Chapter 23

Synthetic Cell-Based Sensors with Programmed Selectivity and Sensitivity

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Abstract

Bacteria live in an ever changing environment and, to adapt their physiology, they have to sense the changes. Our current understanding of the mechanisms and elements involved in the detection and processing of these environmental signals grant us access to an array of genetic components able to process such information. As engineers can use different electronic components to build a circuit, we can rewire the cellular components to create digital logic and analogue gene circuits that will program cell behaviour in a designed manner in response to a specific stimulus. Here we present the methods and protocols for designing and implementing synthetic cell-based biosensors that use engineered genetic logic and analogue amplifying circuits to significantly increase selectivity and sensitivity, for example, for heavy metal ions in an aqueous environment. The approach is modular and can be readily applied to improving the sensing limit and performance of a range of microbial cell-based sensors to meet their real world detection requirement.

Key words Cell-based biosensor, Synthetic gene circuit, Selectivity, Sensitivity, Heavy metals

1 Introduction

To adapt their physiology to the changing environment, bacteria have developed a plethora of sensors to probe their milieu. The different signals gathered through these sensors are processed and integrated by complex genetic networks involving the similar type of logical operations we can find in a computational circuit. Hence, similarly, such biological components (genetic sensors and circuits) could be rewired to generate modular and programmable biosensors [1].

A typical biosensor consists of three cascaded modules: an input sensor, a regulatory circuit, and an output actuator (Fig. 1). A huge variety of genetic sensors have been developed through the evolution and virtually all stimuli could be sensed by an organism or another. Beside the large range of light-based outputs that have been developed during the last few decades, alternative outputs like

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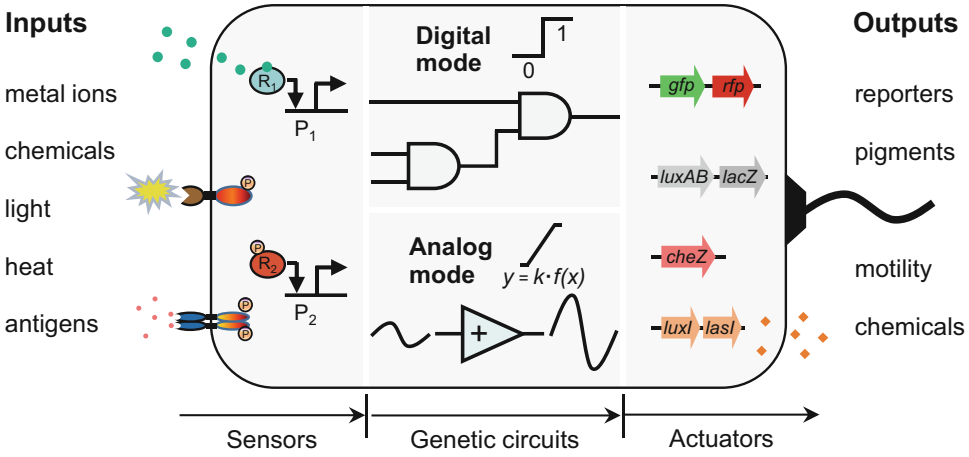

Fig. 1 Architecture of a modular synthetic cell-based biosensor. The cellular sensor comprises three interconnected and exchangeable modules, i.e., the input sensors, the internal genetic information processing circuits and the output actuators. The cells are engineered using various natural or synthetic sensors such as sensor kinases or intracellular receptor proteins to detect environmental signals and genetic circuits such as analog transcriptional amplifiers or digital-like AND logic gates to modulate and integrate these multiple input signals. The programmed cells can then initiate customized responses by activating different output genes according to the logic decision transmitted upstream. Adapted with permission from [4]

the production of specific chemicals or the change of motility or morphology may also be generated. One big challenge in the development of advanced cell-based biosensors is the design of embedded genetic information processing circuits but great progress has been made in the last decade and the toolbox for engineering gene circuits continuously expands and more complex circuits has become possible [2, 3]. Moreover, as we will exemplify later, by carefully designing the embedded genetic circuit, we can engineer a biosensor with sophisticated function.

By taking advantage of the ability of specialized bacteria to sense particular compounds in their environmental niche, a range of single input-sensors have been constructed to detect pollutants like arsenic [4], xylene, or even explosives [5]. Multi-input biosensors have also been constructed and found their utilities in the identification of complex conditions such as the precise detection of a cancer disease [6]. For instance, we may connect the inputs of a multi-input AND logic gate to pathogenicity-related cellular signals and couple the device output to a therapeutics such as a suicide gene to achieve specific in vivo cell targeting and killing.

Here we describe the strategies and methods for designing and characterizing highly sensitive and selective synthetic cell-based sensors that use engineered digital-like genetic logic gates or analogue transcriptional amplifiers to process the transduced sensory signals. Cellular sensors containing relative small circuits have been chosen below to illustrate the design method on purpose.

