ELSEVIER

Designer cell signal processing circuits for biotechnology

Robert W. Bradley[1,2] and Baojun Wang[2]

[1] Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, London SW7 2AZ, UK
[2] Centre for Synthetic and Systems Biology, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3JR, UK

Microorganisms are able to respond effectively to diverse signals from their environment and internal metabolism owing to their inherent sophisticated information processing capacity. A central aim of synthetic biology is to control and reprogramme the signal processing pathways within living cells so as to realise repurposed, beneficial applications ranging from disease diagnosis and environmental sensing to chemical bioproduction. To date most examples of synthetic biological signal processing have been built based on digital information flow, though analogue computing is being developed to cope with more complex operations and larger sets of variables. Great progress has been made in expanding the categories of characterised biological components that can be used for cellular signal manipulation, thereby allowing synthetic biologists to more rationally programme increasingly complex behaviours into living cells. Here we present a current overview of the components and strategies that exist for designer cell signal processing and decision making, discuss how these have been implemented in prototype systems for therapeutic, environmental, and industrial biotechnological applications, and examine emerging challenges in this promising field.

Contents

Corresponding author: Wang, B. (Baojun.Wang@ed.ac.uk, wbjzju@gmail.com)

http://dx.doi.org/10.1016/j.nbt.2014.12.009

Introduction – biological signal processing

Signal processing circuits are widely used in electronic systems to modulate the electrical signal flows necessary to achieve particular desired applications. Similarly, cells employ sophisticated gene regulatory networks to continuously process biological signals for their survival and reproduction [1]. Microorganisms possess the capabilities to sense a myriad of signals, but to coordinate an appropriate response this information must be processed: various types of signal must be transformed to enable interaction between data flows; crosstalk must be prevented between some, whilst others need to be composed to allow combination or comparison; digital and analogue behaviour from different processing units may require assimilation (Fig. 1a). These concerns are central to the goals of synthetic biologists: signal processing behaviour defines the function of the system, so rational design of a biological system is the ability to predictably coordinate the interactions between, and conversion of, various input signals. The term 'synthetic biology' broadly describes the development of tools and techniques that facilitate the rational design and construction of new biological devices and systems for use in biotechnological applications (and arguably also facilitate basic research) [2–4], hence the motivation for examining how designer cellular signal processing has been used to build prototype biotechnological applications.

Signal processing arises from the characteristics of the interactions (abstracted to 'transfer functions') between information carriers: activation of transcription by regulators [5], small-RNA-mediated translation inhibition [6], protein–protein interaction [7], etc. Reasonably accurate design of biological information processing networks therefore depends on knowledge of the kinetic parameters of these interactions, a task that is being made easier through the development of part libraries [6,8]. Incomplete understanding of how parts interact with each other and their genetic, cellular, and environmental contexts [9] limits the extent to which behaviour can be predicted. Minimising or removing interactions between the designer circuit and its cellular context often aids performance, but the ability to tune elements (an activity facilitated by having parts that are easily exchangeable) is often required to enable refinement of the system.

Digital and analogue biological information processing

Biological systems are inherently analogue; though the physical state of cellular components could be considered to encode digital information, their interactions (the basis of computation) are based on probability [10], leading to graded responses at the level of a population of biomolecules or cells. Analogue signal processing uses the characteristics of these graded responses as the basis for computing, for example, two positive log-linear inputs that stimulate the same log-linear output are summed [11]. Digital characteristics are a special case of the standard analogue behaviour, where the transition between distinct low and high levels of the output occurs over a relatively narrow range of input. Components which exhibit digital behaviour can be combined to produce Boolean logic-based decision circuits (Fig. 1b) [1,8,12,13]. There are pronounced energetic and orthogonal part limitations on digital systems when scaling-up biological computing. Whilst efforts in synthetic biology have thus far tended to emulate the digital paradigm of electronic engineering, future advances in the complexity of synthetic biological systems are likely to require more analogue-based computing (Fig. 1c) [10,11]. Nonetheless, small-scale logic circuits are tractable and well suited to a range of applications, such as threshold detection [14] and environmental sensing [5].

Effective digital cellular signal processing results in a single output changing between distinct OFF and ON (low/high) states in response to one or more input signals. Better digital signal fidelity exists in signals with lower noise (e.g. higher concentration of participating molecules [15]), and larger ON:OFF ratios, that can be produced by lowering the level of the output in the OFF state, for example, by reducing leaky transcription at a promoter [16], or raising the level of the ON state, for example, through increasing translation rates by using a stronger RBS [17]. The change in output state must switch sharply as the input crosses a defined threshold level to avoid ambiguity. Input–output functions that have Hill coefficients greater than one generally show this behaviour, as do those which receive positive feedback from the output [18,19]. A major concern when designing digital-based circuits is matching the input and output thresholds of linked components so that downstream components are activated appropriately [20].

The transfer functions of analogue genetic signal processing ideally have well-defined response curves that operate (i.e. remain unsaturated) over a wide dynamic range of input [11] and exhibit low noise. The dynamic range of analogue functions can be extended by introducing a negative feedback loop [11,21] or by attenuating the gain in the transfer function [22].

Strategies for designer cell signal processing

The components and mechanisms of decision gene circuits can be as varied as the biochemical basis of the organism itself. Whilst many examples use the regulation of gene expression as the basis for information flow, data can be carried by all biological molecules, from nucleic acids to proteins to metabolites. Not all signal types are composable, however, and signal processing circuits generally transform diverse inputs into a common medium for computation. Below we summarise and provide our own perspectives on the range of strategies and tools available for performing various types of signal processing in synthetic biological systems.

