Time-sharing of synthetic opto-genetic circuits for multiple channel access communication

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TIME-SHARING OF SYNTHETIC OPTO-GENETIC CIRCUITS FOR MULTIPLE CHANNEL ACCESS COMMUNICATION

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SUMMARY

Quantitative understanding of complex biological systems has been a major challenge. The synthetic biology toolkit helps in engineering genetic circuits to design such complex systems. To develop effective precisely-controlled circuits, rapidly regulate-able gene expression systems are necessary. Opto-genetic modules offer unprecedented ways to control cellular functions in precise spatial and temporal resolution. However, current opto-genetic systems have been limited to low dynamic expression and switchable control. Henceforth, the design of synthetic circuits for coordinated cell-cell communication has been difficult.

A novel blue light-regulated genetic system is characterised in this work that demonstrates precise and stable switchable gene expression control with light intensity and pulse-width percentages. The system provides effective means of coordinated functions and thus, is applied to optically synchronize cells to perform different control functions. With such precise control, the system serves as a basis for underlying biological patterns and new developments in Systems biology and Biotechnology.

LIST OF ABBREVIATIONS

i.	QS	Quorum Sensing
ii.	AHL	Acyl Homoserine Lactones
iii.	GFP	Green Fluorescence Protein
iv.	RFP	Red Fluorescence Protein
v.	mRNA	Messenger RNA (Ribonucleicacid)
vi.	DNA	Deoxy-Ribonucleicacid
vii.	SNR	Signal-to-Noise Ratio
viii.	TDM	Time Division Multiplexing
ix.	FDM	Frequency Division Multiplexing
x.	TDMA	Time Division Multiple Access
xi.	FDMA	Frequency Division Multiple Access
xii.	SDMA	Space Division Multiple Access
xiii.	CDMA	Code Division Multiple Access
xiv.	MIMO	Multiple-Input-Multiple-Output
xv.	rpm	Revolutions per minute
xvi.	OD	Optical Density

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Chapter 1

Introduction

1.1 BACKGROUND

Synthetic biology is an emerging interdisciplinary field that employs, primarily the concepts of Biotechnology, Electrical and Computer engineering to transform organisms into novel biological circuits. It uses the principles of abstraction, modularity and standardisation to deliver tools to design, construct, and engineer biological systems for useful purposes with ease and reliability [36]. In the existing trend of this discipline, the paramount application is to equip microbes with novel human-defined functions.

Over the past few decades, engineers have built standardized bio-constructs and genetic circuits in bacteria that can perform logical and control functions independently. Upcoming research is now targeting to systematically assemble these biological parts to build artificial biological systems, analogous to the approach of Electrical and Chemical Engineering in building systems using individual logic circuits and operation units, respectively. Figure 1.1 illustrates the modularity in design in building synthetic biological systems along with the common symbol representations used for its components.

The development of these enabling technologies requires Systems Biology tools such as computational modelling and simulation. The quantitative tools will be used to predict the behaviour of biological devices and allow us to model and program new biological systems without doing a large number of trial-and-error wet-lab experiments. Taking advantage of these engineering design techniques enables us to generate efficient and systematic methods for increasing the speed, yield, and precision with which we engineer biological systems.

A greater challenge, however is for the synthetic gene systems to be modelled and tuned to meet specific performance criteria that could mimic the greatly complex behaviour of biological systems and diverse biological processes. This has also

raised the possibility of engineering a microbial consortium involving an association of diverse bacterial culture that can perform even more sophisticated tasks than a single bacterial culture [13].

Though Synthetic biology is still at its primitive state, the current and ongoing research promises advances in engineering microorganisms to create potential applications such as novel artificial biological pathways, devices, new biomolecular outputs and redesign of natural biological systems enabling improved products across many industries, including medicine, energy and the environment [7].

Figure 1.1
Fig.1.1 Principles of Synthetic Biology Engineering

1.2 SENDER-RECEIVER SYSTEMS IN SYNTHETIC BIOLOGY

Sender–receiver systems are abundantly present in Biology with communication systems sending information in cellular networks. Communication theory not only provides a quantitative basis for studying and analysing these processes, but also offers the possibility to control the behaviour of an entire cell system by modifying the functions of individual cells in specific ways. Recent advances in synthetic biology have built various artificial Sender–Receiver systems, based on bacterial cell-cell communication using well-characterized components [10].

The implementation of communicating synthetic Sender-Receiver circuits is a promising approach towards the engineering of sophisticated devices based on precisely functioned cells. With the growing complexity of the functions performed by such synthetic circuits, the risk of circuit crosstalk and the competition between them are increasing. Therefore, Sender-Receiver systems based on single cell functionalities are no longer feasible [10]. Multiple subpopulations of specific functional cells, wired by artificial cell-cell communication systems are necessary. In this manner, there is a need to introduce novel communication concepts to multiple subpopulations in synthetic bacteria and enable them to open new communication channels within microbial consortia, which leads to the motivation of the thesis work.

1.3 MOTIVATION

Synthetic Biology provides opportunities for advanced chemical processing from bacteria which are inexpensive renewable substrates and provides efficient microbial capabilities [12]. Thus far, conventional engineering practices have facilitated this field to generate biological parts that perform intracellular operations of computing, logic, switching and single population communication systems [10]. However, the next-generation production programs of gene circuits need to be directed to dynamically sense and regulate different bacteria systematically to function in coordination [43].

This is due to the reasons that diverse bacteria will enable creative programming of interactions to achieve complicated tasks that are difficult or impossible to be completed within a single bacterial culture. Furthermore, a consortium of bacteria is more robust to environmental fluctuations and offers new behaviour of interacting species not found in nature [13]. The involvement of many bacteria to produce complex functions also gives them ability of division in labour and dedicated coordination for efficient community functions. The engineering of multi-cell microbial consortia instead of a single-cell population also enables the division of over-all metabolic workload [13,16].

Communication is the first step to create interacting bacterial species for a microbial consortium. Natural microbial communities have demonstrated

preliminary social behaviour where individual cells cooperate at many levels through signalling to perform group activities and form complex structures together. Such a regulatory cell-cell communication predominant in bacteria is *quorum sensing*, which involves the ability of bacterial cells to monitor their cell numbers and induce collective behaviour above a threshold population-level [28]. Here, the signals used by the bacteria are typically chemicals that function as pheromones to express specific genetic traits. These chemical signals, termed as ‘auto inducers’ enable two-way communication between bacterial communities with their orthogonal properties [29]. The natural capability of the quorum sensing system provides bacteria to coordinate their functions without any crosstalk. It has been challenging to achieve corresponding artificial communication and new microbial networks using conventional biotechnology due the limited orthogonal auto inducer compatibilities in engineered bacteria [31] [28].

New abilities of communication in bacteria have been researched and developed in bacteria by modifying the different genetic components of its natural quorum sensing system. These are by the modifications of auto inducer message signal, sender cell, receiver cell or the environment. The first modification, i.e. with the auto inducer message signal, very few developments have been possible due to the limitation of orthogonal auto inducers [31]. With the receiver and sender cell modifications a variety of applications such as logic gates, prey-predator systems and oscillatory circuits have been designed [32] [16]. Otherwise, with the alteration of the diffusion and cell motility of the medium, spatial-temporal patterns have been produced [14]. All the types of developments have been restricted to new forms of intra-cellular communications and could not completely provide coordinated control among different bacteria. New forms of inter-cellular communication have to be addressed. From literature summary it was seen that the communication medium has not been exploited much, apart from the concepts of cell motility and diffusion. Hence, the possibility of enabling new means of communication should be explored in the communication medium.

Synthetic biology, can provide better opportunities to introduce more advanced techniques from various disciplines to model new inter-cellular systems in the communication channel. From an engineering perspective, the generation of a

system (microbial consortium) from various functional units (different subpopulations of bacteria) requires communication flow and programmed control among the different operating units. Thus a communication control in the different operating bacterial units has to direct them to interact in a programmed fashion and achieve coordination as a systematic network. Providing avenues for the synthetic bacteria to communicate with one another would allow us to build large-scale systems to step into a microbial consortium developing better products [12].

However, there are many challenges, mainly four problems to be accounted for such a coordinated system behaviour.

1. Firstly, the existence of different bacteria in a co-culture will not lead to a cooperative behaviour, instead it will lead to the competitive inhibition of one type of bacteria over another [13]. Since the different bacterial population will compete with one another and not coordinate in natural circumstances, they require to be re-introduced or eliminated as and when needed and in which their behaviour will be required to be monitored with time [12].
2. Secondly, gene transfers between microbes in the same network, i.e. crosstalk has to be avoided [14].
3. The third challenge is to incorporate new ways of stable control in the genes to ensure proper mechanisms of interactions [13].
4. Finally, a fine-tuned performance among the multiple populations in the consortium has to be ensured [13].

To address the above challenges, it was inferred that in order to eliminate crosstalk, competition and enable stable control and time-monitoring in the co-culture of multiple bacteria, the different bacteria had to be in coordinated action. To coordinate actions, the transmission and processing of information require the existence of a signalling system, wherein the signals are directed in an appropriate way.

One way to coordinate signals is for the signalling system to control specific functions only at certain times and switched off during other times. Communication engineering is known to have achieved such organized communications and control in multiple users through the implementation of

multiple channel access [8] [18] [19]. In this thesis, Time Division Multiple Access (TDMA) is taken as an inspiration for coordinated communication between multiple cells.

This thought has motivated me, for the application of a Time Division Multiple Access inspired concept in Synthetic circuits, to form a robust bacterial communication set-up. In this conceptualization, the synchronization for the coordinated of synthetic circuits will be provided by a blue light clock signaling system for the timely channel access of the individual population of bacteria. This light-regulated module can offer unprecedented new ways to control cellular behaviour in precise spatial and temporal resolution.

With the advantages of TDMA and the new direction of approach, it is envisioned that the application of time-division multiple access inspired techniques will overcome the challenges posed for an artificial microbial consortium. It will also help to predict the way to maximise signal robustness, and enhance the prospect of increasingly complex communication capabilities in synthetic systems.

1.4 OBJECTIVE

This thesis aims to improve the communication capabilities in a microbial system through the introduction of a novel cell-cell communication concept, which is inspired by TDMA telecommunication technique.

A few specific scientific aims are listed as follows:

- i. It is aimed to provide a stable control system that can synchronise the communications of the different circuit systems and prevent cross-talk.
- ii. A rapidly reversible system that can precisely express genetic functions at specific time durations is to be designed.
- iii. The cell-cell communication capabilities and capacity in a microbial population are also aimed to be improved with the new concept.

1.5 CONTRIBUTIONS

The thesis proposes a time-sharing concept for improved cell-cell communication in synthetic microbial system. A two-way channel access communication system comprising of two synthetic receivers is designed and simulated to receive signals from a sender using blue light clock signal.

The biological construction for the synthetic circuits and the experiments concerned have been made and conducted by the post-doc (Dr.Prem) working with my co-supervisor. The following were presented in the thesis in order to achieve the time-sharing communication model

- i Mathematical modelling using Systems Biology tools was implemented to simulate gene expressions that can direct time-dependent harvesting of synthetic proteins based on blue light clock. For this, a new set of ordinary differential equations (ODEs) was specifically proposed to model the binding of light transcriptional factor to the regulatory promoter and activate transcription process. The receiver cells were programmed to emit fluorescence and their gene expressions were obtained based as a system of ordinary differential equations.
- ii In order to characterise the system and obtain the quantitative parameters of the synthetic circuits, experiment results (performed by the post-doc) were fitted to the model equations using non-linear least-square parameter fitting. Thereby, the transfer function equations for the system with respect to the optical parameters such as pulse width light illumination and intensity of light illumination had been made for the newly engineered optical synthetic system. Using this, it was shown that dose-dependent blue light illumination pulses or intensity can precisely control the extent of induction or repression.
- iii Based on the characterisation results, prediction of the system for different time period lengths as well as for different light ON cycles were performed and validated with another set of experiments.
- iv Moreover, extended predictions for long time durations to study oscillatory behaviour of the system was simulated.

- v The parallel working of the receiver systems was demonstrated and was used to construct two semi-orthogonal receivers that operated in alternate half-cycles of the blue clock signal. Using experiments and simulation, it was shown that the receivers perform time-sharing and output their respective messages in their corresponding read-out time slots correctly. The performance of the system was also analysed under various clock time period and pulse width illumination conditions.

1.6 SIGNIFICANCES

The work presented in this thesis presents a multiple channel access cell communication framework that provides microbes with multi-control functions without crosstalk and competitive interference. This contributes to the groundwork for building microbial consortium systems. The benefit of the system can be attributed to the application of a novel engineered light-mediated promoter which demonstrates rapid 'ON' and 'OFF' kinetics and overcomes the shortcomings of previously characterised opto-genetic modules in literature [22,23] such as lack of rapid and switchable control, less portable, low dynamic expression and limited parts.

No such direct light-inducible promoter has been reported to rapidly and reversibly induce gene expression using a single transcription factor or one-component system in prokaryotes or eukaryotes [40, 27, and 35]. The robust performance of this new light-mediated promoter has facilitated the use of light pulse as molecular clock signal to direct gene expressions with time. Here, we have programmed a dual channel access light-controlled gene expression system which ideally requires both light-dependent inducible and repressible device able to function in parallel with rapid and reversible control of gene expression in space and time. A semi-orthogonal behaviour is exhibited with the characterised opto-genetic system that has enabled, for the first time, to opto-genetically synchronize two receiver cells performing different logic behaviours over time using blue light as a molecular clock signal.

Further, the availability of such opto-genetic system tools would facilitate synthetic biologists to modulate multiple endogenous gene expressions in a more

controlled fashion with superior spatio-temporal resolution [26]. In bacteria, fundamental processes such as gene expression and cell-cycle events are largely controlled by spatial and temporal oscillations. The novel opto-genetic system not only demonstrated that it can rapidly and precisely be switched ‘ON’ or ‘OFF’ at will, but also demonstrated that by modulating the dosage of light pulses or intensity it could control the level of gene expression precisely. It is shown that both light-inducible and repressible system can function in parallel with high spatial precision in a single cell and can be switched stably between ON- and OFF-states by repetitive pulses of blue light. Hence, the precise spatial and temporal regulation of inducible and repressible gene expression systems would enable dynamic gene regulation for optimal production of bio-commodities at desired levels.

Overall, the rapid and semi-orthogonal capabilities of the light-regulated tools provide multiple channel access abilities contributing to the expansion of communication capacity in the synthetic biology. Also, the ability to process and program the opto-genetic toolbox with precise tune-ability combined with its superior resolution would be highly beneficial to expedite our ability to solve complex biological problems. This synthetic communication framework layers a transformative platform for next-generation synthetic biology microbial networks in prokaryotes.

1.7 THESIS OUTLINE

The thesis consists of seven chapters. In Chapter 1, background information, motivation, the specific objectives, contributions and significance of the works presented in the thesis are described.

Chapter 2 is a literature review of papers on recent synthetic biology research and advancements in cell-cell communication. The review evaluates the methodologies and applications that were considered to achieve communication concepts in Synthetic circuits.

This is followed by Chapter 3 which deals with the theoretical aspects of bio-circuit design. Here the focus is on the regulatory communication mechanism quorum sensing, the central dogma concept of molecular biology and enzyme kinetics that characterise the development of engineered bacterial systems.

In Chapter 4, the types of telecommunication principles are discussed. Then, the concept of Time Division Multiple Access (TDMA) which has inspired the programming of multiple cell-cell communication in Synthetic Biology is studied in detail. Following that, the time-sharing concept model is introduced in the quorum sensing mechanism of bacteria. The working of the model, the components used to for its development and the sub-units used to design the model are discussed here.

Chapter 5 revolves around the characterisation of the novel engineered opto-genetic synthetic circuits by characterising the enzyme kinetics and transfer functions in response to incident light. Further, validations for synchronisation of the light-inducible and light-repressible genetic modules are made. Part of these results have been published in the recent Nucleic Acids Research Paper [43].

Following this, in Chapter 6, the characterized engineered opto-genetic system is constructed as two quorum sensing receivers that respond to AHL chemical message and perform logic functions. Here the implementation of opto-genetic control on receiver systems is made to achieve the multiple channel access capabilities and synchronization of their functions over time. The experiment performed on this concept is validated for one light pulse cycle. Then the prediction of the system behaviour for longer time durations and light pulse cycles is performed.

Finally, in Chapter 7, the summary of the findings is analysed and inferences are drawn about the hypothesis. The implications and future works are concluded from the previous chapters.