However, readers interested in advanced sensors with more complex gene circuits such as a 3-input AND gate [4, 7] or a tunable transcriptional amplifier [8] may refer to our previous published works [4, 7–9]. In the following examples, we used a fluorescent reporter as the sensor output but it can be readily swapped to a more application-oriented output if needed.

1.1 Design and Engineering of Synthetic Cell-Based Sensors

In general, synthetic cell-based sensors could be built in two different manners [10, 11]. In the first case, we can use the host endogenous genetic pathways and rewire the final output of a relevant pathway to a desired reporter gene. Because the whole pathway comes from the same organism as the one where the cellular sensors will operate, it is unlikely that these sensors will not be functional. However, the cell native signalling systems have evolved to respond to their cognate ligands with a particular sensitivity, selectivity and dynamic ranges, and are therefore not optimized for direct reuse in environmental biosensing. Among the issues we may encounter, the sensor high basal activities and low output dynamic range may be addressed by tuning the translational rate of the output reporter gene or its protein lifetime e.g., using a degradation tag. Another potential issue in using inherent signaling sensor is the lack of sufficient sensitivity. As we have shown previously, this may be addressed by tuning the concentration of the cognate sensor receptor protein in the cytoplasm [12]. Since the different signalling components of the cellular sensors are inherent to the host, the sensor circuits may be crosslinked to other components present in the same organism and thus could be more prone to variation in response to change in the environment or the growth condition.

In the second case when the host chassis could not be able per se to sense the signal we intend to detect, we may resort to importing heterogeneous signalling pathway and sensors from other specialized bacterial species. Indeed, many microorganisms have evolved to use different substrates present in their native environment. However, to avoid unnecessary energy spending, most of these modules will be only induced in presence of these substrates. Since the different imported components are not derived from the host, their compatibility with the endogenous machineries and their functionalities are not guaranteed in the new host chassis. Each part needs to be characterized and optimized separately. On the other hand, as the genetic circuit is not an integrative part of the host, the potential interference between the synthetic circuit and the endogenous pathways present in the host should be low or negligible compared to an endogenous circuit.

As shown in Fig. 2, the simplest synthetic cell-based sensors may only consist of an output reporter expressed from a signalling promoter or with the further incorporation of a receptor gene. Here we present exemplar results for four different single-input sensors that have been constructed in *E. coli* TOP10 for sensing

