FEBS Letters 586 (2012) 2122–2128

journal homepage: www.FEBSLetters.org

Review

Engineering and control of biological systems: A new way to tackle complex diseases

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ARTICLE INFO

Article history:
Received 17 March 2012
Revised 25 April 2012
Accepted 25 April 2012
Available online 10 May 2012

Edited by Thomas Reiss and Wilhelm Just

Keywords:
Synthetic biology
Modelling
Network motif
Systems biology
Control engineering

ABSTRACT

The ongoing merge between engineering and biology has contributed to the emerging field of synthetic biology. The defining features of this new discipline are abstraction and standardisation of biological parts, decoupling between parts to prevent undesired cross-talking, and the application of quantitative modelling of synthetic genetic circuits in order to guide their design.

Most of the efforts in the field of synthetic biology in the last decade have been devoted to the design and development of functional gene circuits in prokaryotes and unicellular eukaryotes. Researchers have used synthetic biology not only to engineer new functions in the cell, but also to build simpler models of endogenous gene regulatory networks to gain knowledge of the "rules" governing their wiring diagram.

However, the need for innovative approaches to study and modify complex signalling and regulatory networks in mammalian cells and multicellular organisms has prompted advances of synthetic biology also in these species, thus contributing to develop innovative ways to tackle human diseases.

In this work, we will review the latest progress in synthetic biology and the most significant developments achieved so far, both in unicellular and multicellular organisms, with emphasis on human health.

1. Introduction

The possibility of modifying, or creating de novo, cells and organisms with the aim of solving the open challenges in human health and in biotechnology will open up completely new avenues of research and applications, as well as safety and bioethical issues (a detailed discussion of bioethical issues can be found in [1]).

Such a possibility is becoming a reality thanks to the merging of engineering and physics with molecular and cell biology, which gave rise to synthetic biology. The defining feature of this new discipline is a striving to apply engineering principles and practices to molecular and cell biology; these include the principles of abstraction and standardisation of biological parts, decoupling between parts to prevent undesired cross-talking, and the application of quantitative modelling of the synthetic genetic circuits, prior to their implementation, in order to guide their design.

It is worth briefly considering the relationship between systems biology and synthetic biology: systems biology can be thought of as the other side of the coin, in that it aims at developing a formal understanding of biological systems through the application of engineering and physics principles (for a detailed discussion refer to [2]). Synthetic biology can be used as a tool in systems biology, by applying the famous Feynman quote "What I cannot create, I do not understand": that is, in order to understand a naturally occurring biological system, a simpler version of it can be built using synthetic biology, to identify which are its essential features. Also the contrary can be true, i.e. principles underlying the functioning of biological systems identified by systems biology can be used to design novel circuits in synthetic biology.

In what follows, we will review the most significant developments achieved so far in synthetic biology, with emphasis on applications relevant to human health, starting from unicellular organisms and concluding with early attempts to engineer mammalian cells and multicellular organisms. We will also describe some fascinating, albeit immature, avenues of research in synthetic biology, such as the application of control engineering to living organisms, and discuss the challenges lying ahead.

2. Engineering of unicellular organisms for disease prevention, diagnosis and treatment

Most of the efforts in the field of synthetic biology in the last decade have been devoted to design and develop functional gene

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0014-5793
http://dx.doi.org/10.1016/j.febslet.2012.04.050

circuits in prokaryotes and unicellular eukaryotes [3]. Starting from the seminal works describing a "toggle switch" to turn gene expression off or on [4] to the "repressilator", capable of driving periodic gene expression [5], engineering of biological systems has evolved to include circuits of ever increasing complexity, including logic gates, clocks, counters, and inter-cellular communication [6,7]. More recently, efforts are underway in creating "minimal organisms" by completely replacing their genome with a synthetic one, rather then adding new synthetic circuits to an existing genome. The aim is to engineer minimal organisms acting as "chassis" able to perform new complex functions, encoded in the synthetic genome, yet unencumbered by functions encoded in their native genome.

