1.

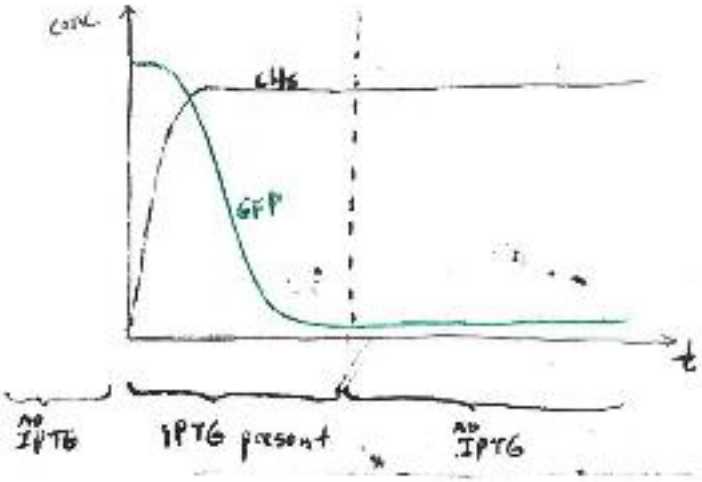
a.

Heat	IPTG	GFP
1	0	1
0	1	0
0	0	reverts to previous state
1	1	indeterminate

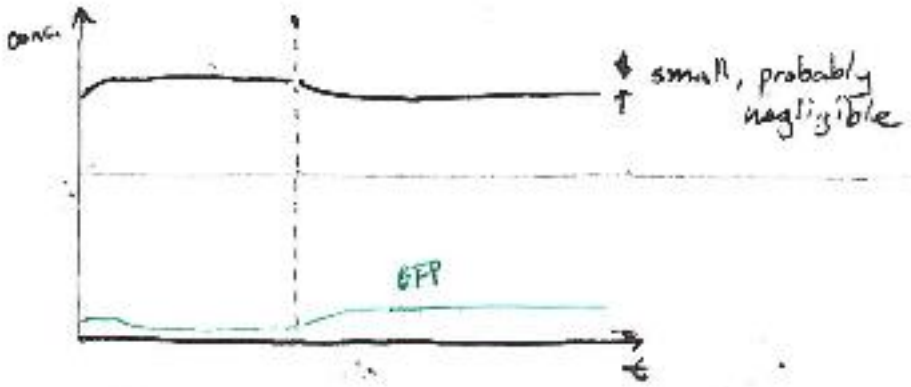
In this problem, order (sequence of input states) matters: Let's work through this step by step. The PL promoter initiates transcription of the LacI gene, whose translated product acts as a repressor of the cl-ts gene by binding to the Ptrc2 promoter upstream of the gene. Conversely, the Ptrc2 promoter initiates transcription of the cl-ts gene whose translated product is a temperature sensitive protein that acts as a repressor of the LacI gene by binding to the PL promoter upstream. Presenting a heat stimulus to the system represses the temperature sensitive protein cl whereas presenting IPTG to the system represses the product of the LacI gene; consequently, the presence of heat alone drives transcription of GFP since heat quenches the activity of LacI's repressor. On the other hand, the presence of IPTG alone quenches the activity of LacI, thereby permitting the uninhibited transcription of cl-ts, which then supresses transcription of LacI-GFP. If we then remove IPTG from the system (still no heat), then the system would revert to some basal production of GFP; if on the other hand, we decided to apply heat to the system while already in the presence of IPTG, then we can expect some production of GFP since heat11 would override the repression of LacI. However, if we added both inputs simultaneously, the output is indeterminate. To understand this, consider the digital equivalent of this gene circuit, an SR latch that is constructed by cross-coupling a pair of NOR gates. Simultaneously initializing both R and S with 'true' or 'false' for that matter, would break the inverse relationship between the outputs of both gates. Consequently, in this scenario, the output (an unstable equilibrium) would slide to either true (GFP = 1) or false depending on the conditions of the environment1. (Part a is from Pradeep Ramesh and Yinqing Li:PoSB TA's from Fall 2012) [5pt]

b. Depending on what you assume the initial conditions to be, the GFP timecourse may be slightly different. If we assume that GFP is on initially, IPTG will repress LacI (which is initially high). This allows clts to repress P_L, causing GFP expression to decrease to a low level. After IPTG is removed, if LacI has been sufficiently repressed by the IPTG pulse, clts will remain high and continue to repress the GFP output. (Alternatively if the IPTG pulse is to short or at too low concentration, GFP expression will bounce back to a high level). [3pt]

