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Advances in Synthetic Biology

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About the Editor

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Dr. Singh has designed and characterized a number of synthetic oscillators, gene regulatory networks, and biosynthetic pathways in *Escherichia coli*. Currently, his laboratory is focusing on the construction of a novel biosynthetic pathway for the production of pigments and chemicals. Additionally, his laboratory works on development of CRISPR-based platform for the diagnosis and eradication of multidrug resistant pathogens. Dr. Singh has published 72 articles, 23 book chapters, and 2 books. He currently serves as a member of the editorial board and reviewer for a number of peer-reviewed journals.

Introduction to Synthetic Biology

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Vijai Singh

Abstract

Synthetic biology is a newly growing field which allows us to design non-natural parts, devices and circuits for biotechnological applications. These novel systems can help to find a solution for current challenges that we are facing in context to fulfilling the demand of drugs, vaccines, precise diagnosis, fine chemicals, biofuels and so on. In the past decade, a number of parts, devices and systems have been engineered and characterized in many organisms. Currently, a number of research groups are focusing on the development of new technologies/assays, including CRISPR-Cas9, riboregulators, riboswitches, cell-free protein synthesis and microfluidics that can accelerate synthetic biology research and its applications. This chapter highlights the progress, challenges and applications of parts, devices, circuits and tools towards biological, biomedical, therapeutic and industrial purpose.

Keywords

Promoter · Transcription factor · CRISPR-Cas9 · Riboregulators · Riboswitches · Circuits · Gene regulation · Gene network

1.1 Introduction

Synthetic biology is a new field that incorporates engineering principles in biology. In the past decade, biological parts, devices and systems have been engineered and tested in many organisms (Endy 2005; Purnick and Weiss 2009; Khalil and Collins 2010). Novel small genetic parts such as promoters (Lutz and Bujard 1997; Alper et

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al. 2005), proteins, RNAs (Basu et al. 2005; Pfleger et al. 2006) and scaffolds (Park et al. 2003; Dueber et al. 2009) have been engineered and well characterized. These parts are reusable and can be assembled together to build novel devices and complex circuits such as oscillators (Elowitz and Leibier 2000; Stricker et al. 2008), biologic gates (Tamsir et al. 2011; Moon et al. 2012), riboswitches (Bayer and Smolke 2005; Wang et al. 2008; Patel et al. 2018) and riboregulators (Isaacs et al. 2004; Callura et al. 2010; Patel et al. 2018) for controlling the cellular behaviour and use them for biotechnological applications.

In recent times, developing synthetic biology technologies is on high priority. It has gained much scientific and public attention towards building or modifying organisms with highly predictive phenotype. A pressing need arises to build advance systems that can solve major issues of health, environment and energy. A wide range of gene cloning methods (Li and Elledge 2007; Sleight et al. 2010), genome assembly (Gibson et al. 2009), genome designing (Gibson et al. 2010), cell-free protein synthesis (Carlson et al. 2012; Moore et al. 2017), microfluidics platform (Balagaddé et al. 2005; Marcus et al. 2006; Maerkl and Quake 2007; Stricker et al. 2008) and CRISPR-Cas9 system (Jinek et al. 2012; Mali et al. 2013; Cong et al. 2013; Jiang et al. 2013; DiCarlo et al. 2013; Bikard et al. 2014; Jakočiūnas et al. 2015; Singh et al. 2017, 2018) have been introduced. This chapter highlights the recent advances, challenges and future potential of synthetic biology for industrial, therapeutic, biomedical and biotechnological applications.

1.2 Engineering and Characterization of Synthetic Parts

Currently, a wide range of synthetic parts including promoter, RBS, transcription factor, small non-coding RNA and transcription terminator have been engineered and tested in many model organisms and cell types.

1.2.1 Synthetic Promoter

A promoter is a portion of specific DNA sequences where RNA polymerase binds and starts transcription process. The main component of *Escherichia coli* promoter is −35 and −10 (Pribnow box) region and operator region (repressor or activator binds). These play a key role in activating or repressing gene expression. Inducible, constitutive and hybrid promoters are mainly used in synthetic biology for designing and construction of synthetic gene circuits, biosynthetic pathways and complex devices for biotechnological applications. An inducible promoter is an important promoter for gene expression. A number of wild types of inducible promoters (pLlac01, pLtet01, pBAD, etc.) have been modulated and re-designed for holding tight control over the gene expression. The pLlac01 promoter contains the operator for LacO where LacI repressor binds and stops transcription. It can be activated by using the IPTG inducer molecule. Similarly, pLtet01 is regulated by TetR and

is induced by anhydrotetracycline (aTc), while pBAD is regulated by araC and activated by arabinose (Lutz and Bujard 1997; Gardner et al. 2000; Khlebnikov et al. 2001; Guet et al. 2002; Stricker et al. 2008).

Constitutive promoters do not possess any operator site for binding of repressor or activator. This promoter is unregulated by any transcription factor; therefore, it continuously expresses the gene. Seven constitutive promoters have been identified in *E. coli* (Liang et al. 1999). Registry of Standard Biological Parts has a series of constitutive synthetic promoters (BBa_J23100 to BBa_J23119) that are regularly used in synthetic biology and metabolic engineering for constructing tunable circuits (Carrera et al. 2011). In the past decade, number of hybrid synthetic promoters have been designed and characterized in many organisms. These promoters contain operators for two transcription factors. In the absence of one inducer molecule, the gene will not be fully expressed. In order to get full activation, both the inducers molecules are required (Kuhlman et al. 2007). pLlac_lux is one such example that contains two operators sites, LacI and luxR, which are induced by IPTG and CO6HSL molecules. pLac_Tet that is repressed by LacI and TetR is in turn induced by IPTG and aTc molecules in order to get full gene expression. Therefore, promoters are essential components in synthetic biology and metabolic engineering. In order to make more standard biological parts, it should be more standardized and modulated so that it can be easily used in biotechnological, therapeutic and industrial applications.

1.2.2 Ribosome-Binding Site

A ribosome-binding site (RBS) is present upstream of messenger RNA and allows ribosome to bind there and begin translation. RBS is also known as Shine–Dalgarno sequence (SD) or Shine–Dalgarno box (Shine and Dalgarno 1975). A single mutation can affect the translation efficiency by weaker binding of mRNA-ribosome pairing efficiency. SD sequences are present both in bacteria and in archaea. The SD sequences regulate the rate of translation. In a study, sequence AAAGAGGAGAAA is considered as a stronger RBS of *E. coli* and was used for building a synthetic oscillator (Elowitz and Leibier 2000). A library of synthetic RBS has been engineered and tested in a number of organisms with different efficiencies. The different strength of RBS can help to regulate the single gene expression or cluster of genes with a wide range of biological functions.

1.2.3 Transcription Factor

A transcription factor is a protein that directly binds with the promoter sequences of the operator region and starts or stops the gene expression. It can perform its function either alone or with the help of complex protein molecules by activating or repressing target gene (Roeder 1996). In synthetic biology, LacI, TetR, Lambda CI and AraC are used for the construction of circuits (Gardner et al. 2000; Guet et al.

2002). For instance, lac operon in *E. coli* contains a number of genes including lacZ, lacY and lacA. These genes express enzymes β-galactosidase, lactose permease, and thiogalactoside transacetylase or galactoside O-acetyltransferase, which is mainly repressed by LacI repressor and activated by lactose or IPTG molecules (Jacob and Monod 1961). Similarly, TetR is another repressor that can bind with the tetO operator region and is induced by aTc molecule (Helbl et al. 1995). TetR is extensively used in synthetic biology for construction of gene networks because of its tight control for fine-tuning of expression of gene (Elowitz and Leibier 2000; Gardner et al. 2000; Swinburne et al. 2008). In addition, luxR is modulated by *N*-acyl-L-homoserine lactone (AHL) molecules. It is considered as either activator or repressor (Koch et al. 2005; Gohil et al. 2018). Another important transcription factor AraC can bind to the araBAD promoter and get activated in the presence of arabinose (Lee et al. 1981). All these above transcription factors are useful in building of regulatory synthetic devices, circuits and pathway or to re-design an existing pathway.

