Contents

1 Experimental characterisation of the genetic toggle switch

1.1 Introduction

In this chapter, I aim to study the genetic toggle switch experimentally. This chapter is organised as follows: In the first section I provide an overview of the circuit used and then outline the methods used for the experiments carried out. In the subsequent section I investigate the effect that the switch has on the growth rate of the bacteria. Then I examine the concentrations of the inducers and the time needed to flip the switch.

1.2 Circuit overview

The toggle switch plasmid I used here was provided by Litcofsky et al. (2012). All the switch components were contained in one plasmid, pKDL071. An overview of the plasmid is shown in Figure 1.1A and the sequence given in Appendix (XXX). The circuit consists of two promoters, Ptrc2 and PLtetO-1 (Lutz & Bujard 1997). Ptrc2 is a constitutive promoter, repressible by LacI. PLtetO-1 is also a constitutive promoter, repressible by TetR, as shown in Figure 1.1B. mCherry (Shaner et al. 2004) and GFP (Shimomura, Johnson, & Saiga 1962) are fluorescent proteins, that were added under the control of the same promoters as the repressors, and thus reflect the levels of TetR and LacI in the system. The plasmid contains kanamycin antibiotic resistance and is high copy (ColE1 origin of replication).

This system is capable of two states, GFP high and mCherry high. When IPTG is added to the system, it represses the repression of TetR and mCherry and thus the cells end up in the mCherry high state. When ATc is added to the system, it represses the repression of LacI and GFP and thus the cells end up in the GFP high

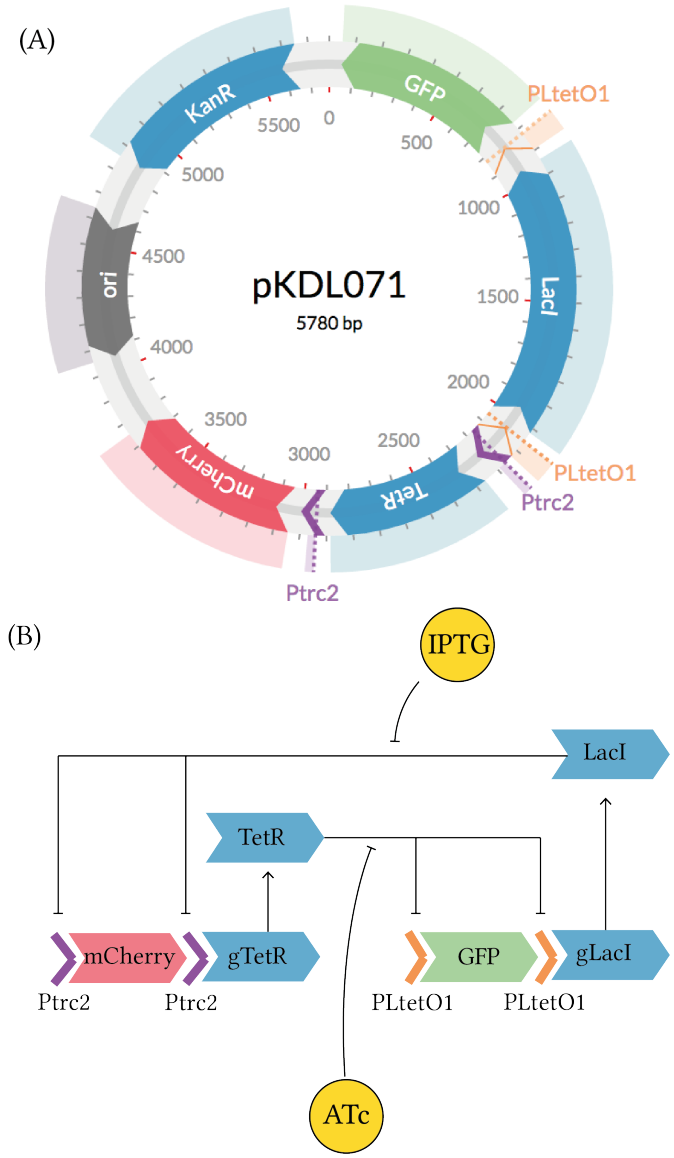

Figure 1.1 : The genetic toggle switch circuit used in this chapter. (A) The plasmid map of pKDL071, the plasmid containing the genetic toggle switch used in Litcofsky et al. (2012) (B) The interactions between each element of the circuit.

state. If no inducer is added to the system it will randomly go to ~~one or the other.~~

1.3 Methods

The toggle switch plasmid was provided by the James J Collins lab in the form of a stab culture in *E. coli* K-12 MG1655.

1.3.1 *Escherichia coli* culturing conditions

Lysogeny broth (LB) was made by diluting LB in deionized water to a concentration of 25 g L^{-1} and subsequently autoclaved. LB agar plates were made by adding bacteriological agar to the above solution to a concentration of 45 mg mL^{-1} before autoclaving. The solution was then cooled down to 55 °C using a water bath. If antibiotic was required it was added to the correct concentration to the cooled solution. The solution was then aliquoted to plates and left to solidify in room temperature. The plates were stored in the fridge for up to 1 month.

Overnight cultures were made by picking a single colony from a static culture in an agar plate. Each colony was placed in 15 mL Falcon tubes (Fisher Scientific, MA, U.S.A) with 5 mL LB with kanamycin antibiotic at a concentration of 50 µg mL^{-1}. The tubes were then screwed loosely and taped securely in order to allow for aeration. The falcon tubes were put in an incubator at 37 °C with orbital shaking at 200 rpm for 12-16 hours.