In what follows, we describe some of the recent applications of synthetic biology relevant to human health, employing engineered unicellular organisms (refer also to [8] for a thorough review). We will conclude the section with a brief overview on the on-going efforts to create a "minimal organism".

2.1. Application of synthetic biology to infectious diseases

Infectious disease prevention usually relies on different routes: host-pathogen interaction inhibition and pathogen neutralization can be listed among them. Both of these approaches have been exploited from the standpoint of synthetic biology to tackle highly aggressive diseases (Fig. 1a). Duan and March investigated the possibility of using the probiotic *Escherichia coli* (Nissle, 1917) as signal mediators for inhibiting cholera by hijacking *Vibrio cholerae* quorum sensing (QS) [9]. QS is used by the pathogen to sense high cell densities in its population and to inhibit its virulence genes. A strain of *E. coli* was engineered to express the *V. cholerae* QS autoinducer molecule cholera-autoinducer-1 (CAI-1) (known to prevent virulence when present at high concentrations in conjunction with autoinducer 2 also present in *E. coli*). This engineered strain was administered to mice following ingestion of the pathogen *V. cholerae*, resulting in a significantly higher survival rate (92%) compared to control mice.

The second route of disease prevention, namely pathogen inhibition, has been explored by Rao et al. [10]. These authors genetically modified the same *E. coli* strain (Nissle, 1917) to secrete HIV-gp41-hemolysin-A hybrid peptides known to block HIV fusion and entry into target cells. In this case, the authors were able to demonstrate a significant reduction in infected cells as a result of bacterial activity.

While these works have been primarily devoted to disease prevention, diagnosis has been one of the most exciting outcomes of engineered *E. coli*. In the project "*E. chromi*", winning the International Genetic Engineering Machine (iGEM) competition in 2009 (University of Cambridge team), *E. coli* bacteria were engineered to express different pigments when different compounds were sensed (thus changing their color). This "lab-in-a-cell" approach could be a suitable and cost effective way to carry out diagnosis for a range of human diseases from human derived samples.

In another seminal work [11], the authors provided evidence for the feasibility and benefits of using engineered enzymatic bacteriophage to reduce bacterial biofilms. Bacteriophages were engineered to express a biofilm-degrading enzyme during infection. This led to the simultaneous attack of bacterial cells in the biofilm and of the biofilm matrix, which is composed of extracellular polymeric substances. This work demonstrated for the first time that a synthetically engineered enzymatic phage substantially reduced bacterial biofilm cell counts by ≈4.5 orders of magnitude (≈99.997% removal), which was about two orders of magnitude better than that of the non-enzymatic phage. In a follow up study