1.2.4 Protein Degradation Tag

Protein degradation plays a crucial role in the reduction of protein overload in the cellular systems. It is regulated by degradation machinery which can clear the protein and maintain the cellular physiology and regulation. The turnover rate is important for balancing the protein functioning and visualizing signal. In the case of synthetic circuits for measuring network dynamics, the transcription factor and fluorescence encoding genes should be fused with degradation tag ssrA. The ssrA is a common degradation tag that is degraded by ClpXP degradation machinery. It decreases the protein half-life and is used for measuring network dynamics (Elowitz and Leibier 2000; Stricker et al. 2008; Danino et al. 2010). Registry of standard biological parts (http://parts.igem.org/Main_Page) has a wide range of weak and strong degradation tags. It is physically available and also can be designed in the form of genes or primers. In order to make a fast dynamics cellular system, degradation tag should be incorporated and used for measuring cellular network function.

1.2.5 Transcription Terminator

Transcription terminator is an important component of the cells. It can stop the running RNAP for transcription process. In prokaryotes, it is mainly rho-dependent and rho-independent types. Rho-independent transcription comprises a palindromic region that creates a G-C rich base pair stem loop followed by T bases. This loop causes RNAP to pause transcription of poly-A tail (Watson 2004). Rho-dependent transcription terminator requires Rho factor which can form a hexameric ring wherein two RNA-binding sites are involved (Skordalakes and Berger 2003). In *E. coli* chromosome, two sets of unidirectional DNA replication pause (Ter)

sites are present that contain the replication fork which controls the termination of chromosome replication (Duggin and Bell 2009). A library of synthetic terminators is available that may be used for designing and construction of synthetic circuits.

1.3 Engineering and Characterization of Synthetic Devices and Systems

1.3.1 Small Non-coding RNA

Small non-coding RNA is a key element present in prokaryotic and eukaryotic organisms. It can either activate or repress gene expression. These small regulatory elements are commonly known as riboswitches or riboregulators, which have been described below.

1.3.1.1 Riboswitches

A riboswitch is a small non-coding regulatory RNA which is mainly present upstream of mRNA and forms a loop that can be dynamically changed in the presence of ligand molecules and activate or repress gene function (Nudler and Mironov 2004; Vitreschak et al. 2004; Tucker and Breaker 2005; Batey 2006; Patel et al. 2018). Riboswitches contain two parts: (1) an aptamer which is responsible for the binding of small ligand molecules and (2) an expression platform which can undergo dynamic changes in response to modulations in the aptamer that allow either activation or repression of a gene function. There are different types of ligand molecules, including protein, amino acids, chemicals, metals, antibiotics, etc. that can bind with small regulatory RNA and dynamically change the secondary structure of RNA allowing them to activate or repress gene function, detect metals, chemicals, etc. There are a number of well identified and characterized riboswitches in wide range of organisms. One such example is that of cobalamin riboswitch that binds with adenosylcobalamin and regulates the biosynthesis and transport of cobalamin (Nou and Kadner 2000).

Glycine riboswitch is known to regulate glycine metabolism upon the binding of glycine molecules (Sherman et al. 2012). Similarly, lysine riboswitch is regulated by binding of lysine molecule, and it regulates lysine biosynthesis. NiCo riboswitch binds with two metals ions nickel (Ni) and cobalt (Co) and is used for metal detection and biosensing. A wide range of naturally occurring riboswitches has been identified and tested. Many riboswitches have been artificially designed, characterized and used in metabolic engineering for monitoring lysine concentration (Yang et al. 2013), therapeutics, biosensing and synthetic biology applications.

1.3.1.2 Riboregulators

A riboregulator is a small non-coding RNA molecule that responds to signal by Watson–Crick base pairing. Under normal condition, mRNAs are cis-repressed and the small non-coding RNA can open the secondary structure allowing them to bind to the ribosome and start the translation process. It can act at different

stages of transcription, translation and post-transcription. It plays a key role in biotechnology, therapeutic and industrial applications (Patel et al. 2018). The first synthetic riboregulator was designed, constructed and characterized in *E. coli*. Small cis-regulatory complementary sequences were inserted upstream of the target gene. In transcription, cis-repressed sequences form a stem loop at the 5′ untranslated region (UTR) of mRNA which tends to interfere with ribosome binding. A small RNA is expressed in trans target which binds with UTR and alters the stem-loop structure, allowing the activation of target gene function (Isaacs et al. 2004).

Similarly, riboregulator has been designed that can confer biologic gates function (Rinaudo et al. 2007) in mammalian cells. Nechooshtan et al. constructed a pH-responsive riboregulator that can respond to change in the pH, thereby causing activation or repression of gene function (Nechooshtan et al. 2009). In metabolic engineering, for increasing the carbon flux or redirecting carbon flux towards desired products, it is important to knock-out non-essential gene or down-regulate essential genes (Gohil et al. 2017, 2019; Panchasara et al. 2018). Na et al. designed and constructed a library of small synthetic RNAs in *E. coli* for increasing tyrosine and cadaverine production (Na et al. 2013). Therefore, riboregulator can be designed to accomplish wider applications in the biomedical, diagnostic, therapeutic and industrial setting.

1.3.2 Synthetic Oscillator

The first synthetic circuits such as repressilator (Elowitz and Leibier 2000) and toggle switch (Gardner et al. 2000) were designed and characterized in the year 2000 in *E. coli*. This groundbreaking work laid the foundation of synthetic biology. Over the past decade, number of synthetic devices and systems have been designed and characterized in different organisms and cell types. It is widely used in a number of applications in synthetic biology and metabolic engineering for fine-tuning or up-regulation of production of the metabolites, biofuels, chemicals, therapeutics and much more.

Ever since its discovery, synthetic oscillators have opened up a new avenue for a digitalized biological living system. It has shown predictive behaviour. The oscillator has the potential to deliver ‘one dose per day’ in a time-dependent manner which rather depends on the programmed circuits. Building oscillators mainly requires negative or positive/negative feedback in circuits. Elowitz and Leibler engineered three genes-based synthetic oscillator which they called as repressilator. It contains LacI, TetR and lambda CI transcription factors and its corresponding promoters that were induced by IPTG. They used GFP as a reporter to monitor oscillatory behaviour, and it was found that 40% cells were oscillating with a period of 160 ± 40 (mean ± s.d.) min and cell division took 50–70 min (Elowitz and Leibier 2000). Repressilator was constructed based on negative feedback, and taking advantage of this, Stricker et al. built a fast, robust and tunable synthetic oscillator in *E. coli*. They used LacI as a repressor and AraC as an activator and pLac-ara as a synthetic promoter. For monitoring the oscillation, GFP with degradation tag

was used to obtain fast dynamics of systems. They observed robust oscillation at 2 mM IPTG at 37 °C within 13–58 min of period by changing 0.1–3.0% of arabinose concentrations. Oscillation was independent of cell cycle (Stricker et al. 2008).

Similarly, a synchronized synthetic oscillator has been built based on quorum-sensing molecules in *E. coli*. A mean oscillatory period of 90 ± 6 min and mean amplitude 54 ± 6 GFP arbitrary units were obtained during high flow rate. At a low flow rate, 55 ± 6 min oscillating period with the amplitude of 30 ± 9 GFP was obtained. This oscillation was based on degrade-and-fire dynamics (Danino et al. 2010). A wide range of synthetic oscillators has been designed and constructed in prokaryotic and eukaryotic organisms. It can be used and expanded towards therapeutic, industrial and biotechnological applications.

