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Review

Recent advancements in synthetic biology: Current status and challenges

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ABSTRACT

Synthetic biology is the design and construction of new biological parts, devices and circuits not existing in nature. It provides a novel solution to imminent challenges in a wide variety of fields, including the discovery of new drugs, production chemicals, renewable biofuels, value-added products and cellular reprogramming. Many efforts have been made to design and characterize synthetic genetic parts, including promoter, transcription factors, RBS, degradation tags and transcriptional terminators, among others. These genetic parts have been assembled for construction of a number of synthetic devices and circuits like oscillators, toggle switches, amplifiers and biologic gates; they play a vital role in cell reprogramming for better understanding of cellular mechanisms and control of biological process. They are also useful for the periodic and tunable production of drugs, fine chemicals, vaccines and much more. It is the goal of this review to aid and accelerate future research in synthetic biology.

Contents

Abbreviations: AHL, acyl-homoserine lactone; ATc, anhydrotetracycline; AU, arbitrary units; C4-HSL, *N*-butyryl-homoserine lactone; CAP, catabolite activator protein; CRP, cAMP-receptor protein; HTH, helix-turn-helix; IPTG, isopropyl β-D-1-thiogalactopyranoside; PFLs, positive feedback loops; RBS, ribosomal binding site; RNAP, RNA polymerase; TF, transcription factor; TSS, transcription start site; yemGFP, monomeric yeast-enhanced green fluorescent protein; σ factor, sigma factor.

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1. Introduction

Synthetic biology is defined as "the design and construction of new biological parts, devices, and systems, or the re-design of existing, natural biological systems for useful purposes." It is a new field of biological science in which many disciplines such as physics, chemistry, mathematics, engineering and computer sciences are applied simultaneously. It will play a vital role in the improving and establishing of synthetic gene networks and biosynthetic pathway for better understanding of cellular mechanisms and to provide valuable products for disease prevention.

American and European scientists have designed a number of synthetic gene circuits using synthetic genetic parts with well defined functions. In recent years, research groups around the world have used synthetic biology to better understand diseases as well as food and energy production. It is a rational design of synthetic gene circuits using modularized, standardized parts, which are DNA traits with well-defined functions (Endy, 2005; Marchisio and Stelling, 2011). The first synthetic genetic devices – "Repressilator" (Elowitz and Leibier, 2000) and "Toggle switch" (Gardner et al., 2000) – were successfully constructed and characterized in *Escherichia coli*. Subsequently, a number of synthetic parts including promoters (Alper et al., 2005; Baron et al., 1997; Lutz and Bujard, 1997), regulatory proteins and RNAs (Bayer and Smolke, 2005; Dueber et al., 2003; Isaacs et al., 2004; Pfleger et al., 2006), and scaffolds (Dueber et al., 2009; Park et al., 2003), have been successfully engineered and characterized in a number of hosts.

Synthetic parts have been assembled to construct novel genetic devices and circuits such as oscillators (Danino et al., 2010; Elowitz and Leibier, 2000; Stricker et al., 2008), riboregulators (Isaacs et al., 2004; Lou et al., 2012), riboswitches (Blount and Breaker, 2006; Tucker and Breaker, 2005; Winkler et al., 2002) and biologic gates (Bonnet et al., 2013; Moon et al., 2012; Tamsir et al., 2011). Friedland et al. (2009) constructed and characterized digital circuits that can program and design cells using the principles of modern computing — such as counting. These devices count various user-defined inputs with a range of frequencies that can be expanded to count higher numbers. In the present review, the recently engineered and characterized genetic parts, devices and circuits are highlighted – especially those in *E. coli* – for a better understanding of the cellular mechanisms and opportunity and challenges in the future.

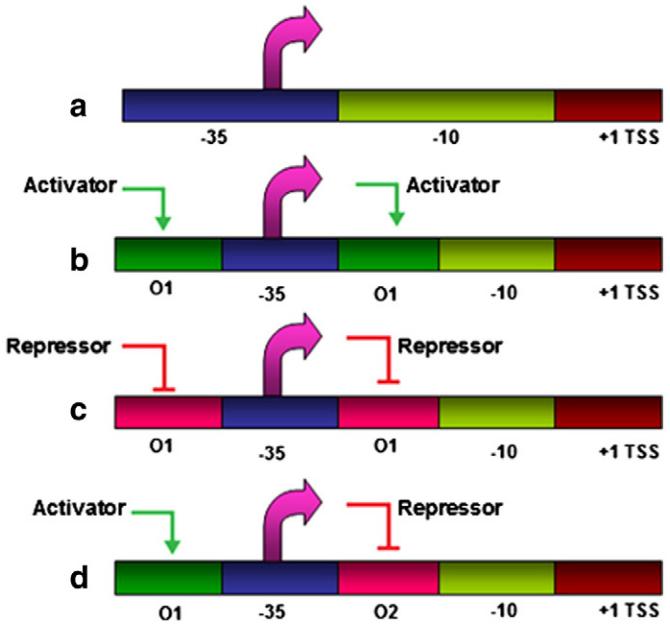

Fig. 1. Schematic representation of different elements present (−35, −10, +1 TSS, Operator site) in *Escherichia coli* based synthetic promoter. (**a**) constitutive, (**b**) inducible, (**c**) repressible and (**d**) combinatorial promoter.

2. Design, construction and characterization of genetic parts

In a recent study Canton et al. (2008), suggested that the ability to quickly and reliably engineer many-component systems from libraries of standard interchangeable parts, is a hallmark of modern technologies. There is a requirement to refine, standardize and modularize the biological parts, devices and circuits.

2.1. Design and modulation of promoter

A promoter is a specific DNA sequence that recruits the transcriptional machinery and facilitates the transcription of a desired gene. The specific sequence of promoters determines their strength by high or low binding with RNA polymerase (RNAP). The major components of *E. coli* promoter sequences are −35 and −10 (Pribnow box), region and operator for repressor or activator or both proteins binding site that can tune the promoter strength. RNAP is associated with the sigma factor for binding at specific regions of the promoter (−35 and −10). When the σ factor and RNAP are combined, a holoenzyme is formed. The σ factor is a protein required for initiation of transcription (the 'transcription initiation factor'), that enables specific binding of RNAP with the promoter. Each molecule of RNAP contains exactly one σ factor subunit, which is dependent on the gene and on the environmental signals (Gruber and Gross, 2003; Sharma and Chatterji, 2010).

There is no operator site which presents a constitutive promoter. It means that there is no effect of the transcription factor (TFs). The promoter contains −35, −10 and the transcription start site (+1 TSS) thus; it can constitutively express the protein (Fig. 1a). An inducible promoter has one or two operator sites for same TF; it binds onto the operator which is induced by the inducer (Fig. 1b). As shown in Fig. 1c, this repressible promoter is repressed by same, or two different TFs, and is activated by inducer molecules. The combined promoter has at least two operator sites for different repressors, which can be activated by using both inducers (Fig. 1d). A number of synthetic promoters could be designed by the promoter engineering to fine-tune gene expressions and help control gene networks.

2.1.1. Constitutive promoter

The unregulated constitutive promoter allows for continual transcription of the desired gene. Liang et al. (1999), identified the seven constitutive promoters in *E. coli*; they include (i), the spc ribosomal protein operon promoter Pspc (ii), the beta-lactamase gene promoter Pbla of plasmid pBR322 (iii), the PL promoter of phage lambda (iv), and (v), the replication control promoters PRNAI and PRNAII of plasmid pBR322 and (vi) and (vii), the P1 and P2 promoters of the rrnB ribosomal RNA operons.