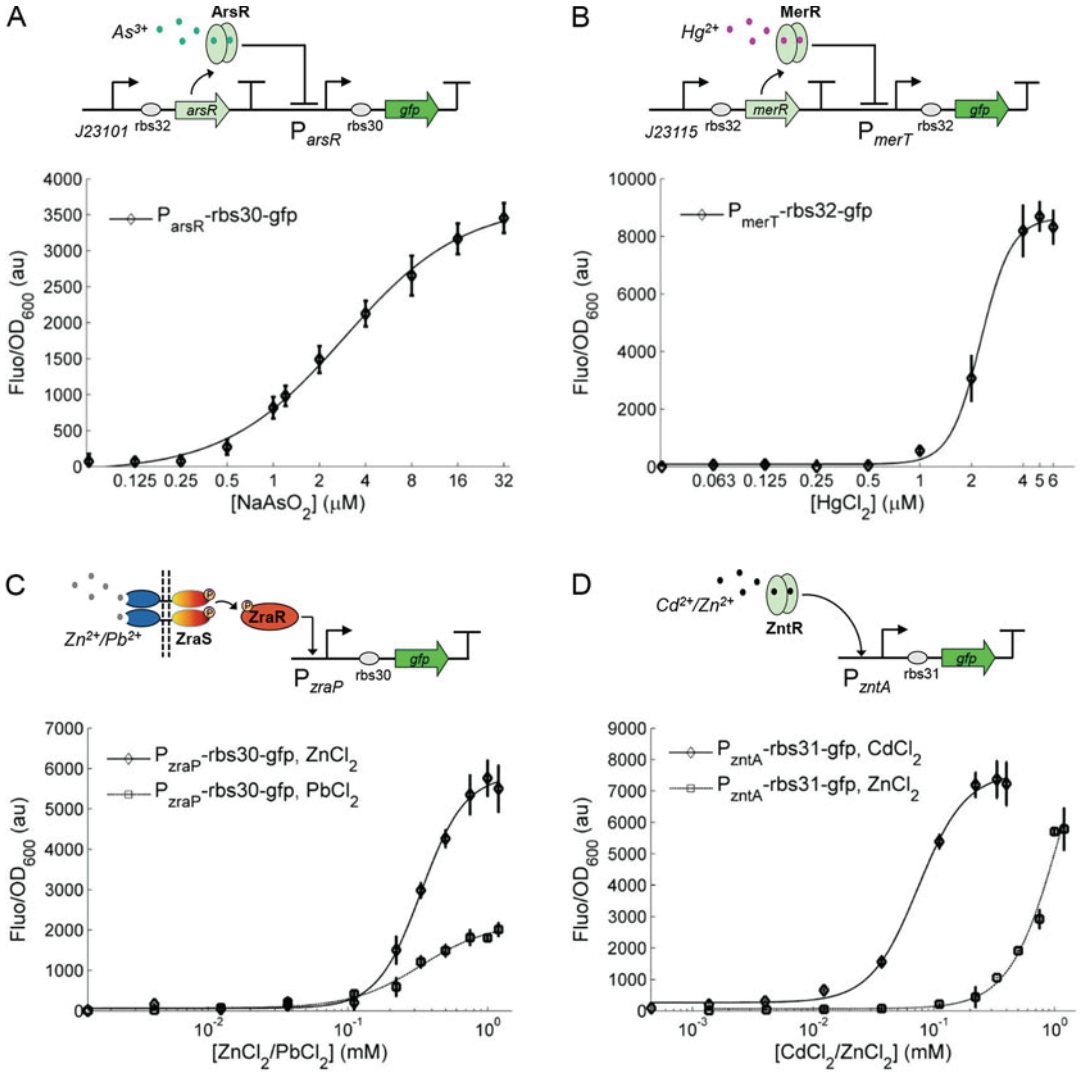

Fig. 2 Design and characterization of a set of single-input cellular biosensors. (**a**) The arsenic sensor were characterized under various arsenite concentrations (0, 0.125, 0.25, 0.5, 1.0, 1.2, 2.0, 4.0, 8.0, 16.0, 32 μM $NaAsO_2$). (**b**) The mercury sensor was characterized under various $HgCl_2$ levels (0, 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 5.0, 6.0 μM). (**c**) The zinc or lead sensor was characterized under various $ZnCl_2/PbCl_2$ levels (0, 0.0041, 0.0123, 0.037, 0.111, 0.222, 0.333, 0.5, 0.75, 1, 1.2 mM). (**d**) The cadmium or zinc sensor was characterized under various levels of $CdCl_2$ (0, 0.00137, 0.0041, 0.0123, 0.037, 0.111, 0.222, 0.333, 0.4 mM) or $ZnCl_2$ as the same as indicated in (**c**). *E. coli* TOP10, LB, 37 °C, 6 h post induction. Error bars, s.d. ($n = 4$). *a.u.* arbitrary units

heavy metal ions in an aqueous environment [3]. The first one is the arsenic sensor (Fig. 2a) derived from the pathway that naturally confers resistance to high concentration of arsenic in *Escherichia coli*. The second sensor is a mercury sensor (Fig. 2b) built using the mercury resistance module present on the R100 plasmid from *Shigella flexneri*. These two examples represent the design of an

endogenous sensor and the design of a heterogeneous sensor respectively. The receptor gene *arsR* is expressed to allow tuning the sensitivity and dynamic range of the arsenic sensor [12] while the mercuric receptor gene *merR* is necessary for the mercury sensor. The last two single-input sensors are two zinc responsive sensors. The construction of these genetic sensors relies on the endogenous signaling systems present in the cell: the two component system ZraSR and the one component ZntR sensor. Under these circumstances, only the cognate regulatory promoter (P_{zraP} or P_{zntA}) is used and coupled to an output reporter (*gfp*). As shown in Fig. 2c, d, the two sensors respond to the presence of not only zinc but also other metals (i.e., lead or cadmium). Such lack of specificity can be addressed by the use of a genetic AND gate as described below.

1.2 Engineered Genetic AND Logic Gates Enable Highly Selective Biosensors

A multi-input AND gate is characterized by the feature that its output is ON only when all the inputs are ON at the same time. Such logic gate can be very useful to increase the selectivity of a cell-based biosensor [4]. As illustrated in Fig. 3a, with the incorporation of more individual nonspecific sensors, the intersection between these sensors will become narrower. Thus increased sensing selectivity can be obtained using an AND logic gate to couple these sensors. Such genetic logic gates may be generated using split or heteromeric activators or specific promoters requiring two or more activator proteins to be active such as the 2- or 3-hybrid system used for protein-protein interaction assay.

Previously we have engineered a modular and orthogonal genetic AND gate in *E. coli* [9]. The modular two-input AND gate comprises two heterologous genes, *hrpR* and *hrpS*, and one σ^{54}-dependent output promoter, P_{hrpL}, from the *hrp* (hypersensitive response and pathogenecity) regulatory system of the plant pathogen *P. syringae* (Fig. 3b). The *hrpR* and *hrpS* encode two regulatory enhancer binding proteins that act synergistically by forming a heteromeric protein complex to co-activate the tightly regulated P_{hrpL} promoter. Both the inputs and output of the AND gate were designed to be promoters to facilitate their connection to different upstream and downstream transcriptional modules. Due to this modularity, the inputs can be rewired to different input sensors and the output can be used to drive various cellular responses.