1.3.2 Inducers

Anhydrotetracycline (ATc) solution was made by diluting ATc from Cayman Chemical Company in 100 % ethanol to a concentration of 1 mg mL^{-1}. Isopropyl-beta-D-thiogalactopyranoside (IPTG) solution was made by dissolving IPTG in deionized water to a concentration of 1 M. The solution was sterilised by passing the solution through a 0.22 µm syringe filter. Both inducers were stored in 1 mL aliquots at −20 °C.

1.3.3 Glycerol stock preparation

To preserve the transformed cultures long [illegible] glycerol stocks were made. 5 mL LB and Kanamycin overnight cultures were made as described in Section 1.3.1. The cultures were kept on ice and 70 % glycerol was added to the cultures in a ratio of glycerol to culture of 1:7. These were aliquoted into cryovials and transferred to a −80 °C freezer for long-term storage.

1.3.4 Revival

For subsequent revival of the frozen cultures, a 1.5 mL eppendorf tube was removed from the −80 °C freezer and put on ice. Small amount was streaked onto an agar plate containing LB and kanamycin. The plates were stored in an incubator at 37 °C overnight. Then the plates were sealed using parafilm and stored at 4 °C for up to two weeks.

1.3.5 Plasmid construction

I constructed two plasmids in order to used them for the flow cytometry experiments. The first plasmid, pSEVA281G contains the promoter PLtetO-1 and GFP and the other, pSEVA281C, contains the promoter Ptrc2 and mCherry from PKDL071, shown in Figure 1.2. I used these two plasmids to determine the appropriate voltages for the lasers that excite GFP and mCherry.

I constructed pSEVA281G by digesting pKDL071 and pSEVA281 using the protocol outlined in Section 1.3.5.2. pSEVA281 is a plasmid backbone containing kanamycin resistance, a high copy origin of replication and a multiple cloning site. I isolated the digested fragments using gel purification (Section 1.3.5.3) and then ligated the isolated fragments (Section 1.3.5.4). I then transfected *Escherichia coli* Dh5α with each plasmid (Section 1.3.5.5). pSEVA281C was constructed via PCR cloning. I carried out a PCR using the pKDL071 plasmid as a template DNA using the protocol outlined in Section 1.3.5.1. I chose the primers so that Ptrc2 and mCherry were copied and a HindIII restriction enzyme recognition sequence added to the fragment. The primers are listed in Appendix (XXX). I purified the amplified DNA using the Qiagen PCR cleanup kit (Qiagen, Crawley, U.K) and then carried out the rest of the cloning procedure as per plasmid pSEVA281G.

Following construction, I isolated each plasmid using the QIAprep Spin Miniprep Kit (Qiagen, Crawley, U.K). I determined plasmid concentration using the Thermo Scientific NanoDrop 1000 Spectrophotometer (Fisher Scientific, MA, U.S.A).

1.3.5.1 Polymerase Chain Reaction

In order to amplify DNA and add the restriction enzyme sites required, I carried out a Polymerase Chain Reaction (PCR) reaction with mutagenic primers. A list of primers can be found in Appendix (XXX). I used the Q5® DNA Polymerase (NEB, MA, U.S.A) with its associated buffer, dNTPs and Q5® enhancer, as specified in

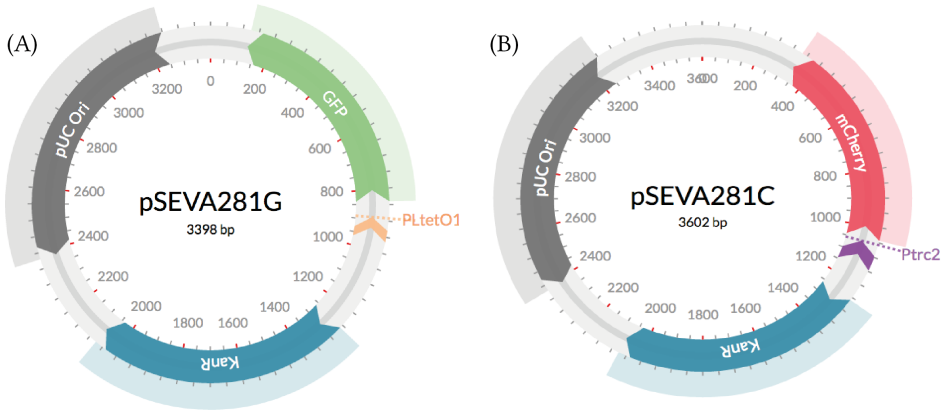

Figure 1.2 : The plasmids used to calibrate GFP and mCherry fluorescence. (A) pSEVA281G plasmid map (B) pSEVA281C plasmid map.

Table 1.1. PCR reactions were run in a T100^{TM} thermal cycler (Bio-Rad Laboratories, Inc., UK) as per the Q5® recommendations, and as outlined in Tables 1.1 and 1.2.

Table 1.1 PCR recipe

Reagent	Final concentration	50 µL reaction
Q5® buffer 5X	1X	10 µL
dNTPs	200 mM each	1 µL
Forward primer	0.5 µM	2.5 µL
Reverse primer	0.5 µM	2.5 µL
Template DNA	2 µg/50 µL	-
Q5® DNA polymerase	0.02 U µL^{-1}	0.5 µL
Q5® enhancer	1X	10 mL
H_2O	-	to 50 µL

Table 1.2 Thermocycling conditions

Step	Cycles	Temperature	Time
Initiation	1	98 °C	30 s
Denaturation		98 °C	10 s
Annealing	30	72 °C	20 s
Extension		72 °C	2 min
Final extension	1	72 °C	2 min
Hold	1	4 °C	∞

1.3.5.2 Digestion

All enzymes, buffers and Bovine Serum Albumin (BSA) were supplied by NEB. I carried out digestion controls by adding H_2O instead of DNA in the digestion reaction. Additionally, during agarose gel electrophoresis uncut plasmid was run alongside the digested plasmid in order to detect the difference.