Chapter 2

Literature Review

2.1 INTRODUCTION

Constitutive Promoter	Regulatory Promoter (Inhibitor/Activator)
Gene	Protein
Regulatory QS Protein	Auto inducer (AHL)
AHL Synthase	AHL Degrading Protein

Fig. 2.1 Common Symbols and Representations in Synthetic Biology

Research on Synthetic Biology has demonstrated inter-cellular communication in synthetic bacteria by altering the different phases of cell metabolism involved in the natural communication mechanism, *Quorum Sensing* (QS). This regulatory sensing method is a means of cell-to-cell communication by which individual cells regulate their biological functions in response to the extracellular concentration of signalling molecules [14]. Figure 2.1 shows the common symbol representations used in Synthetic Biology used to illustrate the Quorum Sensing mechanism. The process involves the secretion of chemical signals called *auto inducers* into the surroundings which get bound to sensory proteins in neighbouring cells and affect their enzyme kinetics as explained in Figure 2.2. With increase in the population of bacteria, “the concentration of inducers called AHLs in the environment increases due to contribution of more bacteria in their synthesis [14].” Thus, through signalling and responding to changes in their local population density auto inducers regulate Quorum Sensing.

Figure 2.2 Quorum Sensing

Fig.2.2 In cell 1, the QS1 activates proteins that produce extracellular chemicals and a type of auto inducers (AHLs) that diffuse out into the environment. The auto inducers (say A1), when produced in high concentrations reach and diffuse into Cells 2 that contains its respective sensory protein receptors. The protein receptors when induced by the auto inducers (A1), in turn activate the QS 2 system of Cell 2 to produce another type of auto inducers (say, A2). The auto inducers (A2) diffuse and bind to its respective sensory proteins in Cell 1 and the cycle repeats. Thus, the auto inducers (A1 & A2) and the QS systems (QS2 and QS1) are orthogonal and correspond to each other respectively.

Having studied the mechanism of quorum sensing, it is now possible to review the modification techniques that can be introduced to the natural mechanism in order to develop synthetic quorum sensing systems. The quorum sensing mechanism involves regulatory processes happening at four phases:

- (1) Sender Cell
- (2) Auto inducer Message signal
- (3) Environment
- (4) Receiver cell

2.2 Modification of Quorum Sensing Capabilities for engineered synthetic Circuits

Research work for synthetic quorum systems has altered these natural controls in either of these phases to create novel functions in engineered bacteria as categorised in Figure 2.3. With the context of which phase was modified to develop new inter-cellular communication in bacteria, literature on this work will be classified and reviewed as follows:

Figure 2.3 Modes of developing engineered quorum circuits

2.2.1 Modification of Auto inducer Signalling molecule

In nature, different and specific QS system chemical inducers are synthesized by different types of bacteria. For example, the bacterium “*Vibrio fischeri*” uses two QS systems, Ain and Lux that employ acyl homoserine lactone (AHL), 3-oxo-C6HSL as its auto inducer; whereas the bacterium *Pseudomonas Aeruginosa* uses the QS systems Las and Rhl that involve the auto inducers, 3O-C12-HSL and C4-HSL. [21] [32]”. In naturally occurring bacterial colonies, only four such quorum sensing auto inducers signals have been identified to be orthogonal to their counterpart QS systems and thus communication between those species is very specific and prevents any cross-talk to other bacterial species. Characterization of the communications of just these four different quorum sensing systems has resulted in the development of many potential circuit components for use in a variety of synthetic biology applications.

Due to the natural abundance of quorum sensing systems, promoter modularity and orthogonality, Quorum Sensing is a very attractive tool for the engineering of next generation genetic circuits and the programming of microbial consortia. Although the completely orthogonal natural systems have straightforward use in this case, there is limited scope of development due to the limited number of orthogonal auto-inducer species [31].

Nevertheless, the introduction of slightly altered mutant forms of auto inducers which were chemically synthesised have been found to function as auto inducer analogues that can induce receptor proteins in the counterpart QS systems. These mutant auto inducers have been considered as tools that can provide additional

control over the natural quorum sensing mechanism [28]. However only very few auto inducers have been well-characterized and chemically identified to explore analogues. Also, the creation of the auto inducer analogues is a very difficult task. Thus, the scope of applications of auto inducer mutants is very narrow and is of little help to construct synthetic circuits [5].

Nonetheless, recent research applying genetic engineering tools has achieved better techniques to overcome this limitation and produce new communication capabilities in bacteria. They have dealt with controlling the mechanism either by outgoing chemicals or processing incoming chemicals sent or received by bacterial cells which will be discussed in the subsequent sections.

2.2.2 Control by Outgoing chemicals from Sender cells

From the quorum sensing mechanism, it is seen that sender cells express proteins when activated by the quorum sensing cascade to produce extracellular auto inducers, chemicals and other proteins. These chemicals diffuse into the environment to affect the surroundings or bind to the neighbouring cells to regulate their biological functions. With the help of biotechnology tools, the sender cells genome sequence can be modified to produce different proteins. The resulting production of the synthetic proteins will then open up new capabilities. For example, sender cells when activated can generate fluorescence through the addition of a Green Fluorescent Protein. The fluorescent protein acts as a reporter that fluoresces when sender cells get activated. The modification of the sender genome in quorum system can even help to achieve new control functions in bacterial communication and promises many practical uses.

One of such applications is the suppression of intercellular communication in pathogenic microbes, a process termed quorum quenching [2]. Along this line, the sender cells can be used to produce proteins that control populations of corresponding receiver cells that cause environmental harm or infections. Especially, when it is required to control small fluctuations in gene expressions, it will be useful to control the population sizes of bacteria with high precision. The “population circuit” developed by You et al, using QS systems LuxR/LuxI was

able to realise this condition (Fig.2.4) [20]. In this system, the sender QS system LuxR when activated during high population levels of other bacteria (high concentration of auto inducers from its complementary LuxI QS), produces a killer protein that kills receiver cells. Thereby, the engineered bacteria producing killer proteins were able to maintain a controlled population of the harmful bacterial population.

Figure 2.4 Population Control Circuit [20]

Fig 2.4 Population control circuit “K is the ‘killer’ gene. S and R represent the AHL Synthase and Regulatory Protein respectively. Inducer signalling molecules AHL are represented by circles.

Experiments by Danino et al. advanced the population control system to generate robust, sustained population oscillations by incorporating control from additional QS systems *aiiA*, and *yemGFP* apart from *luxI*. Their circuit consisted of “interlocked positive-feedback from the activation of *LuxI* QS system and negative feedback loop due to the activation of the second QS System, *aiiA*” [32]. When one QS system was triggered in high cell density conditions it induced a protein that activated the next QS system that expressed killer proteins to degrade the inducers. Again the first system was triggered and the cycle repeated to produce oscillations. Additionally, when performed the experiment in a solid-phase microfluidic device, it was demonstrated that the “synthetic gene circuit produced spatiotemporal oscillations” (Fig. 2.5) [32].

The same group also took their progress forward by expanding on the application of the system to work as “an arsenic detector [1]”. They coupled the oscillatory QS System function with an arsenic sensor component to produce oscillating

fluorescence. (Fig. 2.5 (c)). By reading the fluorescence oscillatory period, it was possible to monitor arsenite concentrations and quantify them reliably down to 0.2 μM .

It can be seen that the above research works in controlling outgoing signals were achieved with the expression of an extra killer or fluorescent reporter gene in sender components that could kill and control the population of the other bacteria. This technology was nothing but the change of the ‘coordinating’ function of the quorum sensing system to a ‘controlling’ function of the quorum system. Further it can be inferred that the responses to bacterial population levels was enabled by the introduced killer protein to control the chemical levels but did not involve any new ways of interactions between the bacterial cells.

Figure 2.5(a) Oscillatory Circuit Figure 2.5(b) Spatio-temporal Oscillations [1]

Figure 2.5(c) Arsenic detector [1]

Fig 2.5(a) Oscillatory Circuit that produces oscillatory levels of fluorescence through three QS systems. AHLs are produced from one QS system whereas degraded through another QS system to produce varying fluorescence levels.

Fig 2.5(b) Spatio-temporal oscillations produced by the interaction of quorum systems at multi-colony levels. Fig. 2.5 (c) Arsenic detector system which when senses *ArsR*, increases the degradation rate that affects the period of oscillation of Green fluorescence produced. Figure on left shows Fluorescence production when no Arsenic is present; Figure on right shows no fluorescence being produced when Arsenic is present.

Another advancement in quorum quenching application from engineered bacteria was achieved by Balagadde et.al to construct the prey-predator system. The difference is that synthetic circuits were constructed using two pairs of orthogonal “QS modules, LuxI/LuxR from *Vibrio fischeri* and LasI/LasR from *Pseudomonas aeruginosa* [11]” to function as predator and prey respectively [14]. Engineered quorum sensing circuits having two QS systems in each cell was constructed such as to achieve a new form of bi-directional communication as shown in Figure 2.6. At sufficiently high concentrations, the LuxI QS system of the prey cells is activated to synthesize 3OC6HSL inducers that get accumulated in the culture. These inducers communicate with the LuxR QS system of the predator bacteria contributing to an “increased expression of an antidote gene (*ccdA*) to rescue the predators [11]”. In turn, the predator bacteria synthesise the inducers, 3OC12HSL by the LasI QS system which diffuse through the prey bacteria to bind to its complementary QS system LasR, and consecutively induce the killer gene for predation. The synthetic ecological system illustrates the prey-predation relationship between a “two-species ecosystem, where one species (prey) suffers from the growth of the second (predator) and the latter benefits from the growth of the former [11]”.

Figure 2.6 Prey-predator system [11]

Fig. 2.6 Prey-predator systems constructed from QS systems LuxI/LuxR that express killer genes *ccdB* for population control. The transcription of *ccdB* gene in the prey system directs itself to death, whereas the expression of *ccdB* gene is inhibited by *ccdA* gene which forms a non-toxic complex with it.

This prey-predator system illustrates two QS systems working together in one cell to regulate the ecosystem, also controlled by expression of killer protein in one cell (Fig.2.6). The incorporation of two QS systems in one cell, though not very useful for practical applications presents a new improvement to study the communication between two species. However, the design of the experiment considers the interactions of the two species by intra-cellular way but not through the external environment and thus is still limited to offer communication regulated by the protein but not any other new form of communication.

2.2.3 Control of Incoming chemicals by Receiver cells

Similar to the control by sender cell through the implementation of additional genes that transcribe protein functions in the previous section, receiver cells can be modulated to perform additional quorum sensing functions. This was achieved by using similar biotechnology tools to alter the configurations of sensory protein receptors that receive the signalling molecules. It was then possible for the receiving quorum sensing cascade to respond differently to the auto inducer concentrations.

A simple example of an experiment that achieved this is the ‘AND’ gate quorum sensing circuit by Brenner et.al [16]. It can be realised that the bi-directional communication of quorum sensing can be achieved only when both bacterial culture densities are high, i.e. only when both bacterial cascades grow to enough population sizes, the auto inducers can be produced in high concentrations to activate both the forward and the feedback components of the quorum sensing cascade. Thus, detection of the bi-directional communication in bacterial cultures was taken to be the output of the AND gate system that detected the combined activation of the two quorum systems. This was enabled by the expression of a fluorescent gene in the receiver genes that got activated by the feedback communication.

Figure 2.7 AND Gate function in Quorum Sensing bacteria [16]

Fig 2.7 QS systems expressing the function of AND gate as a function of fluorescence only when both the systems are activated

Another example that performed more advanced capabilities from the modulation of receiver quorum sensing sequence was the band-patterning system by Basu.et.al (Fig.2.8) and the work by Liu.et.al. Here, the receiver genes were characterised such that they were activated to produce fluorescence only at a particular range of auto inducer concentrations [26]. When the experiment was performed on a solid phase medium, the auto inducers diffused to form spatial band patterns that exhibited fluorescence only in regions of intermediate auto inducer concentration detected by receiver cells [33].

Figure 2.8 Band-patterning system [33]

Fig 2.8 Band-patterning systems formed by synthetic sender and band-detector receiver systems. The receiver cells in close proximity to sender cell produce high amounts of LacI that represses the GFP production. Receiver cells located intermediate to sender cell produce less LacI that do not repress Green Fluorescence but sufficient inducers to produce fluorescence. Receiver cells located far away do not receive enough inducer concentration to activate the quorum sensing system.

A similar but more sophisticated control system was the “synthetic genetic edge detection program” to produce an image from engineered *E. coli* bacteria by Tabor et al. Here the control of bacteria was by light rather than the auto inducer concentrations. The receiver cell bacteria were programmed to produce fluorescence only at intermediate light levels. Thus, at the dark regions and the bright regions of the image light was either too abundant or too little and black pigment was not produced. However, at the edges the light fell in the band detect range of the receiver gene cells to synthesise a ‘black pigment’ [35]. The arrangement of genes in the engineered bacteria (as shown in Figure 2.8) enabled them to give out black pigment only at the edges of image and work as edge detectors. The experiment was able to achieve this new application by implementing an opto-genetic control in the receiver sensory proteins.

Figure 2.9(a) Edge-detecting synthetic circuit

Figure 2.9(b) Output of edge-detect circuit [35]

Fig 2.9(a) Synthetic Circuit that produces black pigment at the edges is controlled by light. The gene LacZ regulates the formation of black pigment when light is present. However, the CI protein represses the black pigment at very bright conditions. Thus, the pigment is produced only at edge of light-dark regions.

Fig 2.9(b): (A) Projection of light into bright and dark regions of bacterial culture to compute output at the edge. (B) Bacteria at the dark regions synthesise inducers that diffuse out of the boundaries

In conclusion, these experiments demonstrate new capabilities of quorum sensing circuit sensing from the alteration of receiver protein configurations that sense auto inducer signals or light. This technology similar to the control by sender cells in the previous section, shows that even receiver sensing components can be modulated to enable new quorum sensing applications. Both types of control were enabled by using biotechnology tools to alter the configurations of sensory protein receptors. It was then possible for the receiving quorum sensing cascade to respond differently to chemicals (auto inducers or light). Though this research technique has potentially enabled different functions from a synthetic quorum sensing system, it is seen that the functions are limited by intra-cellular interactions and do not incorporate any control by inter-cellular communication between the cells.

2.2.4 Control of Communication in Environment

In the quorum sensing mechanism, the environment separating the sender and receiver cells is the medium where the auto inducer message signals flow and can be visualised as the communication channel of the system. Spatiotemporal dynamic studies have been performed for various auto inducer diffusion rates and through its various distances over time [13] (Fig.2.9). For example, the research by Song et al. has pursued the analysis by using the engineered pre-predator

system as a model to study quantitative factors that influence biodiversity in a solid-phase environment [14]. It was investigated that the biodiversity was inversely proportional to cell motility (movement of cells) when the separating distance between the bacterial populations were comparable to the diffusion length of the inducers.

Figure 2.10(a) [13]

Figure 2.10(b)

Fig 2.10(a) Self-organized patterns from the sensing and regulation of cell motility in the QS systems
 Fig 2.10(b) Synthetic Circuit that produces fluorescence based on low and high density sensing.

Apart from the concepts of cell motility and diffusion, it is seen that the communication medium of the bacteria has not been exploited much to offer new ways of interactions. Little research has been performed to study new ways of communication between bacteria by modifying the channel access. However, when visualising the quorum sensing mechanism as a multiple communication system in bacteria, the environment channel is indeed a potential area to be researched on as it is the component that can promise more methods of inter-cellular communication. The controls by modification of sender, receiver and auto inducer signals have proved to offer many additional quorum sensory functions. However, they have been limited to provide new functions controlled by intra-cellular abilities alone. The crosstalk between cell gene expressions are still present in such controls.

Thus, multiple communication in bacteria can be enhanced by providing more access and abilities through the communicating medium. This will enable bacteria to coordinate one another in a programmed fashion so as to make most out of interacting bacteria, and prevent competition between them. The introduction of

more coordinating control in bacteria will further enable them to produce larger systems such as networks or more complex proteins.