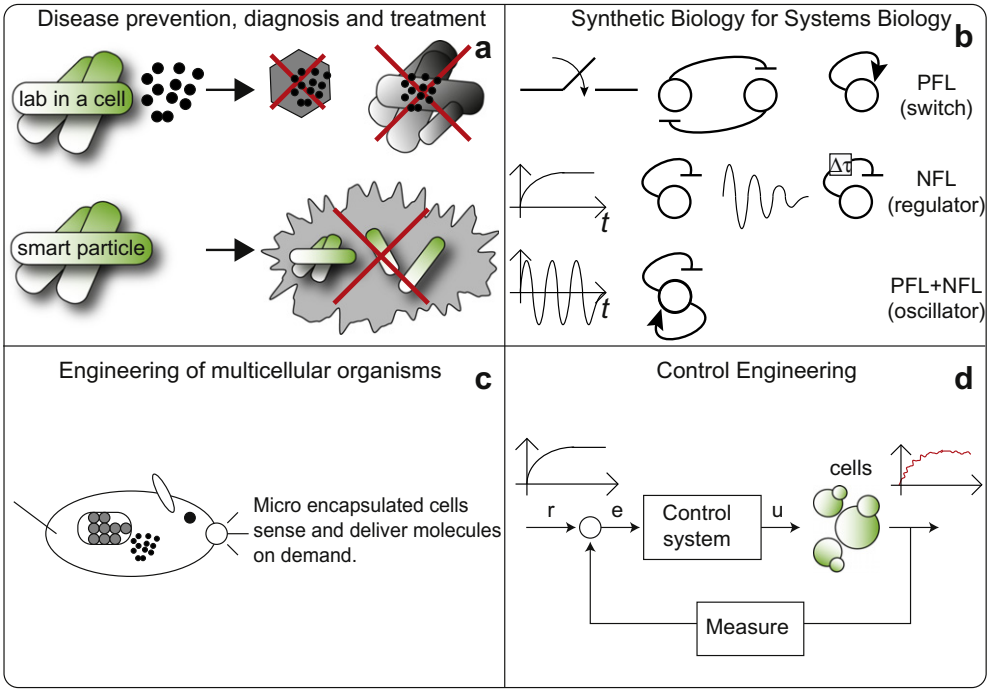

Fig. 1. Overview of the methodologies and applications of synthetic biology and control engineering: (a) Engineered unicellular organisms can be used as "lab in a cell" to combat infectious disease by conditionally producing toxins against pathogens; or a "smart particles" to selectively invade and kill cancer cells. (b) Synthetic biology can be used to probe endogenous "network motifs" and study their function. Positive Feedback Loops have been show to behave as "toggle switches" or to slow down transcriptional response; Negative Feedback Loops induce either robust expression, or when "delayed" spontaneous damped oscillations; combination of these motifs have been show to produce stable oscillations in gene expression. (c) Engineering in multicellular organisms is still at very early stages, however techniques such as micro-encapsulated cells may bring synthetic biology quickly to the clinic. (d) Control engineering aims at precisely controlling gene/protein expression in time, by comparing a "desired" time-course in expression to the measured "time course" and adjusting the stimuli given to the cells (light, induce molecule, etc.).

[12], the same authors showed that suppressing the SOS network in *E.* coli with an engineered bacteriophage enhances killing by quinolones by several orders of magnitude in vitro and significantly increases survival of infected mice in vivo.

Genome scale synthetic biology has been recently proposed as a means to obtain novel vaccines by Coleman et al. [13]; the authors took advantage of the intrinsic redundancy of the genetic code to decrease the efficiency of protein translation thus severely affecting the ability of the poliovirus virus to infect cells. Mice immunized with this engineered poliovirus were found to be resistant to wild type poliovirus infection.

2.2. Application of synthetic biology to cancer and other complex diseases

Other groups pushed the synthetic biology paradigm even further and engineered bacterial strains to selectively invade and kill cancer cells, as shown in Fig. 1a (see [14] for a review). In this application, rather than using bacteria as a ''lab-in-a-cell'' to (conditionally) produce therapeutic compounds, bacteria are treated as ''highly programmable smart particles'' able to (a) target tumours, (b) produce cytotoxic molecules, (c) self-propel, (d) respond to triggering signals, (e) sense the local environment and (f) produce externally detectable signals. Some of the most representative works in this field include the contribution by Anderson et al., who engineered *E. coli* to express invasin, a protein from Yersinia pseudotuburculosis, that allows bacteria to invade tumor cell lines (such as HepG2 and HeLa) only under specific conditions (hypoxia, cell density and external compounds) [15]. Xiang et al. demonstrated how bacteria can be engineered to express short hairpin RNA to elicit RNA-interference in mammalian cells [16].

Ganai et al. engineered non-pathogenic *Salmonella* typhimurium (found to preferentially target tumours over normal tissue) to secrete murine TNF-related apoptosis-inducing ligand (TRAIL) causing conditional caspase-3-mediated apoptosis and death of cancer cells [17].