I set up 2 µg digests by mixing the plasmid with 0.5 µL of each restriction enzyme, 3 µL 10x buffer and 3 µL 10x BSA. I added H_2O to make the reaction to 20 µL. The recipe used is shown in Table 1.3. I placed the reactions in an incubator at 37 °C for 4 hours. Finally, I analysed the solutions using agarose gel electrophoresis (Section 1.3.5.3).

Table 1.3 Digestion recipe

Reagent	Volume
PstI	0.5 µL
HindIII	0.5 µL
NEB Buffer 2.1	2 µL
BSA	0.2 µL
DNA	1 µg
H_2O	to 20 µL

1.3.5.3 Agarose gel electrophoresis

To make a 0.8% agarose gel, I diluted 0.4 g agarose in 50 mL 1X TAE buffer. It was further dissolved by microwaving for 1-3 minutes. I left the solution to cool for 5 minutes and then added 1.5 µL gel red. I prepared gel trays by putting the well comb in place and taping the ends shut. I then poured the solution into the prepared gel trays and left to solidify for 20-30 minutes at room temperature.

I carried out agarose gel electrophoresis by placing the poured gels into the gel tanks. I then flooded the tank with 1X TAE buffer. I prepared the DNA to be analysedI use by adding 4 µL loading dye to 20 µL sample. I also used a negative control with H_2O instead of sample. I prepared the DNA ladder of choice by adding 1 µL H_2O and 1 µL dye to 2 µL ladder. I added each sample to a well by pipetting. The agarose gel was ran at 90 V until the dye was 80% of the way down the gel, approximately 1 hour.

To purify the fragments from the agarose gel, I placed the gel was in a UV box. Using a sterile razor blade, I cut out the desired fragment and placed in a clean

eppendorf tube. I isolated the DNA from the gel using the QIAquick Gel Extraction Kit.

1.3.5.4 Ligation

I used a ratio of 3:1 of insert to recipient plasmid, 1 µL T4® DNA ligase (NEB, MA, U.S.A) and 2 µL ligase buffer. I added H_2O to make the reaction up to 20 µL. The controls I used for each ligation reaction, are shown in Table 1.4. Control 1 is used to detect competent cell viability, control 2 background due to uncut vector, control 3 contamination and control 4 vector re-circularization.

I placed the ligation reactions at 4 °C for 12 hours. I then placed the reactions at 65 °C for 10 minutes to heat inactivate the T4 DNA ligase enzyme. I then carried out a transfection as per Section 1.3.5.5.

Table 1.4 Ligation controls

	Control 1	Control 2	Control 3	Control 4
Vector	Uncut	✓	✓	✗
Insert	✗	✗	✗	✓
Buffer	✓	✓	✓	✓
H_2O	✓	✓	✓	✓
Ligase	✗	✗	✓	✓

1.3.5.5 Transfection

I transfected thermocompetent *E.coli* Dh5αwith the constructed plasmids. I added each ligation reaction to 50 µL of thawed competent cells. The cells were subsequently kept on ice for 30 minutes, then I placed them at a 42 °C water bath for 45 s. I then placed the cells back on ice for 15 minutes. Then I added 500 µL of Super Optimal broth with Catabolite repression (SOC) to each ligation and placed in a 37 °C shaking incubator for 3 hours. I subsequently pipetted 500 µL and 50 µL of each ligation onto petri dishes with LB agar and the appropriate antibiotic. The plates were incubated at 37 °C for 12-16 hours. I used two controls for the transfection protocol, a positive control with no antibiotic in the LB agar and non-transfected cells and a negative control of non-transfected cells and LB agar with antibiotic. These ensure that the cells are viable and not contaminated respectively.

Finally, I counted the number of colonies on each plate. I selected individual colonies from each transfection and grew each separately in 5 mL LB medium for

12-16 hours at 37 °C, 200 rpm. I prepared glycerol stocks from each culture, as per Section 1.3.3.

1.3.5.6 Colony PCR

In order to determine if the fragment was successfully inserted into the vector DNA plasmid, I carried out diagnostic colony PCR. I designed primers that amplified the multiple cloning site of the vector DNA plasmid. These can be found in Appendix (XXX). I made a PCR master mix for the number of colonies to be amplified, 32, with an added 10% to account for pipetting error. I used GoTaq® Flexi DNA polymerase (Promega Corp., WI, U.S.A.) with its associated buffer, dNTPs and $MgCl_2$and H_2O. The recipe for the master mix is shown in Table 1.5.

Table 1.5 Colony PCR master mix recipe

Reagent	Final concentration	Master mix
GoTaq® green Flexi buffer	1X	141 µL
dNTPs	200 mM each	14.1 µL
Forward primer	0.5 µM	1.4 µL
Reverse primer	0.5 µM	1.4 µL
GoTaq® Flexi polymerase	0.02 U μL^{-1}	3.5 µL
$MgCl_2$	1X	42.2 µL
H_2O	-	465 µL

I then added 19 µL from the master mix to each PCR tube. I lifted each of the colonies from the transformation from the agar plate using a 20 µL pipette tip and added it to a PCR mix by mixing. I subsequently used the pipette tip to make a scratch into a clean agar plate, and labelled it. I then carried out a PCR according to GoTaq® Flexi polymerase recommendations, and as shown in Table 1.6.

Table 1.6 Thermocycling conditions for colony PCR

Step	Cycles	Temperature	Time
Cell lysis	1	95 °C	10 minutes
Denaturation		95 °C	30 s
Annealing	35	50 °C	1 minute
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞

Finally I carried out a diagnostic agarose gel electrophoresis as outlined in Section 1.3.5.3.