2.3 OPTOGENETIC CONTROL FOR GENE EXPRESSIONS

In order to incorporate multiple communications in bacteria, the cells have to be signalled appropriately for them to access the channel by expressing their gene expressions at precise conditions. Ideally, biological control in synthetic bacteria that enable them to rapidly and precisely switch gene expressions ‘ON’ or ‘OFF’ at will, would boost our ability to coordinate bacterial functions and prevent cross talk [13]. Typically, exogenous chemicals that bind to soluble transcription factors are used to achieve such systematic control of gene expressions [42]. However, their efficiency is limited because of their potential off-target effects [39], transport process delays [24], toxicity [13] and lack of reversibility of gene expressions [17]. For example, once induced, it is very troublesome to remove the residual chemicals from the growth medium, causing difficulties for precise temporal control of desired gene expression levels [41].

Alternatively, light as a stimulus is non-toxic and can be rapidly delivered to cells in any pattern with precise spatiotemporal control [26]. With light, gene expression levels can be easily tuned and reversed based on the light intensity and pulse illumination. To date, a number of light-switchable transcription factors have been engineered to regulate gene expression in prokaryotes. Nevertheless, there are only a limited number of opto-genetic devices in the current synthetic biology toolbox. Moreover, most of these opto-genetic circuits have major weaknesses such as the dependence of non-endogenous chromophores [15,30], requirement of multiple protein components limiting their portability [40], low gene expression levels [16,17] and suffer potential crosstalk [10]. These drawbacks prevent their incorporation in larger biological systems to perform more complex functions. Hence, an ideal opto-genetic system that requires both light-dependent inducible and repressible devices functioning in parallel with rapid and reversible control of gene expression in space and time will be a key necessity to program further coordination abilities in bacteria.

2.4 MATHEMATICAL MODELLING IN SYNTHETIC BIOLOGY

Synthetic biology uses engineering principles such as standardization, modularity, and Computer Aided Design (CAD). The benefit of these engineering principles enables the development of molecular biology to equip new human-defined functions in bacteria. Such systematic and predictable functionalities in synthetic bacteria require both experimental and modelling approaches. Modelling of biological parts in the system enables the design of complex genetic circuits and analysis of behavior before wet-lab implementation. Biological pathway schematic is a simple example of qualitative models. These models have the drawback of not being able to analysed by computational tools. For this reason, quantitative models are important synthetic biology tools. Further, the advantage of quantitative models is that they can be used to predict the behavior of a system even after some modifications.

Generally, simpler models with fewer parameters that can model the behavior of biological systems are preferred than complex ones since they are much easier to analyze [6]. For the simplification of a model, some assumptions are usually considered. One of the most common assumptions in modelling biological systems is that the molecular constituents are homogeneously dispersed inside the cell. With this assumption, the concentration gradients of some species can be ignored. Another assumption is if that some of the reactions are steady state or are at equilibrium [6]. These assumptions simplify the model without much effect on the observed outputs. If a model cannot model expected behavior, then all of the assumptions are usually revisited and modified.

The most common way of modelling a biological system is to consider it as a system with molecular components and reactions. Each reaction is based on the production and decay of components and the reaction rate, which depends on the component concentration. After the construction of the equations for all the components, the behavior of the system can be observed through either deterministic or stochastic simulations [12]. Usually the equations are of the form of Enzyme (E) converts substrate (S) to product (P) through an intermediate substrate-enzyme (ES) and are governed by enzyme kinetic equations as will be described in the following chapter 3. These reaction forms usually have four components, and the overall reactions are as follow:

2.4 DISCUSSION

The literature review presents recent efforts to construct advanced synthetic gene systems, concerning different levels of complexity and characterization of bacterial communication. The background research has shown that naturally existing genes have either been re-assembled or re-programmed to coordinate advanced cellular behaviour. It is found that synthetic systems were developed from the modification of intrinsic intra-cellular parameters, such as transcriptional kinetics of the sender, receiver or auto inducer gene sequences. Also the alteration of extrinsic QS system factors such as cell motility, diffused inducer concentrations were used to alter the kinetics in communication channel. The parameters affecting the engineered system behaviour have been experimented in different ways to produce a variety of computing, signal and circuit functions. However, they have not been able to achieve any new forms of multiple communication to achieve coordinating functions or interactions among bacteria. A gap to achieve this can be attributed to the unavailability of synthetic tools to control gene expressions with precision and robustness. The availability of such tools would facilitate synthetic biologists to modulate gene expressions among different bacteria in a more controlled and coordinated fashion thereby providing avenues to develop multiple communicating abilities. This would open up the next frontier of distributed computing from many single cells performing different tasks in a cell mixture and reuse of their channel access to increase the combinatorial complexity in a microbial consortium.

Chapter 3

Mathematical Modelling Tools in Synthetic Biology

Majority of the mathematical modelling in the quorum sensing system of the report involves biochemical reactions and associated genetic expressions. Thus, a brief overview of the processes in the central dogma of molecular biology and the model equations of enzyme kinetics of the reaction mechanism are illustrated in this chapter.

3.1 CENTRAL DOGMA OF MOLECULAR BIOLOGY

The central dogma of molecular biology describes the flow of information from genes to protein in a cell [4]. The synthesis of proteins mainly involves three steps, replication, transcription and translation as described in figure 3.1. The rates and kinetics of these processes are governed by enzyme kinetic equations in the different segments of the genetic system. A genetic sequence has three major regions: the promoter, coding region, and terminator as shown in Figure 3.2. The promoter acts as the control for the amount of gene expression i.e. rate of mRNA protein synthesis [4]. It can be constitutively expressed or programmed to be induced by external stimuli such as light to either activate or repress the transcription process as illustrated in Figure 3.2. This is followed by the protein synthesis which is regulated by the coding region that involves two steps: 1) transcription, or production of a messenger RNA (mRNA); and 2) translation of mRNA into a protein [4].

Figure 3.1

Figure 3.1 Central Dogma – Key Processes. I) Replication: Process of copying a molecule of DNA II) Transcription: Process of conversion of a specific DNA sequence into RNA. III) Translation: Process where a ribosome decodes mRNA into a protein

Figure 3.2 Gene Cascade

Figure 3.2 Gene Cascade – Promoter (P) Coding (C) and Terminal (T) Regions. Protein synthesis process in the different segments in a gene. Constitutive promoter initiates transcription and translation of protein B without any stimuli; Regulatory promoter requires a stimuli (B) to induce or repress protein formation (A)

3.2 ENZYME KINETICS

The aforementioned processing steps in the central dogma are governed by a set of enzyme kinetic equations characterizing each type of synthesis. The enzyme kinetics are described as follows [4]:

1) Promoter Region

The promoter is the site which governs the rate of expression of genes and initiates transcription. Promoters are either constitutive or regulatory, i.e. Constitutive

promoters are active all the time without any stimuli, whereas regulatory promoter start transcription only when activated by stimuli.

When in the active state, the promoter region supports the transcription of the mRNA protein bound to it, and the rate of transcription is governed by the following equations:

i) Rate of Gene expression, $p(t)$ by constitutive promoter

$$p(t) = \beta \quad (3.1)$$

where β is the maximum transcriptional rate

ii) Rate of Gene expression, $p(t)$ by regulatory promoters:

a. Inducer - Increases the rate of transcription of promoter

$$p(t) = \beta \frac{[TF]^h}{\gamma + [TF]^h} \quad (3.2)$$

b. Repressor - Decreases the rate of transcription of promoter, $p(t)$

$$p(t) = \beta \frac{1}{\gamma + [TF]^h} \quad (3.3)$$

where

β : maximum transcriptional rate

TF : Transcriptional factor (amount of binding protein)

h : number of binding sites or Hill coefficient (cooperativity)

γ : half-saturation constant

2) Coding Region

The coding region is responsible for transcription of DNA to produce mRNA and the translation of mRNA to a protein. The amount of these components not only depends on the rate of synthesis of the coding region, but also the loss associated with each step of production as illustrated in Figure 3.3.

Figure 3.3

Fig 3.3 Processes involved in Coding region [4]

- a. Transcription to produce mRNA where Regulatory gene expression can be modelled as:

$$\frac{d\mu}{dt} = p(t) - \delta(t)\mu(t) \quad (3.4)$$

where

$\mu(t)$ amount of mRNA

$p(t)$ Rate of gene expression to produce mRNA

$\delta(t)$ Rate of Degradation of mRNA

- b. Translation of mRNA into protein

Protein = production – decay:

$$\frac{d\eta}{dt} = \lambda\mu(t) - \delta_p(t)\eta(t) \quad (3.5)$$

where

$\mu(t)$ amount of RNA

$\eta(t)$ amount of protein

λ Rate of translation

δ_p Degradation constant

The translation rate to express the protein is governed by either Michaelis-Menten equation or the Hill equation and their characteristic responses are illustrated in Figures 3.4(a) and 3.4(b).

Figure 3.4(a)

Figure 3.4(b)

Figure 3.4(a) Michaelis Menten Equation graph for Kinetic rate against substrate concentration [27]

Figure 3.4(b) Hill equation for Kinetic rates with hill coefficient more than 1 [27]

i) Michaelis Menten Equation:

Many enzymes have been known to be characterised using the Michaelis-Menten equation.

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (3.6)$$

where:

V is the reaction velocity (rate of reaction progression per unit time in mol/min)

V_{max} is the highest reaction rate that can be achieved at saturating substrate concentrations.

$[S]$ Is the substrate concentration. (Moles).

K_m is the Michaelis constant.

The inverse proportion of the constant K_m in equation 3.6 shows that the higher the numerical value of K_m , the lower the apparent affinity for the substrate.

ii) Hill Equation:

Some classes of enzymes are not characterised by the Michaelis-Menten equation. The kinetics of such enzymes have the property of an “S-shaped” sigmoidal curve. Sigmoidal kinetic profiles are the result of enzymes that demonstrate positive cooperative binding whereas hyperbolic profiles demonstrate non-cooperativity [27] (Figure 3.4(b)).

The degree of cooperativity is determined by the following Hill equation for non-Michaelis-Menten kinetics [27].

$$\theta = \frac{[L]^n}{K_d + [L]^n} = \frac{[L]^n}{(K_A)^n + [L]^n} = \frac{1}{(\frac{K_A}{[L]})^n + 1} \quad (3.7)$$

where

θ is the fraction of ligand binding sites filled

$[L]$ is the ligand concentration

K_d is the dissociation constant derived from the law of mass action

K_A is the ligand concentration producing half occupation

n is the Hill coefficient that describes the cooperativity.

When $n < 1$, there is negative cooperativity; When $n = 1$, there is no cooperativity; When $n > 1$, there is positive cooperativity

3.3 QUORUM SENSING

Cell communication in some eukaryotic microorganisms such as bacteria, depends on its population density and is based on the release and response of biochemical termed inducers. The term ‘quorum sensing’ has been coined for the mechanism reflecting the need for a minimum population of bacteria to exist [37]. The inducers produced by one bacteria diffuse to the environment and is received by other neighbouring bacteria to activate receptors and produce proteins that express specific bacterial behaviour. The flowchart in Figure 3.5 shows the sequence of quorum sensing activation in bacteria as regulated by the cell densities to express specific biological functions. It shows the differential gene regulation system in bacteria mediated by intercellular signalling provides a control to express particular characteristic behaviour only when growing in large populations. This is also illustrated in Figure 3.6, where it is observed that the transcription of target gene is only expressed in high cell density and high auto inducer concentrations.

Figure 3.5 Quorum Sensing process

Quorum sensing consists of four phases [9]:

- (1) Synthesis of inducers by bacterial cell
- (2) Release of the inducers into the surrounding environment
- (3) Recognition of inducers by other bacteria above a threshold concentration
- (4) Changes in gene regulation to synthesise proteins called synthases

Figure 3.6

Figure 3.6: Conditions for Gene expression in Quorum sensing. At low auto inducer concentration, regulatory protein does not get bound to AHL to transcribe remaining genes in the gene cascade. At high inducer concentration, the AHL-protein complex regulates quorum sensing.

As illustrated in Figure 3.6, the quorum sensing system generally consists of two components, an auto-inducer synthase, which synthesizes AHL auto inducers, and a transcriptional regulator. High cell density induces gene expressions that increase the synthesis of the proteins termed as Synthases that induce the inducer signal production. Increased production of the signal molecule in turn, generates a positive feedback loop, and accordingly, quorum signalling molecules in bacteria are commonly called auto-inducers.

An example of a well characterised bacterium, *Pseudomonas aeruginosa* controls its virulence traits using two different Lux-type quorum-sensing systems, called Las and Rhl [5]. The two systems regulate on another as part of expression of virulence factors. As shown in Figure 3.7, two types of auto inducer molecules form an orthogonal loop between two quorum sensing systems (Rhl and Las) to regulate the quorum sensing mechanism.

Figure 3.7

Fig. 3.7: Las and Rhl quorum sensing systems involved in *P. Aeruginosa* bacteria

3.4 MODELLING OF QUORUM SENSING SYSTEMS

The quorum sensing cascade comprises of different components to regulate the mechanism and it is governed by the enzyme kinetics equation (3.1-3.7) in section 3.2. The equations involved for characterising each of the steps in the process are described as follows:

- (i) Production of inducers by Quorum sensing 1 (sender cell)

The quorum sensing system 1 is activated/repressed/constitutively expressed by the promoter and it determines the rate of transcription. The transcription and translation of the quorum sensing protein (for e.g. LasI) is characterised using equations 3.4, 3.5 and 3.6.

- (ii) Diffusion of inducers in the environment

When the cell populations are grown in a solid agar gel medium, the factor of AHL inducer diffusion has to be considered. Based on the rate of diffusion of the extracellular molecules, the concentration of auto inducer reaching the receiver cells varies. In different environmental conditions, the auto inducers diffuse at

different rates, thereby generating different speeds of microbial intercellular communication. When the diffusion is a slow process, bacteria respond to changes in the auto inducers and regulate functions slowly which is not preferred. Thus, “reduced diffusion” (increased rate of auto-inducer activity) makes a favourable condition for community bacterial functions, for example in liquid media.

When performed the quorum sensing experiments in solid media, the diffusion can be predicted by Fick’s second law of diffusion in terms of concentration, ϕ , time, t , and space, x

$$\frac{\partial \phi}{\partial t} = D \frac{\partial^2 \phi}{\partial x^2} \quad (3.7)$$

where

ϕ is the concentration in mol/m³

t is time in seconds

D is diffusion coefficient in m²/s

x is the position in m

A simple case of diffusion from a boundary located at position $x=0$, where the concentration is maintained at a value n_0 is:

$$n(x, t) = n_0 \operatorname{erfc}\left(\frac{x}{2\sqrt{Dt}}\right) \quad (3.8)$$

This equation can be used to obtain the concentration profile of diffused AHL as similar to figure 3.8 and model the diffused chemical at a certain time or distance.

Figure 3.8

Fig. 3.8: Concentration profile of diffused AHL at various time

(iii) Formation of complex

The auto inducer upon reaching the receiver quorum system, forms a complex with the receptor proteins which is a type of cooperative binding equation characterised by the hill equation 3.7.

(iv) Activation of Quorum sensing 2 (receiver cell) – continuing to step (i) production of inducers.

The auto inducer-synthase complex activates the second quorum system and also produces its respective inducers and the quorum system is regulated as in step (i).

Chapter 4

Theory of Time-sharing Model in Cell-Cell Communication

This chapter describes the basic framework of a telecommunication model and presents the different channel access schemes that are used to improve the communication channel capacity. The mechanism of Time Division Multiple Access (TDMA), which is taken inspiration for this work, is described with its considerations for working. This is followed by the proposal of a time-sharing model and the description of the design parts and logic bio-circuits used to achieve it.

4.1 TELECOMMUNICATION SYSTEM

A simple telecommunication system comprises of three main parts that are present in the form of:

- A transmitter that takes information and converts it to a signal.
- A transmission medium that carries the signal.
- A receiver that takes the signal from the channel and converts it back into usable information.

Figure 4.1 Basic Telecommunication Model

Telecommunications in which multiple transmitters and multiple receivers are involved simultaneously through sharing of the same channel are called multiplex systems. These systems use multiplexing, a technique which allows more than one message to be transferred via the same communication channel with minimum interference. The aim of multiplexing in the communication system is to increase the capacity of the channel. Especially, in wireless telecommunication systems, it is essential for the subscriber to send and receive information simultaneously to

and from the base station. Timing of all operations within a multiplexer is controlled by a high stable oscillator called the master clock. If the digital structure of all the input messages is determined from the same master clock, the multiplexer is called synchronous. Whereas, when different clocks are involved, the multiplexer is said to be asynchronous.