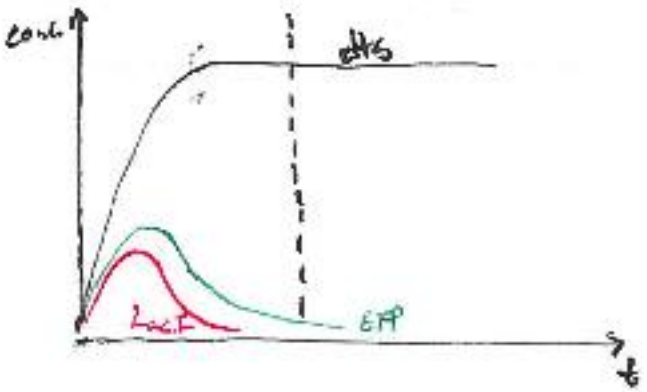
With GFP initially on



With GFP initially off



Both off initially



If we assume GFP is off initially, clts must be high. IPTG represses LacI (which is already low) such that clts is increased very slightly. This will decrease GFP expression slightly. After IPTG is removed, clts returns gradually to its initial value and GFP increases slightly to its initial value (but is still close to zero).

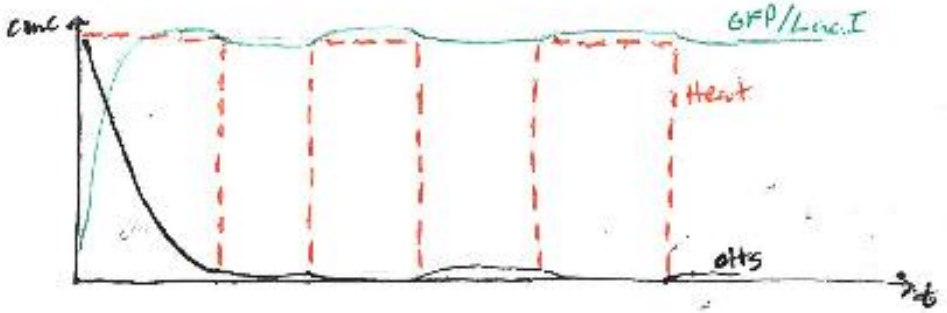
Some might consider a weird case where clts and lacI are both low initially. This case is actually not very biologically relevant since the two steady states are either clts high or lacI high. In this case, clts and lacI (and GFP) concentrations increase in the beginning as neither repressor is at significant enough concentration to do any repressing. However, clts increases faster than lacI since the IPTG represses lacI such that there is less repression of Ptrc-2. Eventually clts reaches concentrations sufficient to repress lacI and GFP. Thus GFP rises initially but then falls to reach a low steady state.

c. The circuit is a genetic toggle switch where GFP latches to either a high or low state if sufficient heat or IPTG is applied respectively. [2pt]

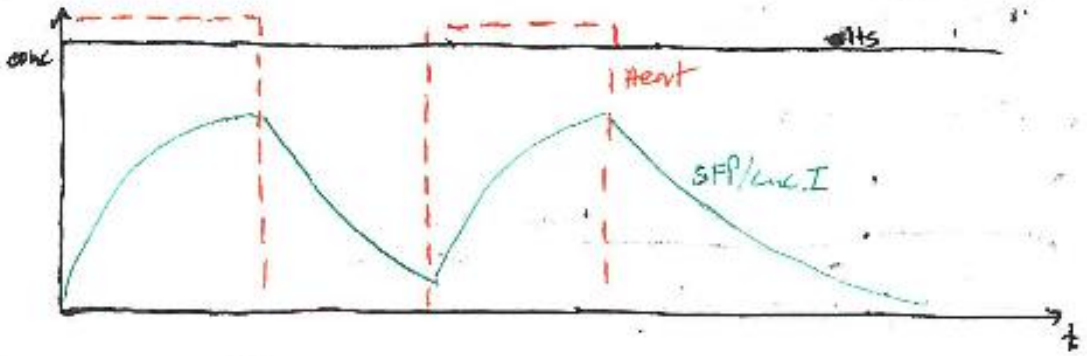
d. This is not the expected behavior. When a bistable toggle switch is switched, the new output should be stable until the circuit is switched again. Decaying GFP output may be due to unmatched clts/LacI repression. For instance if LacI repression is weak, the clts gene product may gradually build up and begin to repress GFP again. For this case, we could add more repeats of the lac operator to the P_{trc-2} promoter to increase LacI repression. We might also adjust the temperature to lower the clts repression to better match LacI. You might also replace the lacI/P_{trc-2} pair with something that behaves better. [5pt]