1.3.5.7 Sequencing

In order to confirm plasmid identity, all plasmids were sequenced using Source Bioscience, Cambridge UK. I submitted 10 µL of each plasmid DNA at a minimum of 100 ng µL^{-1} as per the requirements. I submitted primer sequences that were manufactured by Source Bioscience. Primers can be found in Appendix (XXX).

1.3.6 Growth rate measurement

I carried out a plate reader analysis in order to measure the growth of *E.coli* over time. I made overnight cultures using the method shown in Section 1.3.1. I then diluted the overnight cultures by a 1:1000 ratio into a 5 mL LB + kanamycin solution. The diluted cultures were grown at 37 °C with shaking at 200rpm for 1 hour. I then further diluted these cultures by a 1:100 ratio. I transferred 200 µl aliquots of the dilutions to a clear bottom, black-walled 96-well plate. I also added wells with only LB and kanamycin in order to be used as blanks. I then sealed the plate using a gas permeable membrane and placed it in BMG FLUOstat OPTIMA plate reader to measure absorbance. I set the plate reader to a constant 37 °C, with 30 seconds orbital shaking at 150 rpm and 4 mm shaking width every ten minutes. Absorbance was measured at 540 nm. Data was exported as a CSV file and analysed using Python.

1.3.7 Flow cytometry

I carried out flow cytometry experiments in order to get fluorescent levels in single cells. Flow cytometry allows us to gather this information for thousands of single cells. Flow cytometry data was exported as FCS files and analysed using the R bioconductor package.

1.3.7.1 Concentration assays

I carried out concentration assays to determine the concentration of each inducer (ATc and IPTG) at which the switch flips. I prepared separate overnight cultures as per Section 1.3.1 with added IPTG at a concentration of 1 mM or added ATc at a concentration of 100 ng mL^{-1} (Litcofsky et al. 2012). I then diluted the cultures by 1:1000 into fresh LB medium with varying concentrations of the opposite inducer

than what the cells were grown in overnight. The concentrations used are shown in Table 1.7. For each concentration I made three replicates cultures.

Table 1.7 Concentrations used for flow cytometry assay

ATc (ng/ml)	IPTG (M)
0.05	1e-7
0.06	6e-7
0.07	1e-6
0.08	6e-6
0.09	1e-5
0.1	1e-3
1.0	0.1

I placed the cultures in an incubator at 37 °C, 200rpm for 5 hours. I then placed the cultures in a centrifuge and spun at 13,000rpm for 5 minutes. I discarded the supernatant and replaced it with 1 mL PBS solution. I used the The BD LSRFortessa™ cell analyzer (Becton, Dickinson and Company) at the St. Mary's Flow Cytometry Core Facility at Imperial College London for flow cytometry analysis. GFP was excited using the 488 nm laser and detected using the 533/30 filter. mCherry was excited using the 561 nm laser and detected using the 620/10 filter. Data was obtained at n=10000 events per experiment.

1.3.7.2 Time course assays

I carried out time course assays to measure the time it takes for the switch to flip to each side. I prepared separate overnight cultures of pKDL071 as per Section 1.3.1 with added IPTG at a concentration of 1 mM or added ATc at a concentration of 100 ng mL^{-1} (Litcofsky et al. 2012). I also made overnight cultures of pSEVA281G and pSEVA281C. I then diluted the cultures by a ratio of 1:1000 into fresh LB medium. I made separate cultures for each time point, in triplicates. For cultures grown overnight in IPTG, I added ATc at a concentration of 100 ng mL^{-1} and for cultures grown overnight in ATc, I added IPTG at a concentration of 1 mM. All cultures were placed at 37 °C, 200rpm incubator. At 30 minutes, 1 hour and then every hour up to 6 hours I carried out flow cytometry to the corresponding cultures. I removed three replicates for each induction from the incubator and placed them in a centrifuge at 13,000rpm for 10 minutes. I discarded the supernatant and replaced it with 1 mL PBS solution. I then analysed these cultures in an Attune™ NxT Flow Cytometer (Thermo Fisher Scientific) at University College London. GFP was excited using the

488 nm laser and detected using the 533/30 filter. mCherry was excited using the 561 nm laser and detected using the 620/10 filter. Data was obtained at n=10000 events per experiment. I used pSEVA281G and pSEVA281C cultures to set the laser voltages and pKDL071 cultures to detect the bacteria population.

1.4 Growth rate investigation

I carried out a growth rate analysis to determine whether the ATc or IPTG added to pKDL071 or pSEVA281G *E. coli* cultures affected the growth of the bacteria. Cultures were grown without any inducer overnight as described in Section 1.3.6. I ran assays for the cultures with and without added inducers. As can be seen in Figure 1.3, there is no difference between the conditions. The addition of either ATc or IPTG does not affect the growth rate of *E. coli* K-12 MG1655. Additionally, ATc does not affect the growth rate of *E. coli* Dh5α. Since the addition of ATc flips the switch to the GFP high state, and IPTG to the mCherry high state, we can also conclude that the growth rate of the chassis is not affected by which side of the switch is in the high state. The growth rate of *E. coli* Dh5αwas consistently lower than that of *E. coli* K-12 MG1655.