1.3.3 Biologic Gates

In electrical engineering, logic gates based large-scale digital circuits using Boolean gates are a standard method and have been used for a long time in computer technology. In biology, an orthogonal AND gate was constructed in *E. coli*. Two co-activating genes (hrpR and hrpS) which were controlled by input promoter and a σ(54)-dependent hrpL promoter as output were used. The hrpL promoter was activated when both genes were expressed that allowed AND gate to function. It was modulated by applying another promoter output that connected the output to NOT gate to achieve a NAND gate function (Wang et al. 2011). Similarly, LuxI-LuxR quorum-sensing system (Miller and Bassler 2001; Gohil et al. 2018) was used to generate AND gate function. It could further enhance the specificities by 1.5-fold (Sayut et al. 2011).

In addition, Tamsir et al. designed and constructed XOR logic gate in *E. coli* using orthogonal quorum-sensing sender and receiver devices. Four strains were used that carried different logic gates that had the ability to perform XOR gate function. In the experiment, cell 1 carried NOR and used Ara and aTc input that expressed LasI output. This cell was wired with NOR in cells 2 and 3 through the 3OC12-HSL (N-3-oxododecanoyl homoserine lactone). When, cells 2 and 3 used Ara and aTc as a second input, the output of NOR in cells 2 and 3 was RhlI that produced C4-HSL (*N*-butyryl-homoserine lactone). Cell 4 acted as a buffer gate. The output could be monitored using YFP reporter (Tamsir et al. 2011). In a study, Moon et al. constructed a two input-based AND gate in *E. coli* for creating a more complex programme (Moon et al. 2012). A number of biologic gates have also been designed and constructed in bacteria to mammals. These engineered biologic gates have a wide range of applications in biomedical, therapeutic, reprogramming and also biological computation.

1.4 Advances in Gene Cloning and Genome Assembly Methods

In synthetic biology, gene cloning and expression are key factors that always constrain and limit the proceedings of the experiment. Synthetic gene circuits need to be fine-tuned and optimized in order to attain predictive function. Therefore, apart from the routine gene cloning techniques including sticky end, blunt end and T-A cloning, the recently established gene cloning techniques to accelerate synthetic biology research have been described briefly.

1.4.1 Ligase-Independent Cloning Technique

Ligase-independent cloning (LIC) is restriction enzyme-free cloning technique, which is simple, fast and easy to use it. It uses T4 DNA polymerase for creating a 10–15 bases single overhang in a vector which allows the insert to join easily. LIC was initially used for inter-Alu fragment gene cloning from hybrid and human cells (Aslanidis and de Jong 1990; Haun et al. 1992). Similarly, sequence and ligation-independent cloning (SLIC) has been developed based on homologous recombination for multiple fragments cloning in a single tube reaction (Li and Elledge 2007). In-fusion PCR cloning developed by Clontech Laboratories, USA, is yet another restriction enzyme-free cloning technique, which is simple, rapid and efficient for assembling multiple genes together in a single reaction tube. Primers are designed that contain 15–20 bp homology with the corresponding genes. Insert and plasmid is incubated together for 15 min at 50 °C in the presence of an in-fusion enzyme that can generate overhang and get annealed. This reaction mixture can be directly transformed into the competent cell and clones can be screened based on the marker (Sleight et al. 2010).

1.4.2 Gibson Assembly

Gibson assembly developed by Gibson et al. (2009) is a simple, rapid and efficient technique in synthetic biology. It can be performed in a single tube using three enzymes including T5 exonuclease (chews back DNA), DNA polymerase (adds bases to fill gaps) and Taq DNA ligase (seals nick). Insert and plasmid need to contain 20–40 bases homology, which can be mixed with Gibson master mix and incubated at 50 °C for 30–60 min. Then, this reaction mix can directly be transformed into competent cells and screened for recombinant clones. Currently, Gibson assembly is commercially available in the market and is used for constructing complex circuits (Gibson et al. 2010).

1.5 CRISPR-Cas Systems

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) are found within the genome of bacteria and archaea. It is an RNA-mediated immune system of prokaryotes that protects them from infection of bacteriophage and plasmids (Barrangou et al. 2007; Horvath and Barrangou 2010; Barrangou and Marraffini 2014; Bhattacharjee et al. 2019, 2020). CRISPR-Cas systems are divided into classes I and II, six types and 18 subtypes. All the CRISPR systems contain different types of targeted DNA, RNA or both along with signature proteins (Makarova et al. 2015; Hille et al. 2018).

Currently, the type II CRISPR-Cas9 is widely being used in synthetic biology, metabolic engineering and biotechnology. It has been used for genome editing and regulation in a wide range of organisms (Jinek et al. 2012; Mali et al. 2013; Cong et al. 2013; Jiang et al. 2013; DiCarlo et al. 2013; Bikard et al. 2014; Jakočiūnas et al. 2015). It is a simple, rapid and sensitive tool that requires expression of Cas9 protein, single-guide RNA and PAM sequences at the target region. The Cas9-sgRNA complex binds to the target region and generates a double-stranded break, which later repairs by either the NHEJ or HDR pathway. In this way, genome modification can be executed (Singh et al. 2017). CRISPR-Cas9 system has been used for genome editing of *Drosophila* (Ren et al. 2014; Port et al. 2014), zebrafish (Hwang et al. 2013; Hisano et al. 2015; Liu et al. 2017; Cornet et al. 2018), removal of viruses including human papillomavirus (Kennedy et al. 2014), hepatitis B virus (Lin et al. 2014; Zhen et al. 2015), latent Epstein–Barr virus (Wang and Quake 2014), HIV-1 (Ebina et al. 2013; Zhu et al. 2015), repairing of defective genes (Long et al. 2014; Nelson et al. 2016; Guan et al. 2016) and many more.

By mutating two active regions of the Cas9 moiety, scientists have created yet another variant of Cas9 called the dead Cas9, which has lost its ability to generate a DSB on DNA but it retains binding ability of target DNA. This is known as CRISPR interference (CRISPRi), which has been extensively used for gene regulation, epigenetic modifications, high-throughput screening and imaging genomic loci in wide range of organisms (Qi et al. 2013; Bikard et al. 2013; Chen et al. 2013; Gilbert et al. 2013; Ma et al. 2015).

The type VI class 2, Cas13a has been identified and used for development of simple, rapid and ultrasensitive tool for early pathogens detection. This is attributed as Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK). SHERLOCK has been used for ultrasensitive detection up to attomolar concentration of Zika virus, Dengue virus, *E. coli* and *Pseudomonas aeruginosa* and to discriminate antibiotic-resistant *Klebsiella pneumoniae* as well as mutations in cancer (Gootenberg et al. 2017, 2018; Myhrvold et al. 2018; Khambhati et al. 2019a). CRISPR-based platform can be extended in many more serious pathogens for timely diagnosis in order to properly treat diseases.

1.6 Cell-Free Protein Synthesis System

Cell-free protein synthesis (CFPS) system is used in synthetic biology and metabolic engineering for biomedical, therapeutic and industrial applications. It is membraneless simple, fast and high-throughput tool for production of toxic products, chemicals, assembly of bacteriophages, biosynthetic pathways, incorporation of toxic non-natural amino acids in protein for tagging or improving the potency of protein and many more. CFPS systems, also known as cell-free transcription-translation (TX-TL) systems or cell-free expression systems, have emerged as a powerful tool for performing research without the use of living organisms. It can be used for direct control of transcription, translation, post-translational modification and metabolism in an open source (Carlson et al. 2012; Moore et al. 2017; Khambhati et al. 2019b).

