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Abstract

Prokaryotic whole cell biosensors are effective yet inexpensive, renewable and environmentally substitutes for many contemporary sensors and diagnostic devices. Unfortunately, many previously constructed prokaryotic biosensors are plagued by poor performances, which prohibit their use in real life applications. Biosafety concerns, promiscuous analyte detection, analyte insensitivity, low output dynamic range and high background expression are common issues. Engineering principles and strategies from the emerging field of synthetic biology offer unprecedented solutions. They accelerate new biosensor developments and improve biosensor behaviors. Addition of novel devices and modules from synthetic biology further augments functions beyond sensing and render them safer. Thanks to this, prokaryotic whole cell biosensors have enjoyed a renaissance in recent years, and they hold promises to address the increasing demand for marketable biosensors.

Keywords

Whole-cell biosensor, Synthetic biology, Prokaryotes, Response curve, Sensitivity, Selectivity, Dynamic range, Leakiness

Introduction

Whole cell biosensors are domesticated or engineered cells that detect and report a target or condition of interest (Daunert et al. 2000; van der Meer and Belkin 2010; Wang and Buck 2012; Gui et al. 2017). They are viable alternatives to electronic or chemical sensors and have drawn increasing attention over the last three decades. Whole cell sensors are biodegradable and can be mass produced using inexpensive nutrients. So they are renewable, environmentally friendly, and cost-effective (van der Meer and Belkin 2010; Kim et al. 2018). Both prokaryotic and eukaryotic biosensors have been developed. This chapter limits its scope to only prokaryotic sensors, but the principles covered should also apply to their eukaryotic counterparts.

Prokaryotic biosensors have been researched for various purposes, for instances, environment assessment (Stocker et al. 2003; Wang et al. 2013; Huang et al. 2015b; Hwang et al. 2016; Kim et al. 2016; Cayron et al. 2017), clinical diagnosis (Duan and March 2010; Saeidi et al. 2011; Archer et al. 2012; Gupta et al. 2013; Hwang et al. 2014; Kotula et al. 2014; Courbet et al. 2015; Danino et al. 2015; Cayron et al. 2017; Daeffler et al. 2017; Riglar et al. 2017; Ho et al. 2018; Watstein and Styczynski 2018), and controlled bioprocessing (Zhang and Keasling 2011; Zhang et al. 2012). Some less common applications include mineral surveying and landmine clearing (Cerminati et al. 2011; Belkin et al. 2017).

Despite their advantages and demonstrated successes, many prokaryotic whole cell biosensors fail to survive in or even reach the competitive biosensor market. A recurring concern is biosafety – whole cell biosensors are often subjected to higher levels of legal and ethical scrutiny because there is a risk of releasing genetically engineered micro-organisms into the wild (Dana et al. 2012). Yet with biosafety aside, many prokaryotic biosensors are still leaky with low output dynamic ranges, and suffer from unsatisfactory sensitivity and selectivity (Stocker et al. 2003; Amaro et al. 2011; De Mora et al. 2011; Siegfried et al. 2012; Wang et al. 2013; Huang et al. 2015a; Kim et al. 2016; Merulla and Van Der Meer 2016). Nevertheless, recent advancements in synthetic biology have provided numerous solutions.

Synthetic biology is the rational design of biological systems. It achieves so by applying established principles from engineering to biology, and so is highly interdisciplinary (Endy 2005; Purnick and Weiss 2009; Cheng and Lu 2012; Wang and Buck 2012; Way et al. 2014; Bradley et al. 2016a; Bradley et al. 2016b; Bashor and Collins 2018). These principles include: **1)** Abstraction: complexity is managed through a hierarchy and less relevant information are hidden for clarity. **2)** Standardization: genetic elements are standardized into reusable parts with measurable parameters that can be ranked on a common scale. **3)** Modularization: genetic parts and devices are independent and interchangeable modules with defined functions, and **4)** Rational and quantitative design: behaviors of biological systems can be described and predicted by mathematical models. These principles and concepts enabled synthetic biologists to develop new strategies and devices, which substantially enhanced the performances of existing prokaryotic biosensors. In addition, they paved the way for developing novel biosensors with augmented capabilities. This

chapter thus focuses on how synthetic biology facilitates biosensor development, creates tools for improving biosensors and expands their capacities beyond sensing.

1. Synthetic Biology as an Enabling Platform for Rapid Construction and Optimization of Prokaryotic Biosensors

1.1 A streamlined approach to develop novel prokaryotic biosensors

Before the advent of genetic engineering, the development of a biosensor relied much on serendipity. Many biosensor were byproducts from studying the stress response or metabolic pathways of microbes. For instances, Microtox, one of the earliest biosensor, is a bioluminescent bacteria that glow weaker when its metabolism is harassed by toxic chemicals (Bulich and Isenberg 1981), and a whole cell naphthalene biosensor was developed by random transposition of a luciferase reporter into a naphthalene degradation pathway isolated from a soil bacteria (King et al. 1990). In these examples, the biosensor development processes depended on the fortuitous discovery of a species or a strain with desirable responses towards the targets, and they were hardly generalizable or readily reproducible.

In contrast, synthetic biology offered a formalized approach for biosensor development. A biosensor can be abstracted as a processor with a sensing module, a processing module, and an actuating module (Fig. 1.1a) (van der Meer and Belkin 2010; Wang and Buck 2012; Bradley and Wang 2015; Bernard and Wang 2017; Kim et al. 2018). Any naturally occurring response pathway in a prokaryote can also be dissected and classified in a similar fashion. Therefore, development of a biosensor is reduced to a task of identifying or creating an input module that can respond to the target, and rewiring it to an observable output.

This framework is illustrated by the classical example of the whole cell sensor for detecting arsenic in drinking water. *Escherichia coli* naturally averts high concentrations of arsenic through its endogenous arsenic resistant pathway (Figure 1.1b) (Cervantes et al. 1994; Nealson et al. 2002; Silver and Phung 2005; Andres and Bertin 2016). When bounded by arsenite (III), the ArsR transcription factor would de-repress its cognate promoter P_{arsR} which then triggers expression of molecular pumps that remove arsenite from the cell (Shi et al. 1996; Silver and Phung 2005; Chen and Rosen 2014; Saha et al. 2017). The ArsR-P_{arsR} transcriptional regulation can thus be understood as a sensing module, and the genes coding for the arsenic efflux pump, as an actuating module. The natural sensing module can then be isolated and wired to a downstream reporter GFP and this re-engineered *E. coli* would give higher level of green fluorescence under increased level of arsenic, serving the purpose of a biosensor (Figure 1.1d).

This framework is universal and can be adopted to quickly build new prokaryotic biosensors because many other sensing modules have also been characterized. The majority of these sensing modules worked through either an allosterically controlled transcriptional regulator or a bacterial two-component system. The most well studied examples for the former category include modules for detecting metal ions like

mercury (Figure 1.1c and e) and nutrients such as arabinose and lactose (Misra et al. 1985; Barkay et al. 2003; van der Meer and Belkin 2010; Mahr and Frunzke 2016). Others include modules for reporting quorum sensing molecules or inflammation biomarkers that could indicate infections by pathogens (Lin et al. 2007; Saeidi et al. 2011; Archer et al. 2012; Gupta et al. 2013; Hwang et al. 2014; Courbet et al. 2015; Daeffler et al. 2017; Riglar et al. 2017). There are also examples for hypoxia responsiveness (Anderson et al. 2006; Forbes 2010; Weber and Fussenegger 2012; Yu et al. 2012; Danino et al. 2015), aromatic contamination and DNT/TNT from landmines (Selifonova and Eaton 1996; Belkin et al. 2017). For two-component systems, notable modules include sensors for detecting green, red and blue light respectively (Olson et al. 2014; Fernandez-Rodriguez et al. 2017), as well as sensors for zinc and lead (Fig. 1.3a) (Wang et al. 2013). For both categories of sensing modules, the output from the module is almost always a transcriptional output. Therefore, they can be conveniently connected to an output module in a "plug-and-play" fashion to drive reporter gene expression.

1.2 Efficient sensor optimization by standardized and modularized genetic parts

In biotechnology, whole cell mutagenesis remains the canonical technique to improve the characteristics of a strain. While it is still widely used in whole cell biosensor optimization, they have further benefited from the modularization of genetic elements. The dissection of gene circuitries into standalone parts, e.g. promoters and ribosome binding sites (RBS), encourages the scope of the otherwise global random mutagenesis, as well as their manifested effects, to be confined to a local region. This is exemplified by the work of Li et al. In pursuit of a better sensing module for arsenite, they simultaneously mutated the ArsR coding sequence and the P_{arsR} promoter by error prone polymerase chain reaction, and identified mutations that reduced leakiness and boosted output dynamic range (to be further explained in later sections) (Li et al. 2015).

Part standardization also led to the appearance of part libraries. Since the length of a sequence undergoing mutagenesis is shortened, the combinatorial sequence space to be explored is drastically shrunk. Therefore, sequence variation can be thoroughly exhausted, and this resulted in a large variety of elements with performances that span across the entire activity spectrum. For example, the Anderson promoter library was generated from saturation mutagenesis of a constitutive promoter, and there is a community RBS collection on iGEM that confer different strengths for translation initiation (http://parts.igem.org).

They are particularly helpful if only a single part needs to be optimized – a part that was too strong and led to undesirable behavior in a biosensor could be swapped out by a weaker version effortlessly. This will be illustrated by an example of replacing an RBS to reduce biosensor leakiness later in this chapter. In addition, these libraries enable full exploration of a design space for expression strength. It has

been suggested that a promoter library and an RBS library could be combinatorically combined to optimize expression strength for any gene of interest (Kosuri et al. 2013).

1.3 Biosensor improvement by directed evolution

Synthetic biology has also provided new techniques for optimizing or altering biosensor behaviors through directed evolution, which include phage-assisted continuous evolution (PACE) (Esvelt et al. 2011) and compartmentalized partnered replication (CPR) (Abil et al. 2017). In an example, a biosensor actuated through a split T7 RNAP polymerase had improved signal-to-noise ratio after one split half was subjected to evolution by PACE (Pu et al. 2017). In another example, the transcription repressor for tryptophan has been evolved by CPR to respond specifically to halogenated tryptophan analogs (Ellefson et al. 2018).

1.4 Development of new sensing modules

In biosensor development, there is a perennial and insatiable need for new sensing modules for new chemicals, biomarkers or other targets. Due to strong chemical specificities in regulators and receptors, existing sensing modules intended for one target can rarely fully satisfy the sensing requirement of a different target, even though the two targets may be highly similar in structure. Thus, a search for a specific sensor module is almost always necessary.