Transcriptional control

Transcriptional repressors and activators bind to operator sequences in DNA and respectively inhibit and enhance the transcription of genes by RNA polymerase, thus increasing or decreasing output signal flux. The transcription rate can be controlled by varying the concentration of regulator. For example, the *Pseudomonas syringae* HrpRS-V system tightly regulates expression from the σ^{54}-dependent P_{hrpL} promoter [5,23], making analogue signal modulation possible through graded expression of either activator (positive response), or the HrpS-sequestering HrpV inhibitor (negative response) (Fig. 1c) [22]. The close-to-zero transcriptional output in the OFF state makes the three HrpRS-V inputs ideally suited to building effective digital logic circuits with up to three inputs (Fig. 1b) [7]. A network of three orthogonal repressor-promoter pairs was used to build the classic 'repressilator' [24], but larger biological signal processing systems require more components: a library of regulators based on TetR homologues was

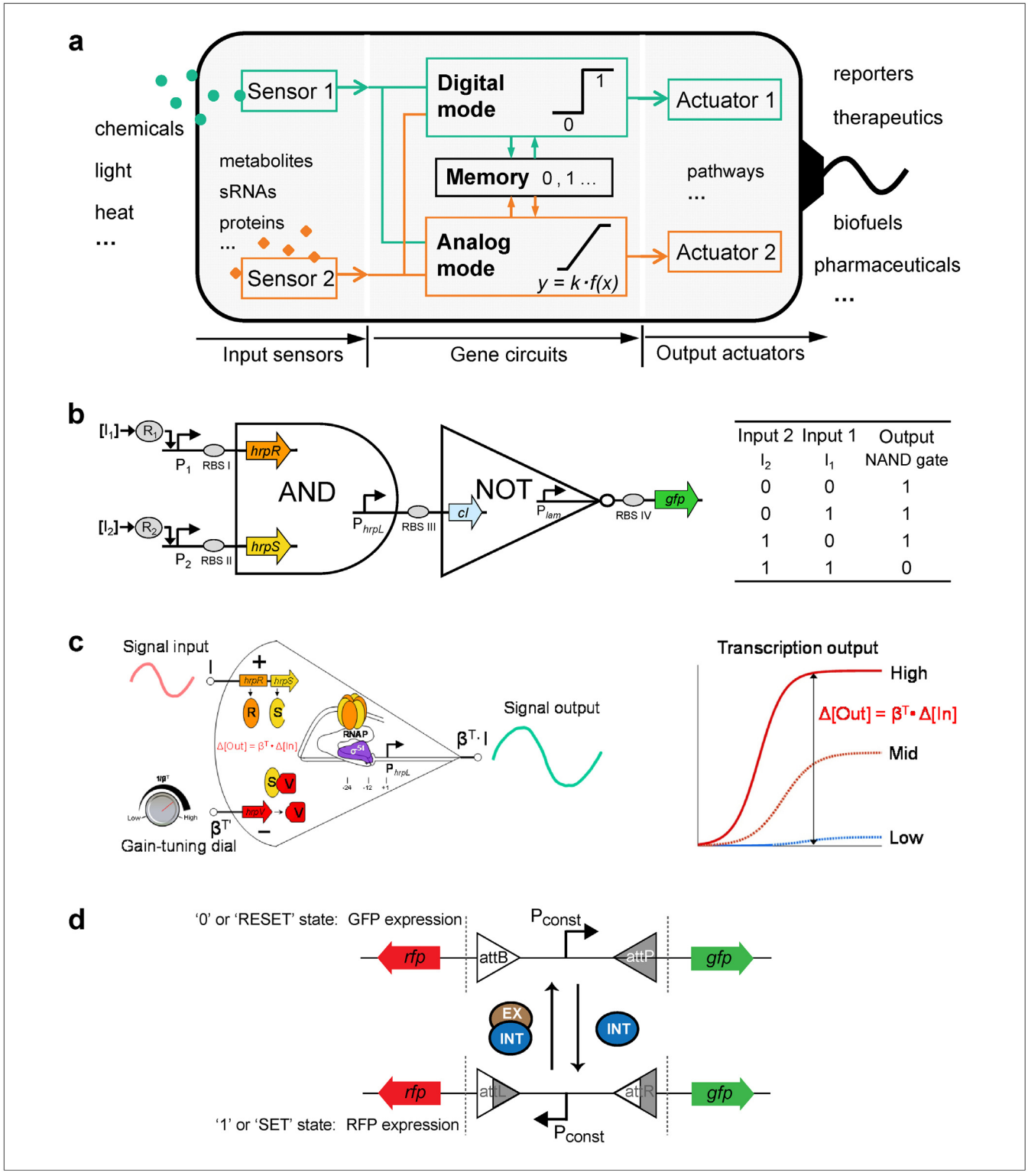

FIGURE 1

Digital and analogue signal processing in cells. **(a)** Two modes of cell signal processing exist in biological systems: digital logic, where signal output switches rapidly between low 'OFF' and high 'ON' states, and analogue responses which are graded transformations of the input signal. Combination and mixing of digital and analogue processing of transduced sensor signals can be useful to drive various customised cellular responses. **(b)** The digital logic mode is exemplified by a combinatorial genetic NAND gate in which the output is only off when both of the two input small molecules (I_1, I_2) signals are present [5]. Expression of both HrpR and HrpS is required to activate expression of the cI repressor, which blocks transcription of the *gfp* output gene. **(c)** The analogue mode is exemplified by a gain-tunable transcriptional amplifier in which the analogue nature of two inputs is combined to control an analogue output [22]. The device functions such that the weak transcriptional input signal (I) scales linearly in response to a second 'gain tuning' transcriptional input (β^T). **(d)** Signals can be stored as digital memory elements. The constitutive promoter P_{const} is flanked by serine integrase attB and attP sites, oriented such that the action of the integrase (INT) flips the memory element (denoted between dashed lines), forming attL and attR sites [51]. Co-expression of the excisionase (EX) partner biases the integrase action in the reverse direction. P_{const} drives transcription of GFP and RFP genes outside of the memory element to report its state.

created by part-mining of bacterial genomes, producing a set of 16 orthogonal repressor-operator pairs [8]. Sets of repressors and activators that target a specific desired regulatory sequence can also be produced through fusions with artificial zinc-finger [25] and transcription activator-like effector (TALE) [26] proteins. Alternatively, the regulator's DNA binding affinity/specificity can be altered by association with a ligand molecule; in these cases the concentration of both regulator protein and ligand may be considered inputs. In addition to classical examples of inducible promoters controlled by regulators that bind metabolites (e.g. LacI, AraC), signalling molecules (e.g. LuxR), and metal ions (e.g. ArsR), more recent CRISPR (clustered regularly interspaced short palindromic repeats)-based tools which require binding of guide RNA (gRNA) sequences have been developed. Nuclease-inactive Cas9 protein can function as a repressor that works by steric hindrance of RNAP at the promoter [27,28], or Cas9 can also be fused with other repressors or activators. Synthetic promoters and genes can be designed to contain multiple different regulator binding sites in order to increase the number of inputs that control the transcriptional output [8].