It requires RNA polymerase, energy sources, cofactors, substrates, DNA or plasmid and translational machinery including ribosome, translation factors and tRNA synthases. CFPS system is commercially available and used for a number of synthetic biology applications. CFPS has been used for incorporation of toxic amino acids for production of proteins such as canavanine (Worst et al. 2015), production of therapeutics (Zawada et al. 2011), assembly of bacteriophage (Shin et al. 2012), building of orthogonal genetic codes (Des Soye et al. 2015; Chemla et al. 2015), diagnostic of Dengue, Zika and Ebola viruses (Pardee et al. 2014, 2016; Gootenberg et al. 2017), testing of synthetic gene networks (Pardee et al. 2014; Takahashi et al. 2015) and many more. CFPS system has the potential to up-regulate and accelerate synthetic biology research in the near future towards biomedical, therapeutic, industrial and biotechnological applications.

1.7 Microfluidics

Microfluidics is a rapidly growing and powerful tool with a number of applications including PCR, cloning, diagnostic and monitoring of single-cell dynamics. It has the potential to reduce cost and reagent consumption. Microfluidics chips are prepared using PDMS (polydimethylsiloxane) and curing reagent, which is a transparent polymer. Microfluidics is getting popular in synthetic biology and has gained a lot of scientific attention. It has been used for continuous measurement of synthetic network by continuous supplying of growth media to the cells (Balagaddé et al. 2005; Stricker et al. 2008).

Microfluidics was used for mRNA extraction from a single cell for synthesis of cDNA followed by PCR. It can allow us to accelerate error-free high-throughput experiment (Marcus et al. 2006; Maerkl and Quake 2007). Currently, microfluidics platform is being used for testing and screening of high-value chemicals and therapeutics and in synthetic biology experiments at single-cell measurement of cellular behaviour (Stricker et al. 2008; Danino et al. 2010; Shen et al. 2015; Rodrigo et al. 2017). A number of companies are developing microfluidics chips for high-throughput detection of pathogens for fitting treatment. Currently, the price of chips

is relatively high because of requisite raw materials and expertise, but in the near future, it is expected to go down due to high market demand and customized chip development.

1.8 Conclusion and Future Remarks

Synthetic biology has the potential to reduce the cost of high-value biomolecules for human and animal uses. Synthetic promoter libraries have been used for a wide spectrum of product production in a tunable way. Small genetic parts can be used for accelerating the construction of biological networks and biosynthetic pathways. Synthetic devices and circuits can be further used for digital control or reprogramming of cellular machinery for tight and tunable gene expression towards healthy cell growth and high production. Currently, burgeoning synthetic biology technologies are on high priority. CRISPR-Cas9 allows us for genome editing towards biotechnological, therapeutic and biomedical applications. Cell-free protein synthesis and microfluidics can be a useful and powerful tool for accelerating synthetic biology research towards finding a solution for food, health and energy.

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Current Progress in Synthetic Genetic Networks

2

Amir Pandi and Heykel Trabelsi

Abstract

Synthetic genetic networks are the main and most-studied area of synthetic biology. Biological networks or circuits provide modular and scalable tools to design-build-test synthetic biological systems for medical, environmental, and industrial applications. This chapter focuses on introducing and discussing the recent progress in design and application of such devices. This chapter starts with the classification of synthetic genetic networks and the role of each and their pros and cons. Then, recent applications of digital/analog genetic/metabolic circuits are presented in three groups of smart therapeutics, diagnosis, and metabolic engineering. Finally, tools and methods of implementing different classes of synthetic gene circuits are presented with covering the majority of the developed methodologies so far. This chapter brings a complete introduction to synthetic genetic circuits and their recent advances to the audience who aim to get familiar with this fast-growing technology.

Keywords

Synthetic genetic networks · Genetic circuits · Digital and analog computation · Gene and metabolic circuits · Genetic circuits applications · Genetic circuits implementation

2.1 Introduction to Synthetic Genetic Networks

Synthetic genetic networks or gene circuits are advanced tools to implement synthetic biological systems for a variety of medical, industrial, and environmental applications (Brophy and Voigt 2014; Purnick and Weiss 2009). The aim of

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these devices is to engineer biological systems receiving multiple inputs such as nutrition and signals, compute them through its artificial networks, and actuate the integrated outputs responding to the environment (Purcell and Lu 2014). The term "computation" which is used in this context means computing biological signals through a synthetic network composed of biological components. As one of the main tools in the field of synthetic biology, genetic networks have been synthesized for the development of (1) biosensors for detection of biomarkers or pollutants, (2) screening or engineering the dynamic regulation of metabolic pathways, or (3) smart therapeutics (Brophy and Voigt 2014).

Inspired by electrical engineering, several synthetic biological devices have been synthesized since the emergence of the field of synthetic biology (Selberg et al. 2018). These devices mimic the digital or analog computation paradigm by applying different classes of cellular components (Purcell and Lu 2014). To name some of the approaches, the synthetic genetic networks implemented so far consist of single or multilayer logic gates (Nielsen et al. 2016; Guiziou et al. 2019), oscillators (Rosier and de Greef 2015), amplifiers (Bonnet et al. 2013; Zeng et al. 2018; Wang et al. 2015), switches (Gardner et al. 2000) and memory devices (Bonnet et al. 2012; Farzadfard and Lu 2014), toehold circuits (Green et al. 2014), CRISPR circuits (Bikard et al. 2013; Nielsen and Voigt 2014), metabolic logic gates (Courbet et al. 2018), and metabolic perceptrons and classifiers (Pandi et al. 2019).

Thanks to the substantial efforts by the synthetic biology community, standard and modular methodologies have been established to engineer different above-mentioned devices (Kelwick et al. 2014; Marchisio 2014). Computer-assisted and bioinformatic tools are the accessory tools through which these methodologies can be generated (Nowogrodzki 2018; MacDonald et al. 2011). These approaches employ cellular components, from gene expression regulators to posttranslational level and metabolic enzymes.

The standardized and modular strategies have led the field to very advanced achievements in building sophisticated genetic networks. However, the next generation of synthetic cellular networks needs to focus on the integration of different approaches enabling hybrid analog–digital computation by the use of several types of cellular machineries (Brophy and Voigt 2014; Goñi-Moreno and Nikel 2019). The integration strategies and cross-species approaches (Kushwaha and Salis 2015; Xiao et al. 2017) empower the potential of artificial genetic networks to be applied for several applications in diverse living species and cell-free systems.

2.2 Classification of Synthetic Genetic Networks

Designed biological circuits can be categorized based on the computational approach that they lay on, digital and analog (Purcell and Lu 2014), or based on the biological functionality of the genes they employ, regulatory and metabolic (Goñi-Moreno and Nikel 2019) (top panel in Fig. 2.1).

