Review

Synthetic Biology is the ‘Engineering of Biology’ – it aims to use a forward-engineering design cycle based on specifications, modelling, analysis, experimental implementation, testing and validation to modify natural or design new, synthetic biology systems so that they behave in a predictable fashion. Motivated by the need for truly plug-and-play synthetic biological components, we present a comprehensive review of ways in which the various parts of a biological system can be modified systematically. In particular, we review the list of ‘dials’ that are available to the designer and discuss how they can be modelled, tuned and implemented. The dials are categorized according to whether they operate at the global, transcriptional, translational or post-translational level and the resolution that they operate at. We end this review with a discussion on the relative advantages and disadvantages of some dials over others.

Introduction

The primary goal of Synthetic Biology is to create new or add additional functionality to biological systems by constructing new parts, or modifying existing biological systems (Purnick & Weiss, 2009). Central to this goal is the idea that the synthetic organism is designed following a systematic design framework with a specific objective in mind designed a priori. Ideally such design objectives can be formulated in a quantitative manner so that the performance of the designed component can be quantified and compared to the original design specification. This design framework is required both to improve reliability of individual biological components and to build functioning genetic systems with a larger number of interconnected parts (Purnick & Weiss, 2009), both considered to be current challenges of Synthetic Biology. Currently, one of the main efforts of Synthetic Biology is on building genetic systems in micro-organisms, not only because of their relative simplicity but as it is envisioned that small genetic circuits can potentially be used as a foundation for building more complex systems (Andrianantoandro et al., 2006).

Although Synthetic Biology has been described as the ‘Engineering of Biology’, a systematic design cycle is still not realized to its full potential, limiting the advancement of the field in terms of functionality, reliability and size of the genetic systems (Purnick & Weiss, 2009). A design framework involves design specifications, modelling, conceptual and detailed design, as well as implementation and testing (Fig. 1). In Synthetic Biology, carrying out conceptual design (e.g. choosing the basic genetic system layout) is currently relatively simple due to the limited size of present-day synthetic genetic systems, but this will become more involved as more complicated systems can be built (Purnick & Weiss, 2009; Slusarczyk et al., 2012). Similarly, methods are being developed to design modules for spatial organization of the cell (Chau et al., 2012; Lim et al., 2012), metabolic pathways and microbial communities (Shong et al., 2012). At the same time, the present design framework needs to be improved with respect to how specifications, more detailed design and robust implementation are performed. An improved forward-engineering framework would consist of a mathematical model of the system chosen in the conceptual design stage,
which can provide a basis for the design, construction, characterization and testing of the developed system. The parameters in this model can then be ‘tuned’ in a systematic manner in order to ensure that the resulting model meets the design specifications. The model with the chosen parameters and predicted performance can be built and its behaviour can then guide subsequent design, implementation and testing.

However, this is easier said than done. Indeed, when ‘tuning’ the different biological dials it is important to fully understand the relationship between specifications, model parameters, biological parts and implementation in order to carry out the design process. The dials used to redesign a biological system can include tuning global parameters or transcriptional, translational and post-translational parameters in the mathematical models. Experimentally this can be achieved by using different plasmid replicons for controlling gene copy number, different promoters to control the rate of transcription initiation, different ribosome-binding sites (RBSs), or different synonymous codons for controlling translation levels or degradation rates of all the species in the systems. The models used for the basic design of genetic systems often contain parameters representing multiple biological parts and so tuning a parameter in a mathematical model can be implemented experimentally in different ways. For example, in the simplest models of gene expression, one parameter often represents many different biological characteristics, such as a ‘transcription’ parameter representing promoter strength, transcription rate and gene copy number. Each of these has different tuning ranges, uncertainties and ease of experimental modification.

In this paper, we present a comprehensive review of ways in which the various parts of a biological system can be modified systematically, focussing in particular on building genetic systems. We first discuss design and modelling of genetic systems, before reviewing in detail the typical dials that can be modified in a Synthetic Biology project. We then present various ways to tune these dials in order to achieve a desirable objective and show how tuning the parameters for each of these dials affects the output of a simple genetic system.

**System design and parameter tuning**

Synthetic Biology aims to be the ‘Engineering of Biology’, where an engineering design cycle is used to systematically improve existing biological systems and create new ones (Anderson et al., 2012). A traditional engineering example is the design of a chemical plant. In this case specifications may include the concentrations of the final products, a conceptual design may determine the order of processes and reactions, while a more detailed design may set variables such as concentrations and flow rates in these processes, followed by further component details based on these variables such as sizes of pipes and reaction vessels (Perry & Green, 1999). Similarly, in a biological system, the specifications may be based on protein concentrations and their response characteristics, while a conceptual design may determine the order of processes and reactions, while a more detailed design may set variables such as concentrations and flow rates in these processes, followed by further component details based on these variables such as sizes of pipes and reaction vessels (Perry & Green, 1999). Similarly, in a biological system, the specifications may be based on protein concentrations and their response characteristics, while a conceptual design may determine the order of processes and reactions, while a more detailed design may set variables such as concentrations and flow rates in these processes, followed by further component details based on these variables such as sizes of pipes and reaction vessels (Perry & Green, 1999). 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specifications. Once a detailed design is completed and verified, the system can be built and then tested to validate the design, with the design cycle repeated if at any stage the resulting functionality is not acceptable or requires improvement (RAEng, 2009).