The rate of RNA production from a gene can also be regulated by the supply or binding kinetics of the polymerase enzyme: use of the phage T7 RNAP allows the activity of transcription itself to be orthogonal to the host transcriptional machinery; part-mining by Temme *et al*. (2012) produced four variants of T7 RNAP that recognise unique targets [29]. It has also been shown that split T7 polymerase fragments can reassemble into a functional enzyme *in vivo*, allowing new orthogonal logic gates to be built based on the differential expression of individual functional fragments [30]. Another different approach to transcriptional control was taken by Rhodius *et al*. (2013), who constructed a library of orthogonal bacterial RNAP sigma factors – proteins that determine promoter sequence recognition by RNAP – and their cognate promoter sequences to enable the modulation of RNAP binding kinetics [31].

Control of RNA stability

Targeted degradation of mRNA can be used to attenuate a signal, with strategies such as small RNA-mediated Hfq protein recruitment [32] and RNA interference [12] both requiring the input of small non-coding RNAs [33,34]. Cys4-based RNA cleavage has been shown to be an effective regulator of signal strength that works well in different sequence contexts [35]: incorporation of the cleavage targeting sequence into a synthetic RNA allows either processing of RNAs for more efficient expression or disruption of coding sequences to prevent translation.

Control of translation

Many classes of RNA can act to prevent translation of an mRNA molecule: RNA-IN–RNA-OUT translation inhibition occurs through the binding of the short non-coding RNA-OUT molecule to the corresponding RNA-IN sequence located at the translation initiation region of an mRNA molecule. Mutalik *et al*. (2012) produced a model that could predict RNA-IN/RNA-OUT binding specificity, enabling the creation of a library of effective orthogonal regulatory RNA pairs [6]. The relative ease of predicting RNA structure means it has also been possible to use rational design to produce other riboregulators [36] including those based on aptamers carrying non-coding RNA fusions [37], though most riboswitch engineering efforts require high throughput selection and screening [38].

Protein signalling

Information flow based on protein–protein interactions can be very rapid and efficient [39,40]. The binding of protein partners may repress the output of one, as in the HrpRS–HrpV interaction [22], or a signal may be passed on via a catalytic interaction, for example proteolysis, that activates an output protein [41]. More recent strategies have made use of inteins, regions of a protein that catalyse their own excision; splitting the intein region and fusing each half to a substrate protein allows trans-splicing of the two substrates upon reconstitution of the complete intein domain [42], with the expression of each substrate protein analogous to the input to an AND gate [43]. The activity of some split inteins can be controlled using small molecule ligands [44] or protein scaffolds [45], allowing rapid induction of catalytic activity from the reconstituted output protein. Further to this, spatial control over protein–protein interactions using engineered protein scaffolds allows for the coordination of multiple signalling components, for example in kinase cascades [46]. Signals carried as proteins can also be modified via degradation, for example using fusions to ligand-induced degradation domains [47]. Prindle and co-workers used competition for protein degradation to temporally coordinate expression of reporter proteins much more closely than is possible via transcriptional control [48].

Memory

A memory element in a signal processing system is one that maintains its output in the absence of an input. Bistable regulation of transcription is an example of epigenetic memory [49,50]. Two output promoters are each repressed by a regulator expressed by the other; in the stable state one promoter is active, and maintains expression of the regulator that represses the other without the need for an input signal (Fig. 2b). The output state changes only when an input signal simulates expression from the down-regulated promoter to the point where it dominates. Signals can be more stably recorded by modifying the DNA molecule itself, for example, through the use of integrases to control the directionality of a DNA section bounded by integrase recognition sites. Bonnet *et al*. (2012) produced re-writable elements using an integrase to flip DNA in one direction, then co-expressing an excisionase to bias the flipping directionality to the original state (Fig. 1d) [51]. Further studies using two integrases demonstrated how the direction of a DNA segment can be controlled in an analogous manner to various two-input logic gates, depending on the arrangement of integrase recognition sites and the genetic elements contained within them [52,53]. Both bistable transcription- and integrase-based memory systems exhibit digital output signal characteristics.

Applications of cell signal processing circuits

Biomedicine and diagnostics

Therapies that act by selectively killing diseased cells require a clear decision to be made. This was reflected in the choice of a digital-logic-based decision circuit by Xie *et al*. for their HeLa cell classifier [16]. Once transfected into human cells the system checks for a HeLa miRNA fingerprint, defined by three high- and three low-abundance miRNAs that were predicted to be able to discriminate

