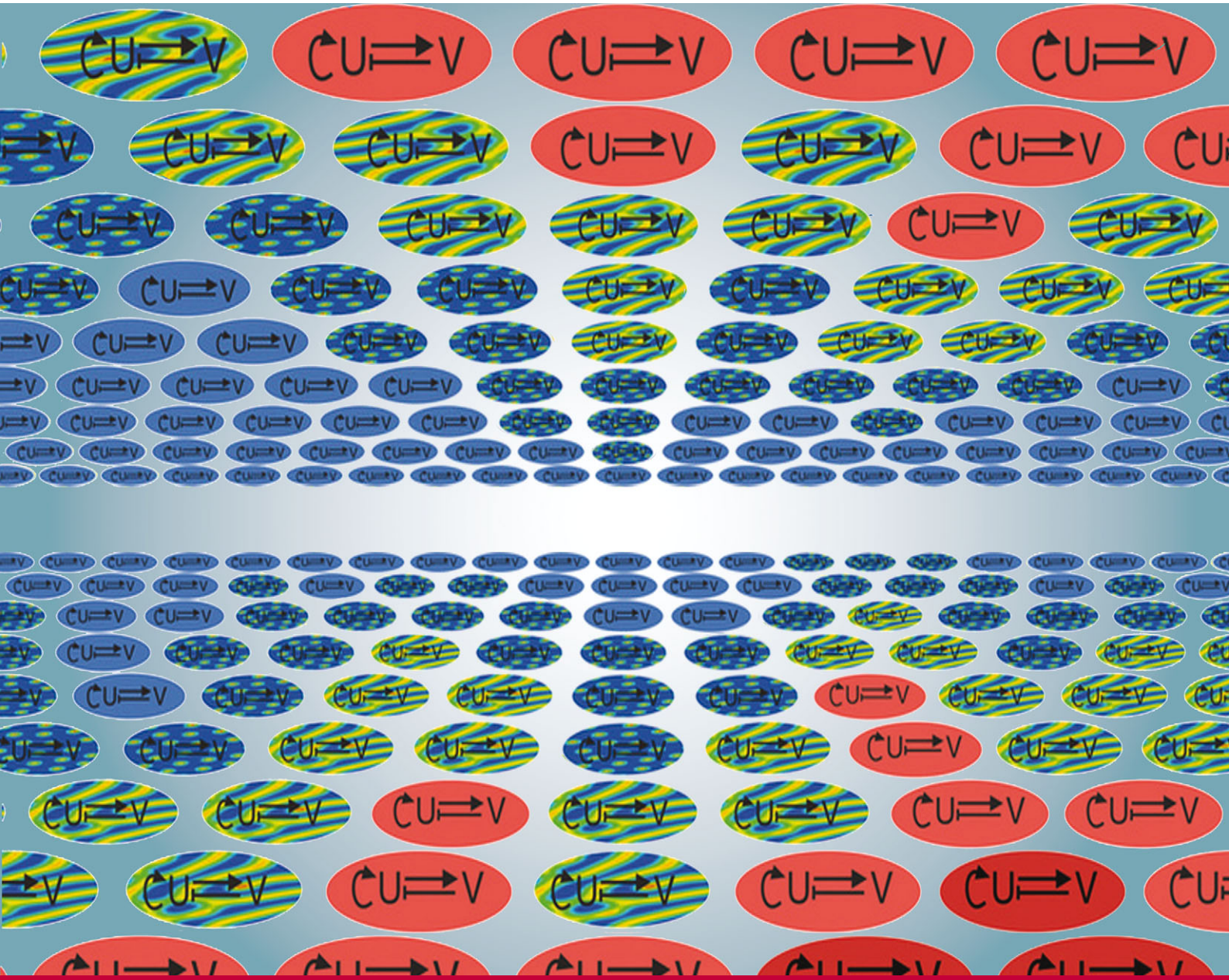
Indexed in MEDLINE!

Molecular BioSystems

Interfacing chemical biology with the -omic sciences and systems biology

www.molecularbiosystems.org

Volume 9 | Number 7 | July 2013 | Pages 1543–1974



Themed issue: Network Biology

ISSN 1742-206X

RSC Publishing

REVIEW
Yolanda Schaerli and Mark Isalan
Building synthetic gene circuits from combinatorial libraries: screening and selection strategies

1742-206X(2013)9:7;1-4

Molecular BioSystems

RSC Publishing

REVIEW

View Article Online
View Journal | View Issue

Open Access Article. Published on 23 January 2013. Downloaded on 11/24/2025 4:30:31 PM.

Cite this: *Mol. BioSyst.*, 2013, **9**, 1559

Building synthetic gene circuits from combinatorial libraries: screening and selection strategies

Yolanda Schaerli[ab] and Mark Isalan[ab]

Received 29th October 2012,
Accepted 20th December 2012

DOI: 10.1039/c2mb25483b

www.rsc.org/molecularbiosystems

The promise of wide-ranging biotechnology applications inspires synthetic biologists to design novel genetic circuits. However, building such circuits rationally is still not straightforward and often involves painstaking trial-and-error. Mimicking the process of natural selection can help us to bridge the gap between our incomplete understanding of nature's design rules and our desire to build functional networks. By adopting the powerful method of directed evolution, which is usually applied to protein engineering, functional networks can be obtained through screening or selecting from randomised combinatorial libraries. This review first highlights the practical options to introduce combinatorial diversity into gene circuits and then examines strategies for identifying the potentially rare library members with desired functions, either by screening or selection.

Introduction

Synthetic biologists apply engineering approaches to build new biological networks. These synthetic circuits are composed of biological parts[1] and are helping us to understand design principles in nature, as well as having numerous potential applications in therapeutics, bioremediation, biofuels, agriculture and biosensing.[2–4] While the field includes designing metabolic pathways[5–8] or even whole cells,[9–11] this review concentrates on transcriptional circuits. We focus in particular on the prospect of applying techniques from protein engineering for building gene networks, such as the screening or selection of combinatorial libraries.

Whereas early synthetic genetic circuit engineering studies began with simple devices like a negative feedback loop[12] and a toggle switch,[13] nowadays the community focuses on circuits displaying more complex behaviours. Recent examples include oscillators comprising thousands of synchronised bacterial colonies,[14] layered logic gates in bacteria,[15] or mammalian cells performing programmable half-subtractor and half-adder calculations.[16] Despite this progress, and the growing interest of the scientific community, it is well-known that synthetic biology faces serious technical difficulties.[17–20] For example, an article entitled "Five hard truths for synthetic biology"[17] outlined several problems in the field: many parts are not well characterised, are incompatible with the host cell and do not work as predicted when assembled into circuits. Therefore, building a synthetic network is usually still a challenging, slow and difficult process, involving "tweaking" and "debugging" the initial design to obtain a working device. The devil is often in the detail of small context-dependent effects, thus limiting our ability to build more complicated networks easily.