e. Case a: This is a simple switch in state as described above. Following the switch, there are only small purturbations in LacI/GFP and clts caused by the heat. Case c: Let's take the extreme case where IPTG is high enough that LacI has no effective repression of Ptrc-2. While the steady-state output is indeterminate if both inputs are high, during the short heat pulses intuitively clts levels should decrease and GFP should increase. Essentially the heat should modulate the concentration of LacI/GFP (likely with some delay) Case b: you should get GFP to be on during the pulses since heat allows the build-up of LacI (assuming the heat is high compared to IPTG). Following the application of heat, IPTG will be intermediate while heat is at zero, causing the state to switch back to an off level. This essentially the same as case c except clts concentration is modulated to some degree in addition to LacI/GFP. Other behavior may be possible depending on the assumptions made. [5pt]

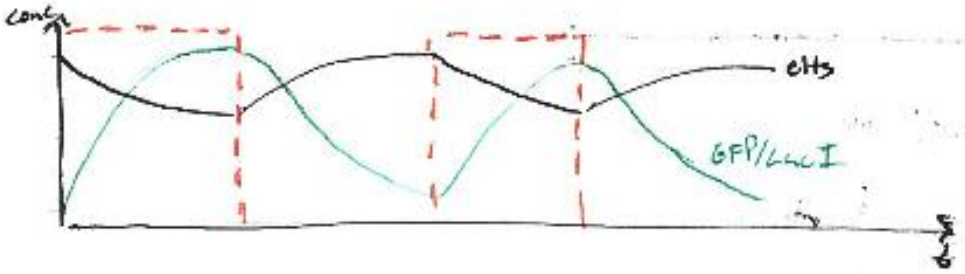
Low IPTG



High IPTG



Intermediate IPTG



2. a and b) [20pt]

Write rate equations for each species

① $\frac{d[DNA_2 \cdot P_1]}{dt} = k_{-6}[P_2][P_1] - k_6[DNA_2 \cdot P_1]$ (P_2 ← DNA_2)

② $\frac{d[P_2]}{dt} = -k_{-6}[P_2][P_1] + k_6[DNA_2 \cdot P_1]$

③ $\frac{d[R_2]}{dt} = k_7[P_2] - k_8[R_2]$

④ $\frac{d[P_2]}{dt} = k_9[R_2] - k_{10}[P_2]$ (no DNA binding term since assuming negligible)

⑤ $\frac{d[DNA_1 \cdot P_2]}{dt} = k_{-1}[P_1][P_2] - k_1[DNA_1 \cdot P_2]$

⑥ $d[P_1]/dt = -k_{-1}[P_1][P_2] + k_1[DNA_1 \cdot P_2]$

⑦ $\frac{d[R_1]}{dt} = k_2[P_1] - k_3[R_1]$

⑧ $\frac{d[P_1]}{dt} = k_4[R_1] - k_5[P_1]$

Find relationship between steady state DNA and protein concentrations

→ steady state when all $\frac{d[\,]}{dt} = 0$

④ at steady state: $[P_2]_{ss} = \frac{k_9}{k_{10}}[R_2]_{ss}$

③ at " " : $[R_2]_{ss} = \frac{k_7}{k_8}[P_2]_{ss}$

$\Rightarrow [P_2] = \frac{k_7 k_9}{k_8 k_{10}}[P_2]$

let's just say all conc are ss from here on for notation sake

① at " " :

$0 = k_{-6}[P_2][P_1] - k_6[DNA_2 \cdot P_1]$

⑨ $0 = k_{-6}[P_2][P_1] - k_6([P_2]_{total} - [P_2])$

since DNA_2 has fixed total conc.