Initial initiative was taken by the MIT Registry of Standard Biological Parts (http://partsregistry.org/Main_Page). A series of constitutive promoters (designated as BBa_J23100–BBa_J23119) have been designed and characterized. BBa_J23119 is the consensus promoter sequence and the strongest member of the family. BBa_J23112, BBa_J23113 and BBa_J23103 are weaker constitutive promoters that can be used in low level expressions of genes — especially toxic proteins. In this series, the two medium-strength constitutive promoters (BBa_J23105 and BBa_J23106), have been used for the construction of synthetic circuits

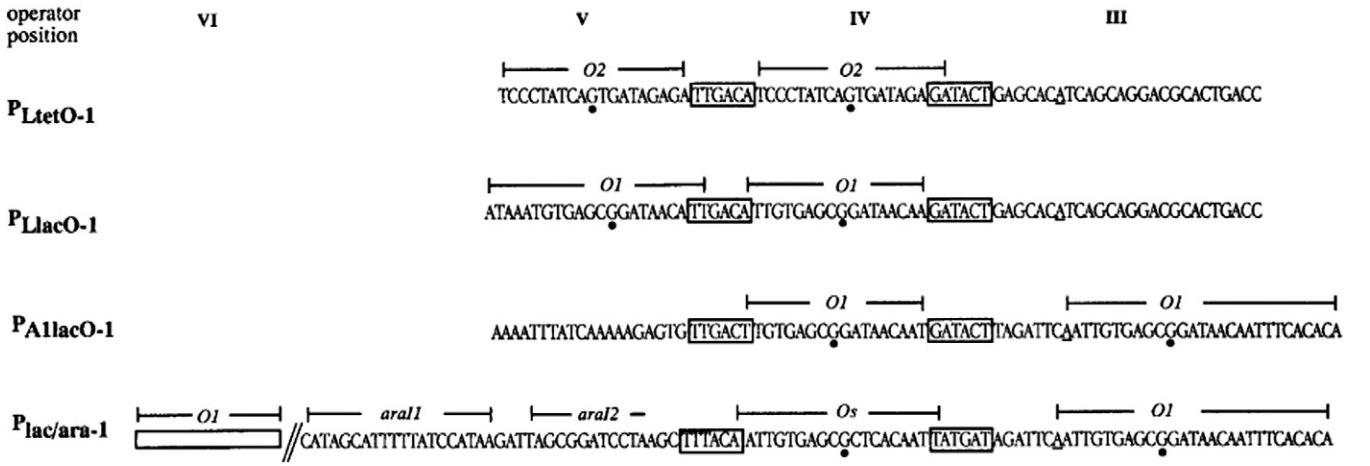

Fig. 2. Topography and sequences of promoters. All promoter sequences which are aligned via the –10 hexamer are flanked by XhoI (upstream) and EcoRI (downstream) cleavage sites whose sequences are not shown. The –10 and –33 hexamers are boxed and the transcriptional start site is underlined. Bars indicate the lac (lacO) and the tet (tetO) operators as well as the I1-I2 binding site of AraC. O1 denotes the corresponding operator sequence in the lac operon, Os is a symmetrical 20 bp synthetic operator. O2 indicates the corresponding operator sequence in the Tn10 tetracycline resistance operon. An additional O1 sequence is indicated upstream of Plac/ara-1 (Lutz and Bujard, 1997).

for heterologous expression in the *E. coli* MG1655Z1 strain. The enhanced yellow fluorescent protein (eYFP), is used to monitor the gene expression (Carrera et al., 2011). When the heterologous gene was expressed, the growth rate was slow because a strong promoter attracts more translation machinery (Carrera et al., 2011).

2.1.2. Inducible promoters

The inducible promoter plays a vital role in high level heterologous gene expression; it is a very powerful tool in genetic engineering, metabolic engineering and synthetic biology. A number of expression systems are now available for use in *E. coli* which is a versatile and extensively used lac expression system based on regulatory genes for arabinose catabolism. These promoters are governed by regulatory elements, including lacI, araC, luxR and tetR; these TFs greatly influence the strength of the promoters. As shown in Fig. 2, the foundation of promoter engineering is the ability to change operator sites and design new synthetic promoters. The new synthetic promoter PLtetO-1 allows the regulation of gene expression up to a 5000-fold range with anhydrotetracycline (aTc), while with IPTG and arabinose the activity of Plac/ara-1 could be controlled 1800-fold (Lutz and Bujard, 1997).

A number of derivatives of the original lactose-inducible Plac promoter are available in a variety of plasmids. The gratuitous inducer IPTG appears to avoid many of the problems of 'all-or-none expression', because it does not require the LacY transport protein for transport across cell membrane 47, which allows its use in organisms without a transport gene (Stricker et al., 2008), and permits variable expression in individual cells. Promoter (lacI regulated, lambda pL hybrid), inverts the regulatory region controlled by lacI. The PLlac0-1 promoter is a hybrid regulatory region, consisting of the promoter P(L) of phage lambda with cI binding sites, replaced with lacO-1. It allows for strong promotion that can be repressed by lacI. The PLlac0-1 promoter is chemically synthesized and well characterized in *E. coli* (Lutz and Bujard, 1997); it is an important promoter used for construction of a number of gene networks in *E. coli* (Elowitz and Leibier, 2000; Gardner et al., 2000; Guet et al., 2002; Rodrigo et al., 2012; Stricker et al., 2008).

PBAD is a promoter for *E. coli* arabinose catabolic genes araBAD. AraC regulatory protein acts as a repressor and also stimulates transcription from PBAD. The promoter system tightly controls gene expression and uncouples the genes for arabinose transporters from arabinose-dependent control, which could lead to homogeneous expression of the genes in all cells of the culture. Arabinose changes the conformation of araC and prevents it from successfully binding to repress pBad. In the absence of arabinose, repressor protein AraC binds to the AraI1 operator site of pBAD, and the upstream operator sites AraO2, blocking transcription. However, in the presence of arabinose, AraC binds and changes its conformation interacting with AraI1 and AraI2 operator sites, which permitted the transcription (Khlebnikov et al., 2001; Schlief, 2000).

Recently the promoter has been constitutively active, and repressed by TetR. The TetR repressor is a protein inhibited by the addition of tetracycline or its analog anhydrotetracycline (aTc). In contrast to tetracycline, anhydrotetracycline is a useful inducer; TetR is used in artificially engineered regulatory networks because of its ability to fine tune gene regulation. In the absence of tetracycline/aTc, the basal expression of TetR is very low, but the expression rises sharply in the presence of even a minute quantity of tetracycline through a positive feedback mechanism. TetR binds to a tet operator of the PL promoter and inhibits the transcription. A very low concentration of aTc inhibits tetR activity in *E. coli*. The <50 ng/ml aTc required for full induction of P LtetO-1 but has no effect on the growth of *E. coli* (Lutz and Bujard, 1997). The PLtetO-1 promoter has been used in the construction of a repressilator (Elowitz and Leibier, 2000; Rodrigo et al., 2012, 2013) and a toggle switch (Gardner et al., 2000). PLtetO-1 is a very important promoter in synthetic biology for the design and construction of novel gene circuits.

2.1.3. Combinatorial promoter

Recently, a number of combinatorial promoters have been engineered and characterized. Combinatorial promoters are regulated by a number of transcription factors; there are many applications for combinatorial promoters in synthetic biology, including the tight control of gene expression. Therefore, in the absence of an inducer, the ideal expression would be a very short time in the presence of the inducer; the expression should vary directly – and preferably linearly – with the amount of inducer time. Combined controls of the lac promoter by the regulators lacI repressor and cAMP-receptor protein (CRP) have been reported (Kuhlman et al., 2007). In this study, promoters occupy a region of 100 bp or less, surrounding the start site (+1), of transcription, approximately from positions −75 to +25. This sequence includes the primary binding sites for the polymerase, the −10 and −35 regions (Hawley and McClure, 1983), while upstream (Chan and Busby, 1989; Ross et al., 1993) and downstream regulatory sequences contain the operators for the activator and/or repressor. Operators within this region facilitate bound TFs to directly contact and recruit the RNAP (activation), or to sterically block RNAP contact with −10 and −35 regions (Browning and Busby, 2004; Busby and Ebright, 1994; Haugen et al., 2006).