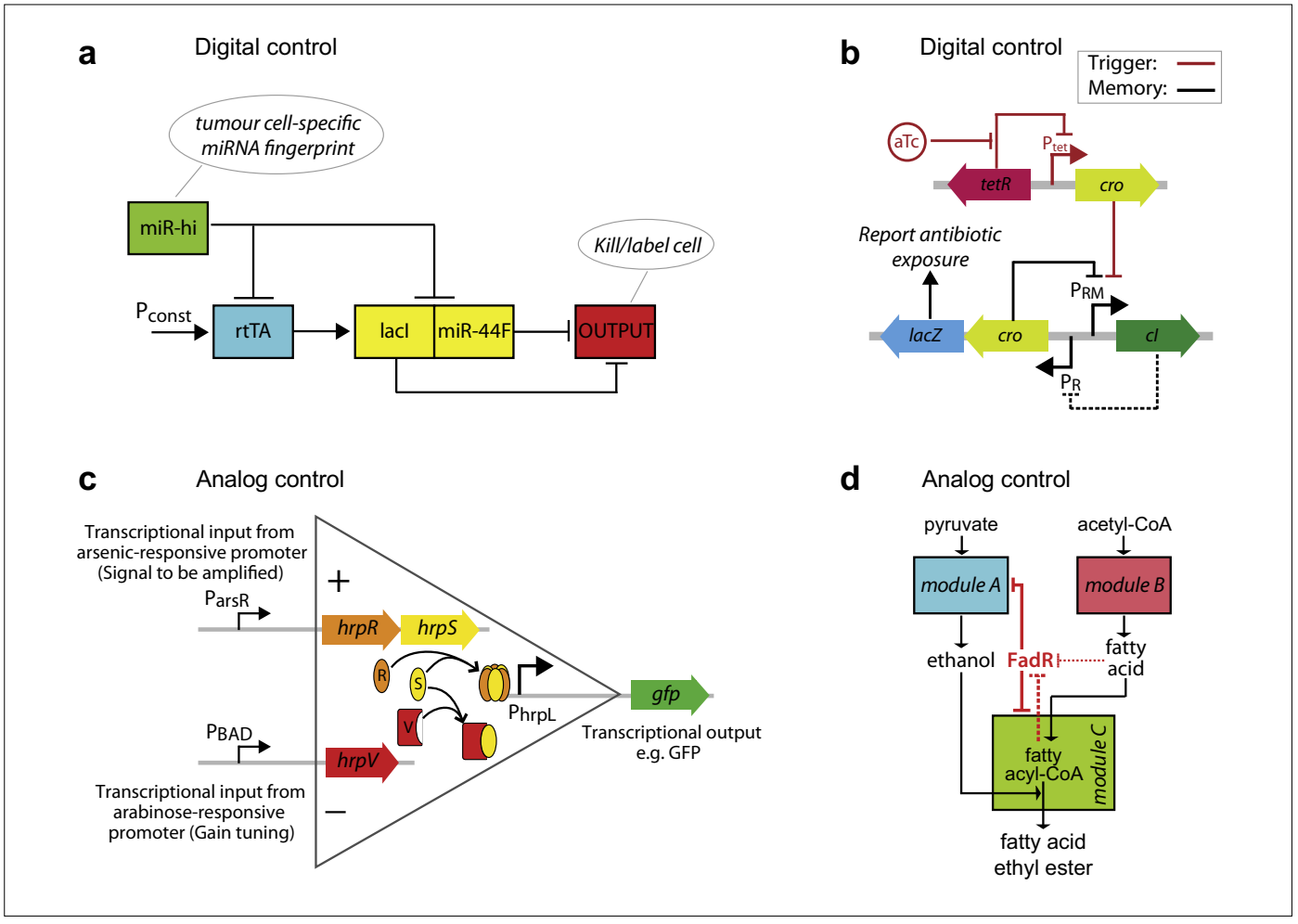

FIGURE 2

Biotechnological applications of designer cell signal processing circuits. **(a)** A high-abundance miRNA sensing module with feed-forward motif, part of the cell-classifier built by Xie *et al.* (2011) [16]. In the absence of the high-abundance miRNA (miR-hi), the reverse tetracycline transactivator (rtTA) drives expression of both LacI and miR-44F, which both block output expression. Presence of miR-hi blocks expression of rtTA, LacI, and miR-44F to de-repress output expression. **(b)** Bistable switch for memory of anhydrotetracycline (aTc) exposure [55]. A 'trigger' circuit (red interaction lines) produces the Cro repressor in response to aTc, as repression of the P_{tet} promoter by TetR is lifted; increased Cro abundance shifts the balance of the cI/Cro bistable memory element (black interaction lines) so that transcription of *cro* and the output *lacZ* is persistently activated. The initial state of cI repression of *cro*/*lacZ* transcription is indicated with a dashed line. **(c)** Tunable transcriptional amplifier based on the *hrp* σ^{54}-dependent activators and inhibitors [22]. A weak transcriptional input signal is used to drive expression of HrpRS, which combine to activate transcription from the strong P_{hrpL} promoter. Gain is controlled through expression of HrpV, which negatively regulates P_{hrpL} activation by sequestering HrpS. **(d)** Dynamic control of fatty acid ethyl ester production (indicated by black arrows) was achieved by Zhang *et al.* (2012) by placing modules for ethanol production and fatty acid consumption under the control of synthetic FadR-regulated promoters [65]. Repression by FadR (full red lines) is relieved in response to fatty acyl-CoA (and to a lesser degree free fatty acid) ligand binding (dashed red lines).

HeLa cells from all other lines in the MicroRNA Atlas database. A positive identification as a HeLa cell results in the decision to express the output protein – either an mRFP fluorescent reporter or the apoptosis-inducing hBax protein. The complete classifier system contains multiple layers of decision making, but here we will highlight features of the HeLa-high-abundance detection subunit.

This subunit takes in three miRNA inputs, and outputs transcription of both *lacI* and a synthetic miRNA (miR-FF4) that go on to synergistically co-regulate the ultimate output (hBax/mRFP); the presence of the three HeLa-high-abundance miRNAs represses transcription of *lacI*/miR-FF4. Rather than having the input miRNAs directly control LacI/miR-FF4 expression, additional motifs were introduced to improve the signal fidelity – important whenever further signal processing is required, and especially so here when the decision is to apoptose or not. Xie *et al.* improved the ON:OFF ratio by introducing feed-forward into the transcription of *lacI*/miR-44F (Fig. 2a) with the rtTA activator and allowing the input miRNAs to repress both rtTA and LacI, thus presumably stabilising the repression of *lacI*/miR-44F by slowing the stochastic re-activation of their transcription [54]. The use of two repressors (LacI plus miR-FF4) to target the ultimate output also improves the ON:OFF ratio by effectively lowering the rate of transcription in the OFF state (reducing leakiness).

At the level of controlling ultimate output expression the decision logic is that of a multi-input NOR gate: the HeLa-low-abundance miRNAs and the LacI/miR-FF4 output from the HeLa-high-abundance miRNA sensing subunit must all be 'OFF' for the ultimate output to be 'ON'. The authors have noted that a real

world implementation of their system as a cancer therapy would benefit from a 'check' function that would highlight cells classified as cancerous using a reporter, before apoptosis is triggered with a chemical stimulus.