To design a logic AND-gated cellular biosensor, we connected two transcriptional inputs of the single input sensors to the modular genetic AND gate with *gfp* as the output readout. Figure 3c shows the design and characterization of a double-input AND gated biosensor that can distinguish between Zn^{2+} and Pb^{2+} or Cd^{2+} [4]. The sensor circuit employs P_{zraP} (responsive to Zn^{2+} and Pb^{2+}) and P_{zntA} (responsive to Cd^{2+} and Zn^{2+}) as the sensory inputs to the AND gate and the *gfp* as the output reporter. Because

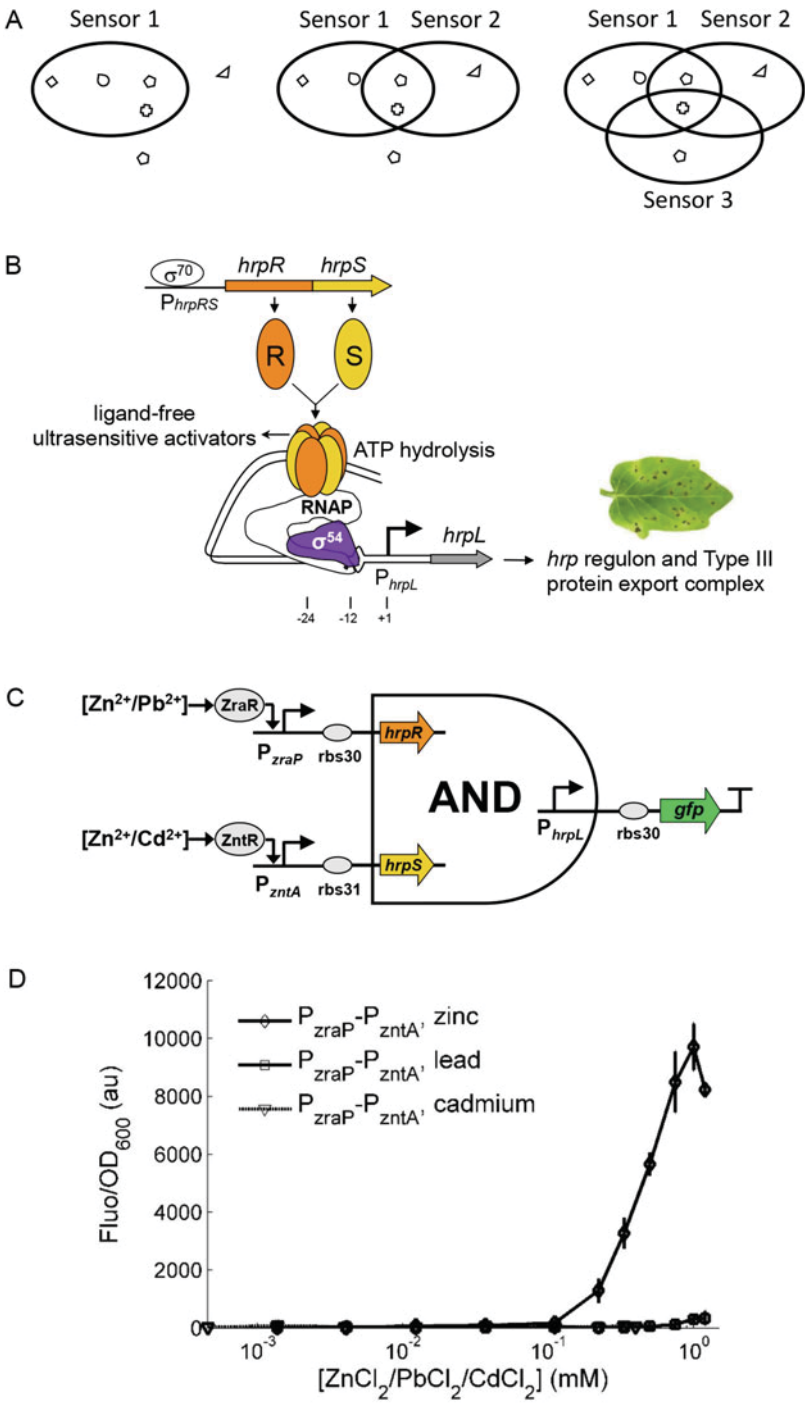

Fig. 3 Design and characterization of a two-input AND logic gated cellular biosensor. (**a**) Venn diagram illustrating a multi-input AND logic gate. The number of substrates (represented by different shapes) recognizable by the whole set of sensors decreases when the number of sensors increases. (**b**) Schematic showing the HrpR/HrpS hetero regulation motif in the *hrp* system of *P. syringae*. The *hrp* (hypersensitive response and pathogenicity) system in *Pseudomonas syring*ae pv. *tomato* DC3000 determines its ability to cause disease in the plant host. The σ^{54}-dependent *hrpL* promoter is the primary regulator of this system and is activated by the hetero HrpR and HprS bacterial enhancer-binding proteins. (**c**) Design of the AND-gated sensor with increased selectivity to zinc ions. (**d**) The AND logic gated zinc sensor was measured using various levels of $ZnCl_2$ or $PbCl_2$ (0, 0.0041, 0.0123, 0.037, 0.111, 0.222, 0.333, 0.5, 0.75, 1, 1.2 mM) or $CdCl_2$ (0, 0.00137, 0.0041, 0.0123, 0.037, 0.111, 0.222, 0.333, 0.4 mM). *E. coli* TOP10, LB, 37 °C, 6 h post induction. Error bars, s.d. ($n = 4$)

both of the two sensory inputs have to be activated in order to generate the fluorescent output, this AND logic gated sensor is only responsive to Zn^{2+} but not Pb^{2+} or Cd^{2+} in conditions containing only a single contaminant of these metals. The dose response curves of the sensor to Zn^{2+}, Pb^{2+} or Cd^{2+} confirm that the sensor is not only highly selective to zinc but also has an increased absolute fluorescent output, i.e., signal-to-noise ratio (Fig. 3d).

