(t).*RNADecay...
         +SuccessfulTranscription2(t).*FullPromotionRate).*Time;
     antigRNA1(t+1)=0;
```

```
        PercentCounter1(t)=SuccessfulTranscription1(t);
        PercentCounter2(t)=SuccessfulTranscription2(t);

end %t

% EquilibriumArray1(n,m)=SuccessfulTranscription1(end);
% EquilibriumArray2(n,m)=SuccessfulTranscription2(end);
%Due to a glitch in matlab, the results array is created in two steps

SuccessfulTranscriptionArray1(1,m)=SuccessfulTranscription1(end);
SuccessfulTranscriptionArray2(1,m)=SuccessfulTranscription2(end);

end %m

EquilibriumArray1(n,:)=SuccessfulTranscriptionArray1;
EquilibriumArray2(n,:)=SuccessfulTranscriptionArray2;

end %n

% figure(1)
% mesh(log10(StartingGRNA1),log10(StartingGRNA2),EquilibriumArray1*100)
% colormap([0 0 0])
% xlabel('log(gRNA 1) (nM)')
% ylabel('log(gRNA 2) (nM)')
% zlabel('Successful Transcription (%)')
% zlim([0 100])
% title('Promoter 1, 10 targets')
%
% figure(2)
% mesh(log10(StartingGRNA1),log10(StartingGRNA2),EquilibriumArray2*100)
% colormap([0 0 0])
% xlabel('log(gRNA 1) (nM)')
% ylabel('log(gRNA 2) (nM)')
% zlabel('Successful Transcription (%)')
% zlim([0 100])
% title('Promoter 2, 10 targets')

Maximum(a)=max(max(EquilibriumArray1))*100;

end %a

figure(3)
plot(1:20,Maximum(1:20))
xlabel('Number of gRNA targets')
ylabel('Successful Transcription Rate (%)')
title('Effect of number of targets on transcription')
```

A3) Lab Construct with defensive anti-gRNA production matched to gRNA

```
clear

%% Constants
%k1=### %Cas9-gRNA Kon rate
%k2=### %Cas9-gRNA Koff rate
FullPromotionRateLac=0.1; %(nM/s) This is made up
FullPromotionRateTet=0.1; %(nM/s) This is made up
KDCas9RNA=0.5; % Kd of Cas9-RNA binding (1/nM)
Kd=0.5; %Kd of specific binding of Cas9 (nM),  Janan has paper
RoadblockDecay=4800; %Derived from Qi 2013, decay constant
Cas9Length=400; %(bp) UNSUPPORTED, NEED SOURCE
```

```
KdTitration=1; %(nM) UNSUPPORTED, NEED SOURCE
RNADecay=0.001283; %(1/s) Rajagopalan 1996, Turnover and Translation ...

Time=50; %s (Iteration time)
Number=3; %Number of gRNA targets
Duration=1000; %Number of iterations

%% Define sizes

[StartingIPTG, StartingaTc] = meshgrid(logspace(-4,2,10), logspace(-1,3,10));
gRNA1=zeros(1,Duration+1);
antigRNA1=zeros(1,Duration+1);
gRNA2=zeros(1,Duration+1);
antigRNA2=zeros(1,Duration+1);
EquilibriumArray1=zeros(length(StartingIPTG));
EquilibriumArray2=zeros(length(StartingIPTG));
SuccessfulTranscription1=zeros(1,Duration);
SuccessfulTranscription2=zeros(1,Duration);
SuccessfulTranscriptionArray1=zeros(1,length(StartingIPTG));
SuccessfulTranscriptionArray2=zeros(1,length(StartingIPTG));

%% Initial Movement from IPTG and aTc levels

gRNA1(1)=0; %(nM)
gRNA2(1)=0; %(nM)
antigRNA1(1)=0; %(nM)
antigRNA2(1)=0; %(nM)

for n=1:length(StartingIPTG)
for m=1:length(StartingIPTG)