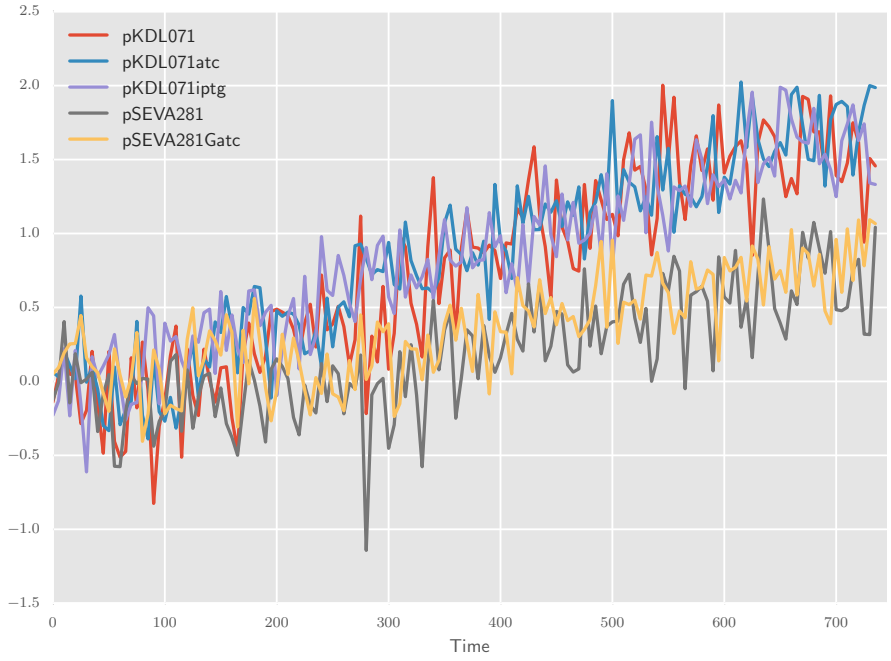

Figure 1.3 : Growth rate analysis of *E. coli* K-12 MG1655 pKDL071 and *E. coli* Dh5αpSEVA281G cultures with and without inducers. The inducers do not affect the growth of the bacteria.

1.5 Toggle switch concentration assays

Here I aim to identify the inducer concentration at which the pKDL071 toggle switch changes state. In order to do that I carry out a concentration assay using flow cytometry, as described in Section 1.3.7.1. As can be seen in Figure 1.4A, during ATc induction the switch flips to a GFP high state when ATc concentration is at $0.09\,\mathrm{ng\,mL^{-1}}$ or higher. We observe a bimodal distribution at concentrations $0.07\,\mathrm{ng\,mL^{-1}}$ and $0.08\,\mathrm{ng\,mL^{-1}}$, which indicates that the switching has begun at these concentrations. Thats why part of the population has switched to the GFP high state but complete switching is not observed until the concentration of ATc is at $0.09\,\mathrm{ng\,mL^{-1}}$. In the case of IPTG induction (Figure 1.4B) we find that the switch flips to the mCherry high state when the concentration of IPTG is higher or equal to 0.001M. A decrease in GFP fluorescence is also observed. We do not observe a bimodal distribution in this case.

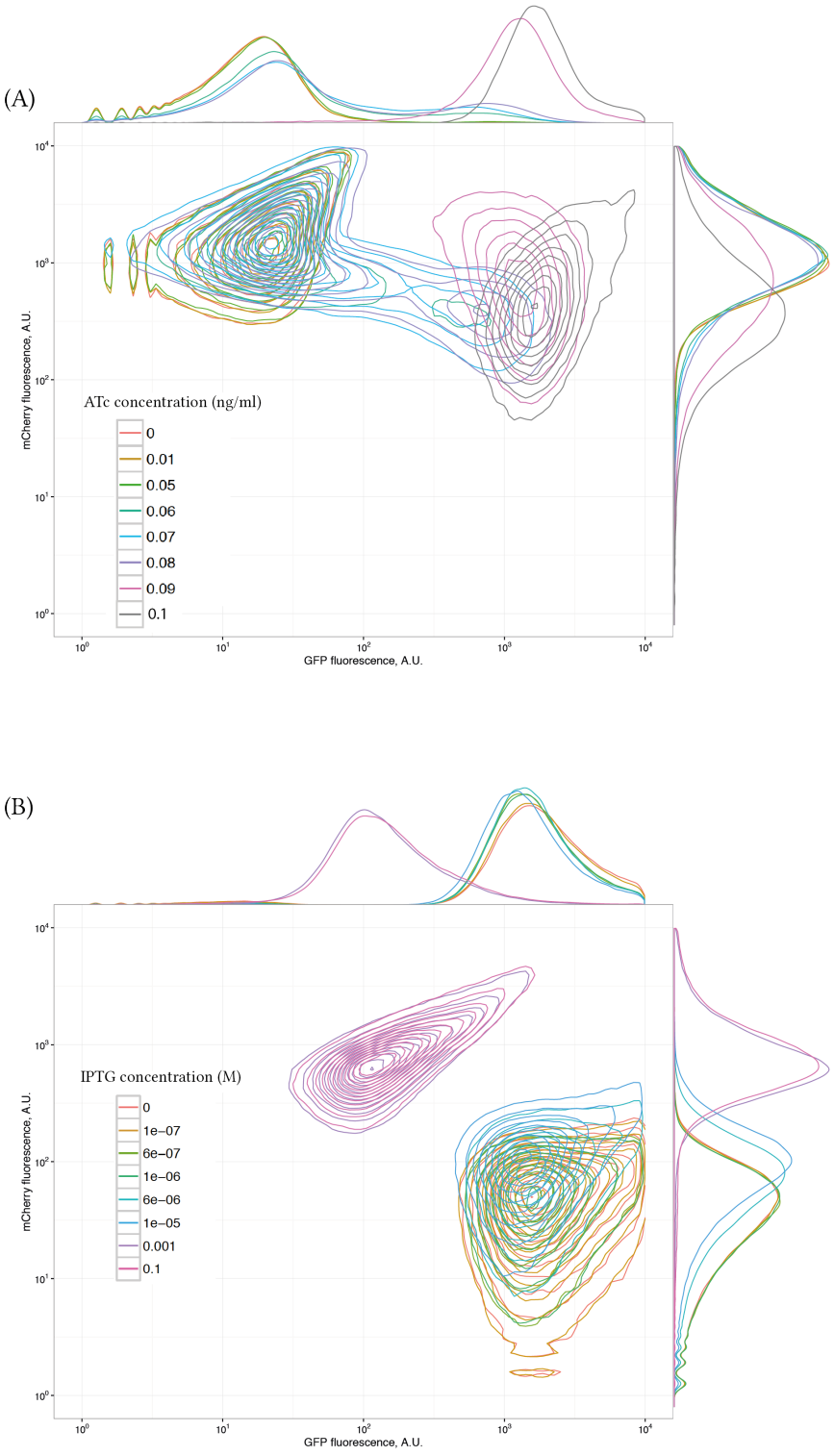

Figure 1.4 : (A) ATc induction at various concentrations (B) IPTG induction at various concentrations.