Royo et al. used Salmonella enterica to engineer an acetyl salicylic acid (ASA) inducible regulatory control circuit with an expression module encoding the 5-fluorocytosine–converting enzyme cytosine deaminase. After infecting mice with tumors by exposing them to engineered bacteria, before 5-fluorocytosine adiministration, these authors found a significant reduction in tumor growth [18].

Given their generally human-symbiotic behavior, gut commensal bacteria are the obvious choice for human disease treatment: this is the case of *Bacteroides ovatus*, used by Farrar et al. to conditionally secrete a biologically active murine interleukin 2 for immunotherapy purposes [19]. More recently, Duan et al. engineered *E. coli* Nissle, 1917 strain to express GLP-1 and PDX-1, two proteins shown to stimulate intestinal epithelial cells to synthesize insulin [20] and thus proposing a treatment for diabetes.

Drug development can strongly benefit from the introduction of synthetic biology, thanks to its ability to provide new means to modify an organism to produce a compound of interest [21] (''lab-in-a-cell'' paradigm), or to use an organism as a vector to deliver the drug only under specific conditions (''smart particles'' paradigm).

Drug production by synthetically engineered cells has its most striking example in the case of artemisinic acid, a precursor of atermisinin, the primary antimalarial drug (a very effective yet expensive treatment for this disease). Ro et al. were able to obtain high titres of artemisinic acid produced by re-engineering the mevalonate pathway within the yeast strain *Saccharomyces* cerevisiae [22].

2.3. Towards a ''minimal organism''

Creating a ''minimal organism'' acting as a living chassis will enable engineering of new complex functions in the cell. The paradigm followed by synthetic biologists is the one used in computer science, where a computer (the minimal organism) is a general-purpose machine and it can perform specific functions using software (the synthetic genome). A synthetic genome should retain only a minimal set of wild-type genes and regulatory sequences that encode for essential functions, thus allowing a desired set of new genes encoding for complex circuits to be encoded in the genome.

Different labs are currently using the *Mycoplasma pneumoniae* [23,24] or the *Mycoplasma mycoides* recipient cells [25], due to their reduced genome size, in order to obtain cells with a minimal genome, by removing non-essential genes from the host genome.

In the case of *M. mycoides*, chosen by the Craig Venter Institute for constructing their ''minimal organism'', Gibson et al. attracted the attention of both the scientific community and the public opinion [25]. As a matter of fact, they demonstrated for the first time that it is possible to ''boot up'' a bacterial cell by replacing the endogeneous genome with a fully synthetic genome replica assembled by DNA synthetisers. The next step will be to synthesise a minimal genome by removing non-essential genes. This contributed to raise public concerns about potentially harmful consequences of synthetic biology to the point that a Presidential Commission has been established in US; the results of the work carried out by this commission is currently accessible through (Presidential Commission for the Study of Bioethical Issues, 2010 http://www.bioethics.gov/).

A complementary approach has been proposed for more complex eukaryotic genomes by developing partially synthetic genome: a new yeast strain has been engineered by partially replacing a chromosome arm with a synthetic version of it including an inducible evolution system, SCRaMbLE (synthetic chromosome rearrangement and modification by loxP-mediated evolution) through which combinatorial mutagenesis can be induced to lead to yeast strains with a broad variety of phenotypes [26].

At the present time, no minimal organism has been created yet, however progress is being made at a fast pace, and applications of this technology may soon impact biomedical research.

3. From unicellular to multicellular organisms: synthetic biology for systems biology

Although most of the studies in synthetic biology are related to prokaryotes and unicellular eukaryotes, the need for innovative approaches to study and modify complex signalling and regulatory networks has prompted advances of this field also in cells from multicellular organisms. The first applications of synthetic biology in mammalian cells aimed at adapting synthetic circuits originally built in bacteria [4,5] by means of well-characterized biological parts and orthogonal elements isolated from the endogenous cellular context. Intelligent use of these techniques allowed researchers to construct ''proof-of-principle'' toggle switches [27,28] and oscillatory networks [29].