The first step in the design of a genetic system is to specify its functionality for defined inputs and outputs. For example, the system may be required to act as a memory device or a switch (Gardner et al., 2000) where the input is the concentration of an inducer and the output is the concentration of a protein. Performance specifications are required in order to determine whether the functionality is met for a particular design (Sen & Murray, 2012). These specifications can be composed of several metrics (Canton et al., 2008; Del Vecchio et al., 2008; Sen & Murray, 2012). For a switch, for example, there could be a requirement for the (time) mean protein concentration to be between set limits when the switch is ‘on’ or ‘off’. Retroactivity specifications, or insulation, may also be required. This ensures that the functionality of the genetic system is not negatively affected when it is connected to another system upstream or downstream (Del Vecchio et al., 2008). Another metric could be the response time: the time it takes for the protein concentration to switch from low to high with a change in inducer concentration (Canton et al., 2008). Also, there may be requirements on the limits of variability or noise around the mean of a protein concentration level (Lestas et al., 2010). Finally, a genetic system design should meet all performance metrics despite noise and uncertainty associated with the components and chassis of the system, as well as the uncertainty in cell size due to growth.

Once specifications are set, the design of a genetic system consists of a conceptual phase (e.g. determining genetic system topology) and then using appropriate models to complete a more detailed design. The latter involves determining model parameters to meet the design specifications set. In the conceptual phase, different system topologies can be used to obtain a desired behaviour, e.g., oscillators (Purcell et al., 2010; Slusarczyk et al., 2012; Strelkowa & Barahona, 2010), switches (Pfeuty & Kaneko, 2009) and adaptive systems (Ma et al., 2009), and more complicated systems can be built to produce more advanced behaviour (Slusarczyk et al., 2012). In the detailed design phase, a mathematical model must first be built and analysed. This model will guide the design but also be used for predicting whether a proposed design meets the required specifications. The same model will also be used for steps after design such as comparison with data from the testing phase of the engineering design cycle. Such models usually take the form of differential equations based on the biochemical reactions defining the designed system (Wilkinson, 2011). These differential equations can be deterministic or stochastic. The design proceeds by using standard optimization and control engineering approaches on the deterministic models to find the best parameter choice that achieves a desired objective. A combination of both simulations (Wilkinson, 2011) and analytical methods (Murray, 2002; Tyson et al., 2003) can then be used to verify the behaviour of the models. In particular, stochastic simulations are very useful in testing the variability of the system due to noise, and to ensure that stochastic effects do not substantially change the system behaviour for low biochemical species numbers (Tian & Kevin, 2006; Wilkinson, 2011). Once design parameters are selected, further models may be required for component design, such as for designing a RBS to match a tuneable parameter (Na et al., 2010). The design process may need iteration, so that if no feasible choice of parameters for a particular system can meet the specifications, then a different topology can be used. Furthermore, once the system is implemented and tested, the process may need to be iterated. In particular, further detailed design or ‘tuning of the dials’ may be necessary for the circuit to function and meet specifications.

Discussion of the genetic system design leads naturally to the question of implementation, the main focus of this review. Which biological components should be modified in order to implement different genetic systems?

A simple genetic system

Before we discuss this question, let us consider a simple, illustrative genetic system and its associated model as an example (Fig. 2). This system will be used throughout the paper to illustrate the engineering design cycle and how ‘tuning dials’ can be performed during the design and implementation stages. The example genetic system contains a constitutive promoter driving the expression of a repressor protein, which in turn represses the expression of a reporter gene from a regulated promoter. The measured output of the system is the concentration of the reporter protein while the input is the concentration of an inducer, which binds to the repressor protein thereby sequestering it away and allowing transcription initiation. The biochemical equations used to model this system are shown in Fig. 2.

The biochemical equations are the mathematical description of the underlying biochemical reactions of the system. From a biological viewpoint, the reactions that must be described are: transcription, translation, repressor–promoter and repressor–inducer interactions, and degradation of species within the system. Equations (1) and (2) describe RNA polymerase binding to a promoter followed by transcription initiation for the repressor and reporter genes, respectively. Initiation of transcription is a reversible reaction (as denoted by the double arrows and forward and reverse reaction rate constants in the equations), whereas extension is considered to be irreversible. Equation (3) is included to reflect the biological reality that most promoters have some basal level of transcription in the absence of an inducer (also called leakiness). Taken together, these equations describe the generation of mRNA species in the system.

Equations (4) and (5) describe the binding of ribosomes to a RBS on mRNA, before translation is initiated for the
repessor and reporter, respectively. These steps have been described as two reactions so that the RBS strength (which is easy to modify experimentally – see below) can be described as two reactions so that the RBS strength (which is easy to modify experimentally – see below) can be described as two reactions so that the RBS strength (which is easy to modify experimentally – see below) can be described as two reactions so that the RBS strength (which is easy to modify experimentally – see below) can be described as two reactions so that the RBS strength (which is easy to modify experimentally – see below) can be described as two reactions so that the RBS strength (which is easy to modify experimentally – see below) can be described as two reactions so that the RBS strength (which is easy to modify experimentally – see below) can be described as two reactions so that the RBS strength (which is easy to modify experimentally – see below) can be described as two reactions so that the RBS strength (which is easy to modify 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these networks. It has been shown that the abundance of RNA polymerases and ribosomes is dependent on cellular growth rates, affecting downstream processes such as transcription rate, translation rate, gene copy number, mRNAs degradation, protein dilution, protein degradation and cell mass (Klumpp et al., 2009). Regulated processes such as promoter repression or activation have also shown growth rate dependence, affecting genetic networks as a result (Klumpp et al., 2009; Scott et al., 2010; Tan et al., 2009). There are a wide range of strains and organisms that can be used to harbour synthetic genetic networks, and in some cases, the networks work predictably across different strains (Prindle et al., 2012) whilst in other cases the behaviour of the network can be drastically altered by changing the host cell (Egbert & Klavins, 2012). In this latter reference, two strains of E. coli were used that both contained a LacIΔ mutation but with slightly different genotypes, exemplifying that even similar strains can have profound effects on network function. Some tuneable elements may also be strain specific and whilst they function in one strain they may be inactive in another, e.g. the DIAL strains used to modulate plasmid copy number (Kittleson et al., 2011).