Although rationally designing and building any given device remains the goal, we have to admit that our current knowledge and understanding of how biology works is frequently insufficient. Therefore, synthetic biologists have started to apply the powerful method of directed evolution to synthetic networks.[21–25] Combinatorial libraries of network variants are produced and then the variants with the desired properties are found by screening or competitive selection. Typically, the identified variants are subjected to one or more rounds of diversification and selection or screening.

In this review, after briefly touching upon the origins of directed evolution, we describe how combinatorial diversity can be introduced into synthetic transcriptional networks. We then focus on selection and screening systems to identify the functional devices in a library. As most of the reviewed work has been carried out in prokaryotes, the emphasis is on bacterial systems but analogous techniques could be applied to eukaryotic cells.

Protein engineering – the precursor of synthetic biology

The idea of synthetic biology has its roots in molecular cloning and recombinant DNA technologies, where genetic components such as transcription promoters and coding regions are now

[a] *EMBL/CRG Systems Biology Research Unit, Centre for Genomic Regulation (CRG), Dr Aiguader 88, 08003 Barcelona, Spain. E-mail: yolanda.schaerli@crg.eu, isalan@crg.es*
[b] *Universitat Pompeu Fabra (UPF), 08003 Barcelona, Spain*

Open Access Article. Published on 23 January 2013. Downloaded on 11/24/2025 4:30:31 PM.

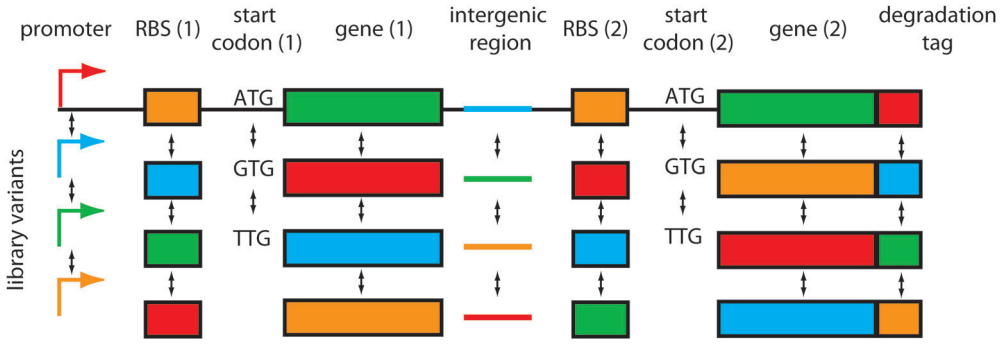


Fig. 1 Where to introduce combinatorial diversity in bacterial gene network libraries. An operon containing two genes is shown as an example. The options of where to introduce variations are numerous: in the network connectivities (not shown), promoters, ribosomal binding sites, (start) codons, genes, intergenic regions, degradation tags, and combinations thereof.

routinely combined to make protein expression constructs or other new plasmids.[26] Making synthetic gene networks is seemingly just one level of complexity higher, simply harnessing the appropriate recombinant constructs to make networks.

One field of biological engineering which is now relatively mature, and where new functional constructs are routinely made, is protein engineering. New proteins are constructed, often using structural information and an element of rational design,[27,28] but also through screening or selecting from large randomised combinatorial libraries. In a screening assay, a specific output of the individual library members (*e.g.* their fluorescence) is measured and the best variants are taken to the next round of randomisation or screening. By contrast, in a selection, the desired behaviour of a variant is linked to a competitive survival advantage, so that only positive clones should ultimately survive the selection procedure. Many highly efficient selection systems (*e.g.* phage display[29]) as well as screening systems (*e.g.* based on flow cytometry[30]) have been successfully applied to protein engineering.[31,32] It is therefore manifest that generating diversity and selecting or screening for the desired variants could also be a powerful tool for the engineering of synthetic networks.

Where to introduce the combinatorial diversity?

One important question is where to introduce diversity in the network. The target sites of mutations should be chosen in order to maximise the success rate of obtaining a functional network (mutational robustness[33]). By analogy to protein engineering, scaffolds such as zinc fingers tolerate mutation while retaining rich functional diversity. It is therefore necessary to find equivalent scaffolds for network engineering.[34]

Another consideration should be the size of the library that can be screened or selected. For instance, when engineering synthetic transcription networks, it would be wasteful to randomise each transcription factor residue; most mutants would be non-functional or similarly functional when compared with the original network, and the library size would quickly become too big to screen. Rather, it would be smarter to mutate around the transcription factor DNA-binding interface, either mutating the key amino acid residues making DNA contacts, or the corresponding DNA bases in the promoter region. Thus, targeted mutations can provide functional diversity in relatively small, easy-to-handle libraries. The options for introducing diversity are numerous (Fig. 1) and include: network connectivities, promoters, ribosomal binding sites, codon variations, intergenic regions, protein parts, degradation tags and others. Here we discuss the different possibilities:

Network connectivities

In the first combinatorial network engineering study, Guet *et al.* built genetic circuits containing three transcriptional regulators (lacI, TetR and cI) and a green fluorescent protein (GFP) reporter.[35] Five different promoters, including binding sites for the transcription factors, were randomly cloned in front of the three genes, thus connecting them in 125 (5^3) different ways. By analysing the behaviour of the resulting networks in the presence or absence of the chemical inducers of lacI (IPTG) and TetR (aTc), the logic gates NAND, NOR and NOT IF could be identified.

While it is conceivable that this approach could be used to identify more new circuits, a different procedure predominates in the literature: for a given desired output a possible network topology is rationally designed and suitable parts are chosen and assembled. This process is potentially guided by a model.[36–41] Directed evolution is then used only if the built circuit is non-functional or needs improvement.[15,42,43]

Promoters

Closely related to changing the network connectivity is altering promoter regions and thereby changing input–output relationships at network nodes. This is achieved by mutating the promoters in one or several of their functional units: RNA polymerase binding site, transcription start site and transcription factor binding sites and enhancers. The tuning of promoters has been used to reduce leakiness and to increase the dynamic range of expression[15] or to fine-tune expression levels of gene parts.[38]

The group of Elowitz built a combinatorial library of random promoter architectures containing up to three transcription

 This journal is © The Royal Society of Chemistry 2013

Open Access Article. Published on 23 January 2013. Downloaded on 11/24/2025 4:30:31 PM.

factor binding sites, which could be placed in the distal, core or proximal regions of the promoter.[44] A subset of the library was characterised and promoter strengths were observed that varied over five orders of magnitude. From this analysis, empirical rules were derived for bacterial promoter design, for example repression is strongest when the repressor binding site is located in the core part of the promoter and is weakest in the distal part.