Building on this early successes, scientists have then used synthetic biology not only to engineer new functions in the cell, but also, in the framework of systems biology, to build simpler versions of endogenous gene regulatory networks to gain knowledge of the ''rules'' governing their wiring diagram (Fig. 1b). Questions such as why some transcription factor regulate their own transcription (positive/negative self-feedback loop), or how the circadian clock

can generate cyclic gene expression, can be tackled using simplified "synthetic" models of these biological processes.

Indeed, over the last few years, the design of simple circuits in mammalian cells has been used to shed light on the biological meaning of recurrent motifs and structures observed in gene regulatory networks.

3.1. Synthetic switches in mammalian cells

The first toggle switch in mammalian cells readapted the basic topology of the *E. coli* toggle switch invented by Gardener et al. [4] and reported in Fig. 1b (PFL). Specifically, Kramer et al. [28] engineered a switch by assembling two transcription factors (PIP-KRAB and E-KRAB) mutually inhibiting their expression. Two antibiotics can be transiently applied to the cells to "switch" the system either on (PIP-KRAB high, E-KRAB low) or off (PIP-KRAB low, E-KRAB high) by transiently restricting E-KRAB repression with erythromycin, or PIP-KRAB repression with pristinamycin. The authors showed that the epigenetic state of the switch can be transmitted to daughter cells after cell division.

3.2. Synthetic oscillators in mammalian cells

The periodic expression of genes underlies the functionality of fundamental processes found in every mammalian cell, such as the circadian clock, and the cell cycle, essential for coordinating the physiology of the whole organism.

To better understand the rules underlying the periodic expression of genes, several synthetic circuits have been built and tested in bacteria, while the stable implementation of a synthetic oscillator in mammalian cells still remains an open problem. Nevertheless, in 2009, the first mammalian oscillator was described [29]. The circuit consists of a sense-antisense expression "pendulum" with the tetracycline-dependent transactivator tTA auto-regulating itself, thereby forming a positive feedback loop (PFL) [29]. The tTA drives also the transcription of the streptogramin-dependent transactivator, which induces the tTA in antisense orientation, thus reducing the tTA levels (negative feedback loop - NFL). This PFL + NFL topology (Fig. 1c) gives rise to periodic fluctuation of tTA levels, which were monitored via a highly unstable form of GFP, whose expression is driven by the tTA. The different components of the genetic oscillator were carried by three different plasmids. Even considering limitations of a transient transfection based approach, the oscillator showed spontaneous, self-sustained oscillations with an average period of 147 min.

3.3. Engineering and analysis of basic networks motifs

Swinburne et al. investigated if and how intron length affects the timing of gene expression [30]. To this aim, they used a synthetic biology approach to engineer and analyse a simplified network motif in mammalian cells isolated from endogenous regulation. Indeed, endogenous intron-containing genes are subjected to multiple transcription and post-transcriptional control, and therefore are not amenable to study the impact intron lengths have on their expression.

The authors engineered a negative feedback loop (Fig. 1c) expressing a humanized Tet repressor (TetR) fused to the fast-maturing Venus variant of yellow fluorescent protein (YFP) under the strong beta-actin promoter, including its first intron. The TetR fusion contains a nuclear localization signal thus allowing TetR to bind the tet-operators (tetO) in its own promoter region and inhibit transcription initiation of its own gene; repression can be relieved by the addition of doxycycline. By varying the size of the first intron of beta-actin upstream of the reporter gene by inserting intron cassettes from 1 kb up to 16-kb, the authors showed an increasing delay in transcription correlated to the intron length. Moreover, this delayed negative feedback loop induced periodic pulses of protein expressing, consistently with mathematical modeling of this simple, but dynamically rich, motif. Interestingly, the delayed NFL is a basic motif also found in the endogenous circadian clock regulatory network.