Gene copy number. The number of gene copies can be used to increase or decrease the amount of available protein over a partially linear range. Gene copy number can be controlled either by changing the origin of replication of a plasmid-bound gene of interest or by increasing the number of chromosomally integrated copies. Plasmid copy number has been shown to play a key role in the behaviour of genetic networks (Atkinson et al., 2003; Mileyko et al., 2008). The range of accessible values is limited to a maximum value that does not saturate the cellular transcription and translation machinery and does not cause undue metabolic burden on cells (‘run-away’ replication) (Nordström & Uhlin, 1992).

Gene copy number is not a continuous number. Chromosomal integration has been shown to tolerate up to five copies of the same gene (Choi et al., 2006), although this could potentially be increased further, and plasmids have a few discrete values that can be accessed (Table 1). Engineering endeavours have produced plasmids with inducible copy number that can be controlled either by the binding of a ligand (Panayotatos, 1984) or through changes in temperature (Sternberg, 1990), allowing dynamic shifts in copy number to be used as a design variable. Alternatively, multiple bacterial strains have been developed (DIAL strains) that maintain the same plasmid at different steady state copy numbers (Kittleson et al., 2011). These techniques give another level of control and tuneability of plasmid copy number in genetic systems.

The potential to maintain multiple plasmids, encoding different components from genetic networks, at different copy numbers within a cell is also possible. This is, however, dependent on the incompatibility group of the plasmid (Table 1) (Tolia & Joshua-Tor, 2006). In addition, multi-copy plasmids are maintained at a mean value in each cell, leading to population variations (Ebersbach & Gerdes, 2005) that will need to be accounted for in modelling efforts. Although plasmid copy numbers have been described here, they are dependent on the context within which they are used. The copy number of plasmids has been shown to have an inverse relationship to the plasmid size (Ebersbach & Gerdes, 2005; Zhong et al., 2011) and cellular growth rate (Klumpp, 2011), and their maintenance within a cell is dependent on the toxicity of the genes encoded on the plasmid (Mileyko et al., 2008).

Transcription level design

Promoter type. Promoters are regions of DNA containing consensus sequences for the recruitment of the transcriptional machinery (sigma factors; RNA polymerase, RNAP) to initiate transcription (Gruber & Gross, 2003). Core promoter sequences are recognized by different sigma factors which are active under different environmental conditions, for example, Escherichia coli σ70, σS, σ32 and σ54 (Table 2) (Gruber & Gross, 2003). The number of σ factors used in bacteria can vary from 1 (in Mycoplasma genitalium) to 63 (in Streptomyces coelicolor) and provides an intricate way for organisms to regulate gene expression in response to specific environmental conditions. Promoters can also be constitutively active or regulated (Fig. 3). Constitutive promoters are active without the need for any transcription factors, whilst regulated promoters have operator-binding sites for repressors or activators that block or assist RNAP binding respectively in the presence of a small molecule (inducer) or under certain environmental conditions (Lloyd et al., 2001) (Table 2). Combinatorial promoter design has been implemented to generate hybrid promoters (also sometimes called logic gates) that are conditionally activated in the presence of multiple inducer signals (Fig. 3) (Cox et al., 2007).

Inducer concentration. Each transcriptional repressor/activator will respond to one or more small molecules known as inducers. There are natural inducers (e.g. allo-lactose for the Lac repressor (Lewis et al., 1996) or tetracycline for the Tet repressor (Orth et al., 2000)), and in some instances non-metabolizable chemical analogues that cause gratuitous induction (e.g. isopropyl-β-D-thiogalactoside, IPTG, for the Lac repressor (Lewis et al., 1996) or anhydrotetracycline, aTc, for the Tet repressor (Lederer et al., 1996)). The advantage of the chemical analogues is that their concentration level remains roughly constant. The level of transcription follows a sigmoidal response to the inducer concentration, which, over a certain range, can be approximated as linear (Table 3). Often the slope of this linear approximation is very large, which may make tuning difficult. Mutations in the small molecule binding site of the repressor could shift the range over which the response is linear (Satya Lakshmi & Rao, 2008), adding further control.
Promoter leakiness and basal expression. The function of promoters is often thought of encompassing two states, either on or off. However, this is very rarely the case, with transcription initiation still occurring even in the repressed state (Lanzer & Bujard, 1988). Often this behaviour is not desirable for inducible promoters. However, it can be beneficial if a particular level of basal expression is required in the repressed state but can be further increased with the addition of an inducer. The leakiness of a promoter can be tuned through the location (Lanzer & Bujard, 1988; Murphy et al., 2007), number (Murphy et al., 2007) and sequence (Alper et al., 2005; Lanzer & Bujard, 1988) of the operator binding sites (Fig. 4) and core promoter elements. A combinatorial TetR repressed promoter library has been described previously with varying operator multiplicity and location resulting in promoters with leaky expression in the repressed state spanning more than two orders of magnitude (Murphy et al., 2007). The strength of repressor binding to its operator sites can affect promoter leakiness and can be tuned through mutation of the operator DNA sequence or the DNA-binding domain of the repressor protein (Nichols & Matthews, 1997; Penumetcha et al., 2010). Another way with which basal transcription can be tuned is through the co-expression of the target gene from a constitutive promoter as well as a regulated promoter, although this will also result in an increase in gene expression level in the induced state.

Promoter strength. Promoter strength can be tuned by creating promoter libraries randomized in the RNAP-binding regions affecting RNAP-binding affinity (Alper et al., 2005; Brewster et al., 2012; Jensen & Hammer, 1998).