1.3 Engineered Transcriptional Amplifiers Enable Highly Sensitive Biosensors

When the sensitivity or output amplitude of a genetic sensor is low, genetic amplifiers can be used to scale up the transduced transcriptional signal from the input sensor. By doing this, the sensitivity and output dynamic range of these sensors can be significantly increased to meet their real world detection limits. For example, the WHO safe limit for arsenic in drinking water is 10 ppb, i.e., 0.133 μM [12].

Previously we have engineered a set of modular genetic amplifiers in *E. coli* capable of amplifying a transcriptional signal with wide tuneable gain control in cascaded gene networks [8]. The fixed-gain amplifier was built by expressing in an operon the cooperative activator proteins, HrpR and HrpS, whose high order functional forms synergistically activate the downstream tightly controlled σ^{54}-dependent P_{hrpL} promoter, thus assisting amplification of the transcriptional input signal (Fig. 4a). To obtain different amplification gains, two configurations of the amplifier ($Amp32^C$ and $Amp30^C$) were designed using two RBS (ribosome binding site) sequences of distinct translational strengths [9] in front of the *hrpS* gene. $Amp30^C$, with a strong RBS sequence (rbs30), should produce a higher signal gain than $Amp32^C$ with a weaker RBS sequence (rbs32).

To verify their amplification capability, we connected the arsenic responsive transcriptional sensor to the input of the fixed gain amplifier with *gfp* as the output. By itself, the arsenic sensor generated a transcriptional output with limited dynamic range and sensitivity in response to varying levels of arsenite (Fig. 2a). When the transduced transcriptional input from the arsenic sensor was connected to our amplifier, the resulting output signal amplitude and dynamic range increased significantly, as well as the response sensitivity to the inducer (Fig. 4b) for both devices ($Amp32^C$ and $Amp30^C$).