In a recent work in our lab [31], we instead investigated the properties of another common motif: the positive feedback loop (PFL), as shown in Fig. 1c. To this end, we engineered Chinese Hamster Ovary (CHO) cells with a cassette composed of the tetracycline-controlled transactivator tTA driven by a CMV-TET promoter containing seven repeats of the Tet Responsive Element (TRE) bound by the tTA protein itself. The mRNA expressed from this promoter contains an Intra Ribosomal Entry Sequence (IRES) in-between the transactivator tTA and a destabilised Yellow Fluorescent Protein (d2EYFP).

The transcriptional activity is restricted by the addition of doxycycline that prevents binding of tTA to the CMV-TET promoter. The PFL is a typical example of a bistable circuit, which can have two equilibria (ON or OFF), as in the case of the toggle switch described earlier.

We demonstrated both experimentally and via mathematical modeling that another property of the PFL is to greatly slow down the transcriptional response to an inducer molecule (doxycycline in our case) compared to control cells, where the PFL is broken by placing the tTA under the control of a constitutive CMV promoter.

Hence, the PFL motif exhibits a dynamic behaviour which is very different from that obtained when auto-regulation is removed, demonstrating that such a behaviour relies on the intrinsic properties of the network topology.

Interestingly, both NFL and PFL are basic network motifs found in clock-like mechanisms giving rise to circadian and ultradian oscillators [32], as well as, in signaling transduction and regulatory pathways [33].

Another fascinating example of the application of synthetic biology to elucidate how endogenous signalling transduction pathways work, it has been reported by O'Shaughness et al. [34]. The authors built an exogenous, minimal mammalian MAPK cascade in yeast to investigate its behaviour in an insulated setting, thus preventing the challenges due to the interconnectivities present in the endogenous environment. The authors show, both experimentally and via mathematical modelling, that varying the relative concentrations of MEK and ERK confers great flexibility and can induce either low or high ultrasensitivity response. They further investigated the effect of scaffolding proteins on the signalling pathway response.

4. Engineering in multicellular organisms relevant to human health: from genetic engineering to synthetic biology

The complexity of multicellular organisms makes the application of synthetic biology seem like a daunting task. The idea of engineering a complete synthetic genome even for the simplest of these organisms is still far-fetched. Nevertheless, when considering the engineering of small synthetic circuits to obtain a desired phenotype, some preliminary results have already been reported, as briefly detailed in the following sections and in Fig. 1c.

4.1. Engineering multicellular organisms: An engineered mosquito to control the dengue disease

Harris et al. have recently reported data from the first open-field trial in which engineered mosquitoes were released in a site on Grand Cayman [35]. This study targets the predominant mosquito vector, *Aedes aegypti*. This novel approach overcomes

the limitations of old methods such as the Sterile Insect Technique (SIT) that was unable to control dengue spreading, since the radiation-mediated sterilization of the mosquitoes made them incapable to compete with wild-type males for mating. The system proposed is based on the RIDL technique (Release of Insects carrying a Dominant Lethal genetic system) [35]. *A. aegypti* mosquitoes were engineered with a simple Positive Feedback Loop containing the tTA protein self-activating its own promoter and causing a highly penetrant, dominant, late-acting lethality in progeny, probably due to the site-specific integration of the construct [35]. Males were reared to maturity through the use of tetracycline in the laboratory, which prevents tTA protein from bind its own promoter, and then were released in the field. The analysis of progenies followed for a four-week period proved that the engineered mosquitoes were able to compete for mates, and that the method is feasible to control dengue through suppression of populations of *A. aegypti*.

Due to the efficiency of this approach, this application has been hailed as a success of synthetic biology; however, it could also be classified as "genetic engineering", due to the lack of quantitative modelling guiding the circuit design prior to its implementation. Indeed, a classic genetic engineering approach was possible since the circuit was very simple (i.e. a positive feedback loop), and the late lethality phenotype was obtained by screening colonies, without identifying the exact mechanism [36].