Table 1. Plasmid copy number and plasmid incompatibility groups

| Plasmid | Replicon | Copy number* | Reference |
|---------|----------|-------------|-----------|
| ColE1   | ColE1    | 15–20       | Schmidt & Inselburg (1982); Shizuya et al. (1992) |
| pBR322  | pMB1     | 15–20       | Balbas et al. (2013); Lin-Chao et al. (1992); Sternberg (1990) |
| pBR3272 | pMB1     | 30–40†      | Hakkaart et al. (1985); Kool & Nijkamp (1974); Lin-Chao et al. (1992) |
| pUC     | pMB1     | >200†       | Figurski et al. (1979); Lin-Chao et al. (1992) |
| pACYC   | p15A     | 18–22       | Chang & Cohen (1978); Hasunuma & Sekiguchi (1979) |
| F1      | F1       | 1–2         | Peterson & Phillips (2008); Shizuya et al. (1992) |
| pNS358  | P1 lytic ori | 1–25 | Peterson & Phillips (2008); Sternberg (1990) |
| pCDF    | CloDF13  | 10–90       | Hakkaart et al. (1985); Kool & Nijkamp (1974); Kües & Stahl (1989) |
| RK2     | RK2      | 4–7         | Figurski et al. (1979); Kittleson et al. (2011) |
| pSC101  | pSC101   | –5          | Hasunuma & Sekiguchi (1979); Kittleson et al. (2011) |
| pJPA12  | pSC101   | 27          | Deuschle et al. (1986); Gruber & Gross (2003); Peterson & Phillips (2008) |
| pJPA13  | pSC101   | ~240        | Becker & Hengge-Aronis (2001); Peterson & Phillips (2008) |
| pRSF    | RSF1030  | 10–60       | Grossman et al. (1987); Kües & Stahl (1989); Wang & DeHaseth (2003) |
| pBjk2992-jtk2541 | ColE2 | 1–60  | Bernardo et al. (2009); Buck et al. (2000); Kittleson et al. (2011) |
| pBjk2993-jtk2541 | R6K | 5–250  | Kittleson et al. (2011); Schleif (2000) |

*Plasmid copy number is dependent on plasmid size, gene toxicity and, in some instances (indicated by †), by temperature.

Table 2. Constitutive, negative, positive and hybrid regulated promoter types

| Promoter | σ factor | Regulatory type* | Regulator | Inducer | Reference |
|----------|----------|-----------------|-----------|---------|-----------|
| Pβlac    | σ70      | Con.            | NA        | NA      | Deuschle et al. (1986); Gruber & Gross (2003) |
| csiD     | σ5       | Con.            | NA        | Starvation/heat shock/nutrient starvation | Becker & Hengge-Aronis (2001) |
| PGroE    | σ32      | Con.            | NA        | Heat shock/nutrient starvation | Grossman et al. (1987); Wang & DeHaseth (2003) |
| Pα       | σ54      | Con.            | NA        | Nitrogen/nutrient starvation | Bernardo et al. (2009); Buck et al. (2000) |
| Pbad     | σ70      | Pos.            | AraC      | Arabinose | Schleif (2000) |
| P1lacO-1 | σ70      | Neg.            | LacR      | IPTG    | Lutz & Bujard (1997) |
| P1tetO-1 | σ70      | Neg.            | TetR      | aTc     | Lutz & Bujard (1997) |
| P1lac/ara-1 | σ70  | Neg./Pos.        | LacR/AraC | IPTG/arabinose | Lutz & Bujard (1997) |

*Con., constitutive; Pos., positive regulation; Neg., negative regulation.
in the operator region, which changes the strength of the interaction of the repressor/activator with the DNA (Alper et al., 2005), or in the DNA sequences flanking the promoter, reducing any upstream/downstream context dependent effects on promoter strength (Davis et al., 2011) (Fig. 4). Mutation of the DNA sequences between important binding motifs has also shown that a linear variation in promoter strength can be achieved (Ellis et al., 2009; Jensen & Hammer, 1998). Although discrete values will be obtained through the creation of a randomized promoter library, the promoter strength can typically be treated as a continuous variable for modelling purposes. Randomized promoter libraries have elicited numerous constitutive and regulated promoters with activities spanning over two orders of magnitude (Alper et al., 2005; Davis et al., 2011; Ellis et al., 2009; Jensen & Hammer, 1998). A limitation in the use of these promoters as predictable parts is the lack of a standardized promoter.

**Table 3. Inducers and their working conditions**

| Promoter type | Repressor | Inducer | Working condition       | Reference                  |
|---------------|-----------|---------|-------------------------|----------------------------|
| Lac           | LacI      | IPTG    | $10^{-4}$–$10^{-3}$ M   | Lutz & Bujard (1997)       |
| Lac           | LacI*     | IPTG    | $10^{-2}$–$10^{-4}$ M   | Satya Lakshmi & Rao (2008) |
| Lac           | LacI*     | Temperature | 30–40 °C             | Yabuta et al. (1995)      |
| Tet           | TetR      | aTc     | 5–50 ng ml $^{-1}$      | Lutz & Bujard (1997)       |
| P$_{BAD}$     | AraC      | Arabinose | $10^{-4}$–$10^{-1}$ M  | Mirasoli et al. (2002)    |
| P$_{gfp/P_{R}}$ | c 857     | Temperature | 30–42 °C             | Menart et al. (2003); Villaverde et al. (1993) |
| P$_{lux}$     | LuxR      | AHL     | $10^{-9}$–$10^{-6}$ M   | Koch (2005); Urbanowski et al. (2004) |

*LacI mutants developed to respond to decreased IPTG concentration or alternative stimulus.*
strength metric. Promoters can often perform differently from how their original characterization would suggest, due to differences in experimental conditions and measurement equipment. Therefore predicting the behaviour of a gene regulatory network component such as a promoter across different laboratories can be difficult. The need for a promoter strength metric for the accurate comparison of promoters produced from different libraries, experimental conditions and laboratories has resulted in the development of a technique to standardize a promoter strength with respect to a reference promoter, and quantifying this relative strength in terms of relative promoter units (Kelly et al., 2009).