In nature, prokaryotes contribute the most in biodiversity. They thrive in all niches, including those inhabitable to other organisms, and respond to countless stimuli. If an environment has long been enriched with a compound, it is very likely to find in vicinity a prokaryotic dweller evolved to detect and metabolize it (Nealson et al. 1991; Cervantes et al. 1994; Nealson et al. 2002). Consequently, a conventional practice to uncover a new biological sensing module is to sample and study bacteria in a target-enriched environment. The aforementioned naphthalene biosensor is one of such examples – the naphthalene metabolic pathway originated from a strain of *Pseudomonas fluorescens* living in the soil of a manufactured gas plant. This is still routinely practiced but it poses limit in biosensor development. Nowadays, there are new strategies to accelerate sensing module discoveries.

1.4.1 Part mining

Part mining is the bioinformatics-guided search for biological parts from sequenced genomes and is a branch of genome mining. Genetic parts or proteins often have orthologs in closely related species which carry out similar tasks and share homology in sequences. Therefore, if a part of interest has a define sequence feature, orthologs that potentially belong to the same family can be identified by performing sequence alignments across multiple genomes. Shortlisted candidates can then be synthesized and characterized, and parts that showed desirable responses can be

grouped to form a new library. Many sequenced genomes, as well as metagenomes from unculturable prokaryotes, have been sequenced and deposited into bioinformatics databases, which become a lucrative resource for parts mining (Johns et al. 2018). For example, using sequences for a cadmium responsive transcription factor from *Staphylococcus aureus*, researchers discovered a new sensing module for cadmium from the genome of *Bacillus oceanisediminis*, a bacterium that resides in sediment under sea (Kim et al. 2016).

In addition, some databases contain annotated proteins or parts, so mining can also be done via search of keywords in described or predicted biological functions. One demonstrated example is the creation of a library of sensing modules for aromatic compounds, obtained through part mining from the Uniprot database (Peking IGEM 2013).

1.4.2 Antibody derived domains as universal sensing modules

Many sensing modules have been derived from natural pathways, but their development into biosensor components often requires thorough understanding of the underlying biochemistry, which is a time-consuming process. Moreover, some targets might not have a natural sensing module responsible for its detection. Some recent researches sought to circumvent these problems by turning to antibody derived protein domains.

Antibody derived domains are protein fragments of antibodies created proteolytically or genetically. The leading example is the single domain antibodies (sdAb) (Holliger and Hudson 2005). They retain the variable region responsible for strong and specific antigen recognition and binding, but their considerably smaller sizes allow stable and soluble expression from bacteria. Most importantly, a novel sdAb for any given epitode can be quickly and inexpensively created by screening a synthetic and combinatorial antibody domain library against the epitope. Identified sdAb can then be fused with other protein domains that could elicit sensing response upon target binding. A proof of concept example was demonstrated by fusion of caffeine binding sdAb to a DNA binding domain (Chang et al. 2018). sdAb colocalized on a caffeine molecule would then allow dimerization of the DNA binding domain and restore its function as a transcription repressor.

2. Tools and Strategies from Synthetic Biology for Optimizing Biosensor Performance

Many early biosensors have subpar performances compared to their electronic or chemical counterparts, rendering them uncompetitive in field applications. In recent years, tools and strategies developed from synthetic biology created new avenues to improve characteristics of whole cell prokaryotic biosensor. There are mathematical models that provide quantitative frameworks for sensor improvement (Ang

et al. 2013; Mannan et al. 2017), but this section focuses on tools and strategies that have been experimentally proven.

2.1 Properties of a biosensor

From the perspective of engineering, it is paramount to define measurable properties so that improvements can be gauged quantitatively. Different metrics are available for defining for a whole cell sensor's performance (Daniel et al. 2013; Mannan et al. 2017). The one adopted in this chapter focuses on four important aspects most pertinent to applications: selectivity, sensitivity, output dynamic range and leakiness (Fig. 1.2). Selectivity is a qualitative property that concerns how well the biosensor distinguishes the target of interest among others that are chemically similar (Fig. 1.2a).

The other three properties define characteristics of the response curve of a biosensor, which mathematically describes how a sensor's output varies with its input (Fig. 1.2b). Most biosensors have a sigmoidal response curve that monotonically increases with the target concentration. In this sense, the sensitivity of a biosensor can be defined as its detection limit – the minimal concentration of target that elicits an observable response. The output dynamic range refers to the ratio between the maximally achievable output and the basal output of the biosensor. Leakiness is the basal level of the biosensor when no target is present, and has been known to jeopardize sensor applications: if colorimetric outputs are used, the response may be easily saturated even in absence of targets, and would restrict titrimetric analysis (Wackwitz et al. 2008).

2.2 Strategies for enhancing selectivity

Some sensing modules are naturally promiscuous and can be triggered by targets with similar chemical properties. This is often the case for heavy metal sensing (Amaro et al. 2011; Wang et al. 2013). For example, CadC from *S. aureus* can sense Cd, Pb and Zn, CmtR from *Mycobacterium tuberculosis* can sense Cd and Pb, and ArsR from *E. coli* can sense As, Sb and Bi (Saha et al. 2017).

These sensing modules are thus non-specific and could give false positives when deployed in the field. Given that those transcription factors are allosterically regulated, an intuitive and routine approach is to randomly mutate their binding pockets, and screen for mutants with increased selectivity towards the ion of interest. A number of successful cases have been reported: a mutated CueR remained sensitive to Cu^{2+}, but no longer responded to Ag^{+} and became more sensitive to Au^{+} (Stoyanov and Browns 2003). In another example, MerR was mutated to detect Cd^{2+} rather than its original ligand Hg^{2+} (Hakkila et al. 2011). RcnR is normally regulated by both Ni and Co but could be mutated to only recognize the former (Cayron et al. 2017). However, as with general mutagenesis, this approach is time consuming and does not guarantee success in identifying a receptor with desirable traits.

A general solution is to employ a genetic logic AND gate with two different receptors that can detect the same ligand (Bernard and Wang 2017). For example,

two transcription factors can detect Zn^{2+} but both are non-specific: ZraR detects Zn^{2+} and Pb^{2+}, and ZntR detects Zn^{2+} and Cd^{2+} (Wang et al. 2013). Implementation of an AND logic using ZraR and ZntR as the inputs will yield a biosensor that responds to Zn^{2+} but not Pb^{2+} or Cd^{2+}, thus increasing its specificity (Fig. 1.3a).

This AND gate example was derived from the HrpRS activator complex and its cognate promoter P_{hrpL} (Fig. 1.3b). P_{hrpL} is a σ^{54}-dependent promoter that is activated only when both HrpR and HrpS are present (Jovanovic et al. 2011; Wang et al. 2011a; Liu et al. 2018). Many others logic gates orthogonal to HrpRS system are also available from the synthetic biology community: a T7 RNA polymerase-amber suppressor system that integrates two input signals (Anderson et al. 2007), activator-chaperone systems (Moon et al. 2012), split T7 RNA polymerase-based systems (Shis and Bennett 2013; Schaerli et al. 2014), and recombinase based Boolean gates (Bonnet et al. 2013; Courbet et al. 2015). These orthogonal logic gates provide means to enhance biosensor specificity in more complicated cases.

2.3 Strategies for increasing sensitivity

Similar to solutions for biosensor selectivity, random mutagenesis on promoters or transcription factors used to be a popular way to generate mutated sensing modules capable of detecting lower ligand concentrations. However, the issue of sensitivity is intrinsically tied to the relative concentrations between the receptor and ligand, so a more rational approach is to tune their respective densities in the cell.

2.3.1 Sensitivity improvement by tuning receptor densities

Depending on their modes of action, a sensing module could be more sensitive by increased or decreased concentrations of receptors (Merulla et al. 2013; Wang et al. 2015). If the receptor is a transcription repressor that could be de-repressed by an inducer (Fig. 1.4a), reducing its concentration could lead to both a higher sensitivity and a higher dynamic range. In the canonical allosteric transcriptional regulation paradigm, the binding between the inducer and the repressor, and between the repressor and the promoter are in equilibrium. A minimum concentration of inducer is always required to sufficiently inactivate the repressor and to allow the promoter to drive an observable expression of the reporter gene. Reducing the repressor concentration would therefore lower the specified demand on the inducer concentration, which effectively translates into a higher sensitivity for the sensing module. This strategy has been demonstrated on the previously described arsenic biosensor, in which its sensitivity was improved by replacing the strong constitutive promoter that drives repressor ArsR by a weaker variant (Fig. 1.4c).

To obtain a higher sensitivity in the opposite scenario, where the receptor is a transcription activator inducible by the inducer (Fig. 1.4b), the receptor density needs to be raised (Wang et al. 2015). In the case of the LuxR-P_{lux} sensor that detects the ligand AHL, more LuxR presented in the cells implies a higher probability to

form LuxR-AHL complexes (Fig. 1.4d). Therefore, the P_{lux} promoter can be activated with a lower concentration of AHL, and so the sensor is more sensitive.

It is noteworthy that receptor densities could also be dynamically controlled to give conditional sensitivity. A cadmium biosensor was integrated with a toggle switch, where the CadR repressor concentration was modulated by both the cadmium concentration and a sensitivity tuning ligand (Wu et al. 2009). Under a moderate level of the sensitivity tuning ligand, an increase in cadmium concentration would in turn reduce expression of the CadR repressor. This positive feedback mechanism increased the sensitivity of the biosensor, and interestingly, the feedback could be quenched by increasing the concentration of the sensitivity tuning ligand.

2.3.2 Sensitivity improvement by tuning intracellular ligand densities

For ligands that interact with sensing modules intracellularly and do not diffuse freely across the membrane, increasing their cytoplasmic concentrations increases their availability and hence their probability to excite the sensing module. In fact, this strategy can be frequently found in nature. In *E. coli* for example, the transportations of arabinose and rhamnose across the cell membrane are governed by positive feedback loops (Altenbuchner and Mattes 2005; Fritz et al. 2014). Induction of the metabolism pathway drives expression of the transmembrane transporter protein, which subsequently facilitates the imports of the sugars.

In biosensor design, if the transmembrane transporters for a ligand could be identified, the same outcome could be reproduced by overexpressing importers and knocking out exporters (Fig. 1.4e). For instance, disruption of the efflux transporters for Zn/Cd/Pb in *P. putida* strain KT2440 improved the detection limits up to 45-fold (Hynninen et al. 2010). In another example, an engineered *E. coli* biosensor achieved better sensitivity through introduction of several foreign Ni-uptake systems and deletion of Ni efflux pump (Cayron et al. 2017).