By taking into account the two induction curves of the switch turning to each high state, we can see the dynamic ranges of pKDL071 in *E.coli*. We can see in Figure 1.5 there is an approximately 100-fold change in fluorescent units during IPTG and ATc induction.

A Hill function was used to model the characterisation curves shown in Figure 1.5. The model used is the following:

$$F = Pmin + (Pmax - Pmin)\frac{\left(\frac{[I]}{Kd}\right)^n}{1+\left(\frac{[I]}{Kd}\right)^n}, \tag{1.1}$$

where F is the median fluorescent unit and [I] is the concentration of inducer. Pmin and Pmax are the minimum and maximum fluorescence respectively, and Kd and n are the dissociation constant, and Hill coefficient. I fit Hill function models using maximum likelihood estimation to the response curves. The values for parameters Pmin, Pmax, Kd, and n are 8, 1600, 0.1, 1.8 respectively for the ATc induction and 8, 700, 0.08, 2.5 for IPTG induction.

For the case of the ATc induction we observe a sharp switch between the GFP low to the GFP high state, as can be seen in the characterisation curve in Figure 1.5B. This is a clear indication of the bistability of this switch.

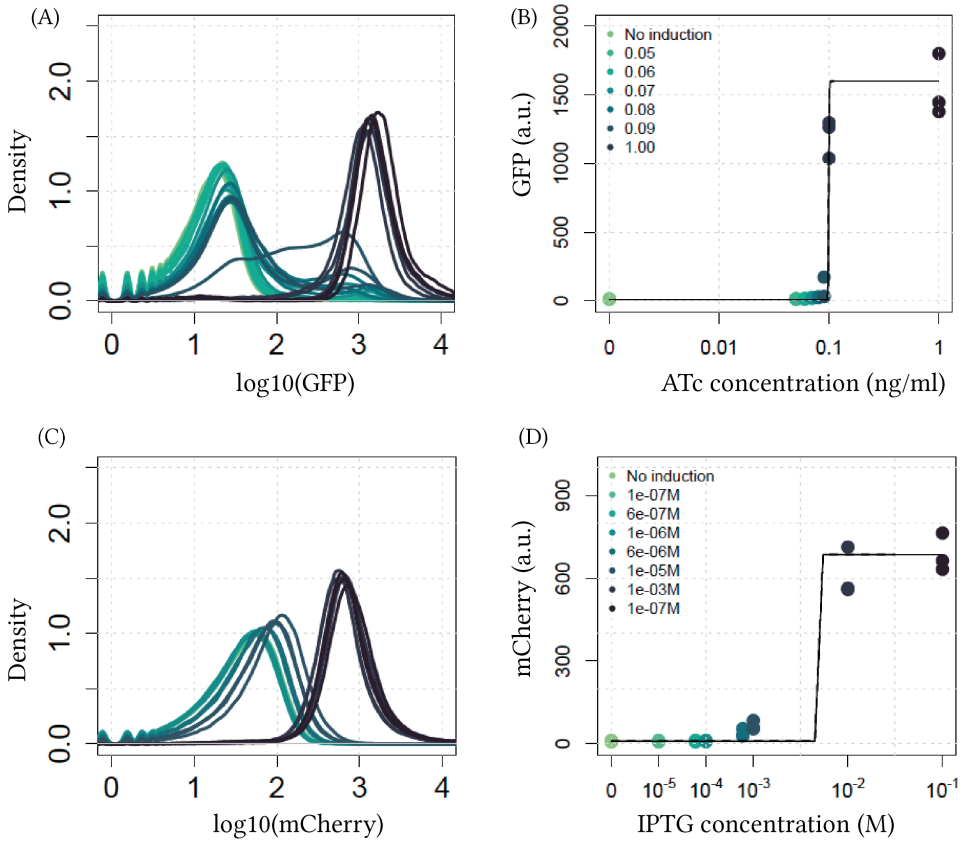

Figure 1.5 : (A, B) ATc induction of pKDL071. (C, D) IPTG induction of pKDL071.

1.6 Toggle switch time course assay

I further analysed the pKDL071 toggle switch by investigating the time it takes for it to switch from one high state to the other. To do that I used the method outlined in Section 1.3.7.2. I obtained separate time courses for the IPTG and ATc inductions.

As can be seen in Figure 1.6 pKDL071 ATc induction begins switching 1 hour after induction. Complete induction is seen at 6 hours. During the IPTG induction (Figure 1.7) we see a bimodal distribution at 4 hours, and induction is complete at 6 hours. We observe that during ATc induction there is an increase in GFP fluorescence and a decrease in mCherry fluorescence, in the case of IPTG induction the increase in mCherry fluorescence is not as prominent. A decrease in GFP fluorescence is observed during IPTG induction.