For instance, PLlac_lux is a hybrid promoter which is repressed by a lacI repressor and induced by IPTG, while it is repressed by a luxR repressor bound to co-repressor CO6HSL. It contains a luxR box and a

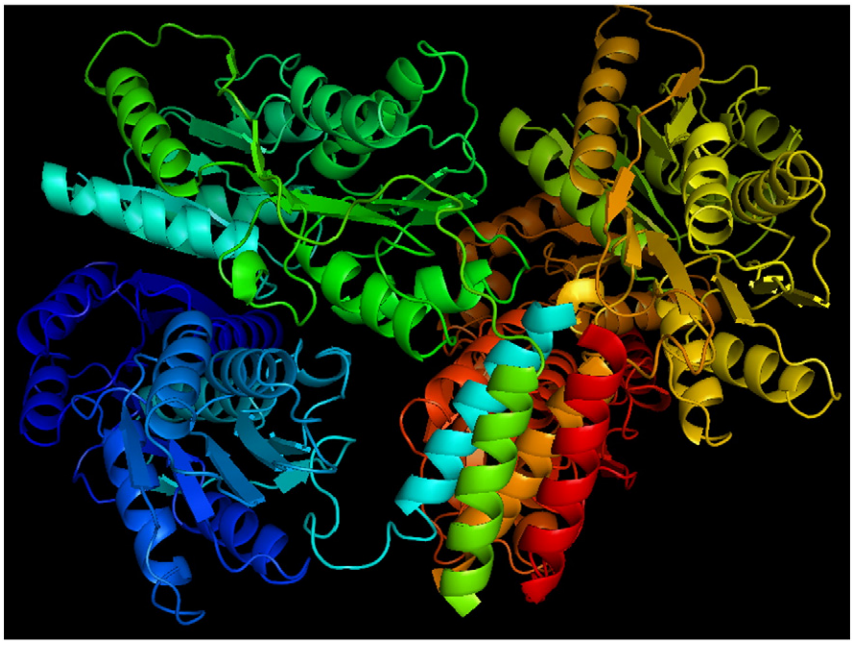

Fig. 3. The 3-dimensional structure of lacI repressor (PDB: 1LBI).

lacI binding site. Promoters control the expression of genes in response to the two TFs. For fine-tuning gene expression, it is required that the native promoter be replaced by the engineering of a synthetic promoter for over expression and regulation of the gene network. Another combinatorial promoter, PLtet_lac, is probably repressed by both tetR and lacI; it contains the two binding sites for tetR and lacI which is induced by aTc and IPTG. It is very important that the promoter can be used in the design of the AND logic gate in *E. coli*. A very strong promoter is required for the heterologous production of protein and enzymes.

In recent years, a number of synthetic promoters were designed using promoter engineering for gene expression and regulation; they also play an essential role in controlling gene networks and biosynthetic pathways. Inducible promoters are among the easiest and most effective for regulating gene expression, but it is essential that the promoter induces consistently in all the cells. While the constitutive promoters respond to change in growth conditions or an important intermediate metabolite, they allow for inexpensive, inducer-free gene expression. The combinatorial promoter is used in designing complex biological systems; therefore, there is a need for the design and characterization of a series of new synthetic promoter libraries for rewiring and reprogramming cellular machinery with well defined behavior.

3. Characterization of transcription factor

A transcription factor is a protein that binds to specific DNA sequences (especially the operator region of the promoter), and controls transcription of the desired gene. It performs this function alone or with other proteins in a complex by promoting (activator), or blocking (repressor), the recruitment of RNAP to specific genes (Roeder, 1996). The major four transcription factors, LacI, TetR, LuxR and AraC, are used for construction of gene networks and synthetic circuits.

3.1. LacI transcription factor

The lac operon of *E. coli* is the hypothesis for gene regulation; the key component is the lacI repressor. The uncontrolled-type DNA sequence of lac operon has three lac repressor recognition sites in a stretch of 500 base pairs. It also has a binding site for catabolite activator proteins (CAP), which is considered to be an activator. The lac operon is required for the transport and metabolism of lactose in *E. coli* as well as some other enteric bacteria. It consists of three adjacent structural genes that include lacZ, lacY and lacA. The genes encode β-galactosidase, lactose permease, and thiogalactoside transacetylase, or galactoside O-acetyltransferase (Jacob and Monod, 1961) as shown in Fig. 3, the three-dimensional structure of lacI of *E. coli*. The lacI repressor protein is a homotetramer as its tetramer contains the two DNA binding subunits composed of two monomers. These subunits dimerize to form a tetramer capable of binding two operator sequences. Each monomer consists of four distinct regions, the N-terminal DNA-binding domain (two lacI proteins bind a single operator site), a regulatory domain, a linker that connects the DNA-binding domain with the core domain, and the C-terminal tetramerization region (Lewis, 2005; Swint-Kruse and Matthews, 2009).

Research performed by Oehler et al. (1990), investigating DNA binding via an N-terminal helix-turn-helix structural motif, targeted one of several operator DNA sequences that included O_1, O_2 and O_3. The O_1 operator sequence overlapped slightly with the promoter, which prevented binding by the RNA polymerase, thereby inhibiting expression. Recently lacI was used to design novel biological gene networks in a number of hosts, including mammals.

3.2. TetR transcription factor

The TetR is a well characterized repressor protein. The TetR family exhibits a high degree of sequence similarity at the DNA binding domain (Helbl et al., 1995; Hinrichs et al., 1994). The intergenic region between the tetR and tetA genes contains two identical operators separated by 11 bp. TetR binds to tetO operators of promoter and prevents transcription. Structurally, the TetR homodimer binds to the operator and each 15-bp operator that shows an internal palindromic symmetry with an extra central base pair. Because of its efficiency in binding onto promoters, which helps in the tight regulation of transcription, TetR has recently been used to design gene networks (Elowitz and Leibier, 2000; Gardner et al., 2000; Swinburne et al., 2008). In this study, combined gene networks were constructed using transcriptional regulators LacI, TetR, and lambda CI with their corresponding promoters. These gene networks displayed the characteristics of digital logical circuits such as NAND, NOR, or NOT IF (Guet et al., 2002).

3.3. Transcription regulator LuxR

The luxR is considered to be a highly useful TF in synthetic biology; its function as a quorum sensors is mediated by binding *N*-acyl-L-homoserine lactone (AHL), signaling molecules to the N-terminal receptor site of proteins. AHLs are generally considered to be activator/repressor proteins (Koch et al., 2005). However, the C-terminal region is a DNA-binding helix-turn-helix (HTH), domain of approximately 65 amino acids present in regulators of the LuxR/FixJ (*Vibrio fischeri*); luxR-type HTH domain proteins occur in a variety of microorganism that controls luminescence. The DNA-binding HTH domain is usually located in the C-terminal region and the N-terminal region often contains an autoinducer-binding domain. LuxR-type regulators act as activators, while some of them can be repressors, or perform a dual role at different sites. LuxR controls a wide variety of biological processes.