Placement of genes in a multi-gene construct or operon. The length of time it takes to transcribe a gene depends upon the gene length (RNAP transcribes at a rate of \(\approx 50 \text{ bp s}^{-1}\) (Gotta et al., 1991; Vogel & Jensen, 1994)) and its distance from the transcription initiation site. Therefore, by placing one or more genes in front of the gene of interest it is possible to introduce a delay in the transcription process. It is important to note, however, that there may be unintended effects on the translation process if the secondary structure of the mRNA molecule produced causes ribosomes to disengage before translation of the gene of interest, or if secondary structure elements are introduced which alter the stability of the mRNA molecule (Carrier & Keasling, 1997a; Newbury et al., 1987). In principle, this transcription delay increases linearly with the length of the superfluous genes added in front of the gene of interest and can be approximated as a continuous variable – although, strictly speaking, this is a discrete variable whose values are multiples of the time it takes to transcribe a single base (though very long mRNA constructs will tend to have larger translational effects). An increase in the length of a transcript also has a positive influence on the amount of translation from the first gene in an operon (Lim et al., 2011). This is due to the fact that transcription and translation take place simultaneously in prokaryotes. Therefore, the first genes in an operon have a longer period for translation during transcription before RNAP dissociation and mRNA degradation (Lim et al., 2011).

Translation level design

Ribosome-binding site (RBS) strength. The efficiency of translation initiation depends on the recruitment of ribosomes to the Shine–Dalgarno sequence, which can be stronger or weaker depending on variations in this sequence and the distance from the translation initiation codon (Chen et al., 1994; Osterman et al., 2013). The RBS strength is also dependent on the upstream (Komarova et al., 2005) and downstream mRNA sequence (Salis et al., 2009) due to the formation of local secondary structures that can influence or inhibit ribosome binding. Prediction

**Fig. 4.** Transcriptional and translational control by riboregulators. A schematic representation of transcriptional control by a riboswitch (a), and translational control by a riboswitch (b) or a trans-activating RNA (taRNA) (c).
of the strength of prokaryotic Shine–Dalgarno sequences can therefore be facilitated by the use of Chris Voigt’s simulation prediction program (RBS calculator) (Salis et al., 2009) or Gyoo Yeol Jung’s UTR Designer (Seo et al., 2013). Over 100 predicted RBSs have been experimentally tested showing that the translation initiation rate can be controlled over a 100,000-fold range (Salis et al., 2009). The Ouyang lab used the RBS calculator to design RBSs with predicted strengths for use in a predetermined bistable toggle switch exemplifying the usefulness of this tool (Chen et al., 2012). Fine-tuning of a genetic toggle switch has also been demonstrated by altering the length of the spacer between the Shine–Dalgarno sequence and the start codon (Egbert & Klavins, 2012). Comparisons of experimental data with RBS calculator predictions were in fairly good agreement dependent on the spacer sequence makeup (Egbert & Klavins, 2012).

**Codon optimization.** Because of the degeneracy of the genetic code, it is possible to create mRNA transcripts with differing sequence that encode the same protein, eliminating rare codons and increasing translational efficiency. An altered coding sequence can also contribute to different mRNA secondary structures and, therefore, translational efficiency. Whilst standard codon optimization techniques aim to maximize protein production through using the most abundant codons observed for highly expressing native host proteins (codon adaptation index, CAI) (Angov, 2011), this technique does not take into account several factors that influence translational efficiency: translational pausing (Angov, 2011), local mRNA secondary structure (Kudla et al., 2009) and tRNA abundances (Welch et al., 2009). Kudla et al. have shown a correlation between codon optimization and the secondary structure of the mRNA at the beginning of a gene (regions −4 to +37) with the translational efficiency in E. coli, with a 250-fold variation in GFP expression across the constructs they tested (Kudla et al., 2009). Progress has been made in predictive algorithms that take into account codon usage and tRNA abundance to optimize a gene’s coding sequence to give a desired translation efficiency (Welch et al., 2009). This codon optimization algorithm could potentially be combined with RNA secondary structure prediction programs in order to facilitate a more accurate prediction in the resulting efficiency of translation.

**mRNA decay rate.** The longevity of the mRNA transcript is controlled by its secondary structure in the untranslated regions, which protect it (Bouvet & Belasco, 1992; Carrier & Keasling, 1997b; Mackie, 2012) or make it more vulnerable (Bouvet & Belasco, 1992) to degradation by RNases, and through efficient binding and translation by ribosomes blocking RNase action (Carrier & Keasling, 1997b; Komarova et al., 2005; Osterman et al., 2013). The half-life for most mRNAs in E. coli is relatively short at ~1–2 min (Mackie, 2012). The longer-lived an mRNA molecule is, the more translation will occur from each transcript. Appending 5’ stem–loop structures of varying sizes to mRNAs has been shown to increase the mRNA half-life between 5- and 10-fold up to a half life of ~24 min (Arnold et al., 1998; Hansen et al., 1994). Appending 3’ REP sequences or insertion of REP sequences into intercistronic regions of polycistronic operons can also stabilize upstream mRNA transcripts by ~3-fold (Newbury et al., 1987).