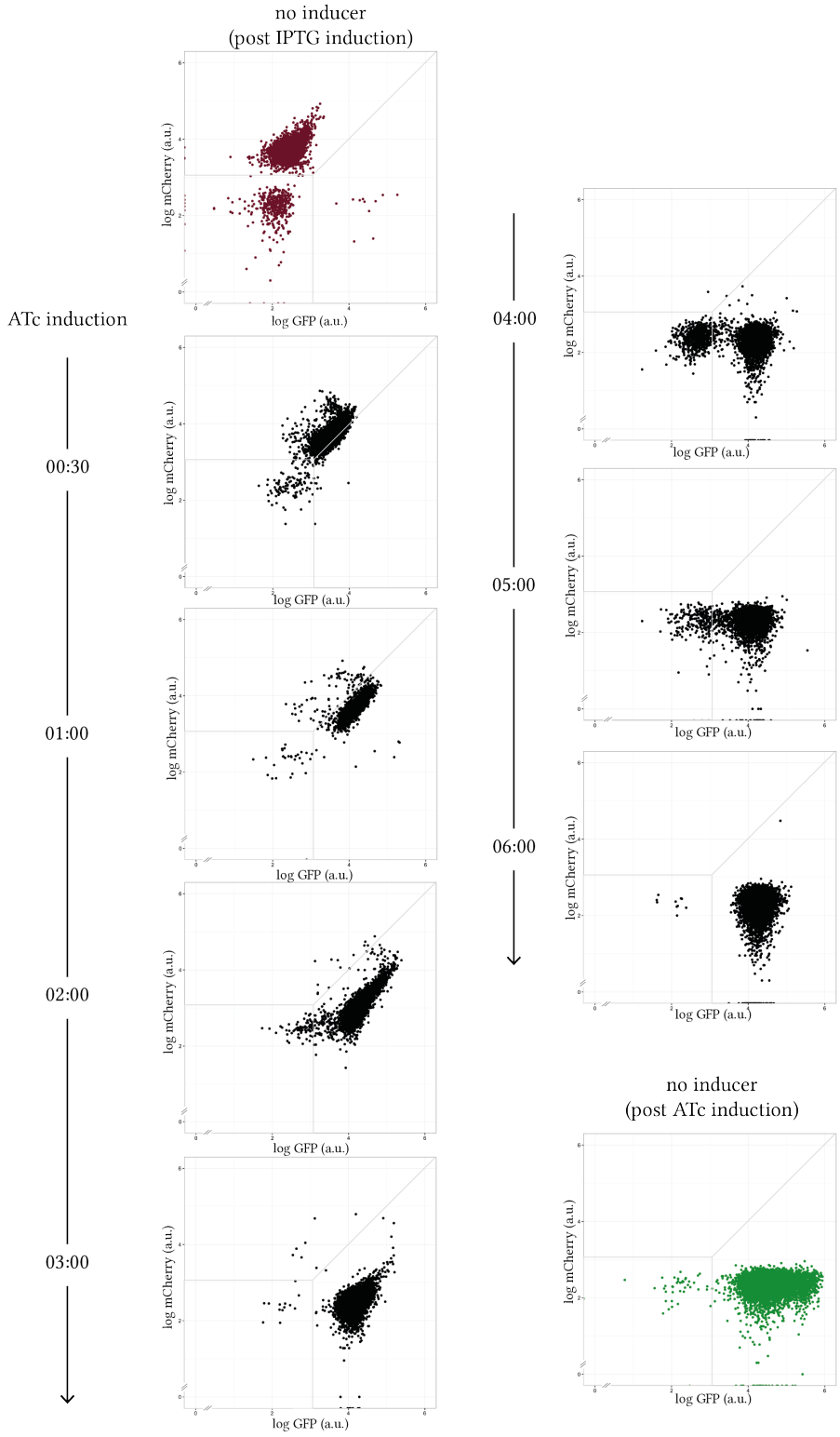

Figure 1.6 : ATc induction of pKDL071 over time

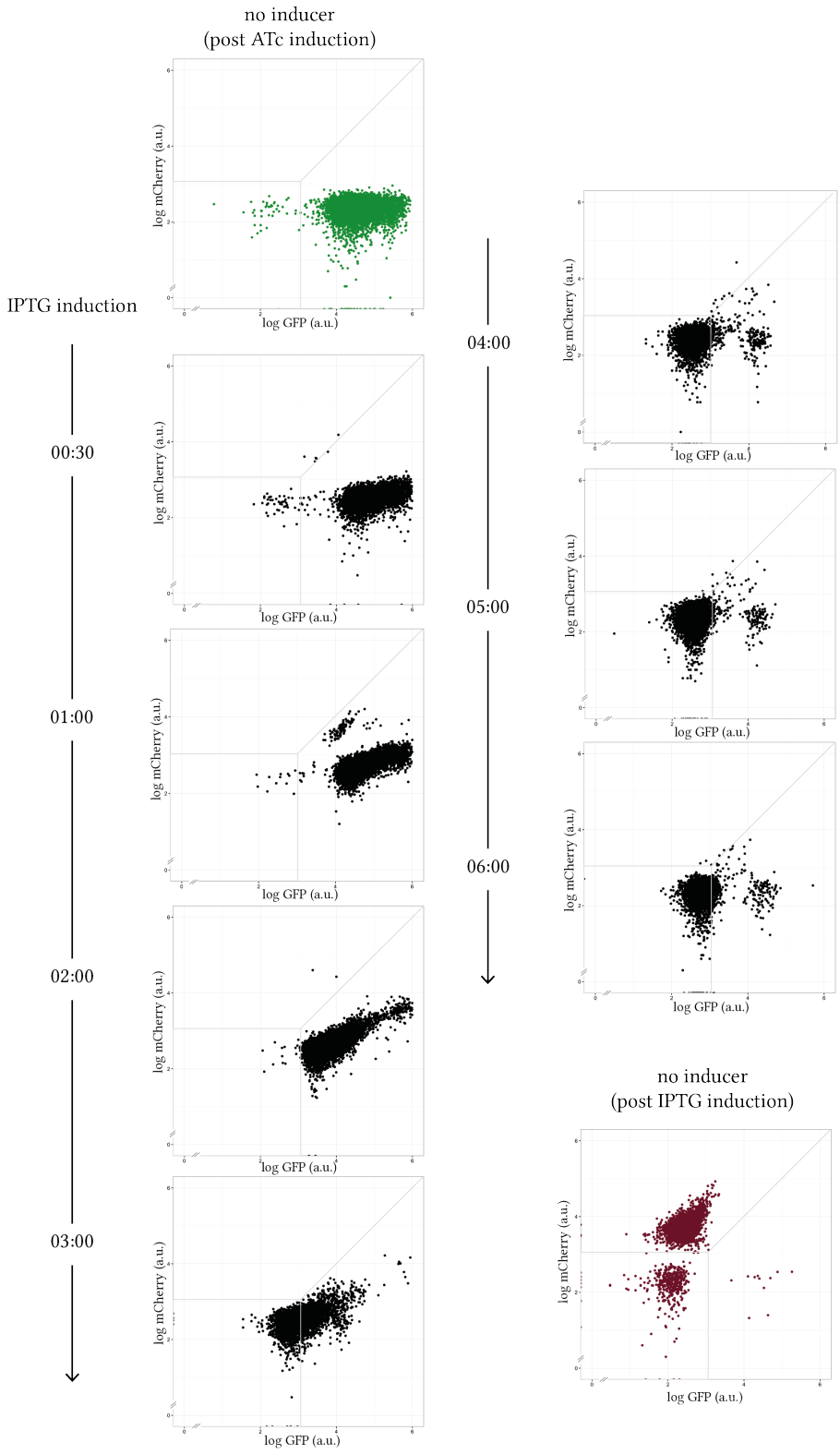

Figure 1.7 : IPTG induction of pKDL071 over time

1.7 Discussion

Here I characterised the genetic toggle switch experimentally. First I study the effect of the two inducers ATc and IPTG on the growth rate of the selected chassis *E. coli* K-12 MG1655. I find that there is no detrimental effect to the bacterium by the inducers. I also find that ~~which~~ state the switch is on has no effect on the growth rate of the bacteria. In order for this toggle switch to be used in a synthetic biology application, it is important that both sides of the switch have an equivalent burden onto the chassis. If one of the steady states creates a larger burden and slows down the growth of the bacteria, this can create an imbalance in the population. If the toggle switch-bearing bacterial population exists in an environment with competing bacteria, for example the gut microbiome, and one of the two states creates a larger burden, this would cause the switch-bearing population to become less competitive compared to the non switch-bearing population. It is therefore crucial that the state of the switch does not affect the competitiveness of the chassis.

I further characterised the switch by determining the minimum inducer concentration necessary to change the state of the switch. I find that for ATc induction, a minimum of 0.09 ng mL^{-1} is required to cause the switch to go to a GFP high state. For IPTG induction I find that a minimum of 0.001 M is required to flip the switch to an mCherry high state. This information is critical for using this switch in other applications. Both sides of the switch are very sensitive to inducer concentrations, as the concentrations required to observe a change in fluorescence are very small.

Furthermore I find that this toggle switch, pKDL071, is faster to respond to a change in ATc concentration that to a change in IPTG concentration. For IPTG induction we observe a change in fluorescence after 3-4 hours of induction. For ATc induction we can see a difference within an hour of induction. This result is in agreement with Litcofsky et al. (2012). This difference in response times must be taken into account when using the pKDL071 switch for other