2.4 Strategies for increasing output dynamic range

A number of native promoters responsive to heavy metals are relatively weak and so their derived biosensors suffer from limited output dynamic ranges (Stocker et al. 2003; De Mora et al. 2011; Siegfried et al. 2012; Wang et al. 2013; Huang et al. 2015a; Kim et al. 2016; Merulla and Van Der Meer 2016). Again, one option is to perform random mutagenesis on the promoter to maximize transcriptional output.

Recently, however, a more reliable approach has been developed for engineering hybrid σ^{70}-based promoters. Chen et al. demonstrated that the dynamic ranges of these promoters could be quantitatively predicted by the choices of their −35 and −10 regions, which dictate the binding affinities between the promoters and the sigma factors required for transcription initiation (Fig. 1.5a) (Cox et al. 2007; Brewster et al. 2012; Guzina and Djordjevic 2017; Chen et al. 2018). By inserting binding sites of inducible transcription factors around the −35 and −10 sequences,

inducible promoters with improved dynamic ranges could be obtained. This example nonetheless is specific for σ^{70} promoters and remains inapplicable to promoters with uninsulated promoter elements.

A more universal solution from synthetic biology is to magnify the initially limited output through transcriptional amplifiers (TAmps). A TAmp is analogous to a buffer gate in electronics – it amplifies a transcriptional input signal before outputting it (Fig. 1.5b). In general, a TAmp takes the form of a transcriptional cascade that comprises a transcriptional factor (TF) and its cognate promoter P_{TF}. TF is the output of the sensing module and the P_{TF} drives expression of the next cascade or the final observable output. To qualify as a TAmp, P_{TF} must have a higher maximal activity than the promoter upstream of TF, so that a tiny transcriptional input could be converted into a huge downstream output. Therefore, mathematically, a TAmp can be understood as a function that receives a transcriptional input from a relatively narrow domain and maps it to a much larger range.

Early examples of TAmps were built using transcriptional repressors. Two examples of repressor based cascades have been built, and one was shown to amplify weak promoter activities that would otherwise be unobservable (Fig. 1.5b-*ii*) (Karig and Weiss 2005; Hooshangi et al. 2005). However, use of repressor-based amplifiers might not be suitable for sensing modules with positive relationships between inputs and outputs, because they would invert the response function, unless they are cascaded in even numbers.

New TAmps based on transcription activators have been developed. This is exemplified by a TAmp built from the HrpRS system described above (Fig. 1.5b-*iii* and c) (Wang et al. 2014). It readily accepts a wide range of transcriptional inputs and linearly amplifies them in an analog fashion. This property proved useful when the amplifier was used to significantly improve an arsenic sensor's output dynamic range. The TAmp is also versatile – the amplification gain can be tuned by regulating the level of HrpS, which could be achieved either translationally, via changing the RBS, or post-translationally, by expressing the HrpS inhibitor HrpV. Another notable example of TAmp was based on the T7 RNA polymerase and its cognate promoter P_{T7} (Kim et al. 2016), which has been shown to boost both the sensitivity and the output dynamic range of a cadmium/lead biosensor.

Provided that the sensing module is tightly regulated, amplification capacities for a TAmp could be further augmented through incorporation of a positive feedback loop. In a proof of concept circuit, the output of a TAmp drove expression of an activator used in the TAmp (Nistala et al. 2010), so signal amplification not only applies on the input but also on the output (Fig. 1.5b-*iiii*). Compared to the standalone TAmp architecture, this coupled TAmp significantly improved the observable output as well as the detection limit of a biosensor. Caution should be exercised when applying this coupled architecture to a potentially leaky sensing module, because a high basal output level could be significantly amplified and would lead to a reduced output dynamic range.

Output amplification can also be realized using recombinase-based memory modules, provided that the maximal attainable transcription activity of the final output can surpass that of the sensing promoter. This will be described further in a later section.

2.5 Strategies for reducing leakiness

Biological processes are evolved to be inherently leaky and noisy, because it leaves room for variation and allows bet-hedging in face of sudden and drastic change in environment (Randall et al. 2011). One would therefore rarely find a natural sensing module that displays little to no leaky behavior. So it is important, and more appropriate, to view leakiness not as a curable property, but as an unavoidable issue to be managed. Leakiness of a biosensor typically originates from the promoter within the sensing module (Wackwitz et al. 2008; Arpino et al. 2013; Adams et al. 2014; Merulla and Van Der Meer 2016), but it could be addressed by strategies that act on levels of transcription, translation and post translational modification.

2.5.1 Managing leakiness on a transcriptional level

2.5.1.1 Receptor and promoter engineering

Since the sensing promoter is almost always the source of leakiness, it is logical to start by engineering the promoter and its cognate transcription factor. Once again, random mutagenesis and random promoter truncation are two popular approaches – libraries of mutant sensing regulator-promoter pairs are screened to obtain less leaky and yet still functional variants (Li et al. 2015; Daeffler et al. 2017). Identified candidates usually have mutations that alter the promoters' transcription initiation rates or the affinities between transcriptional regulators and promoters. The previously described cases of mutagenesis done on the ArsR-Pars sensing module, and the quantitative approach to engineer hybrid σ^{70} promoters are examples of such approaches (Fig. 1.5a) (Li et al. 2015; Chen et al. 2018).

A similar but more predictable method is to vary the number and position of operators (Fig. 1.6a and b) (Murphy et al. 2007; Merulla and Vasn Der Meer 2016; Zong et al. 2017; Chen et al. 2018). Operators are sequences within a promoter that serve as binding sites for transcriptional regulators, and there are some general rules regarding how they affect promoter leakiness. Operators for repressors may be placed in the distal region (upstream of –35), the core region (between –10 and –35), or the proximal region (downstream of –10) (Fig. 1.6a). If only a single operator for repressor exists on the promoter, maximal repression efficiency, and hence minimal leakiness, would be obtained when the operator is placed in the core region (Cox et al. 2007). Repression efficiency is further enhanced if the operator overlaps with part of the –10 or –35 regions (Chen et al. 2018).

Adding an extra operator downstream of a promoter can also reduce leakiness. Should the first operator fail to recruit the repressor, the repressor bound to the extra operator can still inhibit readthrough of the RNA polymerase (Fig. 1.6b). This effect is known as "roadblocking", and its efficiency can be tuned by varying the distance between the extra operator and the core region (Murphy et al. 2007; Hao et al. 2014; Merulla and Van Der Meer 2016). Having multiple extra operators could increase the effect (Murphy et al. 2007). Though, it should be noted that the roadblocking efficiency depends on the maximum strength of the sensing promoter, the repressor concentration and the affinity between the repressor and the operator, and so the effects on different operator-repressor pairs would likely be variable.

Unlike their repressor counterparts, operators for activators can only be functional when placed in the distal region (Cox et al. 2007). They remain much unstudied, but it was known that if an inducible activator can bind its operator in the absence of its target ligand, the resulting promoter will likely be tightly controlled (Chen et al. 2018).

2.5.1.2 Antisense transcription

Antisense transcription refers to the strategy of inserting a second promoter downstream of the sensing promoter, but in an opposite direction (Pelechano and Steinmetz 2013). The second promoter will drive transcription of an RNA that is partially or fully complementary to the RNA produced from the sensing promoter. Antisense transcription interrupts RNA polymerase reading from the first promoter (Fig. 1.6c-*iii*) (Brophy and Voigt 2016), thus reducing its basal expression level and leakiness. If the antisense promoter is located at the 3' end of the target gene, the antisense RNA will be long enough to form a double-stranded RNA with the target mRNA and then triggers its degradation (Fig. 1.6c-*ii*) (Lasa et al. 2011). The efficiency of this strategy can be tuned by regulating the strength of the antisense promoter (Fig. 1.6c) (Brophy and Voigt 2016), but the maximum output expression from the first promoter may also significantly decrease if the antisense promoter is too strong.

Antisense transcription may also exert translational interference: an anti-sense promoter that is positioned immediately downstream of the target promoter may lead to transcription of a short antisense RNA that inhibits translation initiation of the target gene (Fig. 1.6c-*i*) (Kawano et al. 2007).

2.5.2 Managing leakiness on a translational level

The expression rate of any gene of interest can be superficially regarded as a product of its transcription rate and translation rate. Reducing the translation efficiency is therefore an indirect way to counteract transcription rates that are either too high or too low. This is most helpful in scenarios where transcription elements

are fixed and cannot be readily replaced. For example, in a transcriptional cascade, the upstream and downstream promoters are defined. Yet the effective intracellular concentrations of the transcriptional regulators can be modulated by changing their translation rates. This would alter their input-output characteristics and in turn impact the final observable leakiness of the biosensor (Wang et al. 2011a; Nielsen et al. 2016). In a specific example, an active recombinase under the control of a leaky inducible promoter might switch on the downstream signal even in absence of a target. By attenuating its translation initiation rate and hence its concentration, this undesirable effect could be mitigated (Rubens et al. 2016).

The most common way to tune translation is to modify the translation initiation rate, which is to a great extent governed by the sequence of the ribosome binding site (RBS) that dictates it affinity towards the ribosome (Fig. 1.6d). RBS strengths can be described by a biophysical model, and are predictable from the RNA sequences alone (Salis et al. 2009). A few RBS libraries with characterized translation initiation efficiencies are also available (http://parts.igem.org). However, translation rates of a protein have been shown to depend on numerous factors other than the RBS itself. This includes sequences between the RBS and the gene of interest (Kosuri et al. 2013; Mutalik et al. 2013), and codon usage. Additional sequences upstream and downstream of the RBS may also affect the translation initiation process (Salis et al. 2009; Wu et al. 2018), and should be into account when choosing or designing RBS sequences. For example, gene expression could be impaired by an increase in A/T rich repetitive sequences between the RBS and the start codon (Egbert and Klavins 2012), or by having a long mRNA sequence with low GC content (Wu et al. 2018).

2.5.3 Managing leakiness on a post-translational level

In some published biosensors, protein degradation tags were attached to regulator or reporter proteins to promote their clearance from the cell and reduce their effective concentrations (Andersen et al. 1998; Arpino et al. 2013; Cameron and Collins 2014; Bradley et al. 2016b). However, this method comes with a tradeoff with a reduction of maximum output level (Fig.1.6e, grey line).