“A channel-access scheme is based on a multiplexing method, that allows several data streams or signals to share the same communication channel or physical medium” [18]. The use of access methods enables many users to share limited number of channels for the best use of transmission medium. There are four fundamental types of multiple channel access: Frequency Division Multiple Access (FDMA), Time Division Multiple Access (TDMA), Code Division Multiple Access (CDMA) and Space Division Multiple Access (SDMA). In these concepts, two or more users share the same channel (bandwidth of transmission medium), where each user transmits signal only at a specified time, frequency band, spreading code or space.

4.2 TYPES OF MULTIPLE CHANNEL ACCESS IN TELECOMMUNICATION

Fig 4.2(a) FDMA

Fig 4.2(b) TDMA

Fig 4.2(c) CDMA

Fig 4.2(d) SDMA

The various multiple access techniques are described as follows:

4.2.1 Frequency division multiple access (FDMA)

FDMA is frequently used in radio communications systems where a certain frequency band is allocated for a specific use as illustrated in Figure 4.2 (a). A base station allocates a frequency to a terminal to transmit based on their needs. The FDMA is particularly used in mobile communication systems, radio systems and satellite communication systems.

4.2.2 Space Division Multiple Access

When a set of frequencies is reused in another location, it is called space division multiple access. Each region of space called 'cell' is allotted one frequency and can be allotted to another cell provided there is sufficient distance between the cells to avoid interference as shown in Figure 4.2 (d) [25]. The minimum distance is called reuse distance. This type of multiple access is majorly only used in mobile communication system that requires many users separated by considerable distances.

4.2.3 Code Division Multiple Access

CDMA is mainly used for defence application where secure communication is important. They are of two types frequency hopping and Direct Sequence CDMA. In Frequency Hopping, each packet of data is transmitted using a different frequency as seen in Figure 4.2 (c). A pseudo-random sequence generation algorithm decides the sequence of frequency hopping. In Direct Sequence CDMA, each bit is represented and transmitted over multiple bits. The data rate and bandwidth is increased. The information can be decoded only if the chipping rate is known for signal recovery.

4.2.4 Time Division Multiple Access

Time-division Multiple Access (TDMA) is a method to send multiple data streams using a single frequency signal by separating each signal into many segments of a very short duration as illustrated in Figure 4.2 (b) [18]. Each individual data stream is reassembled at the receiving end based on the timing [18]. TDMA, as a technique derived from sampling, has been used to achieve simultaneous transmission of many messages over a single communication link by interlacing trains of sampling pulses. The pulses are delayed with respect to one another to prevent overlap of signals. The time allotted to one sample is called time slot and each station is given a time slot in which it can transmit. Thus, TDMA allows access to entire frequency bandwidth but for a limited amount of time, i.e. all senders are entitled to use same frequency at different times.

In this concept, when two transmissions overlap, co-channel interference occurs. Hence it is required to maintain proper positions of sample pulses in multiplexer and synchronize the sampling process. Synchronization between terminals in TDMA is a major issue. Each station must precisely transmit information only in its assigned time slot, else collision of data will occur. Thus, the complexity of TDMA majorly lies with the synchronization which is governed by the base station that sends a bit pattern (10101010...), used by the terminals to synchronize their clocks [19].

A clock pulse train typically serves as the reference for all samplers. At the receiving station, there is a similar clock that must be synchronized with that of the transmitter. Clock synchronization can be derived from the received waveform by observing the pulse sequence over many pulses and averaging the pulses. Clock synchronization does not guarantee that the proper sequence of samples is synchronized. Proper alignment of the time slot sequence also requires frame synchronization [19]. Figure 4.3 illustrates the concept of TDMA.

Figure 4.3 TDMA Slot Allocation

Fig. 4.3 TDMA time allocation for receiver terminals from base station. Terminal A transmits in time slot 2 using frequency f_1 and Terminal B transmits in time slot 5 using also the same frequency f_1 . Which terminal gets which time slot is decided by the base station.

The time slots used for TDMA systems can be fixed or dynamic. In fixed TDMA, each terminal is given a fixed time even if the terminal system has no information to transmit [19]. Dynamic TDMA systems are conservative as they assign time slots only if the terminal makes a request for it and leads to a more complex system. An advantage of TDMA is the possibility to assign variable number of

time slots per frame to different users. Thus the bandwidth of the frequency can be utilised based on the priority and demand for each user.

4.2.5 Hybrid System

In some radio communication systems, a combination of FDMA and TDMA are used. For example, in cellular mobile communication systems, each base station is given a set of frequencies which is further divided into time slots to be shared by different users [18]. The combination of FDMA and TDMA increases the capacity of the system.

4.3 INSPIRATION OF TDMA IN CELL-CELL COMMUNICATION

The possibility of communication in a system of sender-receivers without cross-talk or interference has been enabled in telecommunication with the provision of multiple channel access. As seen in section 4.2, this has been enabled mainly through the four techniques of FDMA, TDMA, SDMA or CDMA. Similarly, the implementation of multiple channel access capabilities in bacteria will enable programmed communication of sender-receivers without crosstalk. Here, the signal to be communicated will be the quorum sensing signal, auto inducer chemical (3-oxo-C12-HSLAHL). For the application of multiple access, the message signal has to be controlled in the channel either by means of frequency, time or space as seen from the types of Channel access techniques. The cell-cell communication in bacteria considered is the quorum sensing system LasR/LasI system where the LasI gene present in the sender cell is responsible to send the AHL message signal and the LasR gene in the receiver cell receives the AHL message and produce protein expressions.

For multiple cell-cell communication, the simplest organisation of communication model is considered, i.e. with one sender cell and two receiver cells. This communication structure can be compared and conceptually similar to the standard telecommunication model (Fig.4.1) as illustrated in Figure 4.4. Segregating the AHL message signal to the receiver cells without cross-talk from the sender cell will be the objective of the multiple channel access model. For this to be achieved

through FDMA technique poses a difficulty. This is because the auto inducer message signal in quorum sensing from sender must be orthogonal to a specific receiver only. The form of frequency division of the message is not possible as the auto inducer signal can be orthogonal to only one paired sender-receiver system and cannot be extended to more types of auto inducer signal (compared to frequency) within a sender system. Similarly, the application of SDMA poses the difficulty of diffusion of one or more auto inducer (frequency) from a sender system.

Figure 4.4

Fig 4.4 Correspondence of Telecommunication system with multiple cell-communication in Quorum sensing synthetic bacteria

The application of a TDMA concept requires the segregation of auto inducer message signal to the two receiver cells with respect to time. Here, the presence of a timing signal to differentiate the working of the two receiver cells when auto inducer is received is required. This can be facilitated by a clock signal with ON and OFF half-cycles for the two different receiver cells. The ON and OFF of the clock signal must be such that one receiver functions only in the positive half-cycle and the other receiver in the negative half-cycle. This can be achievable when the clock signal is in the form of a suitable input that can interact with the regulatory promoter in the receiver cells and control the working of the receiver gene expressions over time. For rapid and precise expression of the receiver cells, it is also necessary that the clock signal can produce rapid response in the promoter transcription process. A suitable form of a clock signal from the above

considerations will be the implementation of light as a clock to provide the time control of receiver channel in the communication system.

Here, specifically the blue light signal is chosen for the clock as it has shown to provide better response in the transcription process over other colors [46]. Time Division Multiple Access for the receiver cells should be enabled by the blue light clock signal by programming one of the receiver cell's promoter to function in the positive light 'ON' half-cycle, whereas the other second receiver cell's promoter must function in the negative light 'OFF' half-cycle of the clock. Thereby, both receiver cells can differentiate their cycle of working from the clock signal's system of light. This will ensure that the receiver cells' function will not overlap and there will be no cross-talk in the system. This mechanism of working is similar to TDMA concept, however since only one sender is involved and transmits message commonly to two receivers, this form of communication is not regarded equivalent to TDMA and proposed as a time-sharing model of communication.

The overall concept of the time-sharing is illustrated in the Figure 4.5. Here, assuming the message inputs for both receiver cell is 1 over time, in the time-sharing communication model, it is desired that the two receiver cells exhibit their functions without overlap and produce outputs differentiated by time. Thus, the blue light clock signal is introduced with 50% light ON and 50% light OFF, each specific to one receiver cell. Here, the receiver cells are programmed to produce fluorescent proteins (green and red colour) when they receive the message input in their specific light half-cycle. Thus, one receiver (receiver cell 1) produces green fluorescence when the blue light is ON and message is high and another receiver (receiver 2) produces red fluorescence when the blue light is switched OFF and there is auto inducer message signal sent. The receiver output graphs show that the fluorescence are to be produced alternately and hence, overlap of the two cell workings is avoided and multiple communication is enabled.

Figure 4.5

Fig 4.5 Receiver outputs based on AHL message and blue light clock inputs in time-sharing model

4.4 PROPOSAL OF TIME-SHARING MODEL IN BACTERIAL CELL-CELL COMMUNICATION

As discussed in section 4.3, the model of time-sharing that is considered for multiple cell-cell communication in this thesis involves the simplest organisation of multiple communication with one sender cell (comparable to a base station transmitter) and two receiver cells (receiving antennae) illustrated in Figure 4.6. The similarity of the arrangement and working of the two systems, lends way for the quorum sensing system to incorporate multiple channel access function as established in telecommunication systems.

The sender cell is programmed to send message to only receiver 1 in positive half-cycle of the clock signal (blue light ON) whereas, the sender cell message corresponds to only receiver 2 in the negative half-cycle of the clock (light OFF). Since receiver 1 is required to work only in the light ON condition, its regulatory promoter in the cell should activate the transcription of LasR quorum sensing gene only in the presence of light, in other words, it must be inducible to the presence of blue light. Similarly, since receiver 2 is required to work in the light OFF condition, its regulatory promoter in cell should activate the transcription of LasR quorum sensing gene only in the absence of light, i.e. it should be repressible in function to blue light. The bio-design of this receiver system set-up is shown in Figure 4.7. When the LasR gene is activated in the respective light conditions in the receiver cells, the LasR protein is expressed, which when combined with the

autoinducer message signal, activates the reporter signal of message, i.e. expresses fluorescent proteins.

Figure 4.6

Fig 4.6 Time-sharing Synthetic Model for Multiple Channel-Access Communication

Figure 4.7

Fig 4.7 Inducible and Repressible systems output Red fluorescence in response to blue light. The auto inducer message signal involved in the quorum sensing circuit is 3-oxo-C12-HSLAHL.

As discussed, the receiver cells are controlled to function only at their respective half-cycles with the help of a ‘blue light protein’ switch. The clock signal that provides the half-cycles of synchronization is blue light. The time-sharing of the cells to work in their respective time slots, is hence achieved with the help of LasR synthesis either through inducing or repressing by blue light. The LasR production in each receiver system is then open to AHL message input to be received by the Source and produce fluorescence. The LasR synthesis at each half-cycle thus, serves as the time slots for the receiver systems. The output from receiver 1 is

controlled by Green Fluorescence Protein (GFP) that emits green fluorescence in the positive light half-cycle, whereas output from receiver 2 is controlled by Red Fluorescence Protein (RFP) that emits red fluorescence in the negative light half-cycle.

4.5 OPTOGENETIC CONTROL IN SYNTHETIC RECEIVER CELLS FOR TIME-SHARING

The regulation of the receiver system promoters is controlled by blue light illumination. Receiver 1 is engineered such that its LasR QS system is induced and activated in the presence of light to produce fluorescence, whereas receiver 2's LasR protein is repressed in the presence of light and only activated in the absence of blue light. LasR protein transcription rate is thus characterised by the regulatory equations of build-up and decay over time with respect to blue light illumination time as illustrated in Figure 4.8. This regulatory system from blue light provides the means of synchronization of the two receiver cells to function at different half-cycles of time slots and function as the clock synchronization for Time-division Multiple Access. The production of LasR as controlled by blue light illumination for both the inducible and repressible receiver systems are simulated in Figure 4.8.

Figure 4.8 Blue light clock regulation of receiver cells in time-sharing model

Fig. 4.8 Simulation of LasR transcriptional activity from control of blue light for 25 hours' time Period (2 cycles). Positive half-cycle of light activates inducer LasR whereas negative half-cycle of light activates Repressor LasR

In the time-sharing model, the engineered receiver at its time slot when activated, directs the transcription of the genes in LasR protein and forms a complex with the auto inducer molecule 3-oxo-C12HSL. The complex produced in turns activates the expression of fluorescence gene to produce fluorescence protein. Fluorescence is produced only when message is input during the receiver's active time slot. The engineered receiver bacteria hence receive two inputs, one of the optically controlled promoter input and the other, the auto inducer message to produce the output, fluorescence signal. The inputs for the receivers and the corresponding output signal can be tabulated as a truth table and the logic for the receiver systems can be obtained.

Receiver 1 comprises of the blue-light inducible promoter and the downstream Green fluorescent protein. From the logic functions of the system in truth table 4.1, it is seen that its function is the AND logic circuit. Its gene expression is activated only in the presence of both AHL message and blue light exposure.

Receiver cell 2 has a repressible promoter in its genetic cascade and activates its gene expression of red fluorescent protein (RFP) in the absence of light (0) along with the presence of AHL (1). The truth table in Table 4.1 shows the Input/output conditions for the system. These regulatory actions in the receiver cells are possible with the help of blue light protein binding mechanisms involved with the promoter region during the presence and absence of light [23]. From the truth table outputs, it is seen that the receiver 1 demonstrates AND gate logic, whereas the receiver 2 demonstrates N-imply gate logic (negate of implication gate). The corresponding logic representations for both receivers are represented in Figure 4.9.

Table 4.1 Expression of fluorescence in the receiver cells at different conditions

Truth table Outputs for Inducer Receiver 1			Truth table Outputs for Repressor Receiver 2		
No AHL	No Light	0	No AHL	No Light	0
No AHL	Light	0	No AHL	Light	0
AHL	No Light	0	AHL	No Light	1
AHL	Light	1	AHL	Light	0

Figure 4.9

Fig. 4.9 Logic representations for Receiver cells in the time-sharing model

4.6 MECHANISM OF TIME-SHARING MODEL

As the population of sender cells increases, the LuxI QS system produces enough AHL inducers to activate the LasR QS signalling system in the receiver population. In the receiver cell system, after a threshold inducer level is reached and with the clock signal input, the QS system in the receiver cells functions and in turn outputs the transcription of the RFP or GFP genes. Thus, fluorescence is exhibited when a threshold population level of sender cells is signalled by the AHL inducers. The output fluorescence is visualised on a microplate reader over time in Optical Density Units at 600 nm wavelength (OD600). Two receivers involved here receive signals as controlled by inducer and repressor systems in their genetic circuits, and thereby split their channel access according to the control of light. The channel access for the receiver cells will then depend on the half-cycle which is active at the time of operation as described in Figure 4.5.

When modelling each protein/mRNA, their different production and degradation rates have to be fast enough to be expressed maximally only in their respective half-cycles. If not the output will not be constrained to the half-cycle of its corresponding receiver but cross-talk with the other receiver's half-cycle. This will

result in low efficiency of the time-sharing system by contributing to errors in readout signals. However, when the readout fluorescence of the two receivers are of different wavelengths (e.g. green and red) it will not constitute a cross-talk at the outputs (as they can be measured separately). Another way to model synchronous receiver system output with the clock signal, would be to extend the clock signal duration and compensate for the low production and degradation rates. This way the chemical kinetics or clock signal lengths can be manipulated according to ensure maximum signal in the half-cycle.

Also, the basal fluorescence expressed during a receiver's OFF state in a half-cycle must be ensured distinct in order to not mix up with the other active receiver's signal. For example, when some LasR-AHL complex is not fully degraded in the 'OFF' time slot of a receiver it may express basal noise-like fluorescence in the other receiver signal. The basal fluorescence expression in the OFF state has to be well distinguished from the peak fluorescence levels in the ON state so that it can be thresholded out from digitalised receiver output. In this case, the chemical kinetics of the LasR-AHL are to be manipulated with the clock duration for the fluorescence levels to be distinct. This will facilitate the time-sharing synchronisation with the clock between receivers, and ensure that appropriate signals at the ON-states and OFF-states is output without interference. It is also of note that the synchronisation of the receivers with the sender signal is not of concern, as the sender signals have been inserted manually into the channel. Also, the time-readout clock is shifted in sequence to retrieve delayed received signals.