Consideration of real-world applicability was central to the demonstration by Kotula *et al*. (2014) of engineered gut bacteria programmed to sense and record the presence of tetracycline [55]. A simple bistable switch based on the well-defined lambda phage cI/Cro system was adapted so that a 100 ng ml^{-1} dose of anhydrotetracycline (aTc) could perturb the system from OFF (cI-high) to ON (Cro-high, LacZ reporter expression from P_{RM} promoter active) (Fig. 2b). *Escherichia coli* cells hosting the sensing and memory system were introduced into the intestinal tract of living mice, where they were able to detect aTc and remain in the triggered state for at least five days, whilst aTc was cleared after only eight hours. A significant feature of the study in terms of practical applicability was the isolation of, and incorporation of the genetic circuit into, a murine-gut-adapted *E. coli* strain. Whilst lab-adapted *E. coli* were essentially outcompeted by gut flora by eight days, the gut-adapted strain was still present at approximately 1000 CFU ml^{-1}. Throughout the report Kotula *et al*. consider the burden of their system on the host – be it the effect of the genetic programme on *E. coli* fitness, or the engineered strain on the host mouse. The authors deliberately chose the cI/Cro system because of its low demand on the host machinery (order of 10^2 cI/Cro molecules per cell), and indeed no detriment to engineered *E. coli* growth rate was observed in competition assays. At the next level up, carrying the reporter strain had no effect on host mouse fitness as assessed by monitoring host weight. This system could be easily adapted from a diagnostic to a treatment technology by changing the input to a sensor for pathogens or disease biomarkers and altering the output to an appropriate therapeutic response [56].

Environmental sensing and remediation

Cell signal processing circuits are ideally suited to enhance the sensitivity, specificity and robustness of cell-based biosensors for detecting environment contaminants and hazards in a cost effective and environmental friendly manner [1,57]. Whole-cell biosensors have been the subject of much research prior to the synthetic biology movement [58], but innovative approaches are now being applied to the topic: the use of components that are orthogonal to (or even escape from [59]) the possibly confounding context of the host metabolism improves sensor accuracy and robustness, as does the integration of sensors with increasingly complex synthetic signal processing circuits. For instance, digital logic is very useful in applications where detection of threshold ligand concentrations is important, and discrimination between various inputs is required. Metal ion detection for bioremediation technologies is one such application. Wang *et al*. (2013) characterised the dose–response of various metal sensing modules in an *E. coli* host strain, using the orthogonal HrpRS activator proteins as an AND gate [57]. This modular system can be tuned by adjusting RBS sequence strength upstream of *hrpR/S* gene, allowing composition of transcriptional signals of differing strengths from various sensors. The AND gate configuration was employed to greatly improve the selectivity of Zn^{2+} sensing, using two modules that were co-activated by one of Pb^{2+} or Cd^{2+}. An input signal to the AND gate can also be inverted by linking it to HrpV (an antagonist of HrpRS activation) expression [7], thereby allowing detection systems to be built that require the absence of one input to trigger the output. Further refinement of the ArsR/P_{arsR}-based arsenic sensing system via tuning the intracelluar ArsR receptor density has pushed the detection sensitivity below the 10 ppb level set for drinking water by the World Health Organisation [60], making biological detection of arsenic applicable to 'real-world' scenarios. Further gains in the detection limit might be achievable by increasing intracellular arsenic levels using importer proteins [61].

The transcriptional output from the P_{arsR} promoter in response to arsenic is relatively weak; the hypersensitive HrpRS protein complex can also be used as an analogue transcriptional amplifier by simply relaying the signal to the stronger P_{hrpL} promoter [7,22]. Maximum transcriptional output is fixed to the strength of P_{hrpL}, but different gain characteristics when the transcriptional input is non-saturating can be realised by modifying the RBS strength upstream of *hrpR/S*. If we consider the scenario where a digital output from the arsenic sensor is required, the effect of the amplifier is to alter the arsenic concentration that activates the sensor output. Tuneable modulation of the activating and amplifying effects of HrpRS can also be achieved, through controlled HrpV expression (Fig. 2c). The negative influence of HrpV on P_{hrpL} output allows the linear output response range of the amplifier to be shifted, and has applications where impedance matching between the sensor and downstream modules is required, or when prototyping the amplifier with new sensor inputs. Furthermore, connection of the amplifier output (P_{hrpL}) to the gain-control input (*hrpV*) would create a negative feedback loop for robust adaptive control of output response.

Bioprocessing control and optimisation

Applying the paradigm of dynamic analogue control of gene expression to designed biosynthetic pathways will be essential if optimal performance is to be maintained in response to changes in environment and chemical concentrations – especially the concentration of pathway intermediates. Factors such as pH, oxygen concentration, and nutrient availability can all be linked to reporters to provide information about the state of the system, allowing the culturing system to compensate for stress via a detection, analysis and actuation loop [62]. Whilst external systems are arguably necessary for some 'high-level' factors such as culture pH, faster and more precise control is desirable at the level of enzyme concentration in biosynthetic pathways [63]. For example, Fung and co-workers demonstrated the integration of a synthetic regulatory network with host metabolism, using a transcriptional oscillator to control and report on glycolytic flux in *E. coli* [64]. Two more recent studies (below) highlight the benefits of dynamic control of gene expression over traditional optimisation of constitutive promoters in the context of biomolecule synthesis applications.

A positive feed-forward signal was employed to coordinate three modules involved in fatty acid ethyl ester (FAEE) biosynthesis [65]. FAEEs are a potential diesel fuel replacement, formed through condensation of free fatty acid and ethanol (a toxic intermediate). FadR senses free fatty acids, disassociating from its 17 bp regulator region within a promoter upon ligand binding to allow RNA

polymerase progression. Synthetic FadR-responsive promoters were built based on the phage lambda and T7 promoters in order to increase the dynamic range (to allow for higher flux in the engineer strain) compared to weaker native FadR-responsive promoters, and after validation were further modified to contain a lacI binding site to allow for external control using IPTG. Genes coding for both ethanol production and FAEE condensation enzymes were then placed under the control of combinations of the synthetic FadR/LacI-responsive promoters, ensuring ethanol was only produced if fatty acid was also available for the condensation reaction, and that fatty-acid CoA-acylation and subsequent esterification were coordinated for efficient use of CoA (Fig. 2d). A three-fold improvement in FAEE yield over the previous best strain was achieved due to the more effective flux balance through the system.