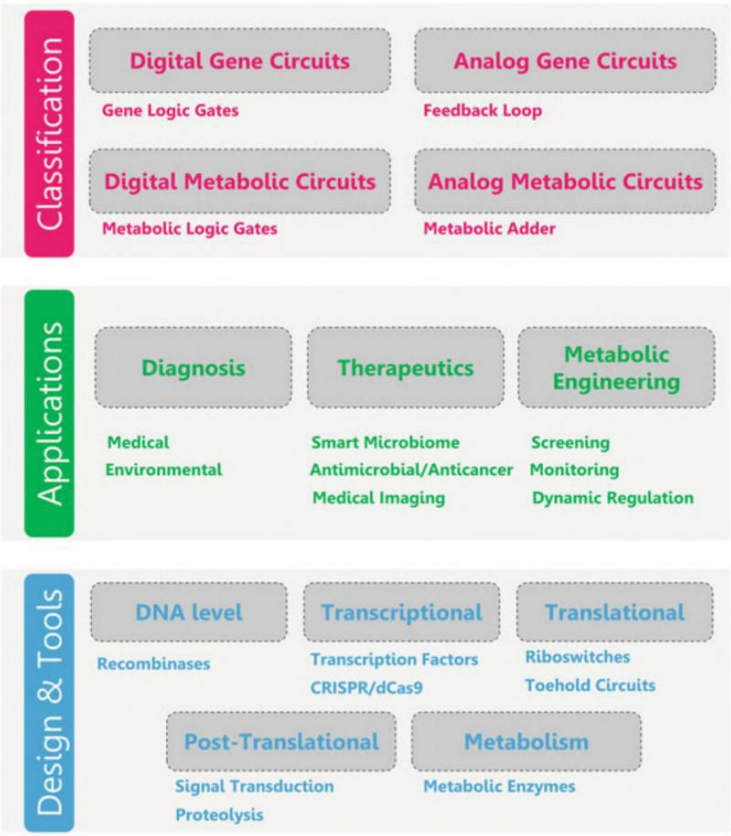

Fig. 2.1 An overview of the whole chapter in a schematic representation. This chapter is presented in three parts to cover the current progress in synthetic genetic networks. *Top panel:* Classification of the synthetic genetic networks in four classes depending on the computation approach that they rely on, digital/analog in gene expression/metabolic layer. *Middle panel:* Applications of synthetic genetic networks in diagnosis, smart therapeutics, and metabolic engineering. *Bottom panel:* Designing strategies and tools to implement synthetic genetic networks using different biological components of the cell in DNA level, transcriptional and translational, posttranslational, and metabolic components. The designed biological networks then will be implemented in eukaryotic/prokaryotic cells or in cell-free systems

2.2.1 Digital and Analog Gene Circuits

As in the electrical circuits engineering, biological gene circuits can perform digital or analog computation depending on their design (Purcell and Lu 2014). So far, the majority of the implementation of genetic networks has focused on digital computation, as it is more standardizable using well-established tools such as Verilog (Nielsen et al. 2016). The digital gene implementation follows the logic functions. For instance, if A and B both are needed to generate an output (either a

reporter in medical or environmental diagnosis or expression of a functional gene), this is an AND logic. The OR gate is the logic gate output of which is active (ON) when two or even of the conditions/inputs of A or B are "ON."

So far, several digital gene circuits have been implemented using different cell components at the level of DNA (Guiziou et al. 2018, 2019; Engelen et al. 2017; Genot et al. 2011), transcription (Nielsen et al. 2016; Kim et al. 2019; Gander et al. 2017; Buchler et al. 2003; Bradley et al. 2016), and RNA (Green et al. 2014; Deng et al. 2014; Martini et al. 2015; Kim et al. 2018; Wu et al. 2019), as well as the protein level (Gao et al. 2018; Fink et al. 2019; Razavi et al. 2014; Fernandez-Rodriguez and Voigt 2016). In cases where there are more than two inputs with complex relationships, their behavior cannot be captured as easy as for simple AND or OR gates. This is a point where computational tools can be used to introduce a complex logic circuit in which the relationship between inputs and output(s) can be computed through multilayer digital gene networks (Nielsen et al. 2016).

Since most of the synthetic biocircuits have been built in the gene expression level, the digital-like behavior (ON/OFF) in the gene expression system has compatibilized the digital computation approach. Therefore, a number of successful digital computation approaches have been introduced during the past few years. However, digital-like behavior is not the only using which cells perform computation. A considerable contribution of biological computation in living cells takes place in an analog manner where the continuous concentrations of the cellular components define the phenotype, not their presence or absence (ON/OFF) (Purcell and Lu 2014; Sauro and Kim 2013).

The substantial contribution of the analog computation in living systems brings the mindset of implementing analog gene circuits. In electronics, analog circuits consume lower energy and require fewer parts to function. In the same way, analog gene networks save cellular energy and avoid the burden (Daniel et al. 2011; Sarpeshkar 2014). This valuable advantage promotes the system orthogonality by using fewer synthetic parts.

There have been only a few studies investigating the analog computation in living systems (Daniel et al. 2011, 2013). Daniel et al. (2013) have developed synthetic analog computation in living cells using a feedback loop inspired by the feedback loop of operational amplifiers in analog electronics. In this study, a simple transcriptional circuit has been designed in a construct such that: (1) in a low-copy plasmid, the transcription factor (TF) is expressed under its cognate promoter controlled by the externally added inducer, and (2) in a high-copy plasmid, the cognate promoter expresses a fluorescent protein reporting the concentration of the ligand. This design alleviates the saturation of the TF (through the feedback loop in the low-copy plasmid that produces more TF and delays its saturation) and the saturation of the cognate promoter (through pulling the flux of transcription to the responsive promoter in the high-copy plasmid). This construction linearizes the dose response of the circuit from a digital-like behavior to an analog behavior (Daniel et al. 2013).

2.2.2 Digital and Analog Metabolic Circuits

Although the analog behavior is one of the characteristics of living cells, it is difficult to implement analog gene circuits which naturally show a digital-like behavior (ON/OFF). However, using other biological mechanisms such as metabolism is more compatible to implement analog computation (Pandi et al. 2019). In this direction, an analog metabolic computation approach has been recently established that is using metabolic enzymes to perform analog biocomputation (Pandi et al. 2019). In this study, metabolic pathways were designed using computer-aided tools (Delépine et al. 2016, 2018) and were implemented in whole-cell and cell-free systems. Multiple metabolic transducers were implemented that are metabolic pathways composed of one or more enzymes transforming a metabolite into another, a product that can be sensed using transcriptional or translational regulators (Koch et al. 2018). By combining metabolic transducers, analog adders were built in both whole-cell and cell-free systems. Cell-free systems enabled performing more complex computations by tightly controlling the amount of DNA of the circuit added to the reaction. This advantage of the cell-free system and high adjustability, along with rapid characterization and possibility of mixing multiple genes at different concentrations, enabled the development of four-input classifiers. In the classifiers, a metabolic perceptron receives four input metabolites and converts them into a common metabolite by model-computed concentrations of their associated enzyme DNA and finally reported through a gene circuit actuator. The metabolic perceptron was inspired by a perceptron algorithm invented in 1960s to mimic human neural systems in information processing and decision-making (Rosenblatt 1958). Since then, perceptrons have become the building blocks of several neural computing and deep learning algorithms (Haykin 2011).

Digital metabolic circuits are other types of biological computation using artificial networks that apply metabolic enzymes to build metabolic logic gates. A number of metabolic logic gates including AND, OR, XOR, NAND, and their combination in order to build complex circuits have been developed (Courbet et al. 2018; Katz 2017). In most of cases, dealing with cellular cofactors and coenzymes for the signal processing makes the application of digital metabolic circuits limited in whole-cell systems and biological samples. Nevertheless, depending on the case, they have a valuable potential to build synthetic genetic network.

2.3 Applications of Synthetic Genetic Networks

The following are the applications of synthetic gene networks in variety of aspects (middle panel in Fig. 2.1).