Alternatively, the architecture of the promoter is kept constant and diversity is introduced by randomising all bases,[45] or only a subset of the promoter while leaving key motifs unchanged.[15,38,46–49] The latter strategy has the advantage that the promoter function is retained in most library members and that the library size can be kept small.

After the generation of the promoter library two different approaches have been pursued. First, the diversity is directly incorporated into the synthetic circuit and a screening or selection is performed to obtain the final working device.[15] Alternatively, members of the library are characterised in the context of a basic device, to obtain a collection of promoters covering a wide range of strengths. The promoter matching the required strength is then used to build the intended network.[38,45,48,49] The second approach requires a good idea of what promoter characteristics will render the device functional, *i.e.* from detailed *in silico* modelling. The advantage is that the collection of well-characterised promoters can be re-used for building different devices. While promoter engineering alters transcription expression levels, it is also possible to tune post-transcriptional processes, as described in the following sections.

Ribosomal binding sites

The translation levels of genes in synthetic circuits are most commonly adjusted by changing the ribosomal binding sites (RBSs) in mRNAs. An algorithm for the forward engineering of synthetic RBSs has recently been developed, for achieving specific expression levels.[50] However, when the optimal expression level of one or more components in a circuit is not known, screening a RBS library is a powerful approach. For example, Anderson *et al.*[51] wanted to engineer bacteria that can invade cancerous tumors upon a signal. For this purpose they put the gene of an invasin, that can initiate bacterial adhesion and invasion of mammalian cells, under the control of a signal-responsive promoter. However, with two of the three tested promoters, the device showed high leaky expression even in the absence of a signal. Functional circuits were ultimately obtained after introducing diversity at the RBS, with a positive selection in the presence of the signal and a negative screen in the absence of the signal.

In another example, mutations were simultaneously targeted to the RBSs of two transcription factors, thus allowing a search for the right balance of their expression levels.[52] Similarly, RBS libraries were applied in the construction of logic gates,[15,43,53,54] orthogonal transcription–translation networks,[47,55,56] a rewritable digital data storage device[42,57] and bistable switches.[57,58]

Codon variations

Moving downstream of the RBS on an mRNA, start codons are where the ribosome begins translation and changing these codons offers another way of modulating translation levels. As prokaryotes mainly use three start codons, these can be rapidly tested. If more start codons are varied simultaneously – or if combined with other library diversity – a more elaborate screening or selection might become necessary. In one study, for example, a library targeted the RBS as well as the start codon of a gene.[54]

Not only can the start codon be exchanged, but also the other codons. While it has long been known that codon usage alters gene expression levels, a recent study quantified this effect and found a 250-fold variation in GFP expression levels in *E. coli*, using different synonymous codons.[59] Design parameters can be obtained to control synthetic gene expression in *E. coli*,[60] and this could provide a source of variation for combinatorial network libraries. Various mechanisms may allow variation, including the use of rare codons or altering RNA secondary structure, *via* the presence or absence of hairpins.

Intergenic regions

The RNA secondary structure of untranslated regions between genes in an operon has been shown to affect transcription termination, mRNA stability and translation initiation.[61] As a result of these combined effects, libraries of intergenic regions can vary the relative expression levels of two proteins in the same operon by up to 100-fold.[61] Such regions therefore provide an alternative site to introduce diversity in a circuit.

Protein parts

Of course it is not only possible to modify regulatory parts, but also the protein parts themselves. Often protein parts are obtained by directed evolution using a suitable screening or selection system that is independent of the rest of the device. Only once a functional protein with the desired characteristics has been found and tested is it incorporated into the synthetic network. This simplifies the screening or selection process and therefore allows the use of bigger libraries. The latter can be necessary because protein sequence space (21^n, where n is a randomised position) rapidly overtakes promoter sequence space (4^n). However, the protein mutagenesis can often be targeted to a subregion more likely to result in useful mutations, by using structural or biochemical information.[31,32] Examples of such part engineering include altering the affinity of a protein to a signalling molecule[62] or to a promoter.[43]

A frequent goal of the directed evolution of protein parts for synthetic biology is to achieve orthogonality, *i.e.* the parts should only interact with their defined molecular partner, but not with other cell components.[63] For example Zhan *et al.* evolved new variants of the lacI repressor to recognise different DNA sequences than the wild-type protein, and which no longer bind to the natural lacI operator (lacO).[64] Collins *et al.* engineered a version of the LuxR transcription factor that activates transcription after binding to a different signalling molecule than its parent protein and which no longer recognises the natural quorum sensing signal.[65] Similarly, orthogonal transcription factors,[66–69] ribosomes,[70] polymerases,[49,71] receptor–ligand pairs[72] and chaperones[15] have been created to expand the

Open Access Article. Published on 23 January 2013. Downloaded on 11/24/2025 4:30:31 PM.

repertoire of available protein parts for synthetic biology. In contrast to standard protein engineering (*e.g.* the improvement in the catalytic efficiency for a new substrate), directed evolution of an orthogonal part not only requires a positive selection or screen for improved binding or activity, but also a negative one in order to exclude undesired cross-talk.

Degradation tags

The half-life of proteins is an important parameter in synthetic networks displaying dynamic behaviour such as switching between different states upon an external signal. Degradation allows an output to be reversible and thus the system can fine-tune responses to varying inputs. The group of Endy recently constructed a circuit that can switch between a red and a green state in response to distinct inputs.[42] In the absence of any input signal the last state is maintained. This was achieved by using two proteins involved in site-specific DNA integration – an integrase and an excisionase. Crucially, the device was only functional after the half-lives of these two enzymes were fine-tuned. To achieve this, the final residues of the ssrA degradation tags[73] were randomised and the library was screened for functional circuits.

Although the randomisation categories described above cover many possibilities, in principle, many other positions and processes could encode useful functional diversity. The main aim of any library design is to cover a useful range of variations (*e.g.* 'low', 'middle' or 'high' activity) with as few variants as possible, so as not to increase library size beyond practical limits. Having designed libraries, the next stage is to search for required outputs under particular conditions.