A solution that rescued the output level but maintained the lower level of leakiness has been recently developed (Fig. 1.6e) (Fernandez-Rodriguez and Voigt 2016). It achieved so by inserting a protease cleavage site between the reporter protein and the degradation tag, and the cognate protease is under the control of the sensing module. At lower target concentrations, small amount of reporter was produced due to leaky expression, but they were quickly degraded due to the presence of the degradation tag. At high target concentrations, both the protease and the reporter would be strongly expressed, so the protease could cleave off the degradation tag from the latter, and the observable output could remain at a high level. (Fig. 1.6e, orange dotted line). This protease-based regulation strategy is flexible and can be effortlessly applied to any leaky biosensors.

3. Functional Expansion of Biosensors by Synthetic Biology

3.1 Memory devices

A biosensor with only sensing and actuating modules suffice to report the immediate availability of a target. Yet when endowed with memory modules that allow record and retrieval of transient detection events, whole cell biosensors become excellent platforms for continuous documentation of surroundings, because they are living organisms that can proliferate and colonize an environmental with minimal maintenance from humans (Burrill et al. 2012). This could be useful in tracking a delayed response in a difficult-to-reach environment, like those in clinical diagnostic settings (Courbet et al. 2015).

Memory devices can be broadly classified according to their reversibility and whether the memory is encoded in the DNA (i.e. genetic versus epigenetic). Synthetic biologists have created an overwhelming number of memory devices and they have been reviewed elsewhere (Inniss and Silver 2013; Roquet and Lu 2014; Bradley and Wang 2015). Given the scope of this chapter, only examples that have been successfully installed and demonstrated in biosensors will be covered.

3.1.1 Toggle switches

The synthetic toggle-switch can be considered as the earliest epigenetic and reversible memory device from synthetic biology. It achieved bistability through two repressors that mutually inhibit each other (Fig. 1.7a) (Gardner et al. 2000). A transient induction that upregulates expression on either one of the repressors (TR1) downregulates the expression of the other (TR2) and allows the latter to dominate, which results in a flip from one state to another. The state of the system will then be stably maintained until the now dominant repressor (TR1) is repressed again. Since only two states are allowed, the toggle-switch converts any graded input response into a digital output, and the readout for a target will no longer be titrated, but this might improve robustness in a sensor and aid decision making processes (Roquet and Lu 2014).

The lambda phage cI/Cro genetic switch is a natural toggle switch which has high repression efficiency and modularity, but other strong repressors that display cooperative binding properties can also be used. Toggle switches have been widely used in whole cell biosensors. For example, *E. coli* biosensors have been engineered to sense and record antibiotic exposure or inflammation in murine guts (Kotula et al. 2014; Riglar et al. 2017). Another toggle-switch was part of a Pavlovian-like conditioning circuit in *E. coli*, where it could memorize a conditioned stimulus (Zhang et al. 2014).

3.1.2 Recombinase based memory devices

Site specific recombinases are enzymes that perform genetic recombination on DNA flanked by specific recognition sites (aka recombination cassettes), resulting in either DNA excisions or inversions (Olorunniji et al. 2016). Biologically, recombinases belong to either tyrosine or serine recombinase classes, and can be further classified by their directionality and their permitted mode of actions (Wang et al. 2011b). For recombinases that can perform both excision and inversion, the mode of recombination depends on the relative orientations of the recombination sites: excision happens between aligned recombination sites and for inversion, anti-aligned sites (Fig. 1.7a). Both allow implementations of memory when the DNA within the recombination cassette is a functional biological part.

A simple memory device using DNA excision can be built by inserting a recombination sites-flanked terminator between a constitutive promoter and a translational unit, which interrupts gene expression until removed (Bonnet et al. 2013). In the case of inversion, the genetic part within the cassette would be purposefully inserted in a non-functional orientation, only to be restored when the recombinase is active. In both cases, the DNA is transformed from one sustained state into another, thereby conferring memory. Since the flanked DNA can only exist in either one of the two states, all recombinase-based memory devices are analog to digital converters. Their genetic nature also implies that the memory is heritable and would last even if the host cell is dead (Siuti et al. 2013).

Many early synthetic memory devices were based on inversions using bidirectional recombinases like the Cre/lox and FLP/FRT systems. They tended to stochastically re-catalyze an inverted DNA back into the original orientation and create a mixed population of cells in either ON or OFF states, which posed an issue to robustness in memory (Schnütgen et al. 2003; Brophy and Voigt 2014). Workarounds to this issue include using mutated recombination sites that drive inversion equilibrium towards the product (Albert et al. 1995; Oberdoerffer 2003), or configurations that would lead to disappearance of some recombination sites in the end state (Fig. 1.7b) (Schnütgen et al. 2003). The same mixed population issue also applies to unidirectional recombinases when their expression is at intermediate levels, but the problem could be alleviated through the use of feedback loops (Moon et al. 2011; Brophy and Voigt 2014; Folliard et al. 2017).

Memory devices engineered from recombinase-recombination cassettes with unidirectionality provide irreversible memories – a temporal exposure to the target will flip the system into an irrevocable state (Siuti et al. 2013). To date, a large number of orthogonal unidirectional recombinases have been identified and they allow different inputs to be remembered within the same cell (Yang et al. 2014). They were demonstrated to record conditions in gastrointestinal tracts, as well as pathogens in human serum and urine samples (Archer et al. 2012; Courbet et al. 2015; Mimee et al. 2015). The sequence of detection events can also be recorded in a state machine, which assigns a unique state to every possible sequence order by coordinating multiple unidirectional recombinases (Roquet et al. 2016).

Biosensors built with irreversible memories would be single-use commodities. In contrast, reusable and rewritable memory devices can be constructed from recombinase-excisionase pairs with conditional bidirectionality (Fig. 1.7c) (Bonnet et al. 2012; Bonnet et al. 2013). The mechanism is illustrated using the integrase Bxb1 gp35 and its cognate excisionase Bxb1 gp47. The integrase alone will drive inversion towards one direction, but the direction is reversed in presence of the excisionase.

3.1.3 CRISPR/Cas-based memory devices

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR associated (Cas) are bacterial immunity systems against phage infections. Their molecular mechanisms have been intensely studied and frequently reviewed elsewhere and so will not covered in this section (Shalem et al. 2015; Jiang and Doudna 2017). CRISPR/Cas systems have innate memory functions to remember previously invaded viruses. They capture exogenous DNA from plasmids or phages and integrate them into the genomic CRISPR arrays as spacer sequences. This adaption process has been repurposed to yield a synthetic memory device, known as the "biological tape recorder" (Fig. 1.7d) (Sheth et al. 2017). A new spacer is incorporated into the array upon induction by a specific target, and multiple events over time can be recorded.

The popularity of the CRISPR/Cas system, nonetheless, remains in its versatility in performing precise DNA cutting and editing. These properties have been also harnessed in a memory device named CAMERA (CRISPR-mediated Analog Multi-event Recording Apparatus) (Fig. 1.7e) (Tang and Liu 2018). The system memorizes signals by one of the two ways: **1)** An incoming signal modulates the activity of a DNA cleavage enzyme, which selectively cuts one of the two recording plasmids and thus alters the ratio between the two. **2)** The signal instructs Cas9-derived base editors to modify DNA sequences. In both examples, the recorded information can be retrieved by sequencing the barcoded DNA sequences or by coupling the resulting change to an observable output.

3.1.4 Other notable memory devices

The three types of memory devices described above were so far the most popular ones. Nonetheless, there are two other noteworthy examples that were also demonstrated in biosensors: **1)** In a device named SCRIBE (Synthetic Cellular Recorders Integrating Biological Events), detection events lead to productions of hybrid RNA-ssDNA molecules that will undergo genetic recombination with the bacterial genome, modifying sequences on the later (Farzadfard and Lu 2014). The strength of sensing correlates with the frequency of recombination and is therefore reflected on a population level, specifically, the proportion of cells that carries the modification.

2) An epigenetic and reversible memory device was constructed by DNA methylase and DNA binding proteins (DBP) that are sensitive to DNA methylation. Target detection therefore triggers DNA methylation and precludes binding of the DBP, and memory reset is carried out via degradation of the methylase (Maier et al. 2017).

3.2 Computation modules to integrate signals

Most biosensors were built to detect a single input, but by receiving and processing multiple inputs, they can be used to sense a complex condition or a global environment (Fig. 1.8) (Wang et al. 2013). Synthetic biology has offered numerous examples on bio-computation and interested readers are advised to consult references cited here (Wang et al. 2011a; Moon et al. 2012; Wang and Buck 2014; Ma et al. 2016; Nielsen et al. 2016; Roquet et al. 2016; Rubens et al. 2016).

Signal integration is of great interest in clinical diagnosis and biotherapy, where multiple signals define a specific disease state and determine if drugs should be administered. To date, no genetic circuits have been developed in prokaryotic biosensors to target multiple clinical biomarkers, but similar ideas have been proposed and tested. For example, a hypothetical *E. coli* that would invade tumor cells would only work when two conditions are satisfied: 1) it reaches a quorum due to colonization and 2) detects a hypoxic environment that is typically found in tumors (Anderson et al. 2006). The two conditions would thus require integration through an AND gate.

3.3 Modules to reshape response function

Other forms of signal processing can remodel the conventional sigmoidal response curve to facilitate biosensor readout. By connecting a sensing module to an incoherent feedforward loop, a biosensor can behave as a bandpass filter and only responds to a limited range of analyte concentrations (Peking IGEM 2013; Rubens et al. 2016). In another example, a coherent feedforward loop successfully transformed the response curve into a semi-log sensing curve (Zhang et al. 2013). These changes in response functions could be helpful when the output sensing modules are wired to specific actuators that are not as responsive to sigmoidal inputs.

3.4 Reporter modules for interfacing with different detection platforms

Many biosensors were first developed using fluorescent reporters as the actuator, which facilitate their characterization in laboratory settings. Yet employing these biosensors in the field would be inconvenient since it must be accompanied with a fluorescent reader. The synthetic biology community has developed a diverse collection of reporters or actuators. They are standardized, and so can easily substitute the fluorescent reporter. This allows the output signals to be observed through other means. For example, some reporter modules can convert detection events into electrical currents measurable by electrodes (Webster et al. 2014; Tschirhart et al. 2017).