2.3.1 Diagnosis

One of the main application of synthetic genetic networks is to develop diagnostic devices (Slomovic et al. 2015). In this context, gene and metabolic circuits have been used to build various genetic networks. For instance, a simple genetic network comprising the quorum-sensing regulatory system of *Pseudomonas aeruginosa* has been engineered in the cell-free system to detect this pathogen in clinical samples (Wen et al. 2017). In a different approach, paper-based cell-free toehold circuits built using RNA switches were utilized to sense RNAs for Zika virus (Pardee et al. 2016), Ebola virus (Pardee et al. 2014), or gut microbiome bacteria in fecal samples (Takahashi et al. 2018). The CRISPR machinery also has been adapted to detect DNA and RNA of viruses and bacterial pathogens in vitro using strategies called SHERLOCK (Gootenberg et al. 2017, 2018), DETECTR (Chen et al. 2018a), and HOLMES (Li et al. 2018). In another approach, applying gene switches built by recombinases in vivo enabled detection of glucose in diabetic clinical samples (Courbet et al. 2015). Using a radically different approach, metabolic enzymes have enabled increasing the number of detectable small molecules. In this work, by plugging metabolic enzyme, a molecule is converted to another which is sensible through transcriptional regulator (Voyvodic et al. 2019). The authors have introduced a modular tool to implement and optimize cell-free biosensors and used this strategy to sense benzoic acid in beverages, as well as hippuric acid and cocaine in clinical samples (Voyvodic et al. 2019).

Biological circuits have also been used for the detection of environmental samples. In a recent study, the authors developed a strategy to build an optimized cell-based biosensor to detect toxic pollutants in environmental samples (Wan et al. 2019). They engineered multilayer amplifiers enabling a high signal-to-noise ratio detection through the transcriptional regulatory system. This promising approach provided facilities to build biosensors for arsenic and mercury with a very high fold-change response to the inducers. Thus, they were able to introduce a strategy to engineer sophisticated gene networks for in vivo diagnosis (Wan et al. 2019). In another work related to environmental diagnosis, a recent attempt used RNA output sensors activated by ligand induction (ROSALIND) in the cell-free system to detect pollutants in water (Alam et al. 2019). ROSALIND consists of three components: highly processive RNA polymerases, allosteric transcription factors, and synthetic DNA transcription templates. These elements together have provided the modular detection of a variety of water pollutants such as antibiotics, toxic small molecules, and metals (Alam et al. 2019).

2.3.2 Therapeutics

Synthetic biological networks provide a new generation of therapeutics called smart therapeutics. One of the earliest attempts was designing a synthetic mammalian circuit to maintain uric acid homeostasis (Kemmer et al. 2010). This synthetic gene

network consists of a uric acid sensor triggering the secretion of a urate oxidase enzyme which eliminates uric acid. In mice harboring this device, the synthetic circuit decreased the amount of blood urate and reduced uric acid crystal in the kidney (Kemmer et al. 2010). In a recent study, Isabella et al. (2018) provided a smart alternative for the protein-restricted diet for phenylketonuria, a genetic-metabolic disorder in metabolizing phenylalanine. For this purpose, the authors have engineered *Escherichia coli* Nissle to actuate phenylalanine metabolizing enzymes responding to anoxic conditions in the mammalian gut (Isabella et al. 2018). Designer circuits can be applied in the development of antimicrobials (Bikard et al. 2014; Bikard and Barrangou 2017), anticancers (Ding et al. 2015; Nissim et al. 2017; Liu et al. 2014; Prindle et al. 2012), microbiome editing (Ramachandran and Bikard 2019; Piraner et al. 2017), or medical imaging (Piraner et al. 2017; Farhadi et al. 2019; Lu et al. 2018; Bourdeau et al. 2018).

2.3.3 Metabolic Engineering

Utilizing synthetic gene networks for bioproduction application has rapidly grown during the last years. Genetic sensors have been applied in the field of metabolic/enzymatic engineering for (1) screening the enzymes and pathways, (2) monitoring the evolution of the products, and (3) dynamically regulating the enzymes or metabolites level (Liu et al. 2015, 2017; de Frias et al. 2018; Rogers et al. 2016; Koch et al. 2019; Venayak et al. 2015). This strategy substantially increases the speed of the design-build-test cycle in improving metabolic pathways and enzymes or exploring novel synthetic enzymes and pathways.

Synthetic gene circuits have shown an increasing potential to engineer dynamic regulation, regulatory cascades to dynamically control and improve the evolution of a product. The dynamic regulation improves the product yield either through directing metabolic fluxes into the direction of the desired product or by adjusting the expression of the enzymes and amount of intermediates as well as preventing the accumulation of a toxic intermediate (Venayak et al. 2015). One of the interests regarding metabolic engineering application is coupling cellular growth and product evolution, which can improve the production as it keeps a balance or controllable switch between growth and target production (Williams et al. 2015; Anesiadis et al. 2013; Gupta et al. 2017; He et al. 2017; Kim et al. 2017; Shong and Collins 2014). This coupling can be implemented using natural (native of the host cell) or synthetic quorum-sensing network regulating the expression of the enzymes in the metabolic pathway.

2.4 Design and Tools

The following are different cellular components providing the implementation of synthetic gene networks (bottom panel in Fig. 2.1).

2.4.1 Transcriptional Level

Undoubtedly, transcriptional regulators are the most studied tools to implement synthetic genetic networks for prokaryotic and eukaryotic applications (Nielsen et al. 2016; Khalil et al. 2012). Since transcriptional regulators are directly in contact with gene expression and DNA, and a number of these regulators are widely studied and characterized, utilizing them has become more scalable and programmable. In this direction, an enormous number of biological parts consisting of promoters, RBSs, terminators, and regulatory transcription factors have been characterized. These parts are characterized natural sequences, or they are synthetic sequences providing the orthogonality, which is of very crucial aspects in developing synthetic biological networks (Stanton et al. 2014; Chen et al. 2018b; Zong et al. 2017; Rudge et al. 2016). Moreover, the community has introduced methodologies for building, automizing, optimizing, and integrating various devices from simple gene networks to complex multilayer circuits (Nielsen et al. 2016; Zong et al. 2017; Rudge et al. 2016; Otero-Muras et al. 2016; Boada et al. 2019). Nielsen et al. have developed a tool called Cello using which complex relationships between a number of inputs could be computed through proposed circuits and the DNA sequence associated to those circuits is also generated (Nielsen et al. 2016).

Apart from transcriptional factors (including activators or repressors), CRISPR/dCas9 also have shown promising characteristics for synthesizing modular transcriptional regulators (Bikard et al. 2013; La Russa and Qi 2015; Kundert et al. 2019). The mutant version of Cas9 or other Cas nucleases which lack the nuclease activity but still maintain the specific binding through their designed gRNA can be used to target anywhere in the genome through highly specific binding of the gRNA-dCas9 complex to the target DNA (Rousset et al. 2018). By targeting desired sequences of the genome, gRNAs can simultaneously block several points in the genome acting as transcriptional repressors (Vigouroux et al. 2018). The CRISPR/dCas9 also can be fused to other proteins such as activators to regulate the activation (Dong et al. 2018; Matharu et al. 2019). There are computational and experimental tools to design such devices by tuning the level of binding through the complementarity of the gRNA and the target sequence (Vigouroux et al. 2018).

2.4.2 Translational Level

Translational regulators are components that control the translation of mRNA through the ribosome. RNA genetic switches or riboswitches are tools that regulate the gene expression in response to their input (Karagiannis et al. 2016; Robinson et al. 2016). However, some riboswitches function in the transcriptional processes such as in termination of the transportation (Serganov and Nudler 2013; Re 2017). Riboswitches consist of an aptamer (sensing) domain and an actuator (regulating) domain for binding to an input molecule and control the gene expression, respectively (Karagiannis et al. 2016; Wittmann and Suess 2012). The binding of an input

2.5 Perspectives

Synthetic gene networks are sophisticated tools to provide facilities in engineering biology. Since the dawn of synthetic biology, modular biological parts and methods have been increasingly equipped scientists toward a future in which cells and biological systems can be engineered for medical, environmental, and industrial applications (Brophy and Voigt 2014). The advances made so far have applied from genetic central dogma level to posttranslational, signal transduction, and metabolic enzymes in different prokaryotic cells, eukaryotic cells, and cell-free systems. Moreover, the experimental and computational approaches provide a potential perspective for the construction of next-generation synthetic biological networks. The next generation of such circuits is the integration of different tools and approaches for mix-hybrid gene circuit implementation (Brophy and Voigt 2014; Purcell and Lu 2014; Pandi et al. 2019; Goñi-Moreno and Nikel 2019; Rubens et al. 2016).