IPTG=StartingIPTG(n,m); %(mM)
aTc=StartingaTc(n,m); %(ng/mL)

for t=1:Duration

       Cas9= 5; %Cas9 (nM)
       FreegRNA1=0.5*(gRNA1(t)-antigRNA1(t)-KdTitration+...
           ((gRNA1(t)-antigRNA1(t)-KdTitration)^2+...
           4*gRNA1(t)*KdTitration)^0.5);
       FreegRNA2=0.5*(gRNA2(t)-antigRNA2(t)-KdTitration+...
           ((gRNA2(t)-antigRNA2(t)-KdTitration)^2+...
           4*gRNA2(t)*KdTitration)^0.5);


       Cas9gRNA1=(Cas9+FreegRNA1+KDCas9RNA- ...
          ((Cas9+FreegRNA1+KDCas9RNA)^2-4*Cas9*FreegRNA1)^0.5)/2*Number;
                           %Derived from KDCas9RNA=[Cas9][Individual gRNA]
                           %                       /[Individual Cas9-gRNA]
      Cas9gRNA2=(Cas9+FreegRNA2+KDCas9RNA- ...
          ((Cas9+FreegRNA2+KDCas9RNA)^2-4*Cas9*FreegRNA2)^0.5)/2*Number;

      PercentOccupied1=Cas9gRNA2/(Kd+Cas9gRNA2); %Hill equation, coeff=1
      PercentOccupied2=Cas9gRNA1/(Kd+Cas9gRNA1); %Hill equation, coeff=1

      TrialProteinProduction1=1; %Assume 100% success
      for k=1:Number
          TrialProteinProduction1=TrialProteinProduction1*...
              (1-PercentOccupied1*exp(-k*Cas9Length/RoadblockDecay));
          %May or may not be linear, unfounded
      end

      SuccessfulTranscription1(t)=TrialProteinProduction1;
```

```
      gRNA1(t+1)=gRNA1(t)+(-gRNA1(t).*RNADecay...
          +SuccessfulTranscription1(t).*FullPromotionRateLac.* ...
          IPTG.^1.84./(0.004422+IPTG.^1.84)).*Time;
      antigRNA2(t+1)=gRNA1(t+1);

       TrialProteinProduction2=1; %Assume 100% success
       for k=1:Number
           TrialProteinProduction2=TrialProteinProduction2*...
               (1-PercentOccupied2*exp(-k*Cas9Length/RoadblockDecay));
           %May or may not be linear, unfounded
       end

       SuccessfulTranscription2(t)=TrialProteinProduction2;
       gRNA2(t+1)=gRNA2(t)+(-gRNA2(t).*RNADecay...
           +FullPromotionRateTet.*SuccessfulTranscription2(t).*...
           aTc.^12.2./(15.27^12.2+aTc.^12.2)).*Time;
       antigRNA1(t+1)=gRNA2(t+1);

end %t

gRNA1(1)=gRNA1(end);
gRNA2(1)=gRNA2(end);
antigRNA1(1)=antigRNA1(end);
antigRNA2(1)=antigRNA2(end);

%% Move to standard conditions equilibrium

IPTG=1000; %(mM)
aTc=100; %(ng/mL)

for t=1:Duration

        Cas9= 5; %Cas9 (nM)
        FreegRNA1=0.5*(gRNA1(t)-antigRNA1(t)-KdTitration+...
            ((gRNA1(t)-antigRNA1(t)-KdTitration)^2+...
            4*gRNA1(t)*KdTitration)^0.5);
        FreegRNA2=0.5*(gRNA2(t)-antigRNA2(t)-KdTitration+...
            ((gRNA2(t)-antigRNA2(t)-KdTitration)^2+...
            4*gRNA2(t)*KdTitration)^0.5);


        Cas9gRNA1=(Cas9+FreegRNA1+KDCas9RNA- ...
           ((Cas9+FreegRNA1+KDCas9RNA)^2-4*Cas9*FreegRNA1)^0.5)/2*Number;
                           %Derived from KDCas9RNA=[Cas9][Individual gRNA]
                           %                       /[Individual Cas9-gRNA]
       Cas9gRNA2=(Cas9+FreegRNA2+KDCas9RNA- ...
           ((Cas9+FreegRNA2+KDCas9RNA)^2-4*Cas9*FreegRNA2)^0.5)/2*Number;