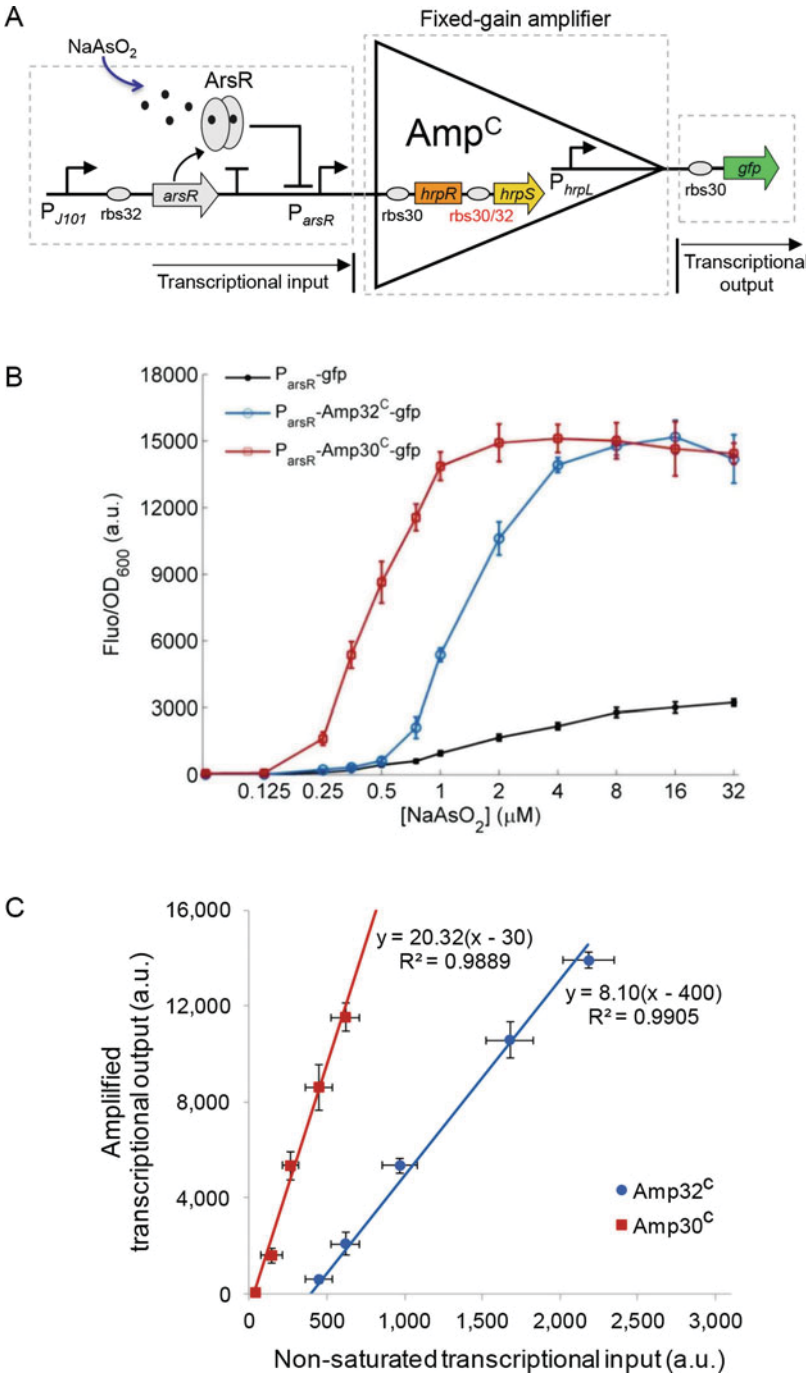

Fig. 4 Engineering and characterization of the arsenic sensor enhanced by transcriptional amplifiers Amp32^C and Amp30^C. (**a**) The transcriptional amplifier comprises two terminals corresponding to the signal input and signal output. Here two amplifiers with different gains, Amp32^C and Amp30^C, are designed by using two different RBS sequences ahead of the *hrpS* gene. An arsenic responsive sensor is the input signal and *gfp* the output. (**b**) Steady state responses of the arsenic sensor without amplification and with amplification by Amp32^C and Amp30^C. The cells are induced by 12 varying concentrations of arsenite (0, 0.125, 0.25, 0.35, 0.5, 0.75, 1.0, 2.0,

2 Materials

1. 96-well microplate (Greiner Bio-One, chimney black, flat clear bottom, Catalog No.655096). To prevent fluorescence spill from neighbouring wells, the wall of the wells should not be transparent and black wall gives better result than the white one. The presence of a lid will reduce the evaporation and also prevent potential contamination of the sample during the growth. The lid (Greiner Bio-One, Catalog No. L3911-100EA) should be transparent to the different lights used during the measurement.
2. Plate reader such as the BMG Labtech FLUOstar fluorometer for repeated absorbance (OD at 600 nm) and green fluorescence (485 nm for excitation, 520 ± 10 nm for emission, Gain = 1200, bottom reading) or red fluorescence (584 nm for excitation, 620 ± 10 nm for emission, Gain = 2000, bottom reading) readings (20 min/cycle) (*see* **Note 1**).
3. Plate shaker such as the BMG Labtech THERMOstar. While this is not mandatory, it allows culturing and characterization up to four 96-well plates at the same time by incubating the plates at appropriate temperature with continuous shaking.
4. A spectrophotometer and associated 1 ml cuvettes.
5. A repetitive pipette (Gilson REPETMAN Electronic Pipette 0.1–50 ml, F164503) for fast loading cell culture into 96-well plates, and associated repet tips (e.g., F164550—5 ml syringe tips; F164560—12.5 ml syringe tips).
6. A multichannel pipette (Gilson PIPETMAN Concept Multi C8x10 1–10 μl, F31032) for fast loading sample inducers into 96-well plates.
7. A range of dilutions of culture inducers. In this study, we use arsenic in its arsenite form (Catalog No. 35000-1L-R, Sigma-Aldrich, St Louis, MO) as inducer. To characterize the sensor cell response to different arsenite concentrations, we use a serial dilution in deionized water, for example, 0, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 μM.
8. LB (Luria–Bertani Broth) media (10 g/L peptone, 5 g/L NaCl, 5 g/L yeast extract) for cell culture.
9. Relevant antibiotics. The antibiotic concentrations used in the final cell culture are 50 μg/ml for kanamycin and 50 μg/ml for