Decreasing the cost of the chemical synthesis and the sequencing of DNA provides a more affordable DNA reading and writing (sequencing and gene synthesis, respectively). Hence, the field of synthetic biology will be rapidly advancing through high-throughput experiments exploring the potential of the synthetic version of the code of life, DNA. The enormous available data of biological datasets and the future data that will be generated could be the training datasets for machine learning and deep learning exploration on these data to learn more and more about biology as well as to predict the future genetic networks (Camacho et al. 2018).

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Current Progress and Limitations in the Design, Construction, and Characterization of Synthetic Parts

3

Vinuselvi Ponraj

Abstract

The field of synthetic biology has grown multidimensionally that we now have a large collection of interchangeable input and output modules. Design and construction of new synthetic parts are no longer a challenging task. However, the performance of a synthetic part generally has a lower accuracy than the corresponding natural system. Characterization of synthetic parts poses the actual drawback as most of the times these modules are studied in isolation and are expected to produce the same result when put together as a part of a large circuit or transferred from one chassis organism to the other. It becomes necessary to develop robust mathematical models and conquer the quantitative aspect of the synthetic parts which could then help improve the performance of the synthetic circuit. In this review, we brief the status and limitations of the design, construction, and characterization of synthetic parts and use "oscillators" as a case study to emphasize the betterment of the abstraction of the synthetic part either in isolation or in combination. The simple "oscillator circuit" was improved over a decade from being a barely visible oscillator to the one that can oscillate for up to 15 generations. The oscillator circuit forms a stand-alone example for the need for the harmony of stochastic chemistry and synthetic biology to achieve the long-standing goal of well-characterized genetic parts analogous to the electronic circuit.

Keywords

Synthetic parts · Quantitative biology · Mathematical models · Oscillator · Decoys

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3.1 Introduction

Realizing the presence of logical operations in the biological circuits is the biggest motivation for the birth of new field of science: "*Synthetic Biology*." The field was inspired by the promise to design well-characterized biological parts that when put together within a living cell could produce favorable and highly predictable output analogs to electronic circuits. Thanks to the efforts of the scientific community across the globe, there is a vast collection of interchangeable synthetic biology toolkits for use in wide range of organisms including both prokaryotes and eukaryotes. In addition, genomic sequencing techniques are gearing up the growth of synthetic parts with the revolutionary collection of sequence data from the depths of oceans to the heights of mountains. Genome engineering and gene cloning techniques (reviewed in Chap. 17 of this book) have now made it possible to make newer synthetic parts within few hours or assembly even the whole genome of an organism easily. Synthetic parts have taken a tremendous improvement from being based on simple DNA-binding transcription factors to those involving complex regulations mediated by sRNA and ligand-controlled riboswitches. Synthetic biology finds its grounding needs in several fields including but not limited to the production of cheaper therapeutics, biofuels, discovery of new class of antibiotics, tissue engineering, targeted drug delivery in the tissue of interest, in bioremediation, etc. (Khalil et al. 2012; Park et al. 2019; Khalil and Collins 2010).

The robust nature of the living systems combined with our limited ability to understand the interplay between gene expression pathways is a potent bottleneck for the rapid growth of biologically inspired synthetic parts. It remains a bold ambition to make independent biological parts to work analogs to electronic circuitry where individual parts' output is accurately predicted. For instance, the technical "0" in an electronic device means the complete absence of input (meaning they have a distinct ON/OFF states) whereas in a biological part "0" translates to the absence of a signal while still experiencing a basal metabolic rate (meaning they produce significant signal in OFF state). In addition to the basal metabolic rate there is a stochastic difference (both intrinsic to the cells and extrinsic from the environment) in independent cells which when put together results in unstable initial (and final) states of the biological parts translated as a technical "0." Inspired by the challenges in the theoretical predictions of the output of a synthetic part, newer mathematical frameworks have been developed to assist the characterization of synthetic parts (Brophy and Voigt 2014; Xiang et al. 2018; Xia et al. 2019). In this review, we highlight the importance of the interplay of synthetic and systems biology to improve the design and characterization of existing biological parts.

3.2 Design of Synthetic Parts

3.2.1 Input Module

A core set of three repressors (CI, TetR, and LacI) were used to design several of the initial group of synthetic circuits. Combination of inputs has increased tremendously: LacI orthologs, TetR orthologs (Stanton et al. 2014), riboswitches, light-induced promoter systems (Levskaya et al. 2005), zinc-finger transcription factors, transcription activator like effectors, several classes of constitute and inducible promoters, T7 RNAP, quorum sensing system, recombinases, etc. Several synthetic circuits have been designed and implemented using a combination of these input modules. Few of the synthetic circuits are listed in Table 3.1. The intrinsic feature of any input module is the proteins involved should be orthogonal, i.e., they cannot cross-talk with each other and with the existing biological machine.

While there are several successful attempts to develop synthetic circuits there are few potential issues to be taken care while choosing the right combination of the input modules. Multiple input module can cause acute toxicity in the cell especially if they are expressed at high copy numbers from the plasmid. It becomes too difficult to counteract the leaky level and steady state expression of multiple inputs. The large repository of input modules challenges the selection of right components for a given synthetic circuit. Components that work in larger dynamic range and with distinct OFF state expression are the high choice for most circuit design. However, there are only handful of components that satisfy these criteria. Computational tools like RBS Designer (Reeve et al. 2014) and promoter analyzer can come in handy to improve the existing array of input module to achieve larger dynamic range, lesser leaky level, and higher threshold/steady state values.

Table 3.1 Examples of common synthetic parts

Synthetic parts	Features	Reference
Pulse generator	Feed forward motif causes spatiotemporal gene expression in bacterial community	Basu et al. (2004)
Bistable switches	Genetic switches with two stable steady states	Lebar et al. (2014)
Oscillators	Circular negative feedback loops function as a biological clock	Elowitz and Leibler (2000)
Logic gates	AND, NOT, OR, and almost any logical operation	Stanton et al. (2014)
Edge detection	Used to detect boundaries of an object in an image	Tabor et al. (2009)
Cancer classifier circuit	Selectively triggers apoptosis in HeLa cells using microRNAs as input module	Xie et al. (2011)
Whole cell biosensors	Visualize and diagnose cancer metastasis	Danino et al. (2015)
Drug-induced kill switches	T cell therapies	Budde et al. (2013)

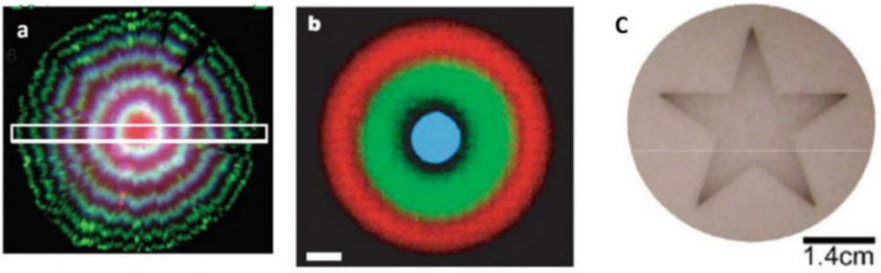

Fig. 3.1 Examples of achievements in different synthetic circuits. A. Synchronized oscillation seen in a colony of E. coli (Potvin-Trottier et al. 2016). B. Pattern formation in *E. coli* (Basu et al. 2005). C. Edge detection in *E. coli* (Tabor et al. 2009)

Several well-defined synthetic parts are in use today and are named based on the inspiration from the analogous electronic devices. Genetic switches work based on the principle of logical NOR gate with two stable steady states and a genetic memory of the current state. Oscillators use circular negative feedback loops to produce an alternating sinusoidal output. Oscillatory circuits are so common form of natural circuits with circadian rhythm patterns being the well-known example. Few of the examples commonly used synthetic parts and its characteristic features are listed in Table 3.1 and in Fig. 3.1.