       PercentOccupied1=Cas9gRNA2/(Kd+Cas9gRNA2); %Hill equation, coeff=1
       PercentOccupied2=Cas9gRNA1/(Kd+Cas9gRNA1); %Hill equation, coeff=1

       TrialProteinProduction1=1; %Assume 100% success
       for k=1:Number
           TrialProteinProduction1=TrialProteinProduction1*...
               (1-PercentOccupied1*exp(-k*Cas9Length/RoadblockDecay));
           %May or may not be linear, unfounded
       end

       SuccessfulTranscription1(t)=TrialProteinProduction1;
      gRNA1(t+1)=gRNA1(t)+(-gRNA1(t).*RNADecay...
          +SuccessfulTranscription1(t).*FullPromotionRateLac.* ...
          IPTG.^1.84./(0.004422+IPTG.^1.84)).*Time;
      antigRNA2(t+1)=gRNA1(t+1);
```

```
        TrialProteinProduction2=1; %Assume 100% success
        for k=1:Number
            TrialProteinProduction2=TrialProteinProduction2*...
                (1-PercentOccupied2*exp(-k*Cas9Length/RoadblockDecay));
            %May or may not be linear, unfounded
        end

        SuccessfulTranscription2(t)=TrialProteinProduction2;
        gRNA2(t+1)=gRNA2(t)+(-gRNA2(t).*RNADecay...
            +FullPromotionRateTet.*SuccessfulTranscription2(t).*...
            aTc.^12.2./(15.27^12.2+aTc.^12.2)).*Time;
        antigRNA1(t+1)=gRNA2(t+1);

end %t

% EquilibriumArray1(n,m)=SuccessfulTranscription1(end);
% EquilibriumArray2(n,m)=SuccessfulTranscription2(end);
%Due to a glitch in matlab, the results array is created in two steps

SuccessfulTranscriptionArray1(1,m)=SuccessfulTranscription1(end);
SuccessfulTranscriptionArray2(1,m)=SuccessfulTranscription2(end);


end %m

EquilibriumArray1(n,:)=SuccessfulTranscriptionArray1;
EquilibriumArray2(n,:)=SuccessfulTranscriptionArray2;

end %n

figure(1)
mesh(log10(StartingIPTG),log10(StartingaTc),EquilibriumArray1*100)
colormap([0 0 0])
xlabel('log(IPTG) (mM)')
ylabel('log(aTc) (ng/mL)')
zlabel('Successful Transcription (%)')
zlim([0 100])
title('Lac Promoter, 3 gRNA pairs, higher Tet transcription')

figure(2)
mesh(log10(StartingIPTG),log10(StartingaTc),EquilibriumArray2*100)
colormap([0 0 0])
xlabel('log(IPTG) (mM)')
ylabel('log(aTc) (ng/mL)')
zlabel('Successful Transcription (%)')
zlim([0 100])
title('Tet Promoter, 3 targets, equal transcription')
```

A4) Lab Construct with exogenously produced anti-gRNA nucleic acids

```
clear

%% Constants
%k1=### %Cas9-gRNA Kon rate
%k2=### %Cas9-gRNA Koff rate
FullPromotionRateLac=0.1; %(nM/s) This is made up
FullPromotionRateTet=0.1; %(nM/s) This is made up
KDCas9RNA=0.5; % Kd of Cas9-RNA binding (1/nM)
Kd=0.5; %Kd of specific binding of Cas9 (nM),  Janan has paper
RoadblockDecay=4800; %Derived from Qi 2013, decay constant
```

```
Cas9Length=400; %(bp) UNSUPPORTED, NEED SOURCE
KdTitration=1; %(nM) UNSUPPORTED, NEED SOURCE
RNADecay=0.001283; %(1/s) Rajagopalan 1996, Turnover and Translation ...