The engineered mevalonate-based pathway for biosynthesis of the toxic isoprenoid farnesyl pyrophosphate (FPP) is linear, making control of toxic FPP accumulation a case of applying both negative feed-back to the FPP synthesis pathway, and positive feed-forward to any downstream FPP consuming enzymes [66]. In the absence of previously identified FPP-responsive elements, the *E. coli* transcriptome was analysed for FPP-responsive promoters. A library of 35 was chosen and screened for the ability to control production of the FPP-derivative amorphadiene. The highest yield using the FPP-responsive promoters resulted from a combination that weakly up-regulated FPP consumption, and weakly down-regulated FPP production. Despite being negatively regulated by FPP concentration, FPP biosynthesis enzymes were produced in higher amounts compared to conditions where expressed from an inducible promoter. Whilst further improvements are possible – some results indicated that higher levels of amorphadiene synthase expression would be beneficial – it was demonstrated that yields were at least 2-fold higher from the strain that self-regulated FPP production compared to those that used inducible/constitutive promoters.

In both examples it was shown that the dynamic response was the key to achieving optimal yield, rather than simply that the pathway enzymes had been tuned to optimal levels; combinations of both stronger and weaker constitutive promoters were less effective. Both studies also showed the dynamically-responsive strains excreted less acetate, implying beneficial effects of improved regulation extend to central metabolic flux. Reviewing their work, the authors look forward to the prospect of much faster post-translational control over metabolic pathways using allosterically regulated enzymes that may be designed *de novo* [63].

Challenges and outlook

The prototype applications described above highlight the great potential of synthetic cell signal processing circuits, but they remain prototypes simply because the creation of new biological functions is not fully an engineering exercise. Though resources of characterised parts are growing, there is not enough information available to generate accurate predictions of the performance of complex synthetic system activities in living organisms. Expanding the range of available orthogonal genetic components remains a key foundational challenge, as there are insufficient orthogonal elements in the current toolkit to design large synthetic biological systems, especially where digital signal processing is used to obtain clear input–output relationships [1]. Digital logic is part-heavy [67] and will encounter problems with scale-up due to the presently limited number of available orthogonal parts and the unpredictable consequences of composing multiple genetic modules. Analogue computing is an attractive alternative strategy for producing high order functions (e.g. linear amplification, addition) without employing a large amount of orthogonal parts. It will be necessary to combine both digital and analogue computing in future advanced signal processing circuits to achieve complex cellular functions, and this requires the development of more part libraries. It is encouraging to observe investigations into new approaches to the engineering of orthogonal regulatory circuit components, such as using splicing protein inteins [43] or CRISPR-Cas9 tools [27], that are highly anticipated to expand the synthetic biologist's tool kit.

Beyond the characteristics of parts themselves, we are just beginning to appreciate the mechanisms behind contextual variance observed in synthetic circuits that gives rise to a large part of their unpredictable behaviour [5,68]. Cardinale and Arkin [69] reviewed three levels of context dependency for synthetic systems: (i) composition – how elements of the system interact; (ii) host – how the system interacts with the cellular machinery; (iii) environment – the influence of external physical and chemical factors, and other organisms. Strategies to measure context dependency are being developed [9,68], and management of some of the interactions is possible: post-transcriptional cleavage of mRNAs, for example, improves translational consistency [35]; use of orthogonal parts [8], dynamic control of pathway flux [65], and use of DNA-based rather than epigenetic memory [51] all minimise interactions with, and the load on, the host machinery. Contextual variance may be significantly reduced through the use of artificial chassis for synthetic gene networks, if the loss of auxiliary biological functions (i.e. anabolic metabolism, ATP production, etc.) is acceptable for the application. Artificial liposomes can be produced with precisely defined contents [70], and recently paper-based systems have been demonstrated as an inexpensive platform for both rapid prototyping and clinical testing [59].

With regard to future implementation, minimising the context dependency of synthetic systems is one way of making them more robust, as there is selection pressure against synthetic systems that cause stress to the host. Depending on the lifetime of the biological device, engineering reduced evolution rates for specific components or the entire host may be required [71]. Robustness as a general feature of designed systems describes the ability to maintain performance in the face of a changing environment. As more complex systems are designed, robustness can be incorporated by using dynamic control mechanisms and adding redundancy to pathways [72].

Beyond the issues surrounding the construction and effectiveness of designer biological systems, real-world deployment of these devices requires them to be safe, and ethically and legally compliant [73,74]. Many safety features can be engineered into [75] synthetic systems, but it is not purely a design problem – the field must continue to foster an open, responsible culture that inspires trust from the public. These issues will be solved in concert: as the behaviour of synthetic biological devices becomes

more predictable and robust, so they become safer. Similarly, repeated demonstrations of the reliability and efficacy of such systems will hopefully improve or maintain public perception of designer biotechnology. Consequently, a cautious, transparent and responsive approach is required for synthetic biological systems to graduate from the lab.

Acknowledgements

This work was supported by a BBSRC project grant [BB/K016288/1] and a Royal Society grant award [RG120527]. B.W. acknowledges the support by the SULSA (Scottish Universities Life Sciences Alliance) and the Wellcome Trust ISSF (Institutional Strategic Support Fund).