3.2.2 Output Reporters

Important aspect of the design of a synthetic part is the presence of a readable output. Widely available readable output includes fluorescence reporters (Suel 2011), luminescence, enzymatic assays, and cell fitness. Fluorescence outputs are a popular choice because it is available in different colors and works well in most organisms. Drawbacks in using fluorescent output are as follows: fluorescence signal across different labs is incomparable because it depends largely on the optical property of the instrument (microscope, plate readers, FACS) used to read the signal, cross talk between fluorescence protein is highly troublesome when more than one fluorescent reporter is used in the same circuit, fluorescence is sensitive to changes in oxygen level, pH, and temperature. Luminescence on the other hand is robust reporter but involves ATP as source of energy and is not an attractive choice among scientist. Enzymatic assays cannot be used to obtain a dynamic profile of the performance of the synthetic part. Most synthetic parts do not impose fitness defects in the organism of choice and hence, cellular fitness is a less common output readout. Surprisingly limited availability of the reporter choice is a biggest limitation in obtaining characteristic details of complex synthetic parts.

3.3 Measurement Techniques

The sensitivity, robustness, and multiplexing of the measurement techniques have made significant progress in the recent years. To begin with only bulk measurements of the output readout were possible which failed to address the actual stochasticity in the data. Advancements in fluorescence microscopy and fluorescence activated cell sorting (FACS) helped look at individual cell's behavior. With the advent of microfluidics and mother machine (Okumus et al. 2018; Potvin-Trottier et al. 2018) it is now possible to monitor the same cell for several generations and even ask cells to process dynamic stimulus. We are starting to realize that most outputs of the bulk measurements are the underpredictions of the stochastic cell-to-cell variations rather than the actual underperformance of the new devised synthetic part. The faster growth of the field of stochastic chemistry and biological noise helps in deeper ability to model the response and behavior of the gene expression. Harmony between the characterization of the synthetic parts and the stochastic chemistry is important to achieve a repository of well-defined genetic parts.

3.4 Case Study: Progress and Limitations in Oscillator Circuit

A simple oscillatory circuit is built by three genes inhibiting each other's production in a single loop (A⊣B⊣C⊣A). TetR, CI, and LacI are expressed on plasmids to achieve oscillation in *E. coli*. However, in the initial attempt a noisy oscillation that lasted for only a few generations was observed (Elowitz and Leibler 2000). The same system was proven to be an effective harmonic oscillator in cell-free extracts (Niederholtmeyer et al. 2015). In another attempt oscillation was tried using the light-inducible promoter system. Long lifetime of the fluorescent proteins resulted in step-wise increase in the output signal instead of the expected sinusoidal harmonic oscillator (Lee et al. 2013). The circuit constructed by Elowitz and Leibler 2000 was later revisited and tested on a microfluidic platform and was shown to be oscillating more uniformly rather than the reported noisy oscillation (Danino et al. 2010). Soon after the brainstorming inputs from stochastic theory it was realized that the three input modules and the output modules would perform better when expressed from a single low copy plasmid rather than two separate plasmids for the input and the output modules as in the original design. Having more than one plasmid type in a cell will raise a stochastic competition between the two plasmid types resulting in greater cell-to-cell variation. Combining the input and output modules in a single plasmid did improve the performance of the oscillators but instead of the expected harmonic oscillatory response, a relaxed oscillation was observed. Again, based on the stochastic theory this could be simple because one of the three proteins (TetR) had a low threshold value and hence the other two proteins (LacI and CI) overrule the circuit for a while until the third protein was sufficiently accumulated. To overcome this TetR threshold was increased by simply adding competing binding sites for TetR on a plasmid theoretically raising the threshold

Recent Progress in DNA Parts Standardization and Characterization

4

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Abstract

Synthetic Biology, which is the 'engineering of biology', depends on well-characterized and standard genetic elements that can be assembled together to construct complex, multi-component genetic circuits that function in a context-independent and predictable fashion. Here, we describe widely used standards employed for constructing DNA parts, and also discuss key assembly methods that can be used to build genetic devices starting from standard parts. Methods used to characterize parts and devices are discussed, and the finally, the need for 'functional standards' is outlined.

Keywords

Part standards · Assembly methods · Genetic devices · Synthetic biology

4.1 Introduction

The implementation of synthetic biology, which is an amalgam of molecular biology and engineering principles, depends on well-characterized genetic elements (e.g., coding sequences (CDS), promoters, ribosome-binding sites (RBS) and transcription terminators) that can be assembled together in different combinations to form genetic circuits that can then be tested. In line with the engineering paradigm that informs the discipline of synthetic biology, there have been attempts to define standards that genetic elements (or 'parts') can conform to, allowing them to be easily joined to each other like nuts and bolts.

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This chapter will review the different standards that have been proposed for the construction of genetic parts, and their relative advantages and disadvantages. As standardization is aimed at ease of assembly, the various assembly techniques that are employed to join parts are also discussed. Approaches used to characterize parts and devices are described. The chapter ends by proposing the need for a new standard that will enable functional comparison of genetic parts, a feature that is currently poor addressed.

4.2 The Need for Standardization of Genetic Parts

Traditional molecular cloning workflows rely on generating sites for restriction endonucleases on either ends of DNA fragments, usually by means of polymerase chain reaction (PCR)-mediated primer extension. This strategy is constrained by the availability of suitable restriction sites in the vector backbone, and of course, the sequence of the DNA fragment to be cloned. While this is usually not an issue for most routine cloning exercises, cloning more than one gene in a single vector (to generate a synthetic operon, for example) or cloning large DNA fragments can be severely hampered due to the increased probability of finding restriction sites within the DNA to be cloned.

One way to circumvent this issue is to modify the DNA sequence to remove restriction sequences that are found within DNA fragments to be cloned. Site-directed mutagenesis can be employed to mutate such sites, or the DNA fragment can be chemically synthesized with the offending sites suitably altered. Both these strategies have their associated disadvantages, which have led researchers to come up with cloning strategies that, to varying degrees, circumvent the problems that traditional cloning strategies pose.

In the synthetic biology approach to molecular cloning, DNA fragments are treated as ‘parts’ or ‘modules’ that can be joined to each other in a standard fashion. Genetic elements are flanked by DNA sequences that are compatible with ends of other DNA sequences, making it possible to stitch together parts in a defined and precise manner. Such treatment of DNA parts opens up possibilities that are difficult, if not impossible, to imagine with traditional cloning strategies.

Standardized structural and functional composition of biological parts is one of the goals of the discipline of biological engineering (Knight 2005). Such parts lend themselves to modular assembly, which allows parts to be shuffled together to generate different genetic constructs that are to be assessed for functionality. Moreover, there can be situations where a single gene or a set of genes may have to be expressed in multiple host organisms, both prokaryotic and eukaryotic, to determine the most suitable host for production of a biomolecule of interest. Standard parts can then be easily swapped and replaced by genetic elements specific to different host organisms, simplifying the process of vector construction.

Parallel to the efforts of part standardization, which, as discussed above, aims at enabling modular construction and reuse of basic DNA parts in order to build systems of increasing complexity, is the development of assembly methods that can