Fig. 4 (continued) 4.0, 8.0, 16, 32 μM $NaAsO_2$). (**c**) The scatter plot shows the linear relationships between the non-saturated transcriptional inputs (the signal inputs that do not lead to maximum output level of the device) and the amplified outputs of $Amp32^C$ and $Amp30^C$ by fitting to a linear function. *E. coli* TOP10, LB, 37 °C, 5 h post induction. Error bars, s.d. ($n = 3$)

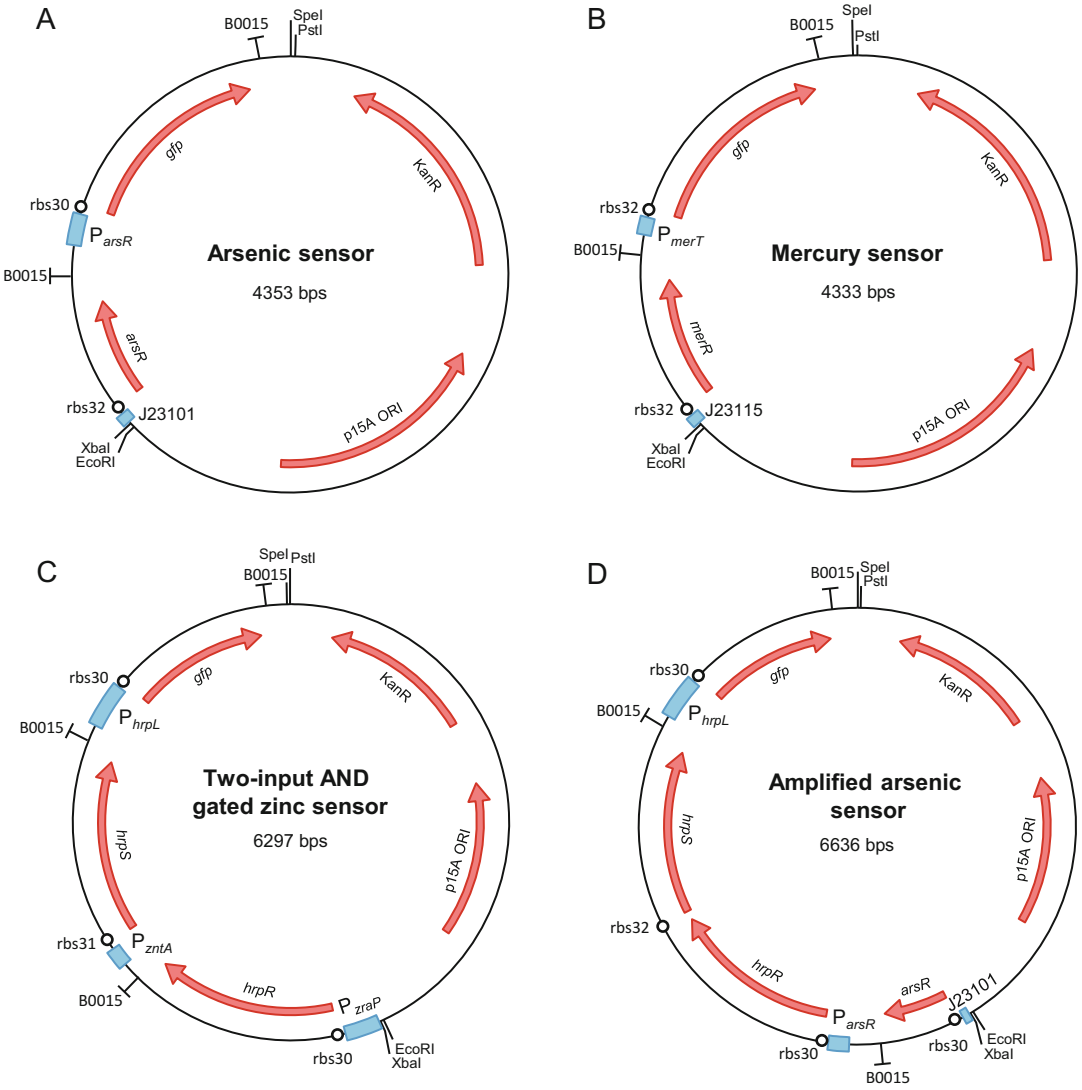

Fig. 5 Plasmid maps showing some representative circuit constructs used in this chapter. (**a**) The plasmid for encoding the single input arsenic sensor with *gfp* as the output (Fig. 2a). (**b**) The plasmid for encoding the single input mercury sensor with *gfp* as the output (Fig. 2b). (**c**) The plasmid for encoding the AND gated zinc sensor with *gfp* as the output (Fig. 3c, d). (**d**) The plasmid for encoding the fixed-gain amplifier $Amp32^{C}$ with the arsenic sensor as input and *gfp* as the output (Fig. 4)

ampicillin. The stock solution is generally prepared 1000× concentrated in deionized water.