⑥ at steady state: $[P_1] = \frac{k_4}{k_5}[R_1]$

⑦ " " " : $[R_1] = \frac{k_2}{k_3}[P_1]$

$\Rightarrow [P_1] = \frac{k_2 k_4}{k_3 k_5}[P_1]$

⑤ " " " :

$0 = k_{-1}[P_1][P_2] - k_1[DNA_1 \cdot P_2]$

$0 = k_{-1}[P_1][P_2] - k_1([P_1]_{tot} - [P_1])$

plug in expression for $[P_2]$ in terms of P_2

(10) $$0 = k_{-1}\frac{k_7 k_9}{k_8 k_{10}}[P_1][P_2] - k_1([P_1]_{tot} - [P_1])$$

we now need to find and plug in an expression for $[P_2]$ in terms of $[P_1]$

From (9): $$0 = k_{-6}[P_2][P_1] - k_6([P_2]_{tot} - [P_2])$$

$$k_6[P_2]_{tot} = (k_{-6}[P_1] + k_6)[P_2]$$

$$[P_2] = \frac{k_6[P_2]_{tot}}{k_{-6}[P_1] + k_6} = \frac{k_6[P_2]_{tot}}{k_{-6}\frac{k_2 k_4}{k_3 k_5}[P_1] + k_6}$$

(11) $$[P_2] = \frac{[P_2]_{tot}}{\frac{k_{-6}}{k_6}\frac{k_2 k_4}{k_3 k_5}[P_1] + 1}$$

Now we can plug the expression for $[P_2]$ from (11) into (10)

$$0 = \frac{k_{-1}\frac{k_7 k_9}{k_8 k_{10}}[P_1][P_2]_{tot}}{\frac{k_{-6}}{k_6}\frac{k_2 k_4}{k_3 k_5}[P_1] + 1} - k_1[P_1]_{tot} + k_1[P_1]$$

cancels based on assumptions $k_2 = k_7$, ...

→ Use your favorite symbolic equation solver to find steady state $[P_1]$

$$[P_1] = \frac{1}{2k_1 k_{-6}}\left(-k_1 k_6 - k_{-1}k_6[P_2]_{tot} + k_1 k_{-6}[P_1]_{tot} - \left\{4k_6 k_{-6} k_1^2[P_1]_{tot} + (k_1 k_6 + k_{-1}k_6[P_2]_{tot} - k_1 k_{-6}[P_1]_{tot})^2\right\}^{1/2}\right)$$

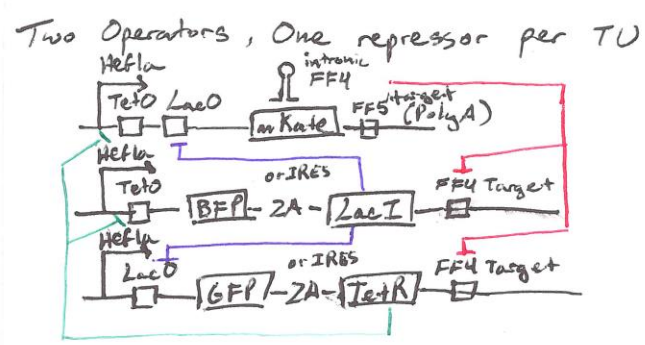
As $[P_2]_{tot}\uparrow$, terms underlined in red dominate, bringing $[P_1]$ towards zero

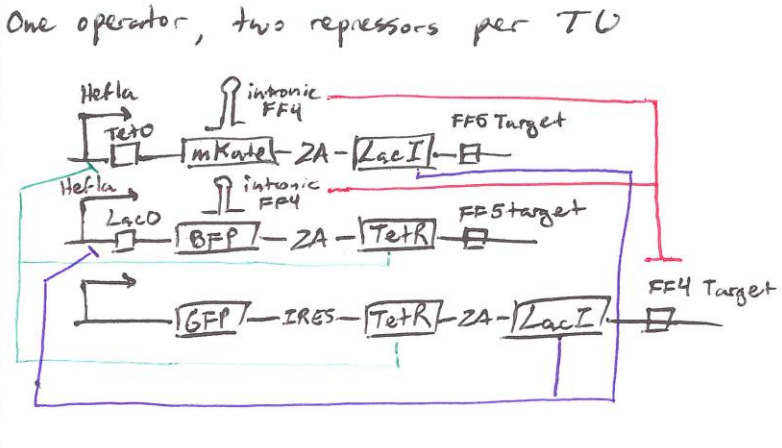
As $[P_1]_{tot}\uparrow$, terms underlined in blue dominate, bringing $[P_1]$ towards $[P_1]_{tot}$