Typically, colorimetric reporters are often used to allow direct observations of sensor output by naked eyes. They include chromoproteins, some fluorescent proteins, and enzymes that produce pigment or catalyzed chromogenic substrates (Biran et al. 2003; Stocker et al. 2003; Fujimoto et al. 2006; Wackwitz et al. 2008; Joshi et al. 2009; De Mora et al. 2011; Joe et al. 2012; Shin 2012; Kotula et al. 2014; Pardee et al. 2014; Courbet et al. 2015; Danino et al. 2015; Huang et al. 2015a; Pardee et al. 2016a; Didovyk et al. 2017; Watstein and Styczynski 2018). This could drive down the operating costs of a biosensor because it obviates additional readout machines.

Yet, there are also a few other interesting reporters that enable biosensors to operate *in vivo*: Acoustic reporter genes were used to encode intracellular gas vesicles in *E. coli* (Bourdeau et al. 2018). These vesicles can scatter ultrasound waves that non-invasively penetrate living tissues and therefore lead to imaging contrast. Another example employs a luciferase reporter: *E. coli* entrapped in an ingestible micro-bio-electronic device (IMBED) can detect analytes that diffuse into its residing chemostat chamber. The cells then respond by emitting light which can converted into digital signals through photodetectors. The signal can then be further relayed to computers via Wi-Fi (Mimee et al. 2018).

3.5 Biosafety enhancing modules

As explained in the introduction, biosafety remains the bottleneck for field applications of prokaryotic whole cell biosensors, and a huge volume of work in synthetic biology was dedicated to address this issue. Some representative work have been selected to illustrate various strategies in managing the biocontamination risks.

"GeneGuard" was a stable and modular system for biosafety control in *E. coli* (Wright et al. 2015). Three safety modules were inserted into the genome: 1) A rich-media compatible auxotrophy selection marker ensures that the host cell would hardly survive if it leaves an industrial closed system. The marker also facilitates their disposables (Moe-Behrens et al. 2013). 2) Host-dependent origins of replication, as well as 3) toxin-antitoxin pairs prevent propagation of episomes that could have accidentally transferred to other organisms.

The host cells can also be programmed to commit suicide after a certain retention time in the environment. This could be achieved by putting genes encoding toxic products under the control of a synthetic timer (Tom Ellis, Xiao Wang 2009) or counting circuits (Friedland et al. 2009; Callura et al. 2010).

Another method is to erect a "Linguistic barrier" between the host cell and other prokaryotes in nature (Pinheiro et al. 2012; Wright et al. 2013). The genes that are expressed in the host cells are refactored so they cannot be transcribed or translated in other prokaryotes in the wild.

Conclusions and Future Directions

Prokaryotic whole cell biosensors are suitable for a broad range of applications and are promising alternatives to other types of sensors. With the support of synthetic biology, the development and optimization of novel whole cell biosensors have been vastly accelerated. Engineering principles facilitate biosensors to be constructed from a bottom up approach and provide means to engineer new sensing modules on top of the myriad available options. Through the lens of rational design, researchers could pinpoint cruxes that limit selectivity, sensitivity, dynamic range and leakiness and apply strategies that precisely tackle each issue and improve the overall sensing behavior of a biosensor. Finally, various parts and devices from the synthetic biology community augment functions of biosensor in memory and signal processing capacities, and make them easier and safer to be used in field applications. More tools and design principles that give rise to robust and reliable sensors will likely be discovered in the future, and they will further encourage whole cell biosensors to be applied in real life scenarios.

Synthetic biology alone however cannot solve all problems present in whole cell biosensors. At the current stage, despite the fact that many prokaryotic biosensors had impeccable performances in laboratory, only a few could enter business. There are three underlying reasons. First, the actual working environments for the biosensors are more far complicated than their laboratory counterparts, so requirements on sensitivity, selectivity and robustness are more demanding. The major solution is to subject the biosensor to multiple rounds of rigorous tests using real environmental or clinical samples, and progressively optimize their response behaviors through genetic manipulation. Approaches described in this chapter should aid this process, but sample preprocessing steps like target purification or concentration might be helpful complementary methods to boost biosensor sensitivity (Wen et al. 2017).

There is also a dearth of durable, inexpensive and user-friendly platforms for whole cell biosensor storage and multiplexed sample testing. Some options are available, but they do not keep cells alive over long periods of time. For instance, hydrogels, like alginate beads, agarose and silica gels, can entrap prokaryotic cells and keep them hydrated and functional for around a month (Chang and Prakash 2001; Nassif et al. 2002; Papi et al. 2005; Sharma et al. 2010; Buffi et al. 2011; Power et al. 2011; Shin 2012; Courbet et al. 2015; Belkin et al. 2017). Better entrapping materials and storage conditions are needed for prolonged shell life of biosensors. An alternative is to find a different chassis that is indifferent to harsh environments. In this sense, the Gram-positive bacteria *B. subtilis* is recently gaining grounds. They can give physically and chemically resistant endospores that can be stored dry in wide range of temperatures for years, but can still return to a vegetative state when nourished with water and nutrients (Joshi et al. 2009). Cheap microfluidic devices have also been developed as platforms for biosensor applications, yet they remain inconvenient for multiplexed sample processing and on-site diagnosis (Buffi et al. 2011; Kim et al. 2016; Volpetti et al. 2017).

Lastly, as long as they are still alive, whole cell prokaryotic biosensors will carry the stigma of being potentially biohazardous when they exit laboratories. Biosafety concerns like ecosystem disruption and antibiotic resistance gene transfer will need to be addressed (Dana et al. 2012). Adequate intrinsic containment (biosafety circuits reviewed in the previous section) and physical containment systems like bioreactive nanofibers (Tong et al. 2014) will need to be in place for whole cell biosensors to be safely deployed in the field.

In recent years, cell free systems have been proposed as a solution to circumvent the biosafety issues associated with whole cell sensing. A cell free system consists of cell extracts or purified transcription/translation machineries mixed with energy supplements and amino acids (Perez et al. 2016). It is cheap and easy to produce (Kwon and Jewett 2015; Didovyk et al. 2017). A number of biosensors using cell free extracts have already been shown to be capable of detecting heavy metals, pathogens, antibiotics and viral RNA (Pellinen et al. 2004; Pardee et al. 2014; Pardee et al. 2016a; Didovyk et al. 2017; Duyen et al. 2017; Wen et al. 2017). Furthermore, they could be freeze-dried on cellulose paper, which increases their stability and portability (Pardee et al. 2014; Pardee et al. 2016b). Theoretically, any whole-cell based biosensors can be converted into this paper-based cell free system and become point-of-care diagnostic devices. Still, there will always be unbridgeable differences between the *in vivo* intracellular environment and a cell extract, which may cause unpredictable behavior during biosensor circuit migration. Therefore, until the moment that all circuits can be transferred systematically and flawlessly across the two platforms, whole cell based prokaryotic biosensors will remain a major player in the field of biosensors.

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Figure Captions

Figure 1.1 Architecture and engineering of synthetic biosensors.

(a) Architecture of a modular synthetic biosensor. R, receptor. P, promoter. *gfp*, gene encoding a green fluorescent protein. *rfp*, gene encoding a red fluorescent protein. *luxAB*, genes encoding a bacterial luciferase for luminescent output. *lacZ*, gene coding β-Galactosidase for colorimetric output. *arg*, acoustic reporter genes which expresses gas vehicles that are detectable by ultrasound. *luxI* & *lasI*, genes encoding synthases for quorum sensing molecules. (**b**) *ars* operon from *E. coli*'s chromosome and its role in arsenic regulation (Silver and Phung 2005; Chen and Rosen 2014). GlpF, an aquaglyceroporin. PST, phosphate-specific transport system. PIT, phosphate inorganic transport system. (**c**) *mer* operon from *Shigella flexneri* R100 plasmid and its role in mercury regulation (Misra et al. 1985; Barkay et al. 2003). CH_3HgX, organic form of mercury. HgX, inorganic form of mercury. (**d**) An engineered *E. coli* biosensor with arsenic sensing and reporting function. (**e**) An engineered *E. coli* biosensor with mercury sensing and reporting function.

Figure 1.2 Metric for defining performances of a biosensor.

(**a**) Schematic illustrating a biosensor's selectivity. (**b**) A biosensor response curve with sensor's leakiness, sensitivity and output dynamic range annotated. The sensitivity can be defined as the limit of detection (LOD).

Figure 1.3 Biosensors specificity enabled by synthetic biology.

(**a**) A zinc-specific biosensor using an AND gate (Wang et al. 2013). (**a-*i***) A zinc/lead biosensor and its response curves for Zn^{2+} and Pb^{2+}. (**a-*ii***) A zinc/cadmium biosensor and its response curves for Zn^{2+} and Cd^{2+}. (**a-*iii***) A zinc-specific biosensor was generated by integrating both sensing modules from ***i*** and ***ii*** into an AND gate. *gfp*, gene encoding a green fluorescent protein. (**b**) The HrpR/HrpS hetero regulation motif in the *hrp* (hypersensitive response and pathogenicity) system of *Pseudomonas syringae* pv. tomato DC3000 (Wang et al. 2011a; Wang and Buck 2014). The *hrp* system promotes pathogenicity of the bacterium in its plant host. The σ^{54}-dependent *hrpL* promoter can be activated by the heterohexamers of the transcription activators HrpR and HrpS.

Figure 1.4 Strategies for improving biosensors' sensitivity.

(**a** and **b**) A transcriptional repressor (TR) and a transcriptional activator (TA)-based biosensor. P_C, constitutive promoter. P_{TR}, TR's cognate promoter. P_{TA}, TA's cognate promoter. Black dots, targets of interest. *gfp*, gene encoding a green fluorescent protein. (**c** and **d**) Improving a biosensor's sensitivity by tuning receptor densities (Wang et al. 2015). **c** shows a TR-based arsenic biosensor, and **d** shows a TA-based AHL biosensor. P_{Strong}, strong constitutive promoter. P_{Weak}, weak constitutive promoter. (**e**) Improving a biosensor's sensitivity by increasing its targets' intracellular density (Hynninen et al. 2010; Cayron et al. 2017). IS, importing system. ES, exporting system. R, receptor. P_R, R's cognate promoter.