10. Cell strain containing the empty plasmids without the reporter gene (negative control).
11. Cell strain containing the circuit plasmids to characterize. Some representative sensor plasmid constructs used in this work are shown in Fig. 5.

3 Methods

3.1 Design and Constructing the Sensor Genetic Constructs

1. The sensor construct design is generally based on various sensory components that have been reported in different bacterial species in the literature. Hence, one can search relevant genomic database to extract the useful related genetic sequences, design the sensor accordingly (*see* **Note 2**) and then synthesize them de novo by a commercial gene synthesis company before cloning them into a customized plasmid (Fig. 5a, b) [4].
2. Alternatively, one can request the reported sensor elements/bacterium from the authors of the relevant literature and then apply standard molecular biology methods (e.g., PCR, restriction enzyme digestion and ligation, sequencing) to clone them into a customized expression plasmid. As an example, for the transcriptional amplifier sensor circuit described in Fig. 5d, *hrpR*, *hrpS*, P_{hrpL} and the arsenic responsive sensor construct *arsR*-P_{arsR} were synthesized by gene synthesis company GeneArt following the BioBrick standard by eliminating the four restriction sites (EcoRI, XbaI, SpeI, and PstI) for the BioBrick standard via synonymous codon exchange and flanking with prefix and suffix sequences containing the appropriate restriction sites and ribosome binding site (RBS) sequences. The double terminator BBa_B0015 from the Registry of Standard Biological Parts (http://partsregistry.org) was used to terminate gene transcription. The GFP (Green Fluorescent Protein, *gfpmut3b*, BBa_E0840) reporter was from the Registry of Standard Biological Parts (http://partsregistry.org). The various RBS sequences (rbs30 and rbs32) for each gene construct were introduced with PCR primers if necessary (amplification utilized high-fidelity Phusion DNA polymerase from NEB and an Eppendorf Mastercycler gradient thermal cycler). The sensor circuit construct was assembled following the three-way BioBrick DNA assembly method into plasmid pSB3K3 (p15A ori, Kan^r) and verified by DNA sequencing prior to its use [8]. For brevity, we will not elaborate on the design and cloning procedure of other sensor plasmid constructs here, but interested readers can refer to our previous publications for details [4, 7, 8, 12].
3. To obtain the final sensor cell strain, the sensor plasmid constructs built above can be transformed directly into a target cell strain (e.g., *E. coli* TOP10) following either a chemical or electroporation transformation protocol. At the same time, a negative control strain will also need to be constructed using the corresponding reporter-free plasmids.

3.2 Preparing Sample Inducers at Different Dilutions/ Concentrations

1. For induction of cell culture samples, we generally add 5 μl inducer to 195 μl cell culture. So the different stock solutions of inducers that will be used should be 40× concentrated of their final concentration in the media.
2. For example, to obtain the 16 μM target arsenite induction, add 16 μl of 50 mM $NaAsO_2$ (stock solution) to 1234 μl of deionized water in a microtube to obtain 1.25 ml of a 640 μM arsenite solution (16 μM × 40).
3. Add 500 μl of the previous solution (16 μM) to 500 μl of deionized water to obtain 1 ml of a 320 μM arsenite solution (8 μM × 40).
4. Add 500 μl of the previous solution (8 μM) to 500 μl of deionized water to obtain 1 ml of a 160 μM arsenite solution (4 μM × 40).
5. Repeat the above dilution process until all the inducer concentrations needed are obtained.
6. Keep the inducers at 4 °C if used in the next few days; otherwise, keep them at −20 °C (to prevent any degradation or contamination).

3.3 Culturing and Assaying Sensor Cell Samples

1. Day −1: Re-streak the different sensor cell strains needed (negative control and the strain(s) to characterize) on fresh LB agar plates containing the appropriate antibiotic(s) (*see* **Note 3**).
2. Day 0: From a single colony, inoculate 5 ml media containing the appropriate antibiotic in a 30 ml sterile Falcon tube and incubate it overnight at 37 °C with continuous shaking (200 rpm). As this stage, it would be preferable to prepare several biological repeats for the overnight culture of each strain to characterize.
3. Day 1: Measure the optical density (OD_{600}) of the overnight culture.
4. Dilute the overnight culture to an $OD_{600} = 0.025$ into 4 ml of fresh medium containing the appropriate antibiotic (*see* **Note 4**).
5. Dispense 195 μl of the appropriate culture in each well, if appropriate, using a repetitive pipette. Figure 6 shows a typical layout including negative control (reporter-free cell culture) and blank (medium only wells).
6. Load 5 μl of the inducer solution prepared the day before into the wells containing corresponding samples, if appropriate, using a multichannel pipette to reduce operation time. Note that the inducer solution needs to be mixed thoroughly by vortexing prior to use.
7. Incubate the plate at 37 °C with shaking (200 rpm, linear mode) in the plate reader. Setup the plate reader for repeated