4.7 EQUATIONS GOVERNING SYNTHETIC RECEIVER SYSTEM IN TIME-SHARING MODEL

Figure 4.10

Fig. 4.10 Equations involved in receiver cell system in communication model

From the theory of mathematical modelling of bio-circuits and Telecommunication system, it is understood that the working of the receiver cell should be modelled with the inputs of clock signal, AHL message signals and chemical kinetics based on Central Dogma theory[4]. The sub-systems and corresponding ordinary differential equations that are to be used in the two light-programmable receivers in the system are illustrated in Figure 4.10. Mainly four processes are modelled in this, i.e. the transcription of light regulatory promoter based on clock input, the synthesis of LasR quorum sensing molecule, the AHL message signal interaction, the LasR-AHL complex formation and its following downstream expression of fluorescent proteins. Based on literature of the kinetic rates of the different chemicals involved, it has been identified that LasR, LasR-AHL complex and fluorescent proteins degrade very slowly [23] and thereby can interfere with the mechanism of time-sharing for short time periods. In ideal conditions, it is desirable to achieve shorter time periods of working. Further, for long hours of experimentation, the system has to be continuously supplied with nutrition and maintained for the survival and working of cells. Hence, the biological chemicals LasR, AHL and fluorescent protein must degrade faster and kinetics of the system has to be modified to model digital pulse expressions. For this reason, a degradation tag (a set of genes that induces fast degradation) is

introduced to the LasR gene (modified as lasR_{LVA} and fluorescent protein genes (modified as RFP_{LVA}). The circuit using this system used in the experiment will be discussed in Chapter 6. Before that, the initial characterisation studies for the transcription in light-inducible and light-repressible promoters in the receiver cells is performed in Chapter 5.

Chapter 5:

Characterization of Engineered Synthetic Opto-genetic Device

In this chapter, the light regulatory promoters of the receiver systems, i.e. the blue light inducible and the blue light repressible promoters are characterised to model the transcription of the receiver cells when blue light is input. An initial model is presented for the light transcription kinetics characterisation and is obtained with the help of a light characterisation experiment using different light pulse widths and intensity parameters. The model thus obtained has also been used to validate further laboratory experiment results using the same system.

5.1 CHARACTERIZATION OF ENGINEERED SYNTHETIC OPTO-GENETIC SYSTEM

A novel bidirectional promoter system that can be induced or repressed rapidly and reversibly using the blue light dependent DNA-binding protein EL222 has been engineered in Escheria Coli bacteria by post-doc from School of Chemical and Bio-Engineering. This has been used for characterisation and implementation in the time-sharing experiment in the bacteria. The system comprised of a synthetic blue-light inducible promoter system that showed minimal leakiness in the dark state and five-fold dynamic range upon stimulation with blue light, and a synthetic blue-light repressible promoter system that constitutively expressed genes in the dark state and repressed with increase in the dosage of light. It was demonstrated that dose-dependent blue light illumination pulses or intensity could control the extent of induction or repression, i.e. the level of gene expression could be modulated precisely with the intensity or duration of light. The genetic engineering procedure for the development of the promoter systems are described

in [43]¹. The characterization and validation results performed as part of work in this chapter has also been published in the referenced Nucleic Acids research paper.

The characterization of this opto-genetic device aims to describe its intrinsic properties to enable the regulated and predictable performance of a synthetic communication device [44]. For this, the light-inducible and repressible gene expression kinetics were quantitatively analysed using a mathematical model. In order to study transfer function of the systems, they were studied under different blue light input illumination conditions performed in two sets of experiments.

Firstly, the light pulse ON–OFF duty cycle was varied within a 60 seconds period, i.e. (OFF, 5 s ON; 55 s OFF (8.33%), 15 s ON; 45 s OFF (25%), 30 s ON; 30 s OFF (50%), 45 s ON; 15 s OFF (75%) and ON) with the light intensity fixed at 12 W/m². Secondly, the intensity of blue light was varied (0, 2, 5, 9 and 12 W/m²) with the illumination duty cycle fixed to 100%. In both experiments, the fluorescence expression outputs were measured for a duration of six hours and the values were normalized to the maximum fluorescence measured within each experimental set of readings. The dose-dependent increase in fluorescence was observed, both against the increase in the duration of the illumination pulse and the intensity of the blue light input. Thus, it was inferred that the engineered opto-genetic system can be switched stably between ON- and OFF-states by repetitive pulses of the blue light.

The experimental data were fitted to a Hill function model for the blue light inducible and repressible promoter transfer functions as follows:

$$f([L]_{ind}) = \alpha + \frac{k([L]^n)}{K_1^n + [L]^n} \quad (5.1)$$

$$f([L]_{rep}) = \alpha + \frac{k}{K_1^n + [L]^n} \quad (5.2)$$

¹ Premkumar Jayaraman, Kavya Devarajan, Tze Kwang Chua, Hanzhong Zhang, Erry Gunawan, and Chueh Loo Poh. Blue light-mediated transcriptional activation and repression of gene expression in bacteria Nucleic Acids Res. 2016 : gkw548v1-gkw548.

Where, α is the basal expression level of the promoter, k is the maximum expression due to induction or repression, $[L]$ is the dose of the blue-light; K_I and n are the Hill constant and co-efficient, respectively.

The hill function model, as described in Chapter 3 defines mathematically, the mechanism by which the enzyme binds to the substrate, i.e. as the substrate concentration increases, the velocity of the reaction increases hyperbolically and approaches a maximum rate (k). At rate k , all enzymes are complexed with substrate, and thus any additional substrate added to the reaction has no effect on the rate of reaction. In the case, the dose of the light illumination acts as the substrate for which the promoter drives the binding of enzyme and substrate at active sites. $f([L]_{ind})$ is the function model for the promoter that induces the binding mechanism in the prescence of light, whereas $f([L]_{rep})$ for inhibitive promoter, in the absence of light.

5.1.1 Blue Light Dose Response Study

Figure 5.1 shows the curve fitting of the dose-response curves to obtain the Hill function model parameters in the form of equations 5.1 and 5.2. The characterisation of pulse illumination dose response has been obtained from a six-hour experiment performed for varying blue light illumination pulses of duration 60 seconds (0% to 100%). Similarly, the characterisation of light intensity response has been obtained for different intensity levels (0 to 12 W/m²). The outputs are normalised to the highest value of Red fluorescence measured in optical density units at 600 nm. The average values of triplicate datasets were obtained from each experiment and good curve-fits were obtained in the first attempt. The curve fit (as represented in solid line) has been performed using non-linear parameter estimation toolbox and exhibits a correlation coefficient value (R^2) greater than 0.97. From the fit, the parameters of the switch points in dose response are obtained and shown in table 5.1.

Figure 5.1 (a)Pulse width dose-response curves

Fig 5.1 (a) Varying blue-light illumination pulse (%), 0% (OFF), 8.33% (5 s ON; 55 s OFF), 25% (15 s ON; 45 s OFF), 50% (30 s ON; 30 s OFF), 75% (45 s ON; 15 s OFF) and 100% (ON).m The error bars indicate s.d. (n=3).

Figure 5.1 (b)Intensity dose-response curves

Fig 5.1 (b) Varying blue-light intensity levels (2, 5, 9 and 12 W/m²). The curve-fits are shown as solid lines to the promoter transfer function. The error bars indicate s.d. (n=3).

Table 5.1

Table with parameters to characterise switch points in opto-genetic system

Promoter/Dose	k (A.U.)	n	K_1	α	R^2 (correlation)
Blue light inducible/illumination pulse	1545	2	6.554 %	444.6	0.9749
Blue light inducible /intensity	1577	2	1.052 W/m ²	441.6	0.989
Blue light repressible /illumination pulse	$2.471e^6$	2	27.93 %	1514	0.9914
Blue light repressible /intensity	$4.11e^4$	2	3.4467 W/m ²	1346	0.997

From the curve-fit results in table 5.1, the switch point of activation of the inducible promoter (K_1) for pulse width illumination is represented at 6.5%, which attributes to the sharp increase in expression of the inducible system when the blue light duty cycle increases from 0 to 8.33% (5s ON; 55s OFF). Gradually, the activation starts to saturate with further increase in the pulse illumination from 25% to 100%. The blue light repressible system also demonstrates that at a low duty cycle of 27.93%, the switch point of repression is happening. It further exhibits exponential decrease in gene expression in the pulse width illumination ranges between 50% and 100% (Fig.5.1).

Similarly, the model suggests that low intensity changes are sufficient for inducible and repressible behaviour. Further increase in the intensity demonstrates saturation in the gene expression levels in transcriptional induction and repression. Both systems exhibit maximum response at 100% illumination pulse ('ON') or 12 W/m² intensity relative to the dark state. Overall, the model shows that considerably short illumination times or low intensity is sufficient for inducible and repressible responses in the engineered opto-genetic device (Table 5.1).

5.1.2 Light Pulse Illumination Dynamics for Gene Expression

From the dose response characterisation results in section 5.1.1, the Hill kinetic parameters have been obtained and is used to get the model equations of the dynamic processes in the system in the form of Ordinary differential equations. The six-hour experiment performed for the different pulse width illumination percentages have been fit to the parametric model to obtain the unknown kinetic parameters of this model given in equations 5.3 to 5.8. The results after parametric fitting to the experiment is represented in Figure 5.2. The dashed – dotted lines represent the experiment data and the solid lines represent the simulation from the ordinary differential equation model.

$$\frac{d(Ind.Trp.rate)}{dt} = K_{Ind.Ts} - D_{Ind.Td} (Ind.Trp.rate) \quad \text{init}=0 \quad (5.3)$$

$$\frac{d[mRNA_{IndRFP}]}{dt} = \left[\frac{(Ind.Trp.rate)L^2}{L^2 + 6.554^2} \right] + Basal_{mRNA_{IndRFP}} - \gamma_{mRNA_{IndRFP}} [mRNA_{IndRFP}] \quad \text{init}=0 \quad (5.4)$$

$$\frac{d[RFP_{Ind}]}{dt} = K_{RFP} [mRNA_{IndRFP}] - \gamma_{RFP} [RFP_{Ind}] \quad \text{init}=5.36e^{-6} \text{ (from expt)} \quad (5.5)$$

$$\frac{d(Rep.Trp.rate)}{dt} = K_{Rep.Ts} - D_{Rep.Td} (Rep.Trp.rate) \quad \text{init}=0 \quad (5.6)$$

$$\frac{d[mRNA_{RepRFP}]}{dt} = \left[\frac{(Rep.Trp.rate)L^2}{L^2 + 27.8^2} \right] + Basal_{mRNA_{RepRFP}} - \gamma_{mRNA_{RepRFP}} [mRNA_{RepRFP}] \quad \text{init}=0 \quad (5.7)$$

$$\frac{d[RFP_{rep}]}{dt} = K_{RFP} [mRNA_{repRFP}] - \gamma_{RFP} [RFP_{rep}] \quad \text{init}=2.77e^{-5} \text{ (from expt)} \quad (5.8)$$

Each system inducible/repressible is modelled as three equations that correspond to the three main processes in Central Dogma following the theory as described in Chapter 3. The process of transcription is modelled as two sub-processes, i.e (i) a transcription factor binding to promoter region that in turn, (ii) governs the rate production of an mRNA molecule. Equations 5.3 and 5.6 model the binding of the transcription factor to the promoter to determine the transcription synthesis rate. They have been proposed for the first time to better characterise the dynamics of transcription rate based on the increasing availability of binding transcription factors to the promoter, over time.

Equations 5.4 and 5.7 model the process of ribosomes binding to the promoter to produce mRNA fluorescence. The dynamics follow the typical Hill equation kinetics as described in equations 3.2 and 3.3. Specifically, these equations quantitatively describe the rate at which the gene is transcribed to mRNA based on the binding of the transcription factor with the promoter binding region.

This is followed by Translation that involves the decoding of the mRNA by ribosomes to produce protein [4]. The rate of protein production is linearly proportional to the amount of mRNA available and each protein is assumed to have a fixed probability of degradation every minute [6]. Hence, the commonly used approach of modelling straightforward protein translation equations coupled to ODEs of mRNA transcription is employed in equations 5.5 and 5.8 [4].

The parameter values obtained from Parameter estimation tool in MATLAB after curve-fit have been given out in Table 5.2. This modelling, overall enables us to describe the quantitative relationship between protein levels produced and the light

characterisations, it is possible to study experiments which involve blue light transcription process which we would like to implement in time-sharing model.

Table 5.2

Table with parameters for light pulse width characterisation to opto-genetic system

S. No	Parameter Abbreviation	Parameter Name	Parameter Value	Unit	Reference
1	$K_{Ind.Ts}$	Increase in transcription rate (inducible system)	0.00015	$\mu\text{M}/\text{min}^2$	Estimated
2	$D_{Ind.Ts}$	Decay rate of transcription rate (inducible system)	0.0001	/min	Estimated
3	$K_{Rep.Ts}$	Increase in transcription rate (repressible system)	1	$\mu\text{M}/\text{min}^2$	Estimated
4	$D_{Rep.Ts}$	Decay rate of transcription rate (repressible system)	0.0377	/min	Estimated
5	$\gamma_{mRNA_{RFP}}$	Decay rate of mRNA RFP	0.346	/min	Bio-number 110055
6	K_{RFP}	Translation rate of RFP	0.001	/min	Estimated
7	γ_{RFP}	Decay rate of RFP protein	0.006	/min	Dilution rate, bio-number 111747
8	$Ind.Trp.Rate$	Transcription rate (inducible system)	--	$\mu\text{M}/\text{min}$	--
9	$Rep.Trp.Rate$	Transcription rate (repressible system)	--	$\mu\text{M}/\text{min}$	--
9	$Basal_{mRNA_{IndRFP}}$	Basal inducible mRNA RFP rate	$3.22e^{-7}$	$\mu\text{M}/\text{min}$	From curve-fit
10	$Basal_{mRNA_{RepRFP}}$	Basal repressible mRNA RFP rate	$8.62e^{-7}$	$\mu\text{M}/\text{min}$	From curve-fit
11	$mRNA_{IndRFP}$	Inducible mRNA RFP	--	μM	--
12	$mRNA_{RepRFP}$	Repressible mRNA RFP	--	μM	--
13	RFP_{Ind}	Red fluorescent protein from inducible system	--	μM	--
14	RFP_{Rep}	Red fluorescent protein from repressible system	--	μM	--

Figure 5.2 (a)

Fig. 5.2(a) Inducible System light pulse width (PW) response characterisation

Figure 5.2 (b)

Fig. 5.2(b) Repressible System light pulse width (PW) response characterisation

5.2 PARALLEL GENE EXPRESSION IN BI-FUNCTIONAL OPTO-GENETIC SYSTEM

In order to validate if the synthetic inducible and repressible systems could function in parallel, the two promoter systems were tested if they could simultaneously and bi-functionally regulated in a single cell. Figure 5.3(a) shows the design and mode of action of the bi-functional promoter system. Here, it is illustrated that the two systems are combined in a plasmid within cells where the inducible promoter system activated red fluorescence gene expression, whereas the repressible promoter system activated green fluorescence gene expression, when induced or repressed by light respectively.

Figure 5.3(a)

Fig.5.3 (a) Design and mode of action of the bi-regulatory promoter system. The blue-light inducible promoter (pBbInd) drives the expression of RFP reporter, which is placed in series with the blue-light repressible promoter (pBbRep) that controls the GFP expression. In the presence of blue-light (12 W/m^2) transcription of the inducible promoter is activated expressing RFP, while the repressible promoter is repressed. Conversely in the dark state, RFP expression is de-activated at the inducible promoter, while repressible promoter constitutively expresses GFP.

The constructed cells with the bi-regulatory promoter were layered on an agar plate and applied blue light through a photomask as shown in Figure 5.3 (b). The areas covered by the photomask, that blocked the light exposure (0% illumination) produced green fluorescence, whereas the areas exposed to light (100% illumination) produced red. The experiment was repeated with the bright and dark areas switched in the photomask, reversing the irradiation patterns produced (Fig. 5.3 (b)). Precise fluorescent patterns that replicated the photomask were produced which demonstrates that the bi-functional opto-genetic device works with high spatial resolution. This also showed that the device can function parallel with minimal crosstalk in a single cell.

Figure 5.3(b)

Fig.5.3 (b) Light-switchable activation and repression in a single cell. Spatial control of gene expression. Cells transformed with opto-genetic plasmids, plated on an agar plate were illuminated with blue-light through a photomask with two different spatial irradiation patterns for 18-20 h at 37°C . Images were taken under UV illumination. The width of the arrow is 1.8 cm.