Figure 1.5 Strategies for improving biosensors' output dynamic range.
(**a**) Different σ^{70} binding sites of a inducible promoter yield different basal levels and output dynamics as a result of relative equilibrium constants of σ^{70} binding to the −10 and −35 regions, $\ln(K_{eq}) = -(\Delta G_{-10} + \Delta G_{-35})$ (Chen et al. 2018). Non-consensus bases are underlined. ΔG_{-10} and ΔG_{-35} correspond to the relative changes in the binding energy due to changes in the −10 and −35 sites. R, receptor. P_R, R's cognate promoter which contains an operator for R (RO). *yfp*, gene encoding a yellow fluorescent protein. (**b**) Biosensors without transcriptional amplifiers (TAmp) (**b-*i***), with two transcriptional repressor-based amplifiers (**b-*ii***), or with a transcriptional activator-based amplifiers (**b-*iii***), and their response curves (Hooshangi et al. 2005; Wang et al. 2014; Kim et al. 2016). ***iiii***. An amplifier with positive feedback (Nistala et al. 2010). P_{TR}, TR's cognate promoter. P_{TA}, TA's cognate promoter. *gfp*, gene encoding a green fluorescent protein. (**c**) A gain-tuneable TAmp based on a HrpRSV system (Wang et al. 2014). This device scales the weak transcriptional input signal (I) linearly in response to a second 'gain tuning' transcriptional input (β^T).

Figure 1.6 Strategies of tuning biosensors' leakiness and output dynamics.
(**a**) A transcriptional repressor (TR)-based inducible promoter, with an operator site (TRO) at the distal, core or proximal region of the promoter. Repression efficiency were shown to depend on the TRO's location, which follows core ≥ proximal ≥ distal (Cox et al. 2007). P_{TR}, TR's cognate promoter. *yfp*, gene encoding a yellow fluorescent protein. (**b**) Transcriptional roadblocking effect. *gfp*, gene encoding a green fluorescent protein. (**c**) Antisense transcription as a tool to tune gene expression. Left panel: antisense promoter (P_{anti}) can reduce P_R's leakiness by blocking the ribosome entry to a reporter's mRNA (**c-*i***), triggering mRNA degradation(**c-*ii***), or blocking the RNA polymerase (RNAP) reading from the sensing promoter P_R (**c-*iii***). Right panel: The sensor's leakiness and output dynamics correlate with the strength of P_{anti} (Brophy and Voigt 2016). R, receptor. P_R, receptor's cognate promoter. (**d**) Modification of ribosome binding site (RBS) for a transcriptional activator (TA), a TR, or the output changes the leakiness and output dynamics of a biosensor (Wang et al. 2011a; Nielsen et al. 2016; Rubens et al. 2016). P_{TA}, TA's cognate promoter. (**e**) Schematics of a post-translational regulation on an IPTG biosensor (Fernandez-Rodriguez and Voigt 2016). *L* represents a protein degradation tag LVA.

Figure 1.7 Diagrams of genetic circuits for memorizing environmental signals.
(**a**) A toggle switch-based memory device. Initially the toggle switch is at an OFF state, where the TR2 is expressed and TR1 expression is repressed. Upon target detection, the sensing circuit express TR1, which flips the device into an ON state. TR1 and LacZ will be continuously expressed even after the target is removed. (Kotula et al. 2014; Riglar et al. 2017). R, receptor. P_R, R's cognate promoter. TR,

transcriptional repressor. P_{TR}, TR's cognate promoter. *lacZ*, gene encoding β-Galactosidase for colorimetric output. (**b**) A recombinase-based memory device. Upon sensing a particular target, the biosensor produces recombinase Cre, which first flips the orientation of the *gfp* flanked by *loxP* sites, and then excises one of the two *loxP* sites through the *lox511* sites (Schnütgen et al. 2003). P_C, constitutive promoter. *gfp*, gene encoding a green fluorescent protein. (**c**) An integrase-based memory device switches the sensor's output from *gfp* expression to *rfp* expression. The integrase and excisionase together restore the *gfp* expression (Bonnet et al. 2012). *rfp*, gene encoding a red fluorescent protein. (**d**) A CRISPR-based ''biological tape recorder' system. The signals are recorded into the genomic CRISPR array (Sheth et al. 2017). When there is no signal, only the reference DNA will be recorded; where there are signals, the trigger DNA will be rapidly replicated and preferentially recorded into the CRISPR array. (**e**) The CAMERA recording system (Tang and Liu 2018) has two possible mechanisms: ***i***, it uses Cas9 nucleases to record signals by shifting the ratio of two recording plasmids; ***ii***, it uses Cas9-derived base editors to change DNA sequences upon sensing a signal.

Figure 1.8 Detecting an environmental condition using multi-input AND gate and cell-cell communication.

A three-input AND logic gate based on two HrpRS-based AND gates are separated in two different cell strains of a consortium (Wang et al. 2013). *luxI*, synthase of a quorum sensing molecule $3OC_6HSL$. *rfp*, gene encoding a red fluorescent protein.

Figure 1.1

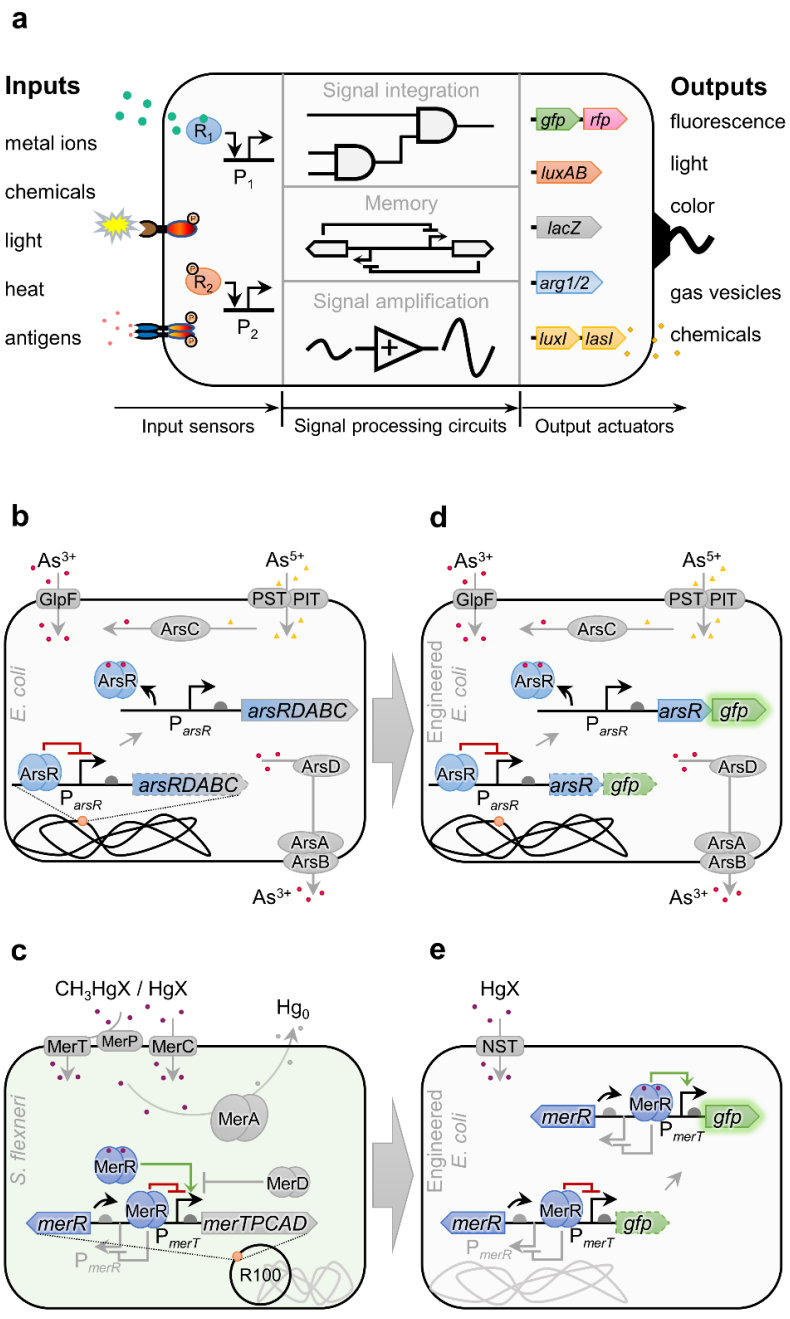

a
Inputs
metal ions
chemicals
light
heat
antigens
Signal integration
Memory
Signal amplification
Outputs
fluorescence
light
color
gas vesicles
chemicals
gfp
rfp
luxAB
lacZ
arg1/2
luxI
lasI
Input sensors
Signal processing circuits
Output actuators
b
d
As3+
As5+
GlpF
PST PIT
ArsC
ArsR
arsRDABC
ArsD
ArsA
ArsB
E. coli
Engineered E. coli
arsR
gfp
c
e
CH3HgX / HgX
Hg0
MerT
MerP
MerC
MerA
MerR
MerD
merR
merTPCAD
R100
S. flexneri
HgX
NST
merTPCAD
gfp

Figure 1.2

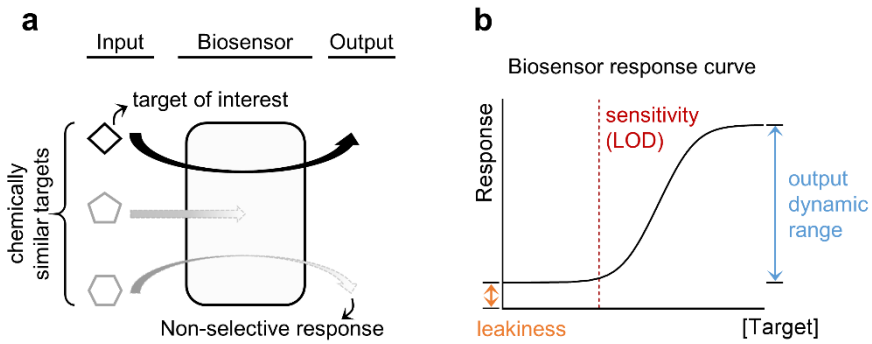

a
Input
Biosensor
Output
target of interest
chemically similar targets
Non-selective response
b
Biosensor response curve
Response
sensitivity (LOD)
output dynamic range
leakiness
[Target]