Time=50; %s (Iteration time)
Number=6; %Number of gRNA targets
Duration=1000; %Number of iterations
AntiAmount=10; %(nM)

%% Define sizes

[StartingIPTG, StartingaTc] = meshgrid(logspace(-4,2,10), logspace(-1,3,10));
gRNA1=zeros(1,Duration+1);
antigRNA1=zeros(1,Duration+1);
gRNA2=zeros(1,Duration+1);
antigRNA2=zeros(1,Duration+1);
EquilibriumArray1=zeros(length(StartingIPTG));
EquilibriumArray2=zeros(length(StartingIPTG));
SuccessfulTranscription1=zeros(1,Duration);
SuccessfulTranscription2=zeros(1,Duration);
SuccessfulTranscriptionArray1=zeros(1,length(StartingIPTG));
SuccessfulTranscriptionArray2=zeros(1,length(StartingIPTG));

%% Initial Movement from IPTG and aTc levels

gRNA1(1)=0; %(nM)
gRNA2(1)=0; %(nM)
antigRNA1(1)=AntiAmount; %(nM)
antigRNA2(1)=AntiAmount; %(nM)

for n=1:length(StartingIPTG)
for m=1:length(StartingIPTG)

IPTG=StartingIPTG(n,m); %(mM)
aTc=StartingaTc(n,m); %(ng/mL)

for t=1:Duration

       Cas9= 5; %Cas9 (nM)
       FreegRNA1=0.5*(gRNA1(t)-antigRNA1(t)-KdTitration+...
           ((gRNA1(t)-antigRNA1(t)-KdTitration)^2+...
           4*gRNA1(t)*KdTitration)^0.5);
       FreegRNA2=0.5*(gRNA2(t)-antigRNA2(t)-KdTitration+...
           ((gRNA2(t)-antigRNA2(t)-KdTitration)^2+...
           4*gRNA2(t)*KdTitration)^0.5);


       Cas9gRNA1=(Cas9+FreegRNA1+KDCas9RNA- ...
          ((Cas9+FreegRNA1+KDCas9RNA)^2-4*Cas9*FreegRNA1)^0.5)/2*Number;
                            %Derived from KDCas9RNA=[Cas9][Individual gRNA]
                            %                       /[Individual Cas9-gRNA]
      Cas9gRNA2=(Cas9+FreegRNA2+KDCas9RNA- ...
          ((Cas9+FreegRNA2+KDCas9RNA)^2-4*Cas9*FreegRNA2)^0.5)/2*Number;

      PercentOccupied1=Cas9gRNA2/(Kd+Cas9gRNA2); %Hill equation, coeff=1
      PercentOccupied2=Cas9gRNA1/(Kd+Cas9gRNA1); %Hill equation, coeff=1

      TrialProteinProduction1=1; %Assume 100% success
      for k=1:Number
          TrialProteinProduction1=TrialProteinProduction1*...
              (1-PercentOccupied1*exp(-k*Cas9Length/RoadblockDecay));
          %May or may not be linear, unfounded
      end
```

```
        SuccessfulTranscription1(t)=TrialProteinProduction1;
       gRNA1(t+1)=gRNA1(t)+(-gRNA1(t).*RNADecay...
           +SuccessfulTranscription1(t).*FullPromotionRateLac.* ...
           IPTG.^1.84./(0.004422+IPTG.^1.84)).*Time;
       antigRNA2(t+1)=AntiAmount;

        TrialProteinProduction2=1; %Assume 100% success
        for k=1:Number
            TrialProteinProduction2=TrialProteinProduction2*...
                (1-PercentOccupied2*exp(-k*Cas9Length/RoadblockDecay));
            %May or may not be linear, unfounded
        end

        SuccessfulTranscription2(t)=TrialProteinProduction2;
        gRNA2(t+1)=gRNA2(t)+(-gRNA2(t).*RNADecay...
            +FullPromotionRateTet.*SuccessfulTranscription2(t).*...
            aTc.^12.2./(15.27^12.2+aTc.^12.2)).*Time;
        antigRNA1(t+1)=AntiAmount;

end %t

gRNA1(1)=gRNA1(end);
gRNA2(1)=gRNA2(end);
antigRNA1(1)=AntiAmount;
antigRNA2(1)=AntiAmount;