applications. This difference could be due to maturation times of the fluorescent proteins. Macdonald, Chen, & Mueller (2012) found that mCherry half-maturation time is 150 mins, whereas the GFP variant used here, GFPmut3b has been especially mutated for fast action (Cormack, Valdivia, & Falkow 1996). Cormack, Valdivia, & Falkow (1996) found that whereas wild type GFP is detectable 1-2 hours after induction, GFPmut3b is detectable 8 minutes after induction. This difference could account for the different response times observed here, but further investigation is required.

1.8 Summary

In this chapter I summarised the experiments carried out for the analysis of the genetic toggle switch. I used the pKDL071 plasmid and characterised its switching behaviour over various inducer concentrations and over time. I found the concentration of each inducer necessary to flip the switch as well as the time it takes for the change to be observed. Furthermore, I investigated the effect of the inducers on the growth rate of the chassis and found that they have no effect. In the next chapter I use the data collected in the chapter to fit to the more realistic toggle switch models used in Chapter (XXX).

Bibliography

Cormack, B. P., Valdivia, R. H., & Falkow, S. (1996). 'FACS-optimized mutants of the green fluorescent protein (GFP)'. *Gene* **173**(1), 33–38.

Litcofsky, K. D., Afeyan, R. B., Krom, R. J., Khalil, A. S., & Collins, J. J. (2012). 'Iterative plug-and-play methodology for constructing and modifying synthetic gene networks.' *Nature Methods* **9**(11), 1077–1080.

Lutz, R. & Bujard, H. (1997). 'Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements.' *Nucleic Acids Research* **25**(6), 1203–1210.

Macdonald, P. J., Chen, Y., & Mueller, J. D. (2012). 'Chromophore maturation and fluorescence fluctuation spectroscopy of fluorescent proteins in a cell-free expression system.' *Analytical Biochemistry* **421**(1), 291–298.

Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N. G., Palmer, A. E., & Tsien, R. Y. (2004). 'Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein.' *Nature Biotechnology* **22**(12), 1567–1572.

Shimomura, O., Johnson, F. H., & Saiga, Y. (1962). 'Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea.' *Journal of Cellular and Comparative Physiology* **59**, 223–239.