Screening and selection systems for synthetic networks

Having decided where to introduce combinatorial diversity in the synthetic circuit, the second important issue is how to identify the (rare) variants with desired properties. While for protein engineering many selections and screening systems are well established, for synthetic networks they are only just emerging and the field could still benefit from innovative new approaches. The challenge is that most synthetic devices not only have one state (*e.g.* being ON), but several (*e.g.* being ON or OFF or changing dynamically). Consequently, to identify a functional circuit, all the states have to be tested. While this might still be quite straightforward in the case of a switch or a logic gate (ON/OFF), it is rather more complicated to set up selections or screening systems for networks with outputs such as spatial or temporal patterns – and these are essential for many key biological processes. For example, the spots, stripes or waves found in Turing or Gierer–Meinhardt patterns[74,75] are thought to be important in developmental patterning, but are difficult to engineer with current approaches. Therefore, the chosen strategy must strongly depend on the desired network behaviour. Nevertheless, the key considerations for choosing the method are common to all synthetic devices and are also shared with protein engineering: what is the throughput of the method? How does this compare to the library size? What is the dynamic range of the screening or selection? What are the enrichment rates? What are the frequencies of false positives and negatives? Next, we give an overview of the selection and screening systems used so far in synthetic biology projects and highlight their advantages and disadvantages.

Screening systems

The majority of synthetic circuits are designed to have a fluorescent output. Therefore, it is often convenient to use a screening system with fluorescence as readout, as no further parts have to be added. For example to screen for an AND gate responding to arabinose and salicylate, a positive screening was performed in the presence of both chemicals.[54] Library members displaying high fluorescence under these conditions were further subjected to three negative screens (no inducer, arabinose only, salicylate only). Only mutants showing the required behaviour under all four conditions were further characterized (Fig. 2).

Such systems generate their own set of practical considerations. Often it might not be clear where to set the fluorescence threshold between positive and negative clones. Cells in the OFF state might already have some fluorescence, but an even higher signal in the ON state. Depending on the application, different thresholds can be acceptable. If so, it might be advisable to measure the ON/OFF ratios for all the members,

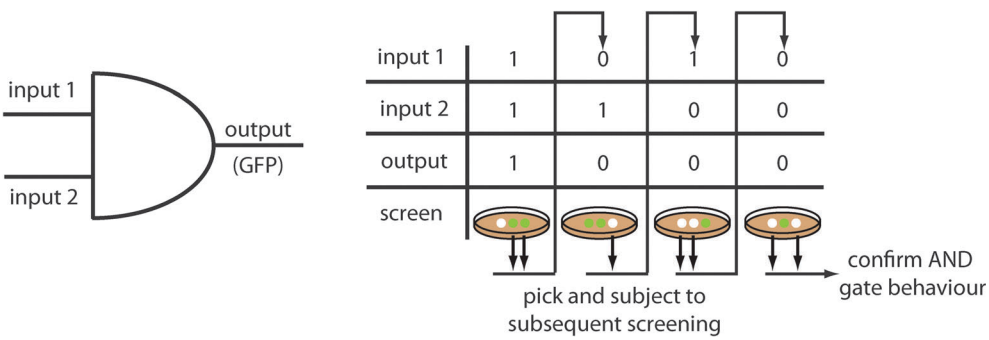


Fig. 2 Example of a screening protocol. Screening for an AND gate, as performed by the Voigt group,[54] requires a positive screen in the presence of the two inputs and three negative screens in the presence of one input or in the absence of any input.

 This journal is © The Royal Society of Chemistry 2013

Open Access Article. Published on 23 January 2013. Downloaded on 11/24/2025 4:30:31 PM.

thus avoiding discarding functional variants. Moreover, there can be a difference in responses at the levels of cell populations and individual cells. Depending on the application, it can sometimes be useful to retain even clones where only a small percentage of cells in the 'clonal' population display the desired behaviour when induced. To understand such systems, flow cytometry measurements can be essential.

As the functions of synthetic circuits become more complicated, the screening systems must also become more sophisticated. Lou and colleagues built a device in bacteria that switches from green to red after a first UV exposure and back to green after a second UV exposure (push-on push-off switch).[52] To screen for this behaviour many steps were necessary: after transformation green colonies were picked and transferred to two agar plates, only one of which was irradiated with UV. Colonies that switched to red after exposure to UV, but did not in the absence of the signal were chosen. Subsequently the same procedure was repeated, but this time red cells switching to green after UV irradiation were selected.

If cells are grown in liquid culture the fluorescence can be measured with a fluorescence spectrometer or a flow cytometer. For cells growing on plates, a fluorescence microscope or an UV transilluminator can be used, the latter only if the excitation range of the fluorescent protein falls in the UV range (*e.g.* GFPuv[78]). Liquid cultures are usually handled in a 96-well format and standard agar plates fit about 200 colonies, meaning that the throughput of plate-based fluorescence screens is commonly 100–2000 library members.

Flow cytometry can be applied in two ways: as a fluorescence measuring tool or as a fluorescence activated cell sorting (FACS) tool. The advantage of using a flow cytometer instead of a fluorescence spectrometer for measurement is that the fluorescence of the individual cells is recorded. This allows one to determine the distribution of the cells within a population, rather than only measuring an average. For example a population might display an unexpected bimodal distribution due to the variable metabolic burden that is imposed on the host by the synthetic device.[79] In other networks, it is only a part of the population that actually responds to the signal.[16,52]

A FACS machine can screen about 10^7 cells per hour[80] and thus enables access to much bigger libraries than other screening methods based on fluorescence. Rather surprisingly, with the exception of few examples,[46,47] this technique has hardly been applied so far to synthetic genetic circuits. The common occurrence of heterogeneous cell populations for one synthetic network, as mentioned above, might be one reason, as these will cause many false positives or negatives in a screening based on single cells. Nevertheless, the advantage of the superior throughput of this method probably outweighs the effort of eliminating errors post-sorting. We therefore expect that FACS will be used more often for the screening of synthetic transcriptional circuits in the future.

Although fluorescent proteins are by far the most commonly used markers for screenings, they are not the only option. Enzymes producing a colorimetric or a luminescent readout are also feasible. For example, in the above mentioned evolution of orthogonal lacI repressors, blue/white colony screening employed β-galactosidase expression and X-gal staining.[64]

Selection systems

By applying a Darwinian selection pressure to the randomised network, so that only the desired variants survive, much bigger libraries can be processed than when having to screen all library members one-by-one. In fact, when using a selection system, the transformation efficiency commonly becomes the limiting factor at around 10^{10} bacterial transformants per μg of DNA (although 10^6 is more achievable in practice[26]). The challenge is how to link the correct readout of a synthetic circuit, in each of its states, to the survival of it hosts; the output of synthetic networks can be coupled to a rescue gene in positive selections and a killer gene in negative selections. This means that under ON conditions only the networks expressing a resistance or a metabolic mutant complementation gene will survive. Conversely, under OFF conditions only the circuits that do not express the (conditionally) toxic gene will grow.