5.3 TIME SHARING IN A BIFUNCTIONAL TRANSCRIPTIONAL CASCADE TO STUDY TEMPORAL BEHAVIOR

The parallel working of the bi-functional opto-genetic device validated from the previous photomask experiment supported the implementation of time sharing concept to achieve coordinated functions using light. This has enabled temporal control of gene expressions using the cells transformed with the bi-functional transcriptional cascade. The experiment for studying the time sharing temporal behaviour was performed by exposing the engineered cells to blue light repeatedly and reversibly in a 2-hour OFF- 2-hour light time period cycle over a time duration of eight hours. This was done in order to program the two independent inducible and repressible functions in a time-sharing fashion with the blue light serving as the clock synchronisation. The molecular clock signal comprised of the light duty cycles of OFF and ON pertaining to the pulse illumination percentage 0% and 100% respectively with light intensity fixed to 12 W/m². Due the parallel and independent working of the engineered device, this would result in the alternate expression of light-repressible system and light-inducible system for every two hours based on the light input.

5.3.1. Experiment and Observations

Figure 5.3 (c) shows the experimental set-up used to perform the time-sharing of the bi-functional light promoters. At time zero, early exponential phase cell culture grown without illumination was transferred into a 12-well plate mounted on an in-house blue light 3 × 4 LED panel system incubated at 37°C shaking at 120 rpm.

Figure 5.3 (c):12-LED Blue Light Source set-up.

Fig. 5.3 (c) The 12-LED blue light source is designed to provide 465nm light for the 12-well culture plate, with adjustable on/off time and light intensity on individual wells. The set-up had been developed by a Master Bio Engineering student from NTU (Hanzhong).

For the first period of two-hour illumination ‘OFF’ cycle, the Green fluorescence protein level produced by the blue light repressible promoter system started to increase in two-fold expression level whereas the Red fluorescence protein level produced from the blue light inducible promoter exhibited low basal activity. In the subsequent two-hour light ‘ON’ cycle period, the blue light inducible promoter demonstrated a two-fold increase in the Red fluorescence expression while the Green fluorescence expression decreased two-fold. This was followed by the next 2-hour illumination ‘OFF’ cycle where the Green fluorescence protein level was restored, resulting in an increase to about 2.5-fold while the Red fluorescence expression from the activated level was declining. Re-illumination of blue light in the final two hours ‘ON’ cycle period, increased Red fluorescence protein expression about one-fold and decreased Green Fluorescence expression for about 1.5-fold. Thus, the overall observations showed that blue light dose-dependent molecular clock input to the promoter systems reflected the time-sharing behavior of the output fluorescent signals.

5.3.2 Proposal of new Ordinary Differential Model to characterise time-sharing of promoters in bright and dark states

The characterisation of the time-sharing system in the single cell bi-functional system was performed again with the proposal of a new simpler mathematical Ordinary Differential equations (as compared to equations 5.1 to 5.8) that modelled the activation and deactivation kinetics of the system using binary inputs i.e. 0% and 100% pulse width illumination values. This binary inputs 0% and 100% in the model is used since from the characterisation the dose response, the promoters showed rapid switching kinetics from 6.55% of light pulse illumination itself. The 0% and 100% light pulse width illuminations are used as bright and dark states in the OFF-ON duty cycles that can provide maximum inducible and repressible responses in the system. Thus, the new set of equations help to model the system better as it illustrates how just the two values of the molecular clock (0% and 100% pulse width illumination) bright and dark states directly affect the transcriptional process in the bio-machinery.

The following set of ODE equations approximate the time-sharing behaviour in the bi-directional opto-genetic system:

$$\frac{d(K_{RFPmRNA})}{dt} = K_{S_{Ind}}(li) - \delta_{S_{Ind}}(K_{RFPmRNA}) \quad (5.10)$$

$$\text{where } li \begin{cases} = 1 \text{ when light is ON} \\ = 0 \text{ when light is OFF} \end{cases}$$

$$\frac{dmRNA_{RFP}}{dt} = K_{RFPmRNA} + K_{IbasalmRNARFP} - \delta_{mRNARFP}[mRNA_{RFP}] \quad (5.11)$$

$$\frac{dRFP}{dt} = K_{RFP}[mRNA_{RFP}] - \delta_{RFP}[RFP] \text{ initial: from expt} \quad (5.12)$$

$$\frac{d(K_{GFPmRNA})}{dt} = K_{S_{Rep}}(lr) - \delta_{S_{Rep}}(K_{GFPmRNA}) \quad (5.13)$$

$$\text{where } lr \begin{cases} = 1 \text{ when light is OFF} \\ = 0 \text{ when light is ON} \end{cases}$$

$$\frac{dmRNA_{GFP}}{dt} = K_{GFPmRNA} + K_{RbasalmRNAGFP} - \delta_{mRNAGFP}[mRNA_{GFP}] \quad (5.14)$$

$$\frac{dGFP}{dt} = K_{GFP}[mRNA_{GFP}] - \delta_{GFP}[GFP] \text{ initial: from expt} \quad (5.15)$$

To construct the ODE model, the system behaviour was understood and attributed to three major dynamic processes involved in the light-inducible/repressible gene expressions similar to equations 5.3 to 5.8.

Firstly, the initial opto-genetic mechanism to activate the promoter, i.e. the interaction of light (transcription factor) with the inducible / repressible promoter of the system was given out. The status of incident blue light (OFF or ON) defines the synthesis rate of mRNA transcription, depending on the binding of blue light to the promoter. Thereby equations 5.10 and 5.13 define the blue light-mediated transcriptional synthesis rates of the inducible and repressible systems respectively. Here, the synthesis rate of mRNA transcription depends on the value of 'l', the parameter for light activation depending on the promoter which is being activated.. The parameters 'li' and 'lr' represents the light activation coefficient, for the inducible promoter and repressible promoter respectively, and parameters Ks and δs determine the steepness of the light activation and deactivation kinetics of the mRNA synthesis rate. These ODE equations are proposed for the first time and characterises the activation and deactivation of the mRNA synthesis rates due to the activation of promoter by binding to the blue light transcription factor.

Following the activation of the promoter, the differential equation for the mRNA transcription driven by the activated promoter is characterised. The transcription follows the standard form of the Michaelis Menten kinetics as given in Chapter 3. The synthesis rate of the mRNA from equations 5.10 and 5.13 is coupled with a fixed degradation rate of mRNA and its initial concentration to construct the overall transcription process. This is described in equations 5.11 and 5.14 which involves the parameters, basal expression of the mRNA transcription as obtained from the six-hour pulse width experiment in section 5.1.2, synthesis rate of mRNA transcription from the earlier ODE equations (5.10 and 5.13) and the degradation rate of mRNA transcription from Bio numbers 110055 reference.

Finally, the dynamics to describe the process of translation from the produced mRNA to Florescence protein is expressed in equations 5.12 and 5.15. The process of translation involves ribosomes to decode the mRNAs into a series of amino acids and fold them into proteins. Hence, the amount of proteins produced is a linear function of the available mRNA as described by Michaelis Menten kinetics [4]. The translation kinetic equations involve the parameters translation rates K_{GFP} , K_{RFP} , and decay rates δ_{GFP} , δ_{RFP} , for the respective fluorescent proteins. The values for the decay rates of GFP and RFP were obtained from the diffusion rates and degradation rates of the protein from Bio numbers 11747 reference and listed out in table 5.3.

Table 5.3 Parameters used in time-sharing model

S. No	Parameter Abbreviation	Parameter Name	Parameter Value	Units	Reference
1	KS_{Ind}	Increase in mRNA synthesis rate (inducible system)	$8.4e^{-7}$	$\mu\text{M}/\text{min}^2$	Estimated
2	δS_{Ind}	Decay rate of mRNA synthesis rate (inducible system)	0.003	/min	Estimated
3	KS_{Rep}	Increase in mRNA synthesis rate (repressible system)	$6.4e^{-6}$	$\mu\text{M}/\text{min}^2$	Estimated
4	δS_{Rep}	Decay rate of mRNA synthesis rate (repressible system)	0.032	/min	Estimated
5	$initial K_{RFPmRNA}$	Initial synthesis rate of mRNA RFP	$2.22e^{-14}$	$\mu\text{M}/\text{min}$	Estimated
6	$initial K_{GFPmRNA}$	Initial synthesis rate of mRNA GFP	$2.22e^{-14}$	$\mu\text{M}/\text{min}$	Estimated
7	$K_{basalmRNARFP}$	Basal synthesis rate of mRNA RFP	$3.22e^{-7}$	$\mu\text{M}/\text{min}$	Estimated

8	$K_{basalmRNAGFP}$	Basal synthesis rate of mRNA GFP	$8.62e^{-7}$	$\mu\text{M}/\text{min}$	Estimated
9	$\delta_{mRNARFP}$	Decay rate of mRNA RFP	0.346	/min	Bio-number 110055
10	$\delta_{mRNAGFP}$	Decay rate of mRNA GFP	0.346	/min	Bio-number 110055
11	K_{RFP}	Translation rate of RFP	0.001	/min	Estimated
12	K_{GFP}	Translation rate of GFP	0.001	/min	Estimated
13	δ_{RFP}	Decay rate of RFP protein	0.006	/min	Dilution rate
14	δ_{GFP}	Decay rate of GFP protein	0.006	/min	Dilution rate, bio-number 105191

Using non-linear least square algorithm in MATLAB Simulink for the best curve-fit to the experiment data (as shown in Figure 5.4 (a), the unknown parameters li , lr , K_S , δ_S , K_{GFP} , and K_{RFP} , were obtained as given in table 5.3. With the following set of parameters, the simulation of the system was performed and characterised the experiment having a cross-correlation value greater than 0.9. Figures 5.4 (a) depicts the simulation (represented as solid line) versus experiment (as dots) after parameter estimation.

The parameterised mathematical model was then used to predict the timesharing behavior of the system for a different OFF-ON duration cycle of three hours simulated for six hours. Similarly, the model was also used to predict the behavior for a different time period duty cycle of 66.67% ON and 33.33% OFF for a time period of eight hours (Fig. 5.4 (c)). The data was normalised with the maximum fluorescence value. The predicted outcome of the simulation correlation well (higher than 0.85) with the experimental results carried out in the same conditions as shown in figures 5.4 (b) and (c). Hence, the opto-genetic system model developed has been validated even with different inputs. The model shows very good correlation with the experiments which validates that our model can approximate the system behavior closely.

Figure 5.4: Temporal control of gene expression by blue-light based bidirectional promoter system

Figure 5.4 (a)

Fig. 5.4 (a) OFF-ON-OFF-ON cycle for every 2 hours over a period of 8 hours with correlation Coefficient -0.9963 (inducible) and 0.873 (repressible)

Figure 5.4(b)

Fig. 5.4 (b) Prediction and Validation of OFF-ON cycle for every 3 hours over a period of 6 hours With correlation coefficient 0.9821(inducible) and 0.89 (repressible).

Figure 5.4 (c)

Fig 5.4 (c) Prediction and Validation of OFF-ON cycle for 2 hours ON 6 hours OFF – correlation coefficient 0.9783(inducible) and 0.9943 (repressible).

Then, the model was used to investigate the mRNA synthesis rates of the two light-dependent promoter systems, mRNA abundance and fluorescent protein production as a function of time and illumination conditions. The intermediates of these kinetic and biological parameters have been simulated in Figures 5.5 (a) – (f) for both the light-regulatory system performed for the three different experiments. Here, it is inferred that the mRNA synthesis rates for the inducible system are varying slowly as compared to the repressible system. Moreover, from the mRNA temporal dynamics, it is also seen that the degradation rate of mRNA is also not rapid enough to model a digital pulse. Thereby, these intermediate mRNA component dynamics makes it a rate-limiting factor to express fluorescence with a sharp rise and fall. In conclusion, the model reveals that the bifunctional promoter system's synchronous and coordinated behaviour can be further optimized with faster degradation rates of mRNA and proteins.

For the case of the inducible system, it can be seen that for activation (blue light 'ON'), there is an observed initial lag in the RFP expression. The model shows that this is due to the gradual increase in the mRNA synthesis rate. On deactivation (blue light 'OFF'), the simulations show gradual decay of the mRNA synthesis

rates (Fig 5.5 (a), (c) and (e)). For the case of repressible system, when the illumination is turned 'OFF', our model exhibits an immediate rise in mRNA synthesis rate before reaching its maximum steady state (Fig 5.5 (b), (d) and (f)). Thus, GFP mRNA level increases rapidly which leads to an increase in Green Fluorescence Protein production. Then, when the illumination is turned 'ON', the mRNA synthesis rate decays rapidly, resulting in a reduction of the total mRNA level. These delays could be due to fast blockage of transcriptional initiation. From the model, it shows that the increase in synthesis rate of the repressible system is more than 1.5-fold faster than that of the inducible system.

Figure 5.5 (a)

Figure 5.5 (b)

Fig 5.5 (a) Temporal dynamics of intermediate parameters and output fluorescence in 2-hour time period experiment involving inducible promoter (b) involving Repressible promoter

Figure 5.5 (c)

Figure 5.5 (d)

Fig 5.5 (c) Temporal dynamics of intermediate parameters and output fluorescence in 3-hour time period experiment involving inducible promoter (d) involving Repressible promoter

Figure 5.5 (e)

Figure 5.5 (f)

Fig 5.5 (e) Temporal dynamics of intermediate parameters and output fluorescence in 2 hours ON 6 hours OFF time experiment involving inducible promoter (f) involving Repressible promoter

5.4 OSCILLATORY BEHAVIOUR SIMULATION

From the simulation model, the time-sharing concept effectively demonstrated synchronous behaviour and was validated for time duration up to eight hours by experiments. Due to experiment constraints, the concept could not be validated experimentally for longer time periods. However, with the mathematical model, the simulation was performed for longer time durations, 24 hours and 48 hours for different blue light illumination conditions assuming constant growth conditions. The simulation was performed for varying time periods of OFF and ON cycles, i.e. 4, 8, 12, 16 and 20 hours' light time periods, and also for various duty cycle lengths of the time periods, from 10% to 90% of every blue light clock signal (Fig. 6.6).

From the simulations, it is seen that for short time ON-OFF time periods (e.g. 4 hours), the oscillatory behaviour is poorly defined, whereas for longer ON-OFF time periods (e.g. 10-20hours), the oscillatory behaviour is defined better, having large peak-to-base output fluctuations. This observation is better reflected in Figure 5.7 that has been simulated for the different time-periods with 50% duty cycle for a time of 24 hours. The model predicts that optimally, the inducible and repressible systems work in synchronisation when the time periods more than 8 hours are used and when the light OFF-ON duty cycles are maintained at 50% or 60%. Overall, the simulation results convey that the different input conditions produce varied amplitude and time-evolution patterns of gene expressions and oscillatory behaviour. This advantage of the light-mediated system gives us a way to control the biochemical levels in a bioprocess.

Figure 5.6(a) Model predicted 48 hours' simulation results of 4 hours' time period

(a) **4 h.** Duty cycle: **10%** - 3.6 h OFF; 0.4 h ON, **20%** - 3.2 h OFF; 0.8 h ON, **30%** - 2.8 h OFF; 1.2 h ON, **40%** - 2.4 h OFF; 1.6 h ON, **50%** - 2 h OFF; 2 h ON, **60%** - 1.6 h OFF; 2.4 h ON, **70%** - 1.2 h OFF; 2.8 h ON, **80%** - 0.8 h OFF; 3.2 h ON and **90%** - 0.4 h OFF; 3.6 h ON.

Figure 5.6(b) Model predicted 48 hours' simulation results of 8 hours' time period

(b) **8 h.** Duty cycle: **10%** - 7.2 h OFF; 0.8 h ON, **20%** - 6.4 h OFF; 1.6 h ON, **30%** - 5.6 h OFF; 2.4 h ON, **40%** - 4.8 h OFF; 3.2 h ON, **50%** - 4 h OFF; 4 h ON, **60%** - 3.2 h OFF; 4.8 h ON, **70%** - 2.4 h OFF; 5.6 h ON, **80%** - 1.6 h OFF; 6.4 h ON and **90%** - 0.8 h OFF; 7.2 h ON.

Figure 5.6(c) Model predicted 48 hours' simulation results of 12 hours' time period

(c) **12 h.** Duty cycle: **10%** - 10.8 h OFF; 1.2 h ON, **20%** - 9.6 h OFF; 2.4 h ON, **30%** - 8.4 h OFF; 3.6 h ON, **40%** - 7.2 h OFF; 4.8 h ON, **50%** - 6 h OFF; 6 h ON, **60%** - 4.8 h OFF; 7.2 h ON, **70%** - 3.6 h OFF; 8.4 h ON, **80%** - 2.4 h OFF; 9.6 h ON and **90%** - 1.2 h OFF; 10.8 h ON.