3.4. Transcription factor AraC

AraC is one of the more important transcription factors and it plays a vital role in the activation of promoter activity. In this study, the DNA-protein contact sites in the araC regulatory region, which is present in the araBAD promoter. DNA protein has been discovered in araC protein, in the cyclic AMP-binding protein and in RNAP by the methylation of protection and DNase I protection assays (Lee et al., 1981). In this experiment, the DNA binding properties of the proteins required for induction by the L-arabinose operon have been measured; but the real mechanisms of induction and repression were studied by observing the multiple interactions of the RNAP, the cyclic AMP receptor protein and araC protein with short DNA fragments containing either the araC or araBAD promoter regions. The binding of the araC protein to the operator site, araO1, directly blocked the RNAP binding at the araC promoter (Hendrickson and Schleif, 1984). Recently, many thousands of homologs of araC exist and regulate the diverse operons in response to numbers of different inducers, or physiological states (Schleif, 2010).

Transcription factors form a growing family of regulatory proteins that can positively or negatively influence transcription by binding to regulatory elements in DNA contacting components of the basal transcription machinery. Thus, there is an urgent need to design and codon-optimize a number of transcription factors that can be used in engineering robust gene regulatory networks.

4. Identification, design and characterization of ribosomal binding site

A ribosome binding site (RBS), is an RNA sequence found in mRNA to which ribosomes can bind and initiate translation; it is also known as the Shine–Dalgarno sequence (SD), or the Shine–Dalgarno box (Shine and Dalgarno, 1975). Translation initiation in bacteria always requires both an RBS sequence and a start codon AUG. The SD sequence exists both in bacteria and archaea that are present; it is also present in some chloroplastic and mitochondrial transcripts. In this study, the RBS sequence (AAAGAGGAGAAA), is stronger and is recognized by *E. coli*; it has been implemented in the construction of oscillatory gene networks (Elowitz and Leibier, 2000).

By aligning with the start codon, this sequence helps to recruit ribosomes to the mRNA for the initiation of protein synthesis. A single mutation in the SD sequence can negatively affect translation efficiency because of reduced mRNA-ribosome pairing efficiency, as evidenced by the fact that complementary mutations in the anti-Shine–Dalgarno (anti-SD) sequence can restore the translation. The complementary sequence (CCUCCU), is called the anti-SD sequence, located at the 3′ end of the 16S rRNA in the ribosome. SD, and anti-SD sequence pairs, initiate the factors IF2-GTP, IF1, IF3 and initiator tRNA fMet-tRNA^{fmet} that recruited the ribosome for protein synthesis (Shine and Dalgarno, 1975). In the relevant study, the development of software can design an RBS by controlling gene expression in a genetic circuit. While the experimental validation of >100 predictions in *E. coli* showed that the method was accurate to within a factor of 2.3 over a range of 100,000-fold, the design method also correctly predicted that reusing identical RBS sequences in different genetic contexts showed different protein expression levels (Salis et al., 2009). Therefore, a stronger RBS has the highest binding affinity with ribosomes, while a weaker sequence has a low binding affinity, knowledge which aids in fine tuning gene expression for a single gene, a set of genes or for a gene network.

5. Design and characterization of protein degradation tag

Protein degradation is important for the reduction of protein overload in a host, for better growth and understanding the real dynamic of the system. Protein decay rates are regulated by the degradation machinery that clears needless housekeeping proteins and maintains appropriate dynamic resolution for transcriptional regulators. Turnover rates are also crucial for fluorescence reporting in order to strike a balance between sufficient fluorescence for signal detection and temporal resolution for tracking dynamic responses; although in the case of fluorescence protein measurement, there is a need to degrade fluorescent protein which is fused with degradation tag ssrA. The ssrA-tagged protein can be degraded by a proteolytic complex ClpXP. Degradation tags are short peptide sequences that mark a protein for degradation using the cell protein recycling machinery; they effectively decrease protein half-life, or the typical lifetime of a protein (Danino et al., 2010; Elowitz and Leibier, 2000; Rodrigo et al., 2012; Stricker et al., 2008).

A similar fledgling initiative has been devised by the MIT registry of standard biological parts (http://parts.igem.org/Main_Page), where a number of degradation tags are freely available to the synthetic biology community. In this study, a small stable RNA (ssRA), tagging occurs when a ribosome becomes stuck on a truncated mRNA. Without a normal termination codon, the ribosome cannot detach from the defective mRNA. The ssRA or tmRNA (transfer-messenger RNA), liberates the ribosome by adding an eleven-codon degradation tag followed by a stop codon; this allows the ribosome to break free and continue functioning (Keiler et al., 1996). A number of designed degradation tags vary in their final three amino acids (AAV, ASV, LVA and LAA) which function suitably in *E. coli* and reduce protein overload (Andersen et al., 1998). There is a need to design and characterize additional degradation tags for the construction of more robust and rapid genetic networks.

6. Characterization of transcriptional terminator

The transcriptional terminator is an extremely important genetic part of synthetic biology research; it is usually present at the end of a gene where it stops the transcription process; in prokaryotes, there are two main types — rho-independent and rho-dependent. A Rho-independent terminator is composed of a palindromic sequence which forms a stem loop rich in G-C base pairs, followed by several T bases. The conventional model of transcriptional termination is that the stem loop causes RNA polymerase to pause, and transcription of the poly-A tail causes the RNA:DNA duplex to unwind and dissociate from the RNA polymerase (Singh, 2012). One of the major transcriptional termination mechanisms requires a RNA/DNA helicase, known as Rho factor. Two structures of Rho, combined with a nucleic acid recognition site, have been determined at 3.0 Å resolutions. Rho forms a hexameric ring with two RNA binding sites-which are primarily responsible for targeting mRNA recognition and which are required for mRNA translocation and for the unwinding-point toward the center of the ring (Skordalakes and Berger, 2003). *E. coli* chromosomes contain two sets of unidirectional DNA replication pause (Ter) sites which hold the replication fork; this controls termination of chromosome replication by restricting replication fork fusion to the terminus region (Duggin and Bell, 2009).

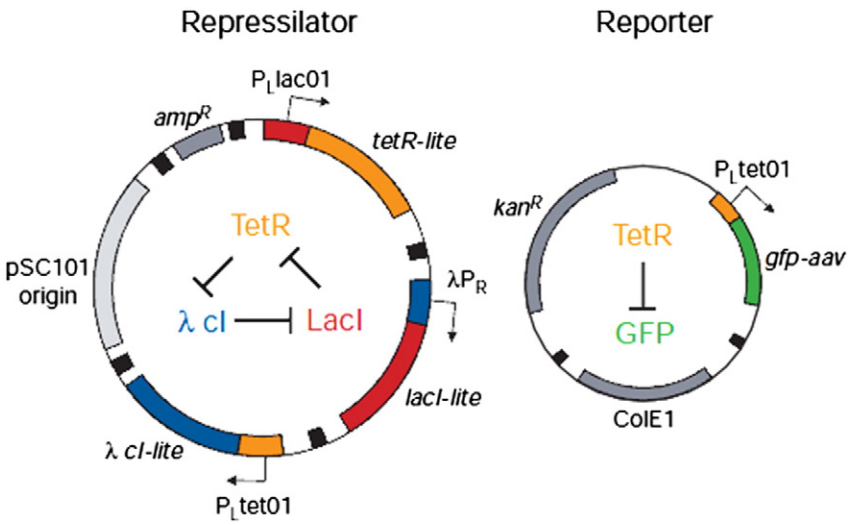

Fig. 4. Design of repressilator is a cyclic negative-feedback loop which composed of three repressor genes and their corresponding promoters, as shown schematically in the centre of the left-hand plasmid. It uses PLlacO1 and PLtetO1, which are strong, tightly repressible promoters containing lac and tet operators as well as PR, the right promoter from phage l. The stability of the three repressors is reduced by the presence of destruction tags (denoted 'lite'). The compatible reporter plasmid (right) expresses an intermediate-stability GFP variant11 (gfp-aav) (Elowitz and Leibier, 2000).