c. This model relies on having large numbers of DNA molecules to avoid stochasticity. However, too many plasmids and DNA binding will reduce free protein concentration. With circuits integrated in the genome, there would only be one copy of the DNA, resulting in stochastic effects that would not be predicted by the above analysis. With high copy plasmids, you would have hundreds of DNA molecules per cell, meaning stochasticity would have less influence. The model might be relevant for predicting some aspects of the system in the case where there are many plasmid copies but even greater concentrations of protein. [3pt]

3.

a. We should have three transcription units (TU: region of DNA usually coding for the promoter – operator – 5' UTR – gene – 3'UTR – terminator), one for each state. Each TU should either make repressors for the other two TU's or each TU should have two operators that are repressed by either of the other two TU's. General solutions are included below. One key part of the solution is how to reset repression in states using FF4 shRNA (or FF5 or TALE14) to repress the other states. This can be accomplished incorporating FF5 target sites into the transcription unit that uses FF4 as a repressor and then using FF5 siRNA to knockdown transcript levels of that TU. [20pt]





In examples above, you can also use TALE14 instead of intronic FF4 and place a TALE14 binding site downstream of the promoter instead of FF4 target site.

b. Tuning the circuit to match repression levels will likely be the major challenge. Even though we can use IPTG, Dox and siRNA to tune the circuit to some extent, there is a potentially large parameter space to scan across for these three concentrations and these experiments could be time consuming. [3pt]

c. The number of elements scales approximately x^2 [closer to x(x+2)] with respect to the number of states. (~15 elements for 3 states, ~8 elements for 2 states etc) [2pt]

d. A hard to transfect cell line means everything should be on one plasmid. This should help reduce heterogeneity in the results you might obtain if you use multiple plasmids, since with multiple plasmids some cells might get multiple copies of some plasmids or not get others. Putting everything on one plasmid means the copy numbers of the genes and promoters should be for the most part equal per cell.

The fastest assembly in this format will be two hierarchical steps. For instance, you could use gateway to assemble the three transcriptional units of (promoter-operator)-(5'UTR-gene-3'UTR) together in 3 parallel steps. If we were to put these into gateway destination flanked by Gibson sites, we could then Gibson the three TU's together [5pt]

e.
A383T: Introduces a new SpeI cut site. This would cause cloning problems for any BioBrick steps involving the SpeI restriction enzyme. This mutation needs to be fixed before proceeding with any BioBrick assembly.
A18G: Mutation does not introduce any cut sites utilized by BioBricks and codes for the same amino acid as the original. This mutation should be fine.
A9G: Mutation does not introduce any cut sites utilized by BioBricks and codes for the same amino acid as the original. (However, the new codon is a rare codon which would affect expression levels of tetR in the final circuit)
G576C: Introduces an EcoRI cut site. This would cause cloning problems for any BioBrick steps involving the EcoRI restriction enzyme. This mutation needs to be fixed before proceeding with any BioBrick assembly.
A604 deletion: While the frameshift mutation will greatly alter the behavior of the repressor in the final circuit, it should not affect BioBrick assembly as restriction sites are not changed [5pt]

g.
Oligo synthesis might have trouble with multiple repeats, though this is improving every day. Synthesis could work, but then again it may not, so we should not rely exclusively on synthesis for this part.

Gibson relies on long and unique overhangs between the parts we are assembling. However the TetO sequence is short and since these are repeated, the overhangs will not be unique. Thus scarless Gibson is not a good choice.

Golden Gate could work. This would involve using PCR to make four versions of TetO with flanking BsaI cut sites different positionalizing overhangs. A final Golden Gate step with BsaI can assemble the four TetO's into an appropriate backbone in a single step. The final result will have scars though.