4.2. A synthetic network to control urate homeostasis in mice

Kemmer and collegues described the development and validation in urate-deficient mice, of a simple synthetic circuit able to sense and maintain constant uric acid levels in the bloodstream making use of control engineering principles [37]. The synthetic circuit is composed by the bacterial transcriptional repressor HucR, optimized for mammalian cells, which binds the SV40 promoter, engineered to contain eight cognate hucO operator sequences (Psv40-hucO), in absence of uric acid. High urate levels induce the de-repression of the promoter that, in turn, triggers the expression of a secretion-engineered Aspergillus flavus urate oxidase that eliminates uric acid. The transplantation of microencapsulated cells stably expressing the circuit, in urate oxidase deficient mice with high urate levels, shows that the device is able to discriminate between mice developing hyperuricemia and mice with low urate levels, fostering the possibility to use this device in a therapeutic approach (Fig. 1d).

Also in this case the circuit was very simple, consisting of an inducible promoter expressing a therapeutic enzyme, however in this case a quantitative model was used to guide the circuit design [37].

4.3. The next frontier: controlling biological systems

A new field of research has been recently established at boundary between synthetic biology and control engineering (Fig. 1d). Control engineering is a well-established engineering discipline, which aims at forcing a physical system to attain a desired behaviour by appropriately modulating "input" signals to which the system is known to respond. Control engineering principles can be used to elucidate how cells and organisms control and fine tune the activity of key pathways in face of fluctuations [38]. In addition, control engineering can be used in conjunction with synthetic biology to regulate at will the dynamics of gene expression or signalling pathways in a cell, opening up a new set of tools for research in systems biology and for biomedical applications.

A great analogy to better understand how a control system works has been presented by Allinson et al. and reported here [38]: the indoor temperature in a house is detected by a thermostat, which then regulates the heat flow to achieve a set, desired temperature. The thermostat operates via a negative feedback loop, where the desired temperature is compared to the measured temperature and based on their difference (i.e. the error), a control system decides whether to switch on or off the heat generator.

In what follows, we present some of the most recent examples of applications of control engineering principles in conjunction with synthetic biology.

4.4. Controlling gene expression in living cells

Inducible expression systems able to switch on or off the expression of a gene/microRNA of interest have revolutionarised molecular biology, greatly simplifying and expanding the experimental tools available to probe gene function. However, these systems suffer from an intrinsic limitation, i.e. the expression level can be chosen to be either be high or low, but the experimenter has no control on the exact level of expression of the gene, neither on its expression dynamics.

Coupling control engineering to synthetic biology allows for the first time in the history of molecular biology to precisely control the amount and the time-course of gene expression, or protein activation, in living cells (Fig. 1d).

Uhlendorf et al. [39] proposed a first step towards in vivo control of protein localisation in real-time, by combining a microfluidic device, an epi-fluorescence microscope and software implementing control approaches. They used the Hyper Osmolar Glycerol (HOG) pathway in the yeast *S. cerevisiae*, which senses osmolarity and triggers osmotic stress responses to maintain water homeostasis via translocation of the effector protein Hog1 to the nucleus. The authors show that they can precisely control the localisation and concentration of Hog1 protein in the nucleus via a feedback control system. The controller changes in real-time the osmalirity level sensed by yeast cells growing in the microfluidics device, by monitoring the concentration of a tagged Hog1 in the nucleus via the microscope.

We described a feedback control system able to regulate at will the expression of a gene of interest embedded in a complex network in yeast by automatically changing the concentration of an inducer molecule in the growing medium [40]. Specifically, we designed a microfluidics platform to grow yeast cells and to change at will, in real-time, the concentration of galactose or glucose in the growing medium. This change in carbon source indirectly affects the level of expression of a fluorescent protein in a complex gene regulatory network [41]. The fluorescence level is monitored by a computer connected to a fluorescence microscope that automatically administers galactose or glucose to the cells to achieve the desired expression level and/or expression dynamics.