Figure 1.3

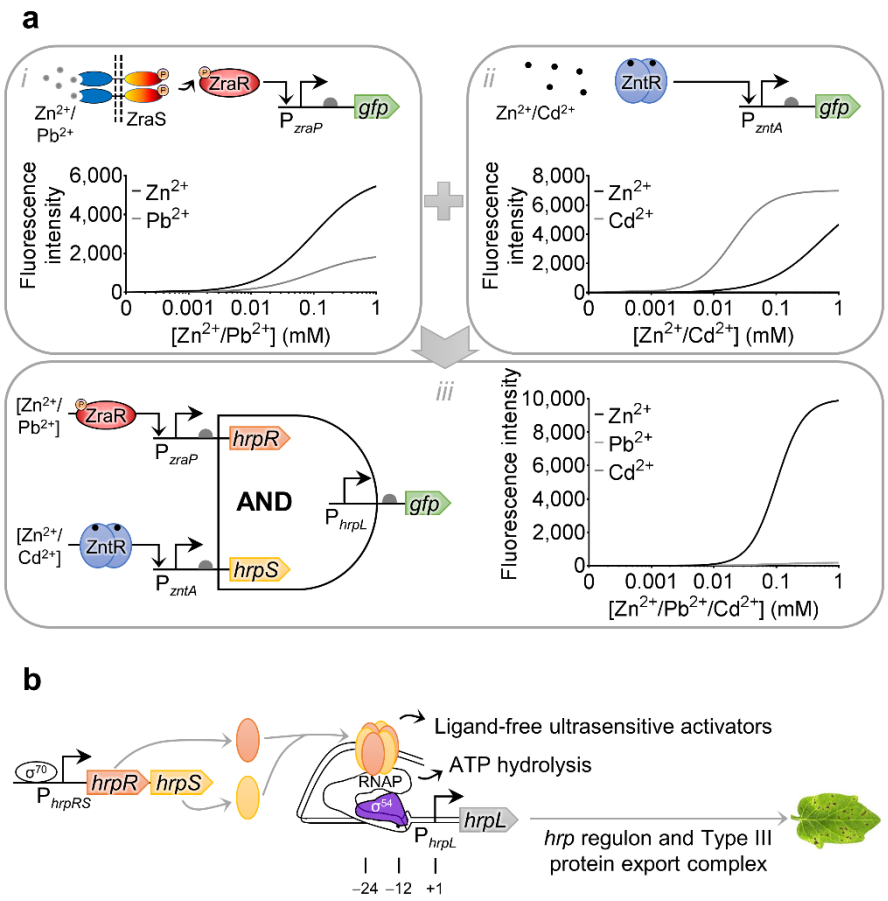

a
i
Zn2+/
Pb2+
ZraS
ZraR
PzraP
gfp
Fluorescence intensity
6,000
4,000
2,000
0
Zn2+
Pb2+
0
0.001
0.01
0.1
1
[Zn2+/Pb2+] (mM)
ii
Zn2+/Cd2+
ZntR
PzntA
gfp
8,000
6,000
4,000
2,000
0
Zn2+
Cd2+
[Zn2+/Cd2+] (mM)
iii
[Zn2+/
Pb2+]
ZraR
PzraP
hrpR
AND
PhrpL
gfp
[Zn2+/
Cd2+]
ZntR
PzntA
hrpS
10,000
8,000
6,000
4,000
2,000
0
Zn2+
Pb2+
Cd2+
[Zn2+/Pb2+/Cd2+] (mM)
b
σ70
PhrpRS
hrpR
hrpS
RNAP
σ54
PhrpL
hrpL
Ligand-free ultrasensitive activators
ATP hydrolysis
hrp regulon and Type III
protein export complex
−24
−12
+1

Figure 1.4

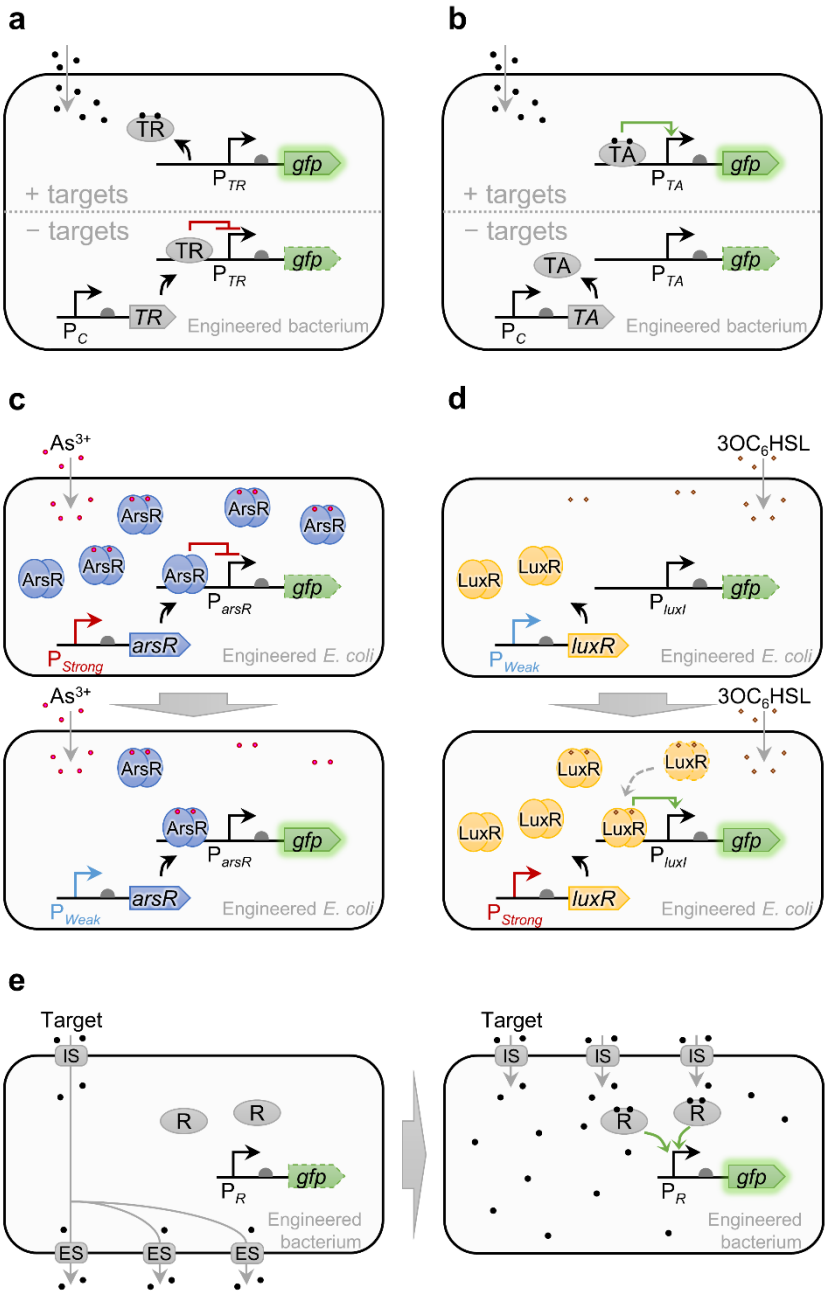

a
TR
gfp
P TR
+ targets
− targets
TR
P TR
gfp
P C
TR
Engineered bacterium
b
TA
gfp
P TA
+ targets
− targets
TA
P TA
gfp
P C
TA
Engineered bacterium
c
As3+
ArsR
ArsR
ArsR
ArsR
ArsR
ArsR
gfp
P arsR
P Strong
arsR
Engineered E. coli
As3+
ArsR
ArsR
gfp
P arsR
P Weak
arsR
Engineered E. coli
d
3OC6HSL
LuxR
LuxR
gfp
P luxI
P Weak
luxR
Engineered E. coli
3OC6HSL
LuxR
LuxR
LuxR
LuxR
LuxR
gfp
P luxI
P Strong
luxR
Engineered E. coli
e
Target
IS
R
R
gfp
P R
Engineered bacterium
ES
ES
ES
Target
IS
IS
IS
R
R
gfp
P R
Engineered bacterium

Figure 1.5

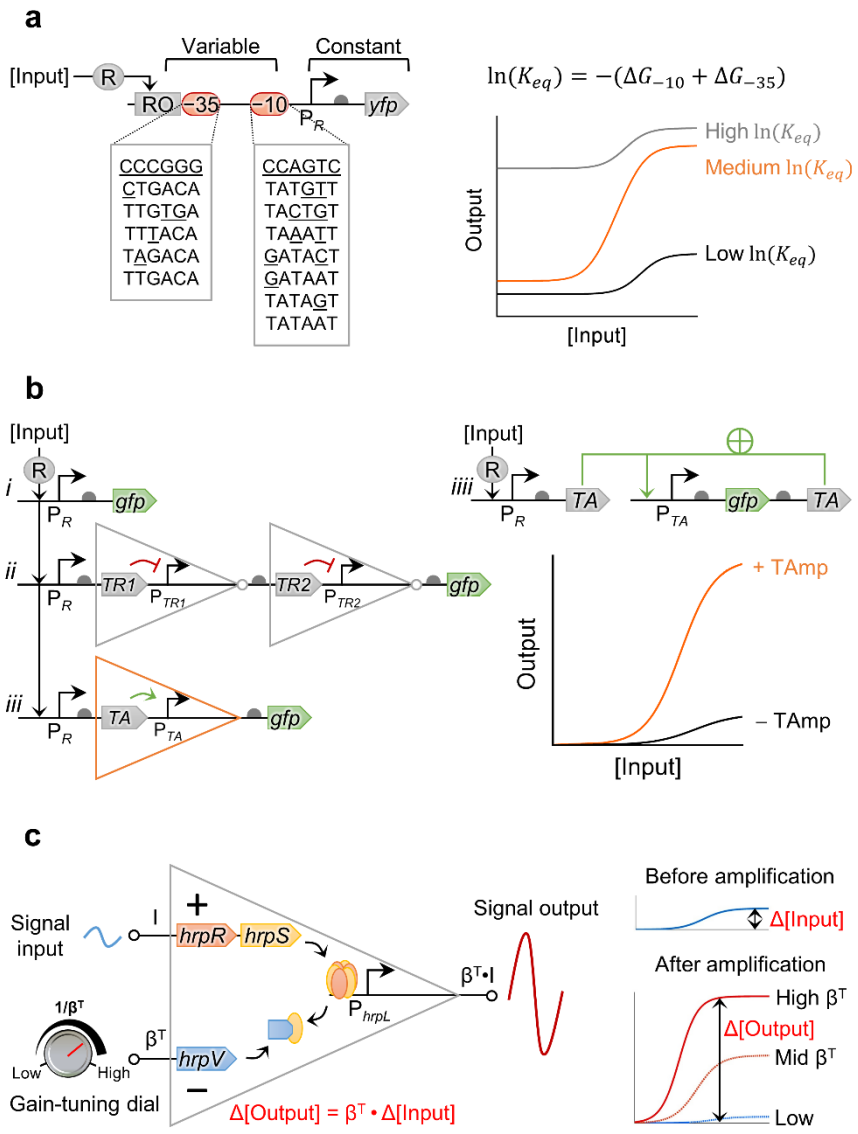

a
[Input]
R
Variable
Constant
RO
-35
-10
P_R
yfp
CCCGGG
CTGACA
TTGTGA
TTTACA
TAGACA
TTGACA
CCAGTC
TATGTT
TACTGT
TAAATT
GATACT
GATAAT
TATAGT
TATAAT
$\ln(K_{eq}) = -(\Delta G_{-10} + \Delta G_{-35})$
High $\ln(K_{eq})$
Medium $\ln(K_{eq})$
Low $\ln(K_{eq})$
Output
[Input]
b
[Input]
i
ii
iii
iiii
gfp
TR1
TR2
TA
P_R
P_{TR1}
P_{TR2}
P_{TA}
+ TAmp
– TAmp
Output
[Input]
c
Signal input
I
hrpR
hrpS
P_{hrpL}
$\beta^T \cdot I$
Signal output
$1/\beta^T$
Low
High
Gain-tuning dial
β^T
hrpV
$\Delta[Output] = \beta^T \cdot \Delta[Input]$
Before amplification
Δ[Input]
After amplification
High β^T
Δ[Output]
Mid β^T
Low