Initial selections of this type used two independent genes on different plasmids,[65] on the same plasmid[81] or as a genetic fusion[70] for the two selection conditions. The disadvantage of this strategy is that any mutation disabling the function of the killer gene results in false positives. Since false positives can quickly outgrow the rare positive library members, especially if the selections are performed in liquid culture, it is important to plate out cells on Petri dishes during selections. Moreover, it is necessary to remove and replace the selection marker plasmid after each round of selection because otherwise this will accrue false positive mutations under the strong selection pressure.[70,81]

To make selections more robust and to simplify processes, systems have been developed where one gene can function as a selection marker for both positive and negative selection. To achieve this, the selection marker gene can either enable cell survival or induce cell death under defined conditions.[53,55,56,76,77] Because any mutation that eliminates the function of the protein will most likely affect both the ON and OFF selections, the chances of the emergence of false positives are much lower. This enables one to perform selections in liquid cultures, further increasing throughput and speeding up the process.

TetA, a tetracycline/H^+ antiporter is just such a dual selector.[55,56,76,77] Its expression confers resistance to tetracycline and can therefore be used for positive selections in the ON state. As a membrane-bound protein, its overexpression also makes the host bacteria more susceptible to toxic metal salts, including $NiCl_2$. Therefore, cells with TetA expression will not grow well in the presence of $NiCl_2$ allowing cells in the OFF state to be selected (negative selection) (Fig. 3A). This strategy was successfully applied in synthetic circuits based on riboswitches.[55,56,77] Additionally, fusing TetA to GFPuv allows a quantitative readout without further subcloning.[55,56]

The *Herpes simplex* virus thymidine kinase (hsvTK) is an alternative dual selector with demonstrated use in *E. coli*.[53] In the presence of a thymidylate synthase inhibitor (5FdU), a thymidine kinase-deficient strain does not grow, due to the lack of thymidine. In ON selections hsvTK expression can

Open Access Article. Published on 23 January 2013. Downloaded on 11/24/2025 4:30:31 PM.

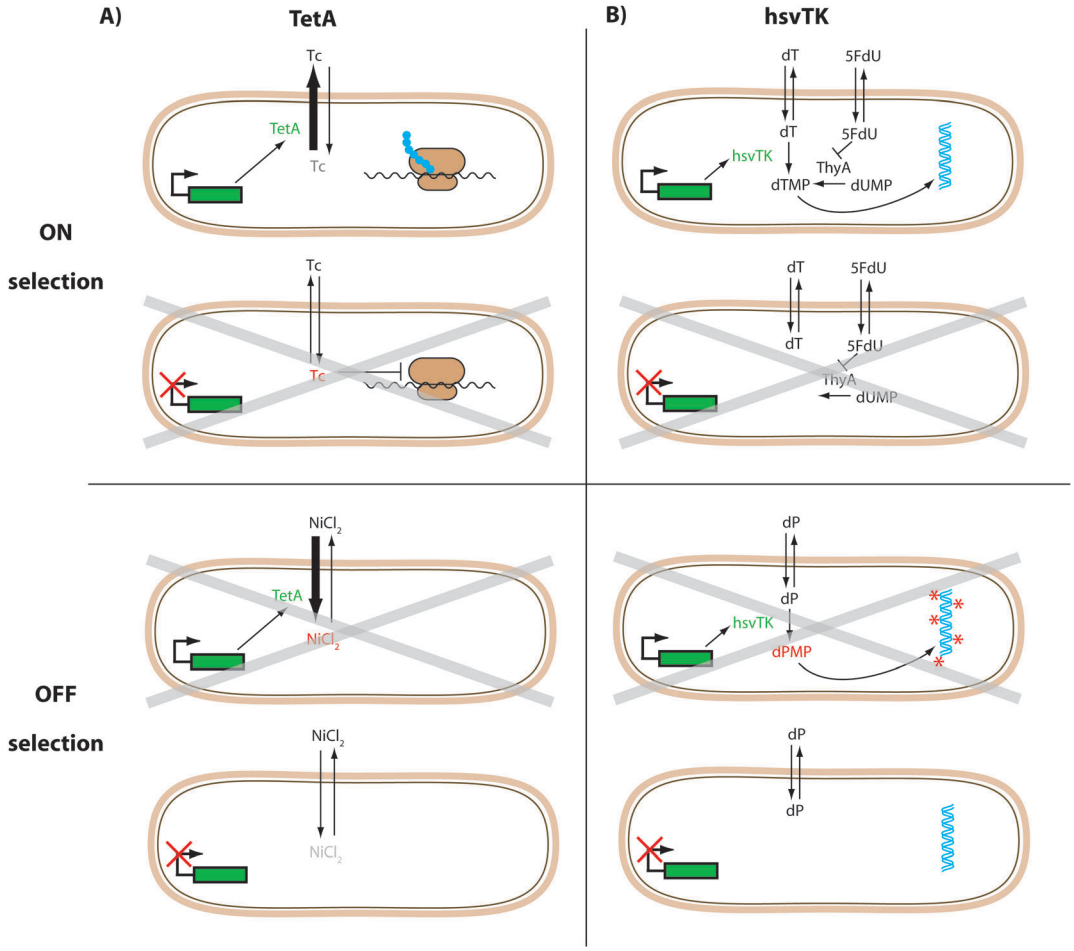


Fig. 3 Dual selection protocols. (A) TetA confers resistance to tetracycline (ON selection) and makes the host bacteria more susceptible to toxic $NiCl_2$ (OFF selection).[55,56,76,77] (B) HsvTK can rescue the thymidine deficiency of a thymidine kinase deficient strain in the presence of a thymidylate synthase (ThyA) inhibitor (5FdU) (ON selection). HsvTK can also make cells sensitive to synthetic dP nucleosides; these become toxic upon phosphorylation by hsvTK (OFF selection).[53] Grey crosses indicate cells where the conditions reduce viability.

rescue this deficiency. The OFF selection is performed in the presence of synthetic nucleosides (*e.g.* deoxyribosyl-dihydro-pyrimido[4,5-*c*][1,2]oxazin-7-one: dP) that only become toxic upon phosphorylation by hsvTK (Fig. 3B). As with TetA, hsvTK can be fused C-terminally to GFP without losing its function (unpublished data).