References

[1] Wang B, Buck M. Customizing cell signaling using engineered genetic logic circuits. Trends Microbiol 2012;20:376–84.
[2] Endy D. Foundations for engineering biology. Nature 2005;438:449–53.
[3] Kitney R, Freemont P. Synthetic biology – the state of play. FEBS Lett 2012;586:2029–36.
[4] Church GM, Elowitz MB, Smolke CD, Voigt CA, Weiss R. Realizing the potential of synthetic biology. Nat Rev Mol Cell Biol 2014;15:289–94.
[5] Wang B, Kitney RI, Joly N, Buck M. Engineering modular and orthogonal genetic logic gates for robust digital-like synthetic biology. Nat Commun 2011;2:508.
[6] Mutalik VK, Qi L, Guimaraes JC, Lucks JB, Arkin AP. Rationally designed families of orthogonal RNA regulators of translation. Nat Chem Biol 2012;8:447–54.
[7] Wang B, Buck M. Rapid engineering of versatile molecular logic gates using heterologous genetic transcriptional modules. Chem Commun (Camb) 2014;50:11642–44.
[8] Stanton BC, Nielsen AAK, Tamsir A, Clancy K, Peterson T, Voigt CA. Genomic mining of prokaryotic repressors for orthogonal logic gates. Nat Chem Biol 2013;10:99–105.
[9] Arkin AP. A wise consistency: engineering biology for conformity, reliability, predictability. Curr Opin Chem Biol 2013;17:893–901.
[10] Sarpeshkar R. Analog synthetic biology. Philos Trans A Math Phys Eng Sci 2014;372:20130110.
[11] Daniel R, Rubens JR, Sarpeshkar R, Lu TK. Synthetic analog computation in living cells. Nature 2013;497:619–23.
[12] Rinaudo K, Bleris L, Maddamsetti R, Subramanian S, Weiss R, Benenson Y. A universal RNAi-based logic evaluator that operates in mammalian cells. Nat Biotechnol 2007;25:795–801.
[13] Tamsir A, Tabor JJ, Voigt CA. Robust multicellular computing using genetically encoded NOR gates and chemical wires. Nature 2011;469:212–5.
[14] Tabor JJ, Salis HM, Simpson ZB, Chevalier AA, Levskaya A, Marcotte EM, et al. A synthetic genetic edge detection program. Cell 2009;137:1272–81.
[15] Viney M, Reece SE. Adaptive noise. Proc Biol Sci 2013;280:20131104.
[16] Xie Z, Wroblewska L, Prochazka L, Weiss R, Benenson Y. Multi-input RNAi-based logic circuit for identification of specific cancer cells. Science 2011;333:1307–11.
[17] Salis HM, Mirsky EA, Voigt CA. Automated design of synthetic ribosome binding sites to control protein expression. Nat Biotechnol 2009;27:946–50.
[18] Becskei A, Séraphin B, Serrano L. Positive feedback in eukaryotic gene networks: cell differentiation by graded to binary response conversion. EMBO J 2001;20:2528–35.
[19] Nistala GJ, Wu K, Rao CV, Bhalerao KD. A modular positive feedback-based gene amplifier. J Biol Eng 2010;4:4.
[20] Brophy JAN, Voigt CA. Principles of genetic circuit design. Nat Methods 2014;11:508–20.
[21] Nevozhay D, Adams RM, Murphy KF, Josic K, Balázsi G. Negative autoregulation linearizes the dose-response and suppresses the heterogeneity of gene expression. Proc Natl Acad Sci U S A 2009;106:5123–8.
[22] Wang B, Barahona M, Buck M. Engineering modular and tunable genetic amplifiers for scaling transcriptional signals in cascaded gene networks. Nucleic Acids Res 2014;42:9484–92.
[23] Wang B, Kitney R, Buck M, Jovanovic M, Joly N, James E. The design and construction of a set of modular synthetic biologic devices for programming cells. IFMBE Proc 2009;25(8):289–92.
[24] Elowitz MB, Leibler S. A synthetic oscillatory network of transcriptional regulators. Nature 2000;403:335–8.
[25] Lohmueller JJ, Armel TZ, Silver PA. A tunable zinc finger-based framework for Boolean logic computation in mammalian cells. Nucleic Acids Res 2012;40:5180–7.
[26] Moore R, Chandrahas A, Bleris L. Transcription activator-like effectors: a toolkit for synthetic biology. ACS Synth Biol 2014;3:708–16.
[27] Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 2013;152:1173–83.
[28] Kiani S, Beal J, Ebrahimkhani MR, Huh J, Hall RN, Xie Z, et al. CRISPR transcriptional repression devices and layered circuits in mammalian cells. Nat Methods 2014;11:723–6.
[29] Temme K, Hill R, Segall-Shapiro TH, Moser F, Voigt CA. Modular control of multiple pathways using engineered orthogonal T7 polymerases. Nucleic Acids Res 2012;40:8773–81.
[30] Shis DL, Bennett MR. Library of synthetic transcriptional AND gates built with split T7 RNA polymerase mutants. Proc Natl Acad Sci U S A 2013;110:5028–33.
[31] Rhodius VA, Segall-Shapiro TH, Sharon BD, Ghodasara A, Orlova E, Tabakh H, et al. Design of orthogonal genetic switches based on a crosstalk map of σs, anti-σs, and promoters. Mol Syst Biol 2013;9:702.
[32] Na D, Yoo SM, Chung H, Park H, Park JH, Lee SY. Metabolic engineering of *Escherichia coli* using synthetic small regulatory RNAs. Nat Biotechnol 2013;31:170–4.
[33] Qi LS, Arkin AP. A versatile framework for microbial engineering using synthetic non-coding RNAs. Nat Rev Microbiol 2014;12:341–54.
[34] Kang Z, Zhang C, Zhang J, Jin P, Zhang J, Du G, et al. Small RNA regulators in bacteria: powerful tools for metabolic engineering and synthetic biology. Appl Microbiol Biotechnol 2014;98:3413–24.
[35] Qi L, Haurwitz RE, Shao W, Doudna JA, Arkin AP. RNA processing enables predictable programming of gene expression. Nat Biotechnol 2012;30:1002–6.
[36] Rodrigo G, Landrain TE, Jaramillo A. De novo automated design of small RNA circuits for engineering synthetic riboregulation in living cells. Proc Natl Acad Sci U S A 2012;109:15271–76.
[37] Qi L, Lucks JB, Liu CC, Mutalik VK, Arkin AP. Engineering naturally occurring trans-acting non-coding RNAs to sense molecular signals. Nucleic Acids Res 2012;40:5775–86.
[38] Sharma V, Nomura Y, Yokobayashi Y. Engineering complex riboswitch regulation by dual genetic selection. J Am Chem Soc 2008;130:16310–15.
[39] Grünberg R, Serrano L. Strategies for protein synthetic biology. Nucleic Acids Res 2010;38:2663–75.
[40] Wang B, Barahona M, Buck M, Schumacher J. Rewiring cell signalling through chimaeric regulatory protein engineering. Biochem Soc Trans 2013;41:1195–200.
[41] Barnea G, Strapps W, Herrada G, Berman Y, Ong J, Kloss B, et al. The genetic design of signaling cascades to record receptor activation. Proc Natl Acad Sci U S A 2008;105:64–9.
[42] Aranko AS, Wlodawer A, Iwaï H. Nature's recipe for splitting inteins. Protein Eng Des Sel 2014;27:263–71.
[43] Lienert F, Torella JP, Chen J-H, Norsworthy M, Richardson RR, Silver PA. Two- and three-input TALE-based AND logic computation in embryonic stem cells. Nucleic Acids Res 2013;41:9967–75.
[44] Mootz HD, Blum ES, Tyszkiewicz AB, Muir TW. Conditional protein splicing: a new tool to control protein structure and function in vitro and in vivo. J Am Chem Soc 2003;125:10561–69.
[45] Selgrade DF, Lohmueller JJ, Lienert F, Silver PA. Protein scaffold-activated protein trans-splicing in mammalian cells. J Am Chem Soc 2013;135:7713–9.
[46] Park S-H, Zarrinpar A, Lim WA. Rewiring MAP kinase pathways using alternative scaffold assembly mechanisms. Science 2003;299:1061–4.
[47] Bonger KM, Chen L, Liu CW, Wandless TJ. Small-molecule displacement of a cryptic degron causes conditional protein degradation. Nat Chem Biol 2011;7:531–7.
[48] Prindle A, Selimkhanov J, Li H, Razinkov I, Tsimring LS, Hasty J. Rapid and tunable post-translational coupling of genetic circuits. Nature 2014;508:387–91.
[49] Gardner TS, Cantor CR, Collins JJ. Construction of a genetic toggle switch in *Escherichia coli*. Nature 2000;403:339–42.
[50] Ajo-Franklin CM, Drubin DA, Eskin JA, Gee EPS, Landgraf D, Phillips I, et al. Rational design of memory in eukaryotic cells. Genes Dev 2007;21:2271–6.
[51] Bonnet J, Subsoontorn P, Endy D. Rewritable digital data storage in live cells via engineered control of recombination directionality. Proc Natl Acad Sci U S A 2012;109:8884–9.
[52] Bonnet J, Yin P, Ortiz ME, Subsoontorn P, Endy D. Amplifying genetic logic gates. Science 2013;340:599–603.
[53] Siuti P, Yazbek J, Lu TK. Synthetic circuits integrating logic and memory in living cells. Nat Biotechnol 2013;31:448–52.
[54] Mangan S, Alon U. Structure and function of the feed-forward loop network motif. Proc Natl Acad Sci U S A 2003;100:11980–85.
[55] Kotula JW, Kerns SJ, Shaket LA, Siraj L, Collins JJ, Way JC, et al. Programmable bacteria detect and record an environmental signal in the mammalian gut. Proc Natl Acad Sci U S A 2014;111:4838–43.
[56] Duan F, March JC. Engineered bacterial communication prevents *Vibrio cholerae* virulence in an infant mouse model. Proc Natl Acad Sci U S A 2010;107:11260–64.
[57] Wang B, Barahona M, Buck M. A modular cell-based biosensor using engineered genetic logic circuits to detect and integrate multiple environmental signals. Biosens Bioelectron 2013;40:368–76.
[58] Hansen LH, Sørensen SJ. The use of whole-cell biosensors to detect and quantify compounds or conditions affecting biological systems. Microb Ecol 2001;42:483–94.
[59] Pardee K, Green AA, Ferrante T, Cameron DE, DaleyKeyser A, Yin P, et al. Paper-based synthetic gene networks. Cell 2014;1–15.