Milias-Argeitis et al. proposed a strategy to control, at the population level, the expression of a fluorescence protein using an opto-genetic approach [42]. The authors use the light-responsive Phy/PIF module to control the level of a PIF3 protein fused to the Gal4 activation domain. By applying pulses of light, they are able to regulate the level of a YFP reporter driven by the Gal1 promoter, which contains Gal4 binding sites. In this case, fluorescent levels were measured via FACS (Fluorescence Activated Cell Sorter).

4.5. Controlling signaling pathways

Toettcher et al. used principles of control engineering to automatically control the amount of light needed to fine tune the intracellular activation of a signalling pathway using genetically encoded light-gated proteins [43]. Also this system relies on the Phy-PIF opto-genetic system. The user provides a desired (time-varying) amount of PIF-BFP fluorescent fusion protein to be localised to the plasma membrane, the controller then compares it to

the measured live-cell PIF-BFP amount (output) in real time and determines the appropriate light input to provide to the cell. Using this strategy, the authors succeeded in achieving desired (time-varying) levels of membrane recruitment of PIF-tagged proteins within seconds.

Application of this technology will allow to derive precise quantitative models of signaling pathways and to study their activation dynamics in unprecedented details, opening up a whole array of innovative experiments.

5. Conclusions

In this review we have first shown examples of state-of-the-art applications of synthetic biology to engineer unicellular organisms towards disease diagnosis, treatment and prevention. Bacteria can be engineered to function as a "lab-in-a-cell" to produce pharmacologically active compounds, as sensors to measure the presence of a diagnostic marker, or modified to work as "smart particles" able to recognise and kill cancer cells. Although many of these applications are only "proof-of-concepts" some may reach practical applications very soon.

There are still, however, a few challenges lying ahead: (a) up-scaling the modelling and construction of synthetic circuits is still very difficult due to a lack of standardisation of biological parts associated to quantitative descriptive parameters and to cross-talking between the different parts. Effective means to decouple the different biological parts (equivalent to operational amplifies in electronics) in order to reduce cross-talking have not been found yet [44,45]. These problems will be mitigated if efforts in creating a minimal organism with a minimal genome will succeed, since it will be easier to engineer orthogonal parts that do not interact with the endogenous components [46]; (b) new experimental techniques are needed for fast "prototyping" of synthetic circuits, such as DNA synthesisers capable of generating long sequences at affordable costs, and innovative modular cloning techniques, which allow to quickly "mix and match" different biological parts [25]; (c) evolution in synthetic biology of unicellular organisms can be used as a tool [46], but it can also be a curse; indeed, if the organism is not properly engineered, evolution will eradicate any modification which decreases the fitness of the individual cells.

Similar considerations apply to synthetic biology in multicellular organisms, with the added difficulty of engineering cell-to-cell signalling, essential for properly inducing a desired tissue/organism-level phenotype. Quantitative understanding of cell signalling is essential for proper engineering, and although this has not been yet achieved, progress is being made at a rapid pace [47,48]. Modelling becomes possible if there are biological parts with quantitative parameters available, and it becomes essential for up-scaling, when synthetic circuits with more than a few parts need to be built. Because of these limitations, we are still in the very early stages of synthetic biology in multicellular organisms. However, techniques such as using engineered encapsulated cells could be a reliable approach to bring synthetic biology approaches to the clinic in a reasonable time [37] (Fig. 1c).

We also reviewed some of the recent developments in the application of control engineering to living organisms. Control engineering offers unprecedented opportunities in the fields of systems and synthetic biology that span from performing highly informative experiments to investigate single cell behavior to turning this knowledge into therapeutic strategies meant to revert pathological states.

Despite the technical and methodological challenges still open, engineering and control of biological systems will likely yield new "synthetic micro-organisms" able to sense "molecular markers" of a disease and perform a therapeutic action in response; or autologous transplants of engineered human cells to restore homeostasis of dysregulated pathways in disease.

The drive in merging engineering and biology has begun and shows no sign of slowing down.

Acknowledgement

This work was supported by an Italian Telethon Foundation Grant (TGM11SB1).

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