Although impressive enrichment factors (1300–33 000 times per ON/OFF cycle) have been demonstrated for these two dual selectors in model selections,[53,56] both systems are still waiting to be applied more widely. One reason might be that it is difficult to match the rather limited dynamic range of these selection systems to the functional range of the synthetic circuits. For example, while it is possible to select circuits that are ON among those that are completely OFF, it is more difficult to select against slightly leaky devices: low expression levels of TetA are already enough to confer tetracycline resistance. The use of less active TetA mutants[82] might alleviate this problem. The upper expression limit of the dual selectors is also constrained as they contribute to the metabolic load imposed on the host cell, even in the absence of the negative selection conditions. Moreover, TetA overexpression is known to be detrimental to cell growth.[84] Another concern is whether the use of mutagenic nucleosides in the hsvTK OFF selection will introduce undesired mutations. Therefore, while the throughput of selection systems is generally higher than that of their screening counterparts, they are often less flexible. However, it is still early days and time will show whether the dual selectors discussed here are robust enough to be adopted by the community.

For devices intended for targeted applications, the selection can be tailored to be more specific for the purpose of the device. An elegant example was demonstrated for the above-mentioned bacteria that can invade cancer cells upon a signal:[51] the bacteria carrying the device library were incubated with the cultured cancer cells, followed by the addition of an antibiotic. Bacteria unable to invade the mammalian cells were killed, but bacteria inside the cancer cells were protected from the antibiotic effect. Internalised bacteria were then released by mammalian cell lysis and grown on plates. Positive clones were subsequently screened for loss of invasiveness in the absence of the signal.

After any process requiring several rounds of selection or screening, the resulting circuits always have to be analysed

 This journal is © The Royal Society of Chemistry 2013

Open Access Article. Published on 23 January 2013. Downloaded on 11/24/2025 4:30:31 PM.

carefully. Controls should ensure a good understanding of the function of the individual circuit components. This analysis should also uncover the cases where the observed behaviour is caused by (unexpected) interactions of the synthetic network and the host cells.[79,85]

The work cited in this section has been mostly carried out in bacterial cells. However, synthetic biology in eukaryotes is fast catching up[86] and we expect to see the development of analogous screening systems in the near future.

Challenges and future prospects

Synthetic biology has made significant advances in recent years and is developing a set of community-based resources.[2] However, the dream that characterised parts can simply be put together to yield new genetic circuits still remains a dream, at least for now. In the meantime, directed evolution is a powerful method to help us bridge the gap between our incomplete understanding of nature's design rules and our desire to build synthetic networks. Techniques to manipulate DNA are already mature[87] and, as we have discussed in this review, many practical options are available to diversify the devices combinatorially. In contrast to its precursor – the field of protein engineering – rather few screening and selection systems have been described so far. This is especially true if the desired circuits are intended to have behaviour that is more complicated than switches or logic gates as, for example, in oscillatory networks or spatial patterns. We expect that the integration of cutting edge technologies within synthetic biology labs will give rise to new ways of performing the screening of genetic circuit libraries. Among these promising techniques are screenings based on microfluidics[88,89] and/or automated high-content imaging.[90–92] For example, the replacement of 96-well plates with a microfluidic device producing water-in-oil emulsion droplets could increase the throughput of screenings by several orders of magnitude.[93] Automated systems, able to take and analyse pictures or movies, could be imagined for screening complicated patterns, such as Turing or Gierer–Meinhardt patterns[74,75] (Fig. 4). However, this remains a truly challenging goal for the synthetic biology community. A combination of rational design, guided by a model, combined with screenings of combinatorial libraries is in our opinion a promising approach to work towards this goal.

Another emerging trend is the engineering of multicellular traits in cell consortia where single cells carry out different simple tasks.[94] This makes particular sense because our ability to increase the size and complexity of synthetic networks in one host cell has begun to stagnate.[17] Two recent studies[95,96] applied this approach in a rather elegant way: cells, each carrying out a simple function, were combined in multiple ways so that the whole consortia carried out far more complicated distributed computational tasks than the individual cells. Perhaps this concept of distributed networks truly represents the future of synthetic biology and individual robust functions could be made relatively easily using the screening or selection of combinatorial libraries. Ultimately, the power of directed evolution has yet to be fully harnessed and should provide us with an efficient new generation of engineering tools in the years to come.

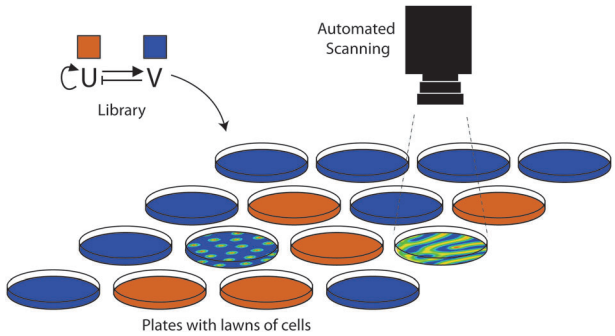


Fig. 4 Automated screening of spatial patterning. The schematic shows a thought-experiment on how one might screen a combinatorial library scaffold for a Gierer–Meinhardt system.[83] Thousands of randomised candidates might have to be tested to find the correct behaviour. The library would comprise variants of an activator (U; red) and an inhibitor (V; blue) which would communicate local, non-linear self-activation and long range inhibition signals to other cells. By plating library members on dishes or multiwell plates (here, one per plate) thousands of randomised parameter sets might be screened for potential patterning behaviour. Reproduced from ref. 34.

Acknowledgements

We thank Javier Macía and Diego Bárcena for critical reading. The group of MI is funded by the European Research Council, FP7-ERC-201249-ZINC-HUBS, the MEC-EMBL agreement and Ministerio de Ciencia e Innovación Grant MICINN BFU2010-17953. YS receives funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme (FP7/2007–2013) under REA grant agreement PIEF-GA-2011-298348.

References

1 B. Canton, A. Labno and D. Endy, *Nat. Biotechnol.*, 2008, **26**, 787–793.
2 J. Peccoud and M. Isalan, *PLoS One*, 2012, **7**, e43231.
3 M. Folcher and M. Fussenegger, *Curr. Opin. Chem. Biol.*, 2012, **16**, 345–354.
4 A. A. Cheng and T. K. Lu, *Annu. Rev. Biomed. Eng.*, 2012, **14**, 155–178.
5 H. H. Wang, F. J. Isaacs, P. A. Carr, Z. Z. Sun, G. Xu, C. R. Forest and G. M. Church, *Nature*, 2009, **460**, 894–898.
6 H. Tsuruta, C. J. Paddon, D. Eng, J. R. Lenihan, T. Horning, L. C. Anthony, R. Regentin, J. D. Keasling, N. S. Renninger and J. D. Newman, *PLoS One*, 2009, **4**, e4489.
7 S. K. Lee, H. Chou, T. S. Ham, T. S. Lee and J. D. Keasling, *Curr. Opin. Biotechnol.*, 2008, **19**, 556–563.
8 H. Alper and G. Stephanopoulos, *Metab. Eng.*, 2007, **9**, 258–267.
9 D. G. Gibson, J. I. Glass, C. Lartigue, V. N. Noskov, R. Y. Chuang, M. A. Algire, G. A. Benders, M. G. Montague, L. Ma, M. M. Moodie, C. Merryman, S. Vashee, R. Krishnakumar,

Open Access Article. Published on 23 January 2013. Downloaded on 11/24/2025 4:30:31 PM.