**Riboregulators.** Riboswitches are RNA genetic control elements that modulate gene expression in response to an inducer molecule (Vitreschak, 2004) or transacting RNA (taRNA) (Isaacs et al., 2004) without the requirement of any RNA–protein interactions. Since their discovery, a number of synthetic riboswitches have been developed that control gene expression by either premature transcriptional termination (Wachsmuth et al., 2013) or by translational inhibition by sequestering RBSs (Dixon et al., 2010; Lynch et al., 2007; Topp et al., 2010) in a dose-responsive manner to specific inducers (Fig. 4). Riboswitches that control premature transcription termination have been shown to elicit up to a 3-fold change in transcription in response to an inducer (Wachsmuth et al., 2013), whilst riboswitches that modulate translation initiation have been developed that span a 2- to 150-fold range in response to an inducer. A model-directed redesign of a translational riboswitch has also been used to predictively adjust its efficiency (Beisel & Smolke, 2009). The taRNA riboregulators work by the binding of the taRNA to a cis-repressed mRNA (crRNA) resulting in the release of the RBS, allowing translation initiation (Isaacs et al., 2004) (Fig. 4). taRNA riboregulators have been utilized in controlling a metabolic pathway and showed a ~1- to 200-fold increase in translation initiation in the presence of the trRNAs (Callura et al., 2012; Isaacs et al., 2004). Whilst the riboregulators described here do not require RNA–protein interactions for their function, the CRISPRi platform for transcriptional repression utilizes ribonucleoproteins (Qi et al., 2013). Briefly, a small guide RNA (sgRNA) is expressed with complementary base pairing to a target DNA sequence and a secondary structural stem–loop that is recognized by a catalytically inactive RNA-binding protein, Cas9. Together the sgRNA-Cas9 ribonucleoprotein binds the target DNA sequence and inhibits initiation of transcription, elongation or transcription factor binding depending on where the sgRNA is targeted (Qi et al., 2013).

**Transcriptional, translational and post-translational design**

**Inteins.** Inteins are the protein-splicing equivalents of introns found in eukaryotic pre-mRNAs. An intein is a genetically encoded element within a target gene and is transcribed and translated together with the target protein before it undergoes autocatalytic self-excision and splicing of the target protein exteins (Gogarten et al., 2002) (Fig. 5). Inteins, therefore, work at both a transcriptional and translational level by increasing the time it takes to
transcribe and translate a target gene. Bacterial inteins range in size from 36 to 1986 amino acids (Perler, 2002), theoretically increasing transcription by ~2–120 s and translation by ~2–100 s. A comprehensive list of inteins and their sizes can be found at http://tools.neb.com/inbase/list_prop.php. Inteins would be beneficial for engineering delay into genetic networks, in particular tuning oscillators that depend on transcriptional and translational delay for their function (Mather et al., 2009; Purcell et al., 2010; Stricker et al., 2008). Split inteins have also been described where the intein domain is transcribed and translated by two separate genes and the resulting proteins can undergo trans-splicing to produce a single functional protein (Elleuche & Pöggeler, 2010; Lockless & Muir, 2009) (Fig. 5). Post-translational control of inteins has been engineered to modulate intein splicing (Skretas, 2005), trans-splicing (Mootz & Muir, 2002) or a combination of both (Shi & Muir, 2005) in the presence of small molecule inducers (Fig. 5).

**Protein level design**

**Protein degradation.** The longevity of proteins within a cell can be tuned by addition of degradation tags at the N (Gur & Sauer, 2008) or the C terminus (Andersen et al., 1998; Flynn et al., 2001; Gur & Sauer, 2008; McGinness et al., 2006) as well as internally (Gur & Sauer, 2008). Different degradation tags can be used to target proteins for degradation to one of several cellular degradation complexes (Kirstein et al., 2009) (Table 4). Modifying the N-terminal residues of a protein can also target it for proteolytic degradation by the N-End rule mechanism (Erbse et al., 2006; Kirstein et al., 2009). Recent work has resulted in degradation tags of varying sequences that tune the rate of degradation by the ClpXP/AP degradation complexes (Andersen et al., 1998; Dougan et al., 2002; Flynn et al., 2001; Hoskins et al., 2000; McGinness et al., 2006; Purcell et al., 2012; Wang et al., 2007). However, Cookson et al. have shown that an abundance of protein targeted to the ClpXP machinery can lead to a queuing effect, which, in turn, leads to a slower rate of protein degradation that is dependent on the overall concentration of tagged species in the system (Cookson et al., 2011). This can be detrimental to genetic network behaviour when a fast turnaround of network elements is required but can also be beneficial in coupling separate networks through the queuing effect (Cookson et al., 2011). By utilizing multiple degradation pathways, the queuing effect could potentially be suppressed.

**Protein activity.** Manipulation of protein activity through point mutations can be used as a means of control with a few discrete values. If the system consists entirely of genetic elements (repressors or activators), then modification of the DNA-binding affinity is best achieved by manipulating the DNA sequence, rather than attempting to mutate the protein. However, for enzymic activities, mutants with altered substrate specificity (Brannigan & Wilkinson, 2002; Wilson & Agard, 1991), kinetics (Brannigan & Wilkinson, 2002) or thermostability (Lehmann & Wyss, 2001) may already be available or can sometimes be created via protein engineering (Brannigan & Wilkinson, 2002; Lehmann & Wyss, 2001; Wilson & Agard, 1991).