7. Engineering and characterization of synthetic devices and circuits

Advancements have been made in synthetic biological experiments for the design and construction of synthetic gene networks in a number of hosts. Repressilator and toggle switches are the first synthetic genetic circuits with predictive functions; subsequently, several devices and circuits have been constructed in *E. coli*, such as amplifiers, oscillators, toggle switch and biologic gates (NOT, OR, XOR, AND, NAND etc.). These genetic devices would be useful in understanding cellular mechanisms, gene regulation and cellular reprogramming; they also help in controlling the production of bio-products such as fine chemicals, bio-fuels, food products and drugs.

7.1. Synthetic oscillator

Synthetic oscillators are powerful tools for predicting and understanding real cell dynamical properties of genetic oscillatory networks. An oscillator can deliver one dose per day in a robust and reliable manner that would closely replicate the natural circadian clock. Noise and time delay in the gene network can be both constructive and destructive in generating oscillations, and stochastic coherence (Purcell et al., 2010). Designing an oscillator requires both positive and negative feedback to show oscillatory behavior in living systems.

7.1.1. Design and characterization of repressilator

Elowitz and Leibier (2000), performed ground breaking research when they engineered and characterized a synthetic oscillator in the

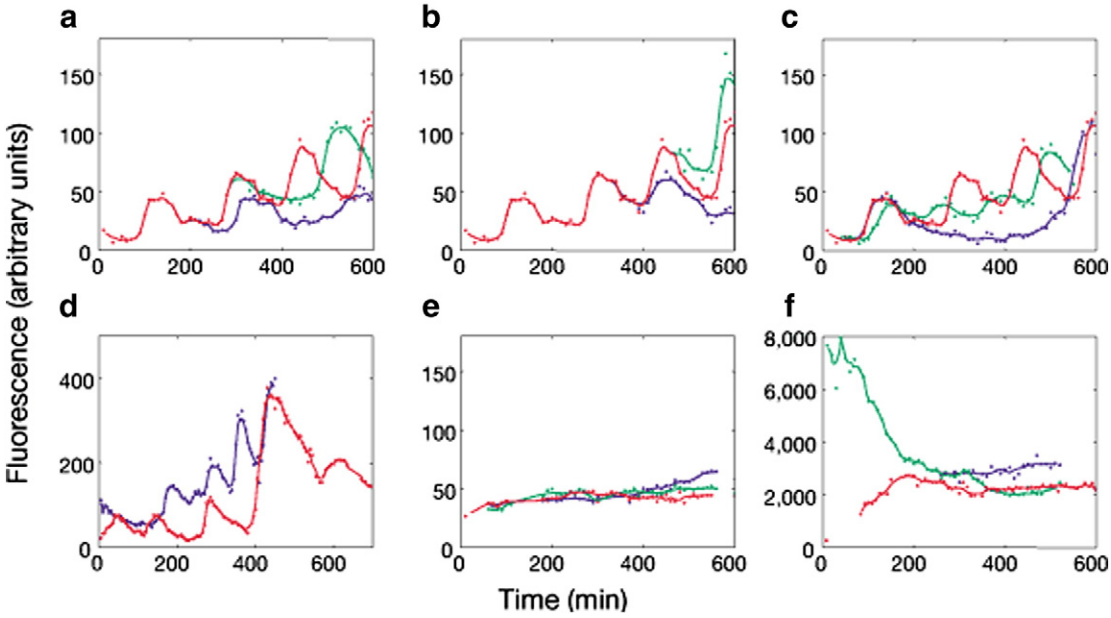

Fig. 5. Comparison of the repressilator dynamics exhibited by sibling cells. In each case, the fluorescence time course of the cells. The red as a reference, and two of its siblings are shown in blue and green. (**a**) Siblings exhibiting post-septation phase delays relative to the reference cell. (**b**) The phase is approximately maintained but amplitude varies significantly after division. (**c**) Examples of reduced period (green) and long delay (blue). (**d**) Two other examples of oscillatory cells from data obtained in different experiments, under conditions similar to those of (**a-c**). There is a large variability in period and amplitude of oscillations. (**e, f**) Examples of negative control experiments. (**e**) Cells containing the repressilator were disrupted by growth in media containing 50M IPTG. **f**, Cells containing only the reporter plasmid (Elowitz and Leibier, 2000).

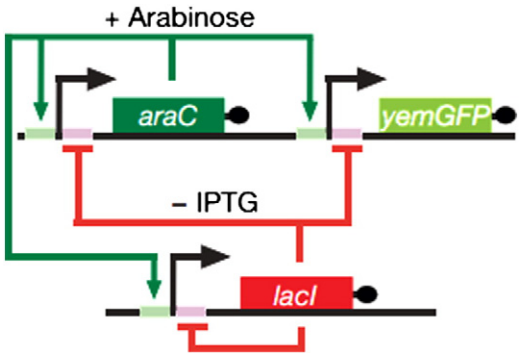

Fig. 6. Oscillations in the dual-feedback circuit. Network diagram of the dual-feedback oscillator. A hybrid promoter plac/ara-1 drives transcription of araC and lacI, forming positive and negative feedback loops (Stricker et al., 2008).

E. coli. Their research was based on three genes, called a repressilator, because each promoter was repressed by a different TF. These genes are lacI from *E. coli*, TetR, from Tn10 transposon and cI from λ phage. LacI represses transcription of tetR; TetR represses transcription of cI, and the cI represses transcription of lacI, which forms a cycle of repression (Fig. 4). In the experimental analysis, about 40% of cells exhibited oscillations within a period of 160 ± 40 (mean ± s.d.) minutes. After septation, GFP levels in the two sibling cells often remained correlated for long periods of time — an average half-time for sibling decorrelation of 95 ± 10 min, longer than cell-division times of 50–70 min under these conditions. This experiment indicated that the state of the network is transmitted to the progeny cells, even though a strong noise component exists. Significant variations have been observed in the period and amplitude of the oscillator output, both from cell to cell and over time in a single cell (Figs. 5a–f). Observation time for oscillations was limited by this entry into the stationary phase to approximately 10 h, allowing three to four oscillations (Elowitz and Leibier, 2000).

7.1.2. Design and characterization of tunable relaxation oscillator

The oscillator was designed using an earlier-modeled network architecture comprising linked positive and negative feedback loops. Hasty and his colleague used a hybrid promoter Plac/ara-1 (Lutz and Bujard, 1997), composed of an activation operator site from araBAD promoter placed in its normal location relative to the TSS; whereas the repression operator sites from lacZYA promoters were placed both upstream and immediately downstream of the TSS. It was activated by AraC in the presence of arabinose and repressed by lacI protein in the absence of IPTG. The araC, lacI and yemGFP (monomeric yeast-enhanced green fluorescent protein), genes were constructed under the control of three identical copies of Plac/ara-1 to form three co-regulated transcription modules (Fig. 6).

In the experimental analysis, the varying of the concentration of IPTG allowed for tuning of the oscillator period (Fig. 7a), mainly at low IPTG concentrations. The period was decreased at high IPTG concentrations (Stricker et al., 2008). The robustness of the oscillator was fixed at 2 mM IPTG and at 37 °C, but the oscillatory period can be used to tune from 13 min to 58 min by changing the arabinose concentrations from 0.1% to 3.0% (Fig. 7b). The oscillatory cells grown in the absence of arabinose could not express the measurable GFP, and high levels of arabinose seemed to saturate the system. Sustained oscillations were observed at a range of temperatures from 25 °C to 37 °C, with a decreasing period as a function of temperature (Fig. 7c); therefore the cell doubling time also decreased with temperature and the oscillatory period increased monotonically with cell doubling time (Fig. 7d). It was observed that the cell doubling time in a minimal medium was significantly longer than in an LB media (80–90 min versus 22–24 min at 37 °C) (Figs. 7c, d). This indicates that a rapid, robust and tunable synthetic oscillator was not coupled with the cell cycle (Stricker et al., 2008).