BioBricks could work as well. We would need to make a BioBrick with TetO, then assemble into EcoRI-XbaI-TetO-TetO-SpeI-PstI with one standard BioBrick step. Then assemble into a four TetO repeat BioBrick product using another BioBrick step. There will again be scars between each TetO. [4pt]

4. Sometimes it's really fun to take stock of the real physical numbers that come from cellular processes when scaled up to large reactors. One thing that is often overlooked when thinking about the dynamics of the cell is heat transport.

a. When dividing, cells require about 32 mmol of ATP as energy input to create 1g of new cell matter. Most of that energy is stored in the chemical bonds that comprise the cell, but as you know, no process is 100% efficient. Assume that the cell is able to convert the energy at 90% efficiency and the rest is dissipated as heat. An *E. coli* contains about 1.0e-12g of biomass and doubles its biomass about every 20 minutes. On average, how much heat is produced by a cell every second. (Hydrolysis of ATP in physiological conditions is about 48kJ/mol.) [3pt]

ANS: $32\ \frac{mmol\ ATP}{g} * 10\%\ \cancel{heat} * \frac{1.0*10^{-12} g}{cell} * \frac{cell}{1200\ sec} * 48 \frac{J}{mmol\ ATP} = 1.28 * 10^{-13} \frac{J}{sec}$

b. A cell is only about a 1e-15L in volume. How much does the temperature increase inside the cell every second if there is no heat transfer out of the cell. Assume that the cell is made of water. (Hint: the definition of a calorie might be useful) [3pt]

ANS: $1.28 * 10^{-16} \frac{kJ}{sec} * \frac{kcal}{4.184\ kJ} = 3.05 * 10^{-17} \frac{kcal}{s}$

One Kcal raises temp of ~1L of water 1 degree C (or K). So the temperature is raised by

$3.05 * \frac{10^{-17}}{10^{-15}} = .03\square/sec$

c. Fortunately, the cells are conductive such that the energy does not increase the internal temperature a lot. In fact, the surface area of a cell is significantly larger than that required for efficient heat transfer. Why do you think cell surface area is not minimized to this constraint? On the other hand, why are cells not usually maximizing their surface area by becoming very long flat sheets? [7pt]

ANS: Mass transfer is a lot slower than heat transfer. Higher surface area allows for efficient mass transfer of nutrients/waste in and out of the cell. Having too high of surface area would make two dimensions of the cell much larger than the 3rd. This would mean that diffusion across those two dimensions (diffusion through the membrane for example as opposed to across it) would be much slower, and the cell would not be able to keep equilibrium throughout its volume.

d. Back to the heat question. A single cell will not boil itself, but a lot, growing and rubbing together might. Imagine a 10,000 L cylindrical reactor of 1m radius filled to the brim with cells making biofuels. Cells in such a system can reach an OD_{600} of 50 ($1OD_{600}$ = 1e9/mL cells). Let's say that each cell is still growing well and is generating the same amount of heat as in part a.

   A reactor that is not cooled will lose most of its heat through conduction with the outside air. A very simple model for this phenomenon is

$$\dot{q} = -kA\frac{\Delta T}{\Delta x}$$

   Where $\dot{q}$ is the rate of heat transfer (watts). k is the thermal conductivity of the material (~20 W m^{-1} K^{-1} for stainless steel), A is the area of the material, Δx is the thickness of the material and ΔT is the difference in temperature on each side of the material (ignore top and bottom of tank surface). Given that the thickness of the reactor would be about 10cm, find out the temperature difference between the inside and outside of the tank if cell propagation is the only source of heat. Ignore transfer from the bottom and the top of the reactor. [5pt]

ANS: $Height_{reactor} = \frac{10\ m^3}{\pi\ m^2} = 3.18m$; $A_{reactor} = 2\pi r * h = 20\ m^2$

$$\dot{q}_{generated} = 1.28 * 10^{-13}\ \frac{J}{\sec \cdot cell} * 50 * \frac{10^9 cells}{ml} * \frac{10^7 ml}{reactor} = 64\ kW$$

At steady-state $\dot{q}_{generated} = \dot{q}_{transported}$; $\Delta T = \frac{\dot{q}_{generated}\Delta x}{kA} = \frac{64*10^3 W * 0.1m}{20\ W\ m^{-1}\ K^{-1} * 20m^2} = 16K$