**Extension to eukaryotic dials**

The majority of the dials described above can also be used in eukaryotes. However, there are additional dials that can be used to tune genetic networks when working in eukaryotes, as follows.
Post-transcriptional modification of mRNA (splicing). In higher eukaryotic chassis, RNA splicing can be used to introduce a delay between transcription and translation (Swinburne et al., 2008). Transcripts of mRNA that have no introns will not need to be spliced, whereas those that have increasing numbers of introns will require longer transcription and processing time before translation. Splicing of mRNA can also be controlled by the addition of morpholinos (synthetic molecules that base pair with target DNA sequences) to block the pre-mRNA splicing machinery (Morcos, 2007). In prokaryotes, group I and II self-splicing introns have been identified, but these appear to have no known specific biological function and are thought to be remnants from an ancient RNA world (Raghavan & Minnick, 2009). Thus, with the current state of understanding, it seems they would not be very easy to target as tuneable dials at the moment.

Translocation. In eukaryotes, transcription occurs in the nucleus and the resulting transcripts must then be translocated to the cytoplasm for translation (Oeffinger & Zenklusen, 2012). This will introduce a delay between transcription and translation (Grünwald & Singer, 2010). Therefore, moving a dial from a prokaryotic chassis to a eukaryotic chassis could be used as a means to introduce delay into a genetic network.

Protein trafficking. In eukaryotic chassis, signal sequences are used to localize proteins to different subcellular compartments/organelles (Alberts et al., 2002; Martoglio & Dobberstein, 1998). Adding a signal sequence to a protein will introduce a delay between translation and function due to the time required for transport of the protein to its intended destination (Hirschberg & Lippincott-Schwartz, 1999). This can also be used as a means to increase the effective concentration of a protein by accumulating it in a smaller volume. Protein trafficking from the cytoplasm to the inner/outer membrane and to the periplasmic space also takes place in prokaryotes, although far fewer subcellular compartments are available for sampling in prokaryotes (Driessen & Nouwen, 2008; Papanikou et al., 2007).

Discussion and perspectives
Tuning parameters in mathematical models will modify the system behaviour; in this way, design specifications can be met. It is often the case, however, that a single design objective can be met via different parameter changes. For example, modifying the gene copy number, promoter strength, RBS strength, mRNA degradation rate or protein degradation rate can all change the steady state protein concentration expressed from a single gene (Fig. 6). To change the dynamics of protein accumulation, the degradation rates of the mRNA and protein can both be modified (Fig. 6) (Alon, 2007). It is, therefore, important to understand how different ‘dials’ affect the variability and noise of the system. For example, low transcription levels and high translation levels will produce a higher variability in protein concentration than high transcription and low translation levels with the same mean protein concentration at steady state (Raj & van Oudenaarden, 2008) (Fig. 6). For the case of high output variability, increasing promoter strength and decreasing RBS strength can be used to decrease variability in the output while keeping the mean output the same. Changing parameters can also modify system behaviour with regard to the output. As an example, negative feedback, either through auto-regulation or indirectly through intermediary genes in the system (Cosentino & Bates, 2012), can be used to improve robustness, reduce output variability and reduce response times of a system. Negative feedback often has trade-offs in the design and there are theoretical limits as to how much negative feedback can improve performance of genetic systems (Lestas et al., 2010), which should be taken into account when tuning parameters. While simple design

| Protease | Cellular localization | Degradation tag/mechanism | Tag position | Approx. half-life (min)* | Reference |
|----------|-----------------------|---------------------------|--------------|-------------------------|-----------|
| ClpXP    | Cytoplasm             | SsrA                      | C-terminus   | 10–110                  | Andersen et al. (1998); Flynn et al. (2001); McGinness et al. (2006); Purcell et al. (2012) |
| ClpAP    | Cytoplasm             | SsrA/Rep                  | C-terminus   | 10–110                  | Dougan et al. (2002); Flynn et al. (2001); Hoskins et al. (2000) |
| ClpAPS   | Cytoplasm             | N-End rule SsrA/non-polar pentapeptide | N-terminus | 2–50                    | Erbse et al. (2006); Wang et al. (2007) |
| FtsH     | Inner membrane        | SsrA                      | C-terminus   | 2                       | Herman et al. (1998) |
| Tsp      | Periplasm             | SsrA                      | C-terminus   | >15                     | Parsell et al. (1990); Silber et al. (1992) |
| Lon      | Cytoplasm             | β20                       | N-terminus, internal, C-terminus | >10        | Gur & Sauer (2008) |

*Approximate protein half-life is dependent on protein size, stability and temperature.

Table 4. Protein degradation pathways

For the case of high output variability, increasing promoter strength and decreasing RBS strength can be used to decrease variability in the output while keeping the mean output the same. Changing parameters can also modify system behaviour with regard to the output. As an example, negative feedback, either through auto-regulation or indirectly through intermediary genes in the system (Cosentino & Bates, 2012), can be used to improve robustness, reduce output variability and reduce response times of a system. Negative feedback often has trade-offs in the design and there are theoretical limits as to how much negative feedback can improve performance of genetic systems (Lestas et al., 2010), which should be taken into account when tuning parameters. While simple design
objectives (e.g. increasing the concentration of protein in the system) can often be designed rationally by an expert, other properties are more complex. For example, to change the system dynamics while keeping the expression level constant involves tuning multiple parameters, such as both the degradation rate and RBS strength (Fig. 6). In engineering design, this is generally achieved by setting such specifications as constraints and searching over all parameters that satisfy those constraints (Perry & Green, 1999). If more than one parameter choice meets all constraints and a benefit or cost is defined then the design can optimize the cost/benefit ratio. This is just one instance where the model can inform the choice between alternative redesigns.