7.1.3. Engineering and characterization of synchronized oscillator

Recently the synchronized oscillator was designed and characterized using global intercellular coupling. Hasty and his colleague used the concept of quorum sensing for the design of the synchronized oscillator.

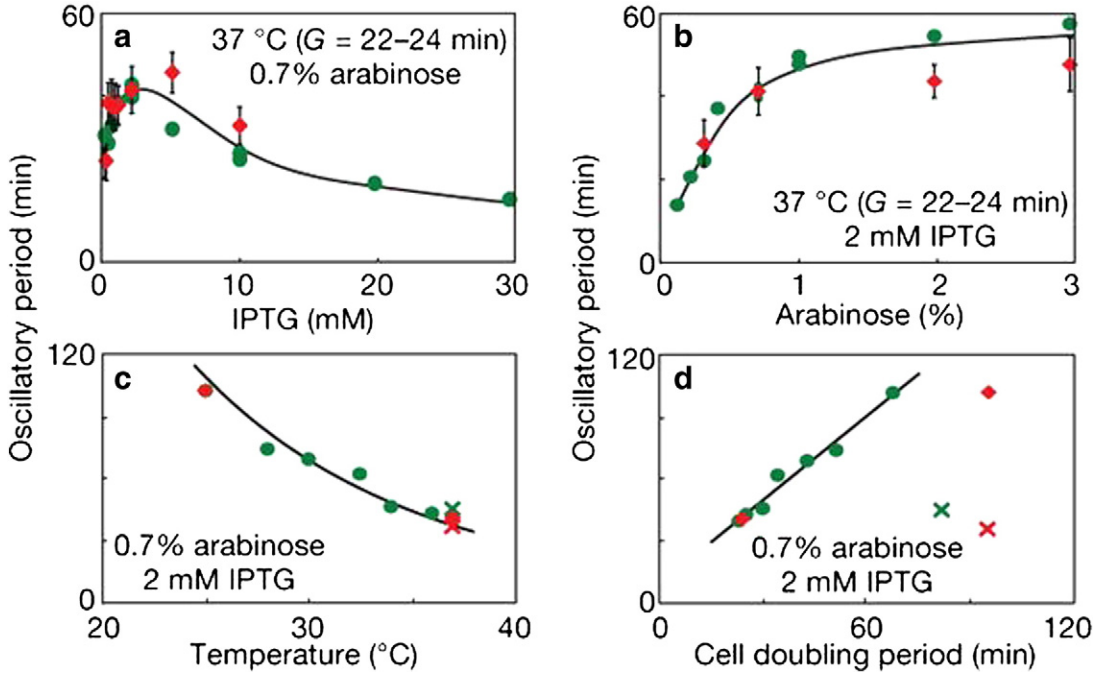

Fig. 7. Robust oscillation. a-c, Oscillatory periods on transects with 0.7% arabinose and varying IPTG (**a**) 2 mM IPTG and varying arabinose (**b**), or 0.7% arabinose, 2 mM IPTG, and varying temperature (c). Mean periods from single-cell microscopy (red diamonds, mean ± s.d.) or flow cytometry (green circles) are shown. Black curves are trend lines in a and b, or represent the theoretical prediction based on reference values at 30 °C. (**c**) samples grown in minimal medium rather than LB are indicated by crosses. G represents the cell doubling period. (**d**) Oscillatory period and cell division time increase monotonically as the growth temperature decreases. Symbols are as described above, and the black line is a linear regression of samples grown in LB (Stricker et al., 2008).

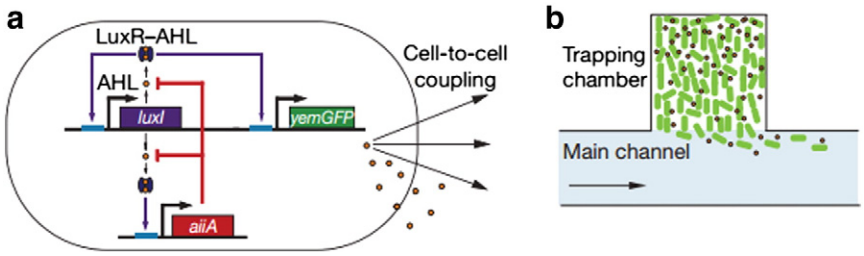

Fig. 8. Synchronized genetic clocks. (**a**) Network diagram. The luxI promoter drives production of the luxI, aiiA and yemGFP genes in three identical transcriptional modules. LuxI enzymatically produces a small molecule AHL, which can diffuse outside of the cell membrane and into neighbouring cells, activating the luxI promoter. AiiA negatively regulates the circuit by acting as an effective protease for AHL. (**b**) Microfluidic device used for maintaining *E. coli* at a constant density. The main channel supplies media to cells in the trapping chamber, and the flow rate can be externally controlled to change the effective degradation rate of AHL (Danino et al., 2010).

As shown in Fig. 8a, the design of the synchronized oscillator was constructed based on elements of quorum sensing machinery in *V. fischeri* and *Bacillus thuringiensis*. The luxI (from *V. fischeri*), aiiA (from *B. thuringiensis*), and yemGFP genes were constructed under the control of three identical copies of luxI promoter. LuxI synthase enzymatically produces an acyl-homoserine lactone (AHL), a small molecule that can diffuse across the cell membrane and which mediates intercellular coupling. It binds intracellularly to constitutively express luxR; the luxR-AHL complex is a transcriptional activator for the luxI promoter (Danino et al., 2010). The AiiA negatively regulates the promoter by catalyzing the degradation of AHL. As in Fig. 8b, the oscillator has been used for monitoring bulk oscillations, which consists of a main nutrient-delivery channel that feeds a rectangular trapping chamber (Danino et al., 2010).

In the experimental analysis, the effective AHL dissipation rate affects the period of the oscillations. Under high flow rate the oscillations stabilize after an initial transient period and exhibit a mean period of 90 ± 6 min and mean amplitude of 54 ± 6 GFP arbitrary units (Fig. 9a). While at low flow rate, a period of 55 ± 6 min and amplitude of 30 ± 9 GFP (arbitrary units), have been obtained. The waveforms have different shapes with the slower oscillator reaching a trough near zero after activation, and the faster oscillator decaying to levels above the original baseline (Fig. 9b). Although the flow rates from 180 to 296 μm min^{-1} were swept, an increasing oscillatory period was observed from 52 to 90 min (Fig. 9c); whereas, amplitude was observed to

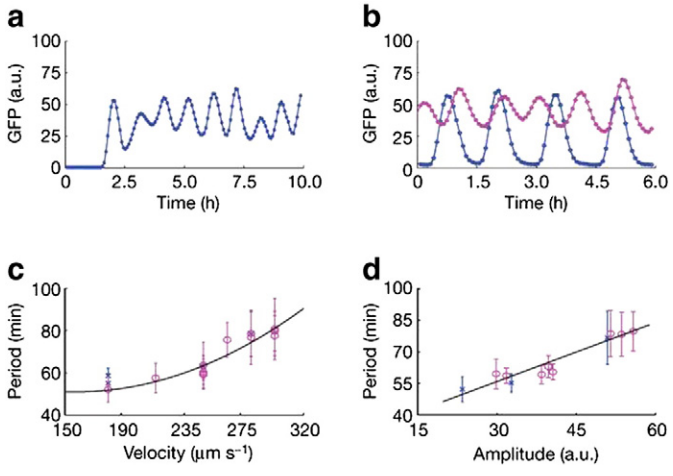

Fig. 9. Dynamics of the synchronized oscillator under several microfluidic flow conditions. (**a**) At around 90 min, cells begin to oscillate synchronously after reaching a critical density in the trap. (**b**) The period and amplitude increase for higher flow rates. Magenta curve is at low velocity (240 μm min-1), blue is at higher velocity (280 μm min-1). (**c**) Period as a function of velocity in the main channel showing tunability of period between 55–90 min. (**d**) Period versus amplitude for all experiments. Magenta circles (**c, d**) are data from 84 and 90 μm traps, blue crosses are 100 μm traps. Error bars in **c** and **d** indicate ±1 s.d. for a single channel, averaged over 10–50 peaks; each data point represents a different run (Danino et al., 2010).

be proportional to the period of oscillations (Fig. 9d), which is consistent with degrade-and-fire oscillations (Danino et al., 2010).