N. Assad-Garcia, C. Andrews-Pfannkoch, E. A. Denisova, L. Young, Z. Q. Qi, T. H. Segall-Shapiro, C. H. Calvey, P. P. Parmar, C. A. Hutchison, 3rd, H. O. Smith and J. C. Venter, *Science*, 2010, **329**, 52–56.
10 T. Oberholzer, R. Wick, P. L. Luisi and C. K. Biebricher, *Biochem. Biophys. Res. Commun.*, 1995, **207**, 250–257.
11 K. Kurihara, M. Tamura, K. Shohda, T. Toyota, K. Suzuki and T. Sugawara, *Nat. Chem.*, 2011, **3**, 775–781.
12 A. Becskei and L. Serrano, *Nature*, 2000, **405**, 590–593.
13 T. S. Gardner, C. R. Cantor and J. J. Collins, *Nature*, 2000, **403**, 339–342.
14 A. Prindle, P. Samayoa, I. Razinkov, T. Danino, L. S. Tsimring and J. Hasty, *Nature*, 2012, **481**, 39–44.
15 T. S. Moon, C. Lou, A. Tamsir, B. C. Stanton and C. A. Voigt, *Nature*, 2012, **491**, 249–253.
16 S. Auslander, D. Auslander, M. Muller, M. Wieland and M. Fussenegger, *Nature*, 2012, **487**, 123–127.
17 R. Kwok, *Nature*, 2010, **463**, 288–290.
18 J. T. Kittleson, G. C. Wu and J. C. Anderson, *Curr. Opin. Chem. Biol.*, 2012, **16**, 329–336.
19 S. Cardinale and A. P. Arkin, *Biotechnol. J.*, 2012, **7**, 856–866.
20 A. L. Slusarczyk and R. Weiss, *Sci. Signaling*, 2012, **5**, pe16.
21 E. L. Haseltine and F. H. Arnold, *Annu. Rev. Biophys. Biomol. Struct.*, 2007, **36**, 1–19.
22 M. J. Dougherty and F. H. Arnold, *Curr. Opin. Biotechnol.*, 2009, **20**, 486–491.
23 M. Porcar, *Syst. Synth. Biol.*, 2010, **4**, 1–6.
24 R. E. Cobb, T. Si and H. Zhao, *Curr. Opin. Chem. Biol.*, 2012, **16**, 285–291.
25 R. E. Cobb, N. Sun and H. Zhao, *Methods*, 2012, DOI: 10.1016/j.ymeth.2012.03.009.
26 J. Sambrook, E. F. Fritsch and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, 1989.
27 B. Kuhlman, G. Dantas, G. C. Ireton, G. Varani, B. L. Stoddard and D. Baker, *Science*, 2003, **302**, 1364–1368.
28 S. J. Fleishman, T. A. Whitehead, D. C. Ekiert, C. Dreyfus, J. E. Corn, E. M. Strauch, I. A. Wilson and D. Baker, *Science*, 2011, **332**, 816–821.
29 G. P. Smith, *Science*, 1985, **228**, 1315–1317.
30 A. D. Griffiths and D. S. Tawfik, *EMBO J.*, 2003, **22**, 24–35.
31 M. Isalan, A. Klug and Y. Choo, *Nat. Biotechnol.*, 2001, **19**, 656–660.
32 G. Winter, A. D. Griffiths, R. E. Hawkins and H. R. Hoogenboom, *Annu. Rev. Immunol.*, 1994, **12**, 433–455.
33 A. Wagner, *FEBS Lett.*, 2005, **579**, 1772–1778.
34 M. Isalan and Y. Schaerli, *SEBBM*, 2012, **171**, 16–20.
35 C. C. Guet, M. B. Elowitz, W. Hsing and S. Leibler, *Science*, 2002, **296**, 1466–1470.
36 K. Wu and C. V. Rao, *Curr. Opin. Chem. Biol.*, 2012, **16**, 318–322.
37 J. M. Carothers, J. A. Goler, D. Juminaga and J. D. Keasling, *Science*, 2011, **334**, 1716–1719.
38 T. Ellis, X. Wang and J. J. Collins, *Nat. Biotechnol.*, 2009, **27**, 465–471.
39 G. Rodrigo, J. Carrera and A. Jaramillo, *Nucleic Acids Res.*, 2011, **39**, e138.
40 G. Rodrigo, J. Carrera, T. E. Landrain and A. Jaramillo, *FEBS Lett.*, 2012, **586**, 2037–2042.
41 A. L. Slusarczyk, A. Lin and R. Weiss, *Nat. Rev. Genet.*, 2012, **13**, 406–420.
42 J. Bonnet, P. Subsoontorn and D. Endy, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 8884–8889.
43 Y. Yokobayashi, R. Weiss and F. H. Arnold, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 16587–16591.
44 R. S. Cox, 3rd, M. G. Surette and M. B. Elowitz, *Mol. Syst. Biol.*, 2007, **3**, 145.
45 H. Alper, C. Fischer, E. Nevoigt and G. Stephanopoulos, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 12678–12683.
46 M. R. Schlabach, J. K. Hu, M. Li and S. J. Elledge, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 2538–2543.
47 W. An and J. W. Chin, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 8477–8482.
48 B. A. Blount, T. Weenink, S. Vasylechko and T. Ellis, *PLoS One*, 2012, **7**, e33279.
49 K. Temme, R. Hill, T. H. Segall-Shapiro, F. Moser and C. A. Voigt, *Nucleic Acids Res.*, 2012, **40**, 8773–8781.
50 H. M. Salis, E. A. Mirsky and C. A. Voigt, *Nat. Biotechnol.*, 2009, **27**, 946–950.
51 J. C. Anderson, E. J. Clarke, A. P. Arkin and C. A. Voigt, *J. Mol. Biol.*, 2006, **355**, 619–627.
52 C. Lou, X. Liu, M. Ni, Y. Huang, Q. Huang, L. Huang, L. Jiang, D. Lu, M. Wang, C. Liu, D. Chen, C. Chen, X. Chen, L. Yang, H. Ma, J. Chen and Q. Ouyang, *Mol. Syst. Biol.*, 2010, **6**, 350.
53 Y. Tashiro, H. Fukutomi, K. Terakubo, K. Saito and D. Umeno, *Nucleic Acids Res.*, 2011, **39**, e12.
54 J. C. Anderson, C. A. Voigt and A. P. Arkin, *Mol. Syst. Biol.*, 2007, **3**, 133.
55 N. Muranaka, K. Abe and Y. Yokobayashi, *ChemBioChem*, 2009, **10**, 2375–2381.
56 N. Muranaka, V. Sharma, Y. Nomura and Y. Yokobayashi, *Nucleic Acids Res.*, 2009, **37**, e39.
57 R. G. Egbert and E. Klavins, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 16817–16822.
58 K. D. Litcofsky, R. B. Afeyan, R. J. Krom, A. S. Khalil and J. J. Collins, *Nat. Methods*, 2012, **9**, 1077–1080.
59 G. Kudla, A. W. Murray, D. Tollervey and J. B. Plotkin, *Science*, 2009, **324**, 255–258.
60 M. Welch, S. Govindarajan, J. E. Ness, A. Villalobos, A. Gurney, J. Minshull and C. Gustafsson, *PLoS One*, 2009, **4**, e7002.
61 B. F. Pfleger, D. J. Pitera, C. D. Smolke and J. D. Keasling, *Nat. Biotechnol.*, 2006, **24**, 1027–1032.
62 D. J. Sayut, Y. Niu and L. Sun, *ACS Chem. Biol.*, 2006, **1**, 692–696.
63 C. V. Rao, *Curr. Opin. Biotechnol.*, 2012, **23**, 689–694.
64 J. Zhan, B. Ding, R. Ma, X. Ma, X. Su, Y. Zhao, Z. Liu, J. Wu and H. Liu, *Mol. Syst. Biol.*, 2010, **6**, 388.
65 C. H. Collins, J. R. Leadbetter and F. H. Arnold, *Nat. Biotechnol.*, 2006, **24**, 708–712.
66 S. K. Lee, H. H. Chou, B. F. Pfleger, J. D. Newman, Y. Yoshikuni and J. D. Keasling, *Appl. Environ. Microbiol.*, 2007, **73**, 5711–5715.