Figure 5.6(d) Model predicted 48 hours' simulation results of 16 hours' time period

d) **16 h.** Duty cycle: **10%** - 14.4 h OFF; 1.6 h ON, **20%** - 12.8 h OFF; 3.2 h ON, **30%** - 11.2 h OFF; 4.8 h ON, **40%** - 9.6 h OFF; 6.4 h ON, **50%** - 8 h OFF; 8 h ON, **60%** - 6.4 h OFF; 9.6 h ON, **70%** - 4.8 h OFF; 11.2 h ON, **80%** - 3.2 h OFF; 12.8 h ON and **90%** - 1.6 h OFF; 14.4 h ON

Figure 5.6(e) Model predicted 48 hours' simulation results of 24 hours' time period

Fig. 5.6 (e) 20 h. Duty cycle: **10%** - 18 h OFF; 2 h ON, **20%** - 16 h OFF; 4 h ON, **30%** - 14 h OFF; 6 h ON, **40%** - 12 h OFF; 8 h ON, **50%** - 10 h OFF; 10 h ON, **60%** - 8 h OFF; 12 h ON, **70%** - 6 h OFF; 14 h ON, **80%** - 4 h OFF; 16 h ON and **90%** - 2 h OFF; 18 h ON.

Fig 5.6: Model predicted 48 hours' simulation results of the oscillations (assuming constant growth conditions) for varying time periods (4, 8, 12, 16 and 20 h) and duty cycles (10% to 90%).

Figure 5.7

Fig 5.7 Simulation of 50% light pulse duty cycle for five different time periods over a time of 24 hours

5.5 OPTIMIZATION OF LIGHT PARAMETERS FOR GENE EXPRESSIONS

In the previous sections, the transfer functions of light inducible/repressible engineered systems have been modelled and studied. It has also been shown that

the systems can be used to produce synchronization and oscillatory patterns. Apart from that, the kinetics of the gene expression can also suggest how to optimise the input light parameters to follow certain constrained patterns. For example, in order to produce protein expressions up to a certain level of threshold only, blue light input has to be exposed only to some limits of pulse widths and intensity levels. Another optimisation problem that can be studied could be the constraint of protein expression above a threshold level of gene expression and for this the requirement of blue light illumination to follow a light exposure pattern. Other specific patterns of desirable interest can also be generated with the optimisation algorithm implementation for the light parameters. Examples for the patterned gene expressions and the optimised light parameters solved are illustrated in Figures 5.8 and 5.9. They have been solved using the Optimization Toolbox in MATLAB Simulink, in which a reference signal is input to the system and the algorithm of trust-region reflective non-linear least square is used to fit the simulation curve to it. These kind of optimisation solutions may be needed in the application of drug delivery systems which have constraints for the drug levels produced.

However, there are some limitations due to the limiting factors of the kinetic rates of the biological system, such as the synthesis rates and decay rates of biological constituents. In this case, the limiting factor is especially the mRNA degradation rate which is also the more sensitive factor in the system. Due to the low rate of degradation, the protein is not able to fall in level quickly and therefore, fast changing patterns with large amplitudes are not feasible to be produced in short time durations. Thus, for large oscillations long time periods with long duty cycles of light are required. Unfortunately, it is also difficult to produce slow varying patterns with large amplitudes due to the relatively high synthesis rates that makes it hard to control the gene expression rates. Furthermore, with small amplitudes of fluorescence, it is difficult to generate large variations (high peak-base values of oscillations).

Therefore, variable patterns with medium amplitudes are easier to generate in this scenario. These signals' amplitudes fall in the range between 1400 to 3000 fluorescence A.U (calibrated with fluorescence OD 600 levels).

Figure 5.8

Fig. 5.8 Specific wave-pattern generated from repressible system model and the pulse inputs derived to create it.

Figure 5.9

Fig. 5.9 Specific wave-pattern generated from inducible system model and the pulse inputs derived to create it.

5.6 CONCLUSION

In conclusion, these results recapitulate that both the blue light activation and repression systems can function coherently in a single cell and can achieve rapid reversible and repeatable gene expression. Further, the rapidly reversible and tune-

able system enables the application of a molecular blue light clock to optogenetically synchronize functions over time. In addition, the ability to optimise the light system to direct gene expressions and characterise patterns creates opportunities to study and design tuning and evolutionary patterns in nature. Hence, the working of the system will prove useful for a diverse range of synthetic biology applications such as the implementation of multiple channel access communication capabilities.

Chapter 6

Application of Time-Sharing in Engineered Synthetic Opto-Genetic System

This chapter demonstrates the engineered opto-genetic device characterised in the previous chapter can be controlled by a blue light molecular clock to synchronize two receiver cells, where one is encoded with light inducible gene strain and the other with light repressible gene strain. As described in the time-sharing model, the light-inducible receiver functions as AND logic circuit, whereas the light-repressible receiver functions as N-imply logic circuit. An experiment is conducted for the first time, applying time-sharing control to synchronise the two receivers performing different logic functions over one-time period. Mathematical models using ordinary differential equations with the help of previous characterisation work are used to validate the system and predict gene expression results for longer time period cycles of working.

6.1 RECEIVER CELLS CONSTRUCTION FOR TIME-SHARING EXPERIMENT

As described in chapters 4 and 5, the time-sharing model for communication in bacteria is enabled by a blue light clock that can activate the receiver systems selectively over time. For this, the two receivers were constructed in such a way that they are activated differently in response to the positive and negative half-cycles of the blue light clock. Receiver cell 1 which functions as AND gate logic circuit as illustrated in Figure 6.1(a) has a plasmid (pBLind – blue light inducible promoter) carrying LasR gene encoded with LVA degradation tag downstream of the light-inducible promoter and degradation encoded Red fluorescent protein under the control of the lasI promoter (pLasI). Transcription of LasR is inducible under blue light illumination (blue light turned ‘ON’) and repressible during dark state. Red Fluorescence reporter expression is activated only in the presence of

AHL input signal by the LasR-AHL complex that binds to the LasI downstream promoter.

Similarly, the N-imply logic gate logic in receiver cell 2 is constructed (Figure 6.1 (b)) in a plasmid (comprising of pBLrep blue light repressible promoter and LasR gene encoded with LVA degradation tag downstream of the light-inducible promoter and Red fluorescent protein with LVA degradation tag under the control of LasI promoter. Transcription of LasR is constitutive during dark state (blue light turned ‘OFF’) and repressible when exposed to blue light. In line with the AND Gate function, only in the presence of AHL input signal (1) and blue light (1), the transcription of red fluorescent protein reporter is activated.

Figure 6.1 (a) Mode of action of AND logic in receiver cell

Fig. 6.1 (a) Mode of action of AND logic in light-inducible receiver cell
pBLind: LasR is expressed under pBLind promoter, while the output RFP signal is under PlasI promoter.

Figure 6.1 (b) Mode of action of N-imply logic in receiver cell

Fig.6.1 (b) Mode of action of N-imply logic in receiver cell
pBLrep: LasR is expressed under pBLrep promoter, while the output RFP signal is under PlasI promoter.

6.2 MODELLING OF RECEIVERS FOR BRIGHT AND DARK STATES

The LasR-AHL quorum sensing system has been simulated for each receiver, repressor and inducer at different conditions, light switched ON and light switched

OFF, to study the difference in fluorescence produced at both these conditions. The simulation was then tuned for kinetic parameters to match the results of the experiment performed in the laboratory (as shown in Figures 6.2(a) and 6.2(b)). Experiment was performed for a five-hour time period with either light OFF or light ON. Both of the bio-circuits involve red fluorescence protein genes as shown in Figure 6.3. AHL message was introduced in the second half-cycle, after 2.5 hours. Results show that the repressor produced more fluorescence in the Light OFF condition, whereas the inducer showed more fluorescence in the Light ON condition. The experiment had been performed with LasR gene tagged with a degradation gene LVA, by which the degradation of LasR was modified to 75 times faster than the original rate to 0.024/min.

Figure 6.2 (a)

Fig.6.2 (a) Inducer during Light Off and Light On conditions

Figure 6.2 (b)

Fig.6.2 (b) Repressor during Light Off and Light On conditions

Figure 6.3

Fig 6.3 Inducible and Repressible receiver systems with LasR-degradation tag LVA output RFP in response to blue light

6.3 MODEL EQUATIONS FOR QUORUM SENSING EXPERIMENT WITH OPTOGENETIC SYNTHETIC RECEIVERS

The blue light activation and deactivation kinetics in response to the digital pulses (0% and 100% light ON and OFF) has been characterized in the Equations 5.3 to 5.8 in Chapter 5. Since the mRNA transcription process of fluorescent protein expression, ribosome used (rbs34) and the size of mRNA/protein of fluorescent protein are similar and comparable to that of the LasR protein, the same set of equations is utilised to model the dynamics of LasR protein production, except for the increase in degradation rate of LasR protein which is increased to 75-fold due to the encoding of LVA degradation tag. This is shown in Equations 6.1 to 6.5.

Following that is the modelling of AHL message signal input and its combination with LasR protein to the downstream express the transcription of pLasI protein. For this, the AHL which is internally manifested in the cell after external AHL is placed in the medium is modelled over time, as described in equation 6.6. The next equation 6.7 models the dimerization of internal AHL with the LasR protein synthesized, to produce LasR-AHL dimer complex. This equation follows the association and dissociation kinetics of the form of equation 3.7.

This leads to the next part in the system which is to activate the downstream LasI promoter. The LasR-AHL complex produced in the previous step, is used to induce the transcription of the LasI promoter. This process is characterised by a Hill function response, derived from Parameter Estimation tool in Simulink. Since the transcription process is not rapid, the maximum transcription rate is not achieved instantaneously. In fact, the delay to reach the maximum transcription

rate can be visualised as the gradual increase in the binding rate of LasR-AHL to the active sites of promoter facilitating the acceleration of transcriptional rate of fluorescent protein mRNA. The kinetics for the varying transcriptional rate to reach the Vmax (maximum rate of transcription) is estimated with Simulink and given out in equations 6.8 and 6.9.

Finally, the translation rate of fluorescent protein is characterised by equation 6.10. Here the parameters of translation rate are derived from the rate of translation with ribose binding rate characterised from Bio numbers reference and the degradation rate of proteins from the use LVA degradation tag [23].

$$\frac{d[KsR]}{dt} = 6.4e^{-6}(lr) - 0.0326[KsR] \quad (6.1)$$

where $lr \begin{cases} = 1 \text{ when light is OFF} \\ = 0 \text{ when light is ON} \end{cases}$

$$\frac{d[mRNAR_{lasR}]}{dt} = [KsR] + 8.67e^{-7} - 0.348[mRNA_{lasR}] \quad (6.2)$$

$$\frac{d[KsI]}{dt} = 8.4e^{-7}(li) - 0.00315[KsI] \quad (6.3)$$

where $li \begin{cases} = 1 \text{ when light is ON} \\ = 0 \text{ when light is OFF} \end{cases}$

$$\frac{d[mRNAI_{lasR}]}{dt} = [KsI] + 3.22e^{-7} - 0.348[mRNA_{lasR}] \quad (6.4)$$

$$\frac{d[LasR]}{dt} = 0.001[mRNA_{lasR}] - 0.024[LasR] \quad (6.5)$$

$$\frac{d[AHLint]}{dt} = 0.00055[AHLext] - 0.00042(AHLint) \quad (6.6)$$

$$\frac{d[LasR.AHL]}{dt} = 60e^6[LasR][AHLint] - 60[LasR.AHL] \quad (6.7)$$

$$\frac{d[Trp.rate]}{dt} = 0.00148[0.0026] - 0.00038[Trp.rate] \quad (6.8)$$

$$\frac{d[mRNA_{RFP}]}{dt} = \frac{Trp.rate[LasR.AHL]^2}{[LasR.AHL]^2 + 1.567e^{-6^2}} - 0.348[mRNA_{RFP}] \quad (6.9)$$

$$\frac{d[RFP]}{dt} = 0.0018[mRNA_{RFP}] - 0.024[RFP] \text{ Initial: from expt} \quad (6.10)$$

6.4 ONE HOUR TIME PERIOD EXPERIMENT TO CHARACTERIZE RECEIVER OUTPUTS

In order to prove the coordinated behaviour of the two logic gates in quorum sensing, the two receiver cells were experimented with two different blue light clock signal pulse inputs (pulse 1: 1 hour ON, 5 hours OFF; and pulse 2: 1 hour OFF, 5 hours ON) and the quorum input signal (5 μ M AHL) was supplied exogenously (Figure 6.3 A and B). Briefly, the exponentially growing receiver

cells 1 and 2 were transferred in two 12-well plates. One of the plates were maintained in the dark and the other was placed onto the custom built 3×4 LED panel (as shown in Fig. 5.3) and the illumination was turned ‘ON’ for a period of one hour. Next, the plates were switched places and were allowed to grow for a period of 2 hours ON/OFF based on the clock signal pulse. At the third hour, the cultures were supplemented with the $5 \mu\text{M}$ AHL chemical message signal. The Red fluorescence was measured for every half an hour for the total study duration of six hours. The data (RFP/Optical Density 600) were normalized to the highest value obtained.

6.4.1 RESULTS

The experiment results along with the simulation data is represented in Figures 6.3 A and B. From the results, it is seen that receiver cell 2 (light-repressible receiver) exhibited the desired response of a digital logic N-imply gate as seen in Figure 6.3 B. This is attributed to the more than 5-fold increase in the Red Fluorescence output observed only when the blue light was absent, and AHL was present. Further, the response to positive clock pulse is a very sharp repressing of the expression of Red Fluorescence even in the presence of AHL. In the case of light-inducible receiver cell 1, the AND gate system showed a leaky response probably due to the leakiness of the light-inducible promoter as seen from Figure 6.3 (A). Compared to the dark state expression, the output Red fluorescence was more than 1.5-fold in the second half-cycle only, when the blue light was turned ‘ON’ and AHL was added.

Figure 6.3A (i)

Figure 6.3 A (ii)

6.3 A (i) Simulation and experiment output of AND Gate logic function.

Figure 6.3 B (i)

Figure 6.3 B (ii)

Figure 6.3. B Simulation and experiment output of N-imply Gate logic function in receiver cell 2

6.4.2 MODELLING RETROACTIVITY IN THE QUORUM CIRCUIT

Literature study [45] has suggested that the dynamics of a modular system in isolation changes its characteristics after interconnection with other modules. This is caused by the reversible association between biomolecules that cause loading effects which is similar in concept to feedback and equivalent to impedance effects in electrical systems. This unavoidable back-action from a downstream component to an upstream component is termed as Retroactivity. To illustrate this, consider the Figure 6.4 which depicts two systems, under two cases, the isolated case versus

the connected case. When connected, a signal, denoted by the dashed arrow (s) uses some information from the downstream component to the upstream, and affects the internal state of the upstream behaviour. This shows that retroactivity is not a consequence of some unknown indirect connection, but the back-effect from the inter-connection of components itself.

Figure 6.4 Model of retroactivity

Fig. 6.4 Isolated and connected system connections of biological modules are represented. The connected system has a feedback component termed retroactivity to the upstream system

The retroactivity effects were especially observed when more binding sites were involved and thus it affects temporal dynamics of systems having binding sites more considerably. The effect of retroactivity was nothing but a time delay in the gene expression level dynamics. The modelling of retroactivity effects is as follows:

In the Figure 6.4, the sub-system from protein Z to protein X constitutes the upstream component while the other sub-system from X to C forms the downstream component. The binding of protein X to promoter p when connected produces a feedback effect as denoted by the connection S .

For the isolated system, the mRNA dynamics can be ignored and the equation considered is as follows:

$$\frac{dx}{dt} = k(t) - \delta(x) \quad (6.11)$$

where δ is decay rate parameter of protein and $k(t)$, the rate of binding of Z to form X .

For the connected system, the protein X has to drive a downstream expression by binding to a promoter p , then the equation for the connected system will have to incorporate both the forward and the reversible binding reaction of protein X with promoter p given as:

where, C is the complex protein promoter formed by the binding of X to p and k_{on} and k_{off} are the binding rates in the forward reaction $X + p \rightarrow C$ and the reversed reaction $C \rightarrow X + p$ respectively.

Since C is not subject to any decay in this example, $p + C = p_{tot}$ where p_{tot} is the total concentration of the promoter.