7.1.4. Synthetic biological clock based oscillator

Hasty and his colleague also constructed a biological clock based on self-sustained oscillators that adjust their phase to daily environmental cycles and external stimuli that map the entrainment region. The oscillators were frequency locked in wide intervals at the external period and showed higher-order resonance (Mondragón-Palomino et al., 2011). In the experimental analysis, the oscillator was coupled with a positive and negative feedback loop; this type of system has been characterized in many circadian rhythms. The monitoring of oscillator dynamics was done by use of GFP. The oscillator genes (araC and lacI), were expressed periodically by the concentration of arabinose, which acts on the positive-feedback loop. Such induction is referred to as "forcing of the oscillator". However, the phase of the oscillations was found with respect to the arabinose signal in ~1600 cell. Thus, the period of oscillations T, was measured as the peak-to-peak interval in the GFP fluorescence time series. However, the phase difference between an oscillator and the arabinose signal has been calculated as $\Delta f = 2p\Delta T / Tf$. Where Tf is the period of forced signal and ΔT was the measured time interval between a peak of arabinose and the immediate following peak of GFP fluorescence (Mondragón-Palomino et al., 2011). Oscillations are intimately linked to timing but little is known about biological time-keeping devices which coordinate periodic physiological activities. Engineered oscillatory gene circuits can help to pave the way for therapy, tissue engineering and biotechnological applications.

Improved understanding of endogenous molecular-level time keeping may reveal therapeutic opportunities for addressing sleep disorders, Huntington's, or Alzheimer's diseases; it can also be used in periodic and tunable gene expression, as per the requirements in cells. The construction of artificial gene regulatory networks has been pioneered by gene circuit engineers, to pave the way for construction of therapeutic gene circuits for next-generation gene therapy, tissue engineering and biotechnological applications.

7.2. Engineering and characterization of the toggle switch

In 2000 Collins and his colleagues borrowed the toggle switch from electrical engineering and re-engineered and characterized it. The toggle switch is composed of two repressors and two constitutive promoters; each promoter is inhibited by the repressor that is transcribed by the opposing promoter. It requires the fewest genes and *cis*-regulatory elements to achieve robust bistable behavior (Gardner et al., 2000). The ideal threshold, or bifurcation, in the pTAK117 toggle switch has been demonstrated both theoretically and experimentally. In this experiment, pTAK117 (initially in the low state), and pTAK102 (as a control) were grown in thirteen different concentrations of IPTG for 17 h to steady state, which is diluted twice (at 6 and 12.5 h), into a fresh medium with the same IPTG concentration. However, the induction of pTAK102 control has a familiar sigmoidal shape. In contrast, the pTAK117 toggle

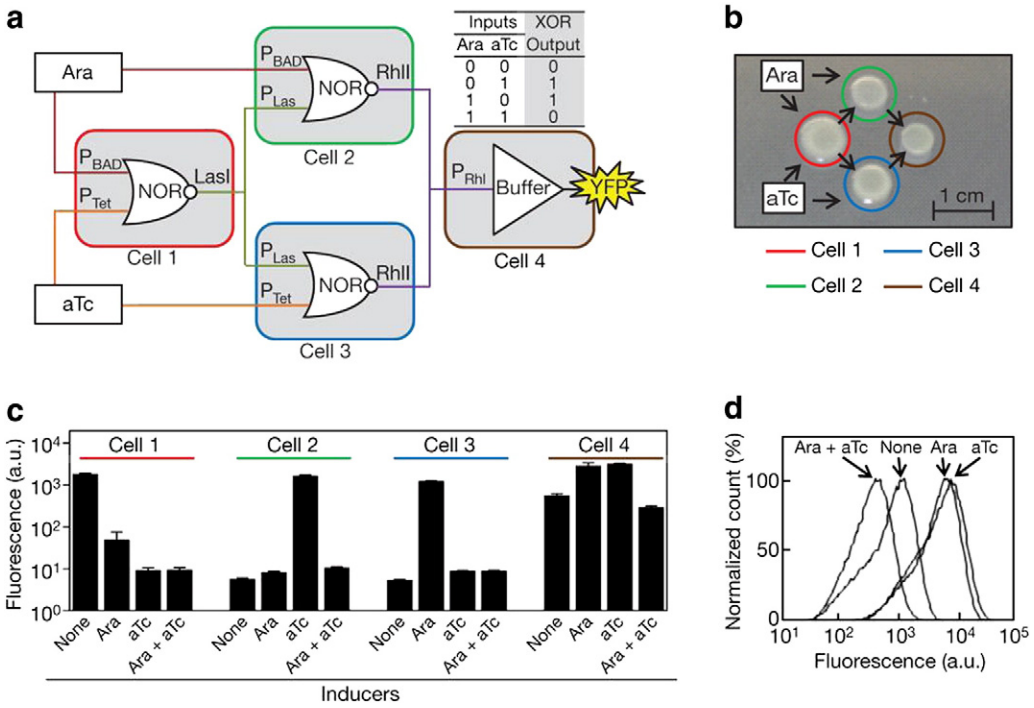

Fig. 10. Construction of an XOR gate by programming communication between colonies on a plate. (**a**) Four colonies-each composed of a strain containing a single gate-are arranged such that the computation progresses from left to right, with the result of each layer communicated by means of quorum signals. The inputs (Ara and aTc) are added uniformly to the plate. (**b**) Spatial arrangement of the colonies. (**c**) Each colony responds appropriately to the combinations of input signals. Fluorescence values and their error bars are calculated as mean ± s.d. from three experiments. (**d**) Cytometry data for the XOR gate (cell 4) (Tamsir et al., 2011).

follows the induction curve of pTAK102, up to an IPTG concentration of 40 μM, at which point it crosses the bifurcation and exhibits a quasi-discontinuous jump to a high expression state (Gardner et al., 2000).

Later, the successful design of the toggle switch (a component of the Lac and Ntr systems) was used for engineering genetic circuits that display toggle switch, or oscillatory behavior. The toggle switch based on lacI has provided constitutively; the level of active repressor has been controlled by lacY mutant and by varying the concentration of IPTG; it has provided nearly discontinuous expression of activator (Atkinson et al., 2003). The toggle switch is considered to be a self-assembled and programmable synthetic gene circuit for controlling cellular behavior that can be useful in gene therapy and tissue engineering.

7.3. Design, construction and characterization of genetic amplifier

In designing electrical circuits, positive feedback is often considered in the design of amplifier; but if there is a need to double, triple, or further increase gene expression in biological systems, a genetic amplifier is required. Positive feedback is a common mechanism used in the regulation of many gene circuits. It amplifies the response to inducers and also generates the binary output. Similar approaches have been used for the design of genetic amplifiers. Recently, amplifier design has utilized a constitutively active, autoinducer-independent variant of the quorum-sensing regulator luxR, which has the ability of the positive feedback module to separately amplify the output of a one-component tetracycline sensor and a two-component aspartate sensor. The positive feedback has amplified the response to the respective inducers; it can be used as a component in the design of more complex synthetic gene circuits (Nistala et al., 2010).