 This journal is © The Royal Society of Chemistry 2013

Open Access Article. Published on 23 January 2013. Downloaded on 11/24/2025 4:30:31 PM.

67 M. Krueger, O. Scholz, S. Wisshak and W. Hillen, *Gene*, 2007, **404**, 93–100.
68 R. Daber and M. Lewis, *J. Mol. Biol.*, 2009, **391**, 661–670.
69 A. S. Khalil, T. K. Lu, C. J. Bashor, C. L. Ramirez, N. C. Pyenson, J. K. Joung and J. J. Collins, *Cell*, 2012, **150**, 647–658.
70 O. Rackham and J. W. Chin, *Nat. Chem. Biol.*, 2005, **1**, 159–166.
71 V. B. Pinheiro, A. I. Taylor, C. Cozens, M. Abramov, M. Renders, S. Zhang, J. C. Chaput, J. Wengel, S. Y. Peak-Chew, S. H. McLaughlin, P. Herdewijn and P. Holliger, *Science*, 2012, **336**, 341–344.
72 K. Chockalingam, Z. Chen, J. A. Katzenellenbogen and H. Zhao, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 5691–5696.
73 J. B. Andersen, C. Sternberg, L. K. Poulsen, S. P. Bjorn, M. Givskov and S. Molin, *Appl. Environ. Microbiol.*, 1998, **64**, 2240–2246.
74 A. M. Turing, *Philos. Trans. R. Soc. London, Ser. B*, 1952, **237**, 37–72.
75 A. Gierer and H. Meinhardt, *Kybernetik*, 1972, **12**, 30–39.
76 Y. Nomura and Y. Yokobayashi, *BioSystems*, 2007, **90**, 115–120.
77 Y. Nomura and Y. Yokobayashi, *J. Am. Chem. Soc.*, 2007, **129**, 13814–13815.
78 A. Crameri, E. A. Whitehorn, E. Tate and W. P. Stemmer, *Nat. Biotechnol.*, 1996, **14**, 315–319.
79 C. Tan, P. Marguet and L. You, *Nat. Chem. Biol.*, 2009, **5**, 842–848.
80 D. Davies, *Methods Mol. Biol.*, 2012, **878**, 185–199.
81 Y. Yokobayashi and F. H. Arnold, *Nat. Comput.*, 2005, **4**, 245–254.
82 P. McNicholas, I. Chopra and D. M. Rothstein, *J. Bacteriol.*, 1992, **174**, 7926–7933.
83 H. Meinhardt and A. Gierer, *Bioessays*, 2000, **22**, 753–760.
84 J. K. Griffith, D. H. Cuellar, C. A. Fordyce, K. G. Hutchings and A. A. Mondragon, *Mol. Membr. Biol.*, 1994, **11**, 271–277.
85 P. Marguet, Y. Tanouchi, E. Spitz, C. Smith and L. You, *PLoS One*, 2010, **5**, e11909.
86 W. Weber and M. Fussenegger, *Chem. Biol.*, 2009, **16**, 287–297.
87 T. Ellis, T. Adie and G. S. Baldwin, *Integr. Biol.*, 2011, **3**, 109–118.
88 B. Lin and A. Levchenko, *Curr. Opin. Chem. Biol.*, 2012, **16**, 307–317.
89 S. Gulati, V. Rouilly, X. Niu, J. Chappell, R. I. Kitney, J. B. Edel, P. S. Freemont and A. J. deMello, *J. R. Soc., Interface*, 2009, **6**(Suppl 4), S493–S506.
90 F. Zanella, J. B. Lorens and W. Link, *Trends Biotechnol.*, 2010, **28**, 237–245.
91 P. Brodin and T. Christophe, *Curr. Opin. Chem. Biol.*, 2011, **15**, 534–539.
92 M. Bickle, *Anal. Bioanal. Chem.*, 2010, **398**, 219–226.
93 Y. Schaerli and F. Hollfelder, *Mol. BioSyst.*, 2009, **5**, 1392–1404.
94 J. S. Chuang, *Curr. Opin. Chem. Biol.*, 2012, **16**, 370–378.
95 S. Regot, J. Macia, N. Conde, K. Furukawa, J. Kjellen, T. Peeters, S. Hohmann, E. de Nadal, F. Posas and R. Sole, *Nature*, 2011, **469**, 207–211.
96 A. Tamsir, J. J. Tabor and C. A. Voigt, *Nature*, 2011, **469**, 212–215.