Figure 1.6

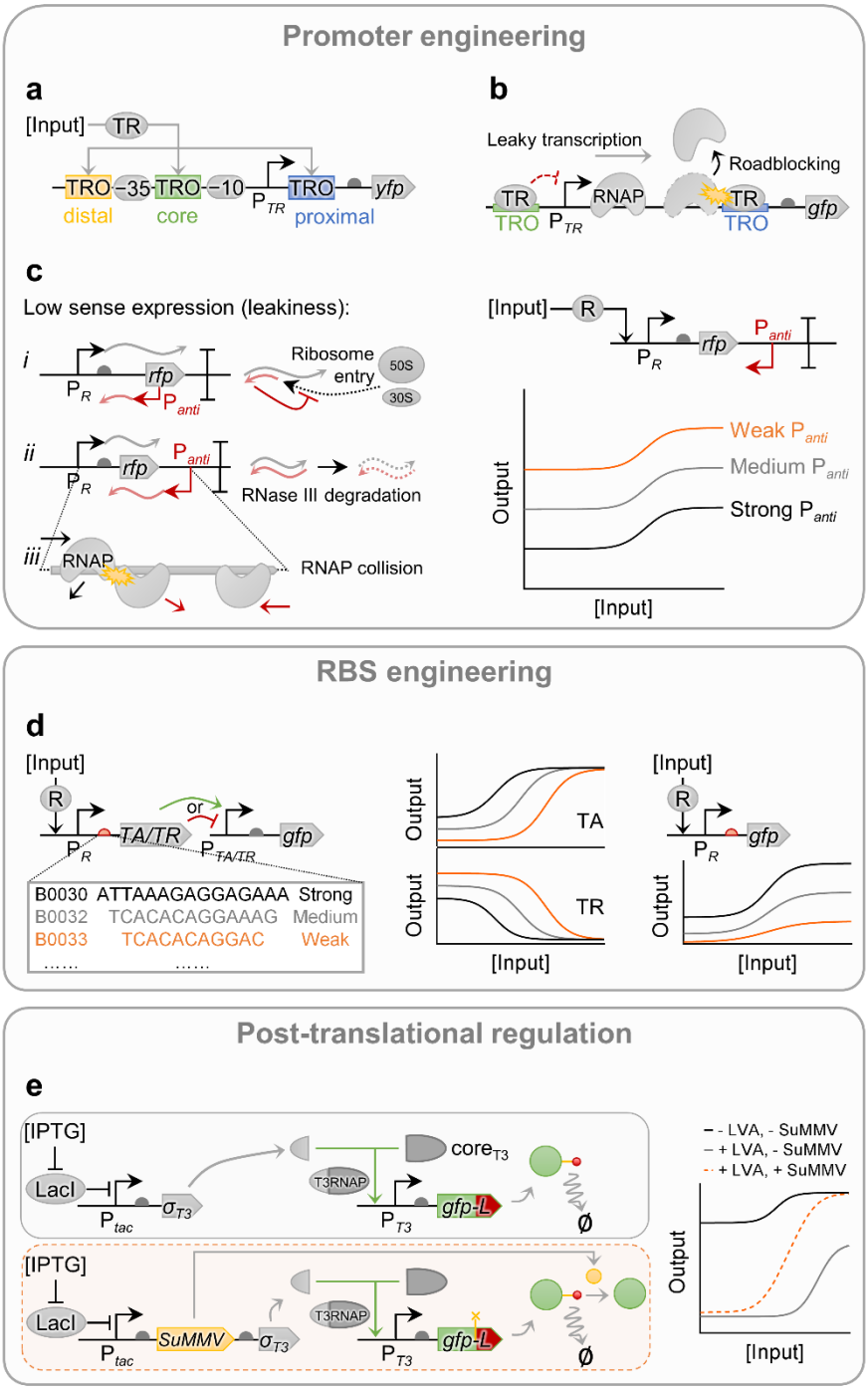

Promoter engineering
a
[Input]
TR
TRO
-35-
TRO
-10
P TR
TRO
yfp
distal
core
proximal
b
Leaky transcription
Roadblocking
TR
RNAP
TR
gfp
TRO
P TR
TRO
c
Low sense expression (leakiness):
i
rfp
P R
P anti
Ribosome entry
50S
30S
ii
P anti
rfp
P R
RNase III degradation
iii
RNAP
RNAP collision
[Input]
R
P R
rfp
P anti
Output
Weak P anti
Medium P anti
Strong P anti
[Input]
RBS engineering
d
[Input]
R
or
P R
TA/TR
P TA/TR
gfp
B0030 ATTAAAGAGGAGAAA Strong
B0032 TCACACAGGAAAG Medium
B0033 TCACACAGGAC Weak
…… ……
Output
TA
TR
[Input]
[Input]
R
P R
gfp
Output
[Input]
Post-translational regulation
e
[IPTG]
LacI
P tac
σT3
core T3
T3RNAP
P T3
gfp-L
Ø
[IPTG]
LacI
P tac
SuMMV
σT3
T3RNAP
P T3
gfp-L
Ø
- LVA, - SuMMV
+ LVA, - SuMMV
+ LVA, + SuMMV
Output
[Input]

Figure 1.7

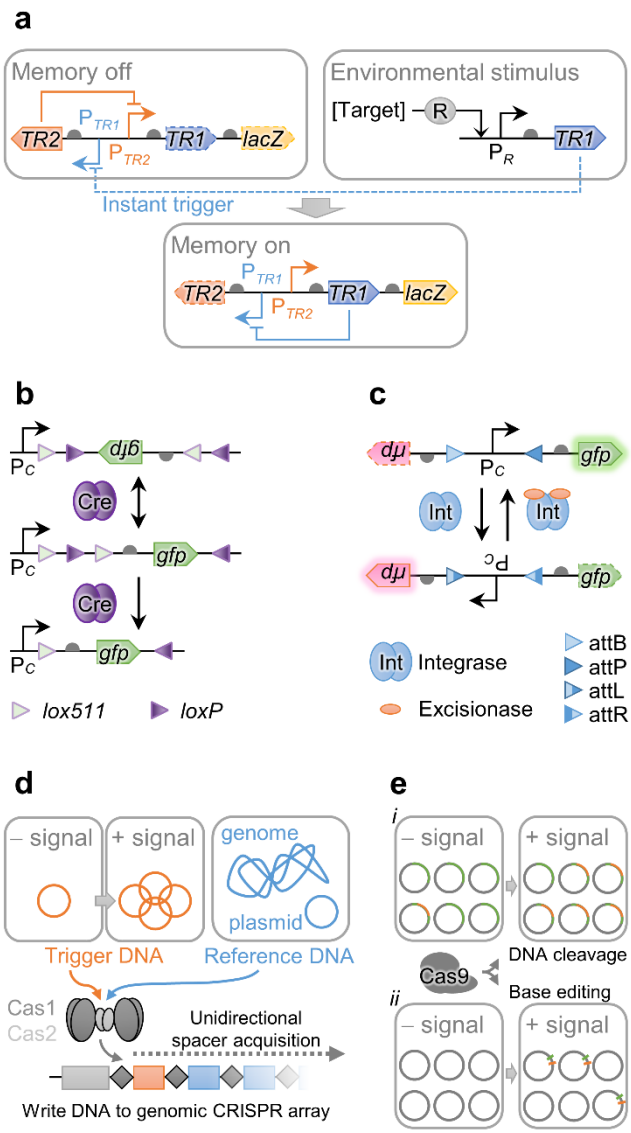
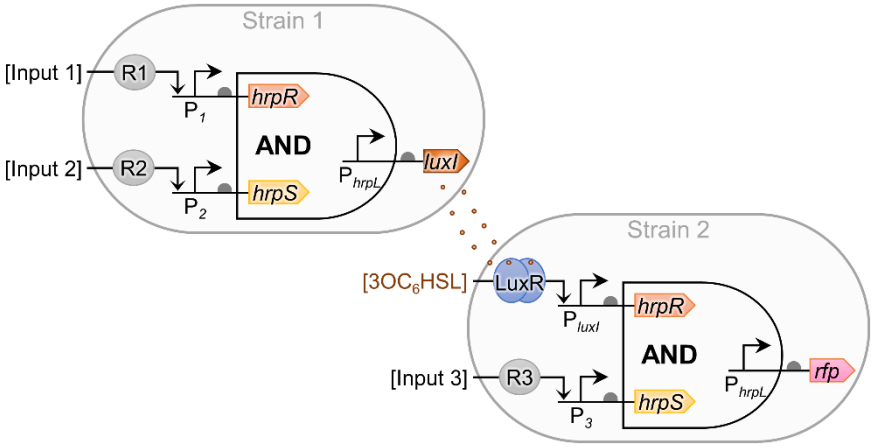

a
Memory off
Environmental stimulus
[Target]
R
TR2
TR1
lacZ
Instant trigger
Memory on
b
Cre
gfp
lox511
loxP
c
gfp
Int
Integrase
Excisionase
attB
attP
attL
attR
d
– signal
+ signal
genome
plasmid
Trigger DNA
Reference DNA
Cas1
Cas2
Unidirectional
spacer acquisition
Write DNA to genomic CRISPR array
e
i
ii
DNA cleavage
Cas9
Base editing

Figure 1.8


Strain 1
[Input 1]
R1
P1
hrpR
AND
[Input 2]
R2
P2
hrpS
PhrpL
luxI
Strain 2
[3OC6HSL]
LuxR
PluxI
hrpR
AND
[Input 3]
R3
P3
hrpS
PhrpL
rfp