In this study, the design and construction of positive feedback loops (PFLs), based on the luxI–luxR quorum-sensing system, have been characterized. PFL's can be used as modular transcriptional regulatory units for the construction of complex artificial genetic circuits; they are highly sensitive and can establish intercellular signaling with the luxR with well-defined functions (Sayut et al., 2007). Therefore, the regulation enables cells to recognize multiple internal expression states in response to a single external input signal. The dynamic property of the positive feedback system, and the synthetic gene circuits in *E. coli* have been designed to measure no-feedback and positive feedback systems (Maeda and Sano, 2006). It was recently predicted that nonlinear interactions between the two positive feedbacks could be produced with ultrasensitive responses. However, the synthetic gene network *E. coli* has been characterized containing two positive feedback loops, which were linked in a coherent fashion. But the concerted action of both positive feedback loops resulted in bistable behavior over a broad range of inducer concentrations. When the feedback loops were removed, the range of inducer concentrations in the system exhibited the bistability (Chang et al., 2010).

7.4. Design, construction and characterization of biologic gates

The concept of designing electronic logic gates (the construction of larger-scale digital circuits from well-defined Boolean gates), is a standard procedure, and several computational methods have been developed. The combination of a simple gene circuit with quorum sensing is used to produce a more complex computation in space. Recently an orthogonal AND gate in *E. coli* was constructed using a novel hetero-regulation module from *Pseudomonas syringae*. The circuits consist of two co-activating genes, hrpR and hrpS, which are controlled by separate promoter inputs, and a σ(54)-dependent hrpL promoter driving the output. In an experimental analysis, the hrpL promoter was activated only when both genes were expressed and also produced a digital AND gate function. The AND gate is demonstrated to be modular by applying newly regulated promoters to the inputs and connecting the outputs to a NOT gate module, to produce a combinatorial NAND gate function (Wang et al., 2011).

In a relevant study, the synthetic multicellular system has been engineered; 'receiver' cells that were programmed to form ring-like patterns of differentiation based on chemical gradients of an AHL signal which is synthesized by 'sender' cells. Nevertheless, in a receiver cell,

'band-detect' gene networks respond to user-defined ranges of AHL concentrations. Although fusing the different fluorescent proteins as outputs of network variants, an initially undifferentiated 'lawn' of receivers was engineered to form a bull's eye pattern around a sender colony. Therefore, an experimental and theoretical analysis reveals that the kinetic parameters most significantly affect ring development over time (Basu et al., 2005).

Recently, a variant of TF lacI and operator lacO that form specifically interacting pairs of NAND gates was designed based on direct protein–protein interactions in coupling with DNA looping. They are reusable, and a multiplex of logic devices can be readily created using the same designs, but with different combinations of sequence variants (Zhan et al., 2010). In a more recent experiment, an improvement was made to the logical responses of a genetic AND logic gate, which is derived from components of the LuxI–LuxR bacterial quorum-sensing system. The properties of genetic AND logic gate use directed evolution and a two-step screening process to alter the activities of the LuxR transcriptional activator. It has enhanced the AND gate logical responses and also increased the specificities of these responses by 1.5-fold (Sayut et al., 2011).

Tamsir et al. (2011) constructed XOR gate in *E. coli* based on different orthogonal quorum-sensing 'sender' and 'receiver' devices (Fig. 10a). There are four strains, each carrying a different logic gate were used to construct an XOR circuit. The strains were spotted onto an agar plate in the spatial arrangement required to perform this function (Fig. 10b). In the experimental analysis, cell 1 carries a NOR gate that uses Ara and aTc as inputs and expresses LasI as the output. This allows cell 1 to be wired to the NOR gates in cells 2 and 3 by means of 3OC12-HSL. Cells 2 and 3 used Ara and aTc as their second inputs, respectively. Similarly, the output of the NOR gates in cells 2 and 3 is RhlI, which produces C4-HSL (*N*-butyryl-homoserine lactone). Cell 4 acts as a buffer gate and integrates the outputs from cells 2 and 3 by responding to C4-HSL. The output of a buffer gate is 'on' only when the input is 'on'. The complete circuit consisting of all four strains behaves as a digital XOR gate with respect to the two inputs (Ara and aTc; Figs. 10c, d). Each intermediate colony performs its digital logical operations appropriately, as tested by replacing each output gene with YFP (Tamsir et al., 2011).

Recently, AND gate has been designed and constructed in *E. coli*. The 2-input AND gate has been designed to be layered to create more complex programs. The construction of 3-input and 4-input AND gates has been used by different permutations of the 2-input gates. In the experimental observation, the 3-input gate has the potential for a fault when shifting from a +Ara/+IPTG/− aTc [110] to a [011] state (removal of Ara and addition of aTc). There are substitute logic combinations that could produce the same functions. These designs have chosen for the studying gate layering. The inputs into these programs were promoters that have been activated by small molecule inducers. In both cases, the output of the program was on only when all of the inducers were present in the medium. But for the 3-input gate, the output when all inducers were present [111] was 4.5-fold above the highest off state [011] (Moon et al., 2012). These biologic gates have been constructed and characterized in *E. coli* which can be useful in reprogramming and also in bacterial computation.

In a recent study, a set of synthetic transcription–translation control devices has been rewired in mammalian cells. The combinatorial circuits have been integrated into two-molecule based inputs. That is performed in digital computations such as NOT, AND, NAND and N-IMPLY expression logic in a single cell. The functional interconnection of two N-IMPLY variants are resulted in bit-wise intracellular XOR operations and a combinatorial arrangement of three logic gates that enabled independent cells by performing the programmable half-subtractor and half-adder calculations (Ausländer et al., 2012). The biologic gates permit the logical transcriptional control in cells and in combination with modern transduction technologies that could serve as versatile tools for regulating the gene expression and as building blocks for complex synthetic gene regulatory networks for biological computation and biotechnology applications. It can be also useful in gene and cell based therapies for controlling of diseases.

8. Conclusion and future perspective

Recently developed synthetic parts, device and circuits can be used for establishing a novel gene network for better understanding of biological complexity. An urgent need arises to re-design/re-engineer the hosts with self assembled gene circuits that play a key role in reprogramming of cellular machinery for improvement of human life in terms of better health, food, drug and energy problems. Wang et al. (2013) suggested that synthetic biology presents much promise in developing systems to address the challenges faced in the area of manufacturing, environment and sustainability, health and medicine. In a recent study, a synthetic gene circuit has constructed a biologic and memory device. In this experiment authors have assembled a synthetic circuit that uses the recombinases to execute the Boolean logic functions with a stable DNA-encoded memory of events. It demonstrated long-term maintenance of memory for at least ninety generations (Siuti et al., 2013).

Therefore, an integrated logic and memory system will enable the implementation of complex cellular state machines, behaviors and pathways for therapeutic, diagnostic and basic science applications. Whereas the processing of biological signal has been performed using the engineered genetic toggle switch (Hillenbrand et al., 2013) and inversion recombination switch (Ham et al., 2008). The synthetic biocomputing devices sense signal input and process a coordinated therapeutic output including sophisticated cancer kill switches integrating multi-input transformation signals (Culler et al., 2010; Nissim and Bar-Ziv, 2010; Xie et al., 2011). Artificial insemination devices (Kemmer et al., 2011), T-cell population controllers (Chen et al., 2010) and blue-light-triggered glucose homeostasis for diabetes therapy (Ye et al., 2011) have been tested. There is further recent implementation of logic gate in mammalian systems by designing of the first mammalian programmable biocomputer (Ausländer et al., 2012). The genetic devices such as oscillator, amplifier, logic gate and toggle switch can be useful to understand the cellular mechanism, gene regulation and cell reprogramming. An urgent need arises to design more novel genetic parts, devices and circuits in a wide range of host for better understanding of biological complexity and biological computation.

Conflict of interest

There is no conflict of interest.

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