[60] Wang B, Barahona M, Buck M. Amplification of small molecule-inducible gene expression via tuning of intracellular receptor densities. Nucleic Acids Res 2015;43(3):1955–64.
[61] Nagy CI, Vass I, Rákhely G, Vass IZ, Tóth A, Duzs A, et al. Coregulated genes link sulfide:quinone oxidoreductase and arsenic metabolism in *Synechocystis* sp. strain PCC6803. J Bacteriol 2014;196:3430–40.
[62] Polizzi KM, Kontoravdi C. Genetically-encoded biosensors for monitoring cellular stress in bioprocessing. Curr Opin Biotechnol 2014;31C:50–6.
[63] Zhang F, Keasling J. Biosensors and their applications in microbial metabolic engineering. Trends Microbiol 2011;19:323–9.
[64] Fung E, Wong WW, Suen JK, Bulter T, Lee S, Liao JC. A synthetic gene-metabolic oscillator. Nature 2005;435:118–22.
[65] Zhang F, Carothers JM, Keasling JD. Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. Nat Biotechnol 2012;30:354–9.
[66] Dahl RH, Zhang F, Alonso-Gutierrez J, Baidoo E, Batth TS, Redding-Johanson AM, et al. Engineering dynamic pathway regulation using stress-response promoters. Nat Biotechnol 2013;1–10.
[67] Moon TS, Lou C, Tamsir A, Stanton BC, Voigt CA. Genetic programs constructed from layered logic gates in single cells. Nature 2012;491:249–53.
[68] Cardinale S, Joachimiak MP, Arkin AP. Effects of genetic variation on the *E. coli* host–circuit interface. Cell Rep 2013;4:231–7.
[69] Cardinale S, Arkin AP. Contextualizing context for synthetic biology – identifying causes of failure of synthetic biological systems. Biotechnol J 2012;7:856–66.
[70] Tan C, Saurabh S, Bruchez MP, Schwartz R, Leduc P. Molecular crowding shapes gene expression in synthetic cellular nanosystems. Nat Nanotechnol 2013;8:602–8.
[71] Renda BA, Hammerling MJ, Barrick JE. Engineering reduced evolutionary potential for synthetic biology. Mol Biosyst 2014;10:1668–78.
[72] Zhu L, Zhu Y, Zhang Y, Li Y. Engineering the robustness of industrial microbes through synthetic biology. Trends Microbiol 2012;20:94–101.
[73] Anderson J, Strelkowa N, Stan G-B, Douglas T, Savulescu J, Barahona M, et al. Engineering and ethical perspectives in synthetic biology. EMBO Rep 2012;13:584–90.
[74] Balmer A, Martin P. Synthetic biology: social and ethical challenges. London: BBSRC; 2008.
[75] Church G. Let us go forth and safely multiply. Nature 2005;438:423.