To derive the equation for dX/dt in the reversible binding reaction, first the forward reaction $X + p \rightarrow C$ in equation 6.11 is considered. Since the concentration of protein X decreases at the rate k_{on} when this reaction is taking place,

$$\frac{dX}{dt} = -k_{on} Xp = -k_{on} X(p_{tot} - C) \quad (6.13)$$

Then the reverse reaction $C \rightarrow X + p$ is considered, where protein X is increasing at a rate of k_{off} which would be modelled as:

$$\frac{dX}{dt} = k_{off} C \quad (6.14)$$

Hence by putting equations 6.11, 6.12 and 6.13 together, the resultant rate equations for protein X and Complex C can be derived as follows:

$$\frac{dX}{dt} = k(t) - \delta X + k_{off} C - k_{on} (p_{tot} - C)X \quad (6.15)$$

$$\frac{dC}{dt} = k_{on} (p_{tot} - C)X - k_{off} C \quad (6.16)$$

The background technique to deduce the equations for the overall rate of change of protein X due to the effect of retroactivity has been given out in equations 6.16 to 6.15. These model equations, can be implemented to derive the kinetic

equations of a similar complex driving the transcription of a downstream promoter. In this experimental consideration, the context of retroactivity falls in the process of activation of the downstream promoter, viz. the *plasI* promoter driven by the LasR-AHL dimer complex to regulate the transcription of fluorescent protein mRNA. Thereby, from the previous set of equations, equation is affected by retroactivity and needs to be tweaked.

With the retroactivity model, the transcription equation 6.9 of the second promoter is modified to

$$\frac{d[mRNA_{RFP}]}{dt} = \left(\frac{Trp.rate[LasR.AHL]^2}{[LasR.AHL]^2 + 1.718e^{-62}} - \lambda[mRNA_{RFP}] \right) - (0.348)[mRNA_{RFP}] \quad (6.17)$$

Parameter Estimation has been performed to estimate the value of λ . After estimation results as 0.06, as expected, there was a time delay in the dynamics of fluorescence protein and the output was validated better with the experiment. The results are illustrated in Figure 6.5

Figure 6.5

Fig 6.4 Results of one-time period experiment with retroactivity model

6.5 SIMULATION OF ENGINEERED QUORUM SYSTEM FOR LONG TIME PERIODS

From the one-time period pulse experiment performed in the opto-genetic quorum sensing device in section 6.3, it is concluded that the time-sharing of receivers has been achieved with negligible crosstalk. Due to experiment constraints such as the very slow AHL degradation and limited nutritional supply in the present set-up, it was hard to experiment the same device for more pulse cycles. However, since the model has been validated with the current experimental results, it can be used to simulate and study the behaviour of time-sharing for longer time durations and more light pulse cycles of the receivers. The simulation result can also help in future experimental study to construct the communication system. Hence, simulation has been performed for extended light pulse cycles to validate if the receiver cells are synchronized by the periodic pulses of the blue light clock signal. The simulation has been performed with the assumption that the AHL signal degrades faster with the rate of 0.02/min to demonstrate the time-sharing. From the experiment results of LasR-AHL system, it is seen that the light-inducible receiver shows leaky behaviour and maintains a considerable high basal value of gene expression even when the light is off. But the repressible system shows good fluorescence difference between the light OFF and the light ON conditions. In the following passages, the simulations performed for extended time periods are described and illustrated for both receiver cells for three cases (a) AHL message input to receiver1 (when light is switched ON) (b) AHL message input to receiver 2 only (when light is switched OFF). (c) AHL message input to both receivers, i.e. when light is switched OFF and ON.

- a. Consider the simulation experiment where, the AHL is input to the receivers only when the light is switched OFF for 80% of eight hours' time period, (i.e. 96 minutes light ON and 384 minutes light OFF) simulated over a time of 24 hours (Fig. 6.6). Here, as expected the light repressible receiver shows red fluorescence of close to $7e^{-5}$ M as it is activated, and the light inducible receiver shows a leaky gene expression level of around $1e^{-5}\mu\text{M}$ when exposed to AHL.

Figure 6.6 Simulation with AHL message sent in absence of blue light

Fig. 6.6 Simulation extended for three eight-hour time periods of time-sharing experiment. AHL message signalled to light-repressible receiver only, when the blue light is switched OFF for 80% of light time period. High fluorescent levels are expressed from repressible receivers (red), whereas basal amounts of fluorescent levels due to leaky behavior are expressed in the inducible receivers (blue) when AHL message is introduced to the system.

The parameters and intermediates of both receiver cells in the simulation experiment in response to blue light and AHL input are shown in Figure 6.7. The Red fluorescence output of receiver 2 (light-repressible) produces maximum fluorescence in its half-cycle of operation and it is threshold to produce the received response of AHL signal as shown in Figure 6.8.

Figure 6.7

Fig. 6.7 Outputs of all parameters from light-inducible and light-repressible systems in the repressible receiver signalling experiment. The sequence of biological interactions in the system from how the light affects the kinetics of the promoter transcription, followed by the production of LasR mRNA and protein is given out. The AHL input is plotted, followed by the lasR-AHL complex that results in the production of RFP mRNA and Red fluorescent protein output.

Figure 6.8 Retrieval of digital received signal from receivers after threshold

Fig 6.8 Output is threshold to value 0.65 in normalised receiver output fluorescence to obtain the digital received signals. The messages sent to the repressible receiver is retrieved correctly in the readout.

- b. Next a similar experiment is performed to simulate the activation of inducible system where the AHL message is input to the receivers only when light is switched ON for 50% light duty cycle of time period eight hours, (i.e. 240 minutes light ON 240 minutes light OFF) simulated over a time of 24 hours (Fig. 6.9). Here, as expected the light inducible receiver shows red fluorescence of close to $2e^{-5}$ M as it is activated. Whereas, the light repressible receiver produces very small quantities of fluorescence in the presence of AHL due to the basal expression of LasR during OFF state. The results of the simulation of the bio-entities and final fluorescence outputs after normalisation and thresholding are shown in Figures 6.10 and 6.11.

Figure 6.9 Simulation with AHL message sent in presence of blue light

Fig. 6.9 Simulation extended for three eight-hour time periods of time-sharing experiment. AHL message signalled to light-inducible receiver only, when the blue light is switched ON for 50% of light time period. High fluorescent levels are expressed from inducible receivers (blue), whereas basal amounts of fluorescent levels due to leaky behavior are expressed in the repressible receivers (red) when AHL message is introduced to the system.

Figure 6.10

Fig. 6.10 Outputs of all parameters in the light-inducible and light-repressible systems in the inducible receiver signalling experiment

Figure 6.11 Retrieval of digital received signal from receivers after threshold

Fig 6.11 Output is threshold to value 0.65 in normalised receiver output fluorescence to obtain the digital received signals. The messages sent to the inducible receiver is retrieved correctly in the readout.

The normalised receiver signal outputs from the experiments after threshold to a value of 0.65, have exhibited that the received signals can be read as digital pulses.

c. The model for the above set-up is simulated for the condition where both receivers receive AHL message from the sender, performed for a 24-hour time-period for two cycles (12-hour time duration). The AHL message input for receiver 1 was given to be [1 1] and for receiver 2, [1 1]. Figure 6.12 illustrates the fluorescence outputs for both receivers corresponding to their time read-out slots. Both Receiver 1 and Receiver 2 receive the AHL message at their respective clock half-cycles throughout the simulation. In order to read out the received information, the analog fluorescence signals have been digitally converted by thresholding. It can be observed that both receivers output their respective messages in their respective read-out time slots correctly, however it is also inferred that a delay in synchronisation of received sequences with clock signal is present, which will be analysed in the subsequent sections.

Figure 6.12 Simulation for 12-hour time period – AHL input for both receivers

Fig 6.12: The fluorescence protein outputs are represented graphically with reference to the blue light clock signal as input. The blue light clock signal is represented digitally. The AHL message signal is sent to for both receivers. Thus, both receivers express high fluorescence levels. Thus, after normalisation of the fluorescence levels both receiver outputs are retrieved digitally with the threshold value of 0.65.

6.5.1 Effect of delay in clock readouts in receiver outputs with Time Periods

The engineered bacterial receiver system simulation was performed for time periods 6 hours, 8 hours, 12 hours, 20 hours and 24 hours with 50% duty cycles (e.g. 12 hours OFF and 12 hours ON for 24-hour time period). After thresholding

the fluorescence signal, the digital fluorescence output was observed to be not in synchronisation with the input blue light clock signal. In order to read the received signal correctly, the clock signal at the output has to be modified to include a delay. As shown in Figure 6.13, a delay in clock readout is included in the output in order to synchronise the fluorescence outputs with the receiver readout times and retrieve the AHL message signal after thresholding. When this clock readout delay was performed for different time periods, the delay for synchronisation for long time period lengths was observed to be lesser than that of the shorter time periods. This observation can be attributed to the fact that the proteins would decay within long half-cycle periods with low degradation rates, as compared to short half-cycle periods.

Figure 6.13 Delay in Clock readout for synchronised message output

Fig. 6.13 Shift in clock readout after two hours delay to obtain received digital pulses in synchronisation with readout clock.

The relation between the time periods and Delay per time period has been studied by fitting the delay hour: time period as a power function using curve-fitting tool as shown in Figure 6.14:

From the curve-fit the power function Relation for Delay in Clock readout is given as:

$$\text{Delay hours: Time period Ratio} = 7.237 \times [\text{Time Period}]^{-1.45} \quad (6.18)$$

Figure 6.14

Fig. 6.14 Relation between delay per time period and time period show that with larger time period, the delay per time period decreases exponentially.

6.5.2 Effect of Signal to Noise Ratio in Receiver Cells with Time period

In a system of two receivers, there is some noise present in output in the form of basal fluorescence expression of the one receiver system when the other receiver system is active. The difference or ratio of the peak fluorescence at one half cycle with respect to the basal fluorescence expression of the other receiver fluorescence can be calculated statistically as Signal to Noise ratio (SNR) as given by expressions 6.19 and 6.20. Receiver 1 system which is induced by blue light expresses more leakiness and thus, due to more basal level of inducible receiver 1, the Signal to Noise Ratio analysis of receiver 2 system is more impacting and proprietarily performed in this section.

Signal to noise ratio of Receiver 2 is calculated as

$$10 \log \frac{\text{Maximum Receiver 2 Fluorescence}}{\text{Minimum Receiver 1 Fluorescence}} \quad (6.19)$$

Signal to noise ratio of Receiver 1 is calculated as

$$10 \log \frac{\text{Maximum Receiver 1 Fluorescence}}{\text{Minimum Receiver 2 Fluorescence}} \quad (6.20)$$

From the graphs of simulation in figure 6.15, with increase in time period durations, better performance of the system is observed, i.e. more Signal to Noise Ratio is output. This can be attributed to the more extensive degradation of

fluorescence during longer time periods to achieve lesser basal fluorescence as well as maximum fluorescence in longer time periods.

The curve-fit to the relation of SNR to time periods and best pulse width performance for time periods are also given out in Figure 6.15, which show a logarithmic linear relationship described by equation 6.21.

Figure 6.15

Figure 6.15: Curve Fitting to SNR trend with Time Period. As time period increases, Signal-to-Noise Ratio increases linearly.

$$10 \log (S/N) = 1.033[Time Period] - 3.875 \quad (6.21)$$

6.6 CONCLUSION

The time-sharing of channel access in two receivers performing two different logic functions has been demonstrated successfully in this chapter. An experiment for one-time period has shown the varied response of receivers corresponding to the light and AHL input. Here, the light-inducible receiver shows some leakiness in the AND Gate function in the dark state, however the gene expression levels can be capped to threshold levels to derive the received pulse response. When the simulation for the experiment is extended for long time durations, it is seen that the received signals contain a time delay for which a shift in clock readout is required as computed mathematically. Further, the relations for clock time readout delay and Signal-to-Noise ratio with respect to time period lengths have been obtained from the study.

Chapter 7

Conclusions and Future Work

7.1 CONCLUSIONS

The incorporation of the novel engineered blue light-inducible/repressible genetic systems has been effectively useful to precisely control gene expressions in response to blue light. The blue light acts as a transcription factor that binds with the regulatory promoter to control the transcription/translation mechanisms in the cell. The thesis studies the dynamics of the blue light binding with promoter through the proposal of a new set of ODEs to characterize it. This has enabled the deeper understanding of molecular kinetics in the underlying light transcription factor activation process.

The simulation model used for characterization study indicated that the opto-genetic receiver systems have a short pulse width illumination switching point of 6.5%. The high correlation coefficient of the simulation model with the validation experiments proves that the model can be used confidently to predict the system behavior for extended time periods, which is usually difficult to study with experiments.

The extended simulation study of the system also suggests that with variable blue light input pulse cycles and time periods, customized patterns of dynamic expression of genes can be produced.

Further, the model lends insight into the rate-limiting steps of the model, which are the mRNA and protein degradation rates, and revealed that the light promoter binding activity was more rapid and reversible.

In addition, the opto-genetic system showed that the cells can operate with negligible interference. Hence, using the mathematically characterized system

model, it was possible to further demonstrate a time-sharing communication model with multiple systems working in parallel, for the first time.

To develop the time-sharing communication model, the light inducible and light repressible promoters were engineered in separate cells to design two parallel receiver systems. The receiver cell with light inducible promoter provided an AND logic, whereas the receiver cell with light repressible promoter provided an N-Imply logic. A common external sender was made to transmit message signals to the two receivers in a shared medium. The corresponding message signals to the different receivers were controlled by the blue light clock, which provided alternate working half-cycles for the two receivers. This way the parallel working of the two logic functions was demonstrated.

The time-sharing design also involved the modeling of LasR protein module prior to the cascade of fluorescent protein synthesis. This involved the connection of an upstream LasR gene cascade and downstream fluorescent gene expression which can affect the system's performance due to retroactivity (back-action from downstream component). Therefore, feedback components of the downstream components were incorporated into the ODE model.

The simulation results have indicated good correlation with the experiment and have been used to study the system under different light input conditions for prolonged durations. It was found that the light-inducible system showed some leakiness in the dark state, however when capped to threshold levels, the basal fluorescence was eliminated.

Following this, the system set-up for the best performance indices of Signal-to-Noise ratio and clock readout delay were given out. It was found that the relation between clock readout delays per time period decreased exponentially with increase in time period hours of light illumination. Moreover, the relation of Signal to Noise ratio was found to increase logarithmically with increase in time period durations.

This coordinated synchronous behavior has been a challenge to achieve between growing populations of cells, but with the implementation of time-sharing control, improved cell-cell communication capacity has been achieved for the first time. Besides, with the help of quantitative tools in modeling, the time-sharing concept can be custom designed and predicted to operate under various illumination and pulse width time durations. This paves way to the next frontier to build microbial consortia networks in synthetic biology.

7.2 FUTURE WORK

i. Modelling Sender Cell in Communication Model

The experiments performed for the time-sharing model has been done with the help of opto-genetic synthetic receivers that have been characterised well with respect to blue light and AHL input message signal. The message signal AHL has been directly input to the receiver cells through a micro-pipette in this set-up, however to implement the communication model a sender cell that secretes AHL would have been ideal. Due to experiment constraints to construct the sender cell, this has not been achieved. In future, with the sender cell in the experiment set-up, the model can study the temporal dynamics of the cell and the AHL diffusion over time. This can help to model a practically feasible system and deduce the merits and de-merits of it.

ii. Implementing other Multiple-Channel Access Communication schemes

Figure 7.1

Fig.7.1 Implementation of FDMA/TDMA hybrid concept in three cells (two cells induced by different frequencies of light and one cell repressed by all lights) to improve channel capacity in communication system

The thesis introduces a new capability of coordination in bacteria inspired by TDMA channel access technique. Here, it presents the simplest application of multiple-channel access communication TDMA in synthetic biology. There are other modes of multiple channel access techniques as described in Chapter 4 such as FDMA or FDMA/TDMA schemes that can be suitably applied to the quorum sensing system. For example, with the role of two different frequencies of light (e.g. blue and red), FDMA can be implemented for coordination of two receiver bacteria cells. This particular application also has the independence of time to coordinate functions. Another way of control, FDMA/TDMA control, as shown in Figure 7.1, can make use of three different bacteria, for example, one induced by blue light, one induced by red light and another repressed by both lights. This extends the communication capacity to more than two cells to be synchronised. The output receiver can be made to fluorescence in three different colours for distinguishing.

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