%% Move to standard conditions equilibrium

IPTG=1000; %(mM)
aTc=100; %(ng/mL)

for t=1:Duration

        Cas9= 5; %Cas9 (nM)
        FreegRNA1=0.5*(gRNA1(t)-antigRNA1(t)-KdTitration+...
            ((gRNA1(t)-antigRNA1(t)-KdTitration)^2+...
            4*gRNA1(t)*KdTitration)^0.5);
        FreegRNA2=0.5*(gRNA2(t)-antigRNA2(t)-KdTitration+...
            ((gRNA2(t)-antigRNA2(t)-KdTitration)^2+...
            4*gRNA2(t)*KdTitration)^0.5);


        Cas9gRNA1=(Cas9+FreegRNA1+KDCas9RNA- ...
            ((Cas9+FreegRNA1+KDCas9RNA)^2-4*Cas9*FreegRNA1)^0.5)/2*Number;
                            %Derived from KDCas9RNA=[Cas9][Individual gRNA]
                            %                       /[Individual Cas9-gRNA]
       Cas9gRNA2=(Cas9+FreegRNA2+KDCas9RNA- ...
            ((Cas9+FreegRNA2+KDCas9RNA)^2-4*Cas9*FreegRNA2)^0.5)/2*Number;

       PercentOccupied1=Cas9gRNA2/(Kd+Cas9gRNA2); %Hill equation, coeff=1
       PercentOccupied2=Cas9gRNA1/(Kd+Cas9gRNA1); %Hill equation, coeff=1

       TrialProteinProduction1=1; %Assume 100% success
       for k=1:Number
           TrialProteinProduction1=TrialProteinProduction1*...
               (1-PercentOccupied1*exp(-k*Cas9Length/RoadblockDecay));
           %May or may not be linear, unfounded
       end

       SuccessfulTranscription1(t)=TrialProteinProduction1;
      gRNA1(t+1)=gRNA1(t)+(-gRNA1(t).*RNADecay...
          +SuccessfulTranscription1(t).*FullPromotionRateLac.* ...
```

```
          IPTG.^1.84./(0.004422+IPTG.^1.84)).*Time;
      antigRNA2(t+1)=AntiAmount;

       TrialProteinProduction2=1; %Assume 100% success
       for k=1:Number
           TrialProteinProduction2=TrialProteinProduction2*...
               (1-PercentOccupied2*exp(-k*Cas9Length/RoadblockDecay));
           %May or may not be linear, unfounded
       end

       SuccessfulTranscription2(t)=TrialProteinProduction2;
       gRNA2(t+1)=gRNA2(t)+(-gRNA2(t).*RNADecay...
           +FullPromotionRateTet.*SuccessfulTranscription2(t).*...
           aTc.^12.2./(15.27^12.2+aTc.^12.2)).*Time;
       antigRNA1(t+1)=AntiAmount;

end %t

% EquilibriumArray1(n,m)=SuccessfulTranscription1(end);
% EquilibriumArray2(n,m)=SuccessfulTranscription2(end);
%Due to a glitch in matlab, the results array is created in two steps

SuccessfulTranscriptionArray1(1,m)=SuccessfulTranscription1(end);
SuccessfulTranscriptionArray2(1,m)=SuccessfulTranscription2(end);


end %m

EquilibriumArray1(n,:)=SuccessfulTranscriptionArray1;
EquilibriumArray2(n,:)=SuccessfulTranscriptionArray2;

end %n

figure(1)
mesh(log10(StartingIPTG),log10(StartingaTc),EquilibriumArray1*100)
colormap([0 0 0])
xlabel('log(IPTG) (mM)')
ylabel('log(aTc) (ng/mL)')
zlabel('Successful Transcription (%)')
zlim([0 100])
title('Lac Promoter, 6 targets, equal transcription')

figure(2)
mesh(log10(StartingIPTG),log10(StartingaTc),EquilibriumArray2*100)
colormap([0 0 0])
xlabel('log(IPTG) (mM)')
ylabel('log(aTc) (ng/mL)')
zlabel('Successful Transcription (%)')
zlim([0 100])
title('Tet Promoter, 6 targets, equal transcription')
```