The dials we have described span a range of scales and levels, and as such provide a series of options, which can be combined to achieve a design objective. Nonetheless, there are caveats: ultimately tuning any dial in a genetic network results in modulating the concentration of mRNAs and encoded proteins in the system. The dynamics of the system, however, depend on the type of control dials that are used. For example, if transcriptional level control dials (regulated promoters, transcriptional riboswitches) are used there will be a longer delay before a functional protein is produced since transcription, translation and protein folding must take place sequentially. On the other hand, if translational or post-translational control dials are used, the functional protein will be produced in a shorter period of time. Therefore if a genetic network with fast dynamics is required, it may be beneficial to factor out post-translational control into the design process rather than transcriptional control. There will typically be a trade-off between the cell producing high levels of protein, poised to carry out its function, and gene expression inducing metabolic burden on the cell. The use of degradation tags on proteins also incurs a high metabolic burden since it decreases the steady state concentration by increasing the protein turnover rate. This results in resources being used to make a protein that is then targeted for fast degradation and is thus short-lived.

Linking multiple dials together can provide a genetic network with several avenues for tuning, providing a high level of control over network behaviour, e.g. coarse tuning through different origins of replication (modulating gene copy number), medium-level tuning through different promoters, and fine-tuning with different RBSs. However, linking different dials together often takes them out of the context under which they were initially characterized, thereby reducing the predictability of their individual behaviour and of their impact on the designed systems. For instance, it has been shown that increasing gene copy number can reduce the dynamic range and increase the leakiness of a promoter (Lutz & Bujard, 1997). Leakiness could potentially be reduced by adding a second layer of transcriptional or translational control by the addition of a third tuneable element, a riboregulator, thus combating the unwanted effects of copy number on promoter behaviour.

Addition and removal of control dials from a genetic network can be experimentally facilitated by the use of modular plasmid designs with large multiple cloning sites, allowing for the sequential addition of network components. Litcofsky et al. demonstrated this by constructing a simple toggle switch and a three-node or four-node feed-forward loop (Litcofsky et al., 2012). Progress has also been made in the use of bio-parts in a plug-and-play methodology through the standardization of plasmid design (Silva-Rocha et al., 2013). Another factor to keep in mind is that, experimentally, some dials are easier to predictably tune than others. Altering gene copy number can be easy to achieve by replacing the origin of replication on plasmid-borne genetic networks or through single or multiple genomic integrations. Whilst the gene copy number can be controlled exactly through genomic integration, plasmid copy numbers can be harder to tune to exact levels given that many factors, described above, can affect plasmid copy numbers. Cell chassis tuning is less simple, potentially requiring genome engineering to achieve particular cell traits that impact on genetic network behaviour. As the effects of different cell chassis on network behaviour are currently not predictable, two approaches are available to aid in network redesign: (1) a genetic network can be characterized in several cell chassis to envisage the differential effects on the network with alternate chassis environments or (2) by using software such as Intermine (Smith et al., 2012) or Ondex (Köhler et al., 2006), developed for searching, data mining and integration of biological databases, which could help in identifying particular characteristics of different cell chassis to help direct and inform the design process. While the use of in silico approaches to design RBSs with predicted strengths can speed up the design and tuning process (Salis et al., 2009), tuning most other dials can be time intensive due to the lack of software to help predict the effect changes on these dials may have. For example, whilst new promoters can be engineered, as described previously, there is often a trade-off between promoter strength, repressor strength, dynamic range and leakiness (Lanzer & Bujard, 1988). Trying to tune one of these parameters can often alter others. Therefore, predictively designing a promoter with specific attributes is not straightforward. However, these trade-offs are common in engineering design for other fields, where they are typically handled using an optimization framework which considers various constraints and objective functions in the design (Boyd & Vandenberghe, 2004; Perry & Green, 1999; Dolan et al., 2012). Directed evolution approaches (Lutz & Patrick, 2004; Neylon, 2004) are available to produce libraries of promoters but they often require extensive screening for desired characteristics and are thus often experimentally time consuming. Likewise, adding transcriptional level control with riboswitches can be relatively easy, whilst using a riboswitch for translational level control is more difficult as its function is often dependent on the RBS
sequence, which cannot be easily tuned without affecting the riboswitch integrity.

Two of the pioneering hallmarks for Synthetic Biology were the realization of simple designs inspired by existing electronic counterparts, i.e. a genetic toggle switch (Gardner et al., 2000) and an oscillator (Stricker et al., 2008). Their designs were inspired by a model-guided approach that provided an in silico assessment of the qualitative behaviour of these simple genetic networks. Further advancements in the field led to the use of a model-guided design (Ellis et al., 2009), which allowed for the tuning of transcriptional layer dials (promoter characteristics) in a reliable and relatively straightforward manner, to achieve a predictable genetic timer that controls yeast sedimentation (Ellis et al., 2009).

Within the scope of cell-based biosensing, model-guided design approaches have been used to inform the development of layered AND gates, housed in separate cell populations, which communicate through quorum sensing to detect specific combinations of metals (Beguerisse-Díaz et al., 2012; Wang et al., 2013). One of the most complex genetic designs achieved to date is exemplified by Moon et al. (2012), who used a combination of computational tools, model-guided design and directed evolution to construct a four-
input AND gate that consists of three circuits integrating four inducible systems within a single *E. coli* cell.

In order to realize the need for truly plug-and-play Synthetic Biology, the designer has to appreciate the types of dials they can use to achieve their design objectives: some are ‘difficult to tune’, some are ‘sensitive’ and some others are ‘uncertain’. In this review, we have described some of the possible dials that are available to the Synthetic Biologist at various organizational layers, thus opening the possibility for a design cycle that will involve mathematical modelling and optimization to produce systems with predictable, robust behaviour.

Acknowledgements

The authors were supported by EPSRC grants EP/031944/1 and EP/I032223/1. K.P. is supported by an RCUK fellowship in Biopharmaceutical Processing. This review forms part of an overall project involving Dr Jorge Gonçalves, Dr Alex A. R. Webb and Dr Ye Yuan from the University of Cambridge and Dr George H. Wadham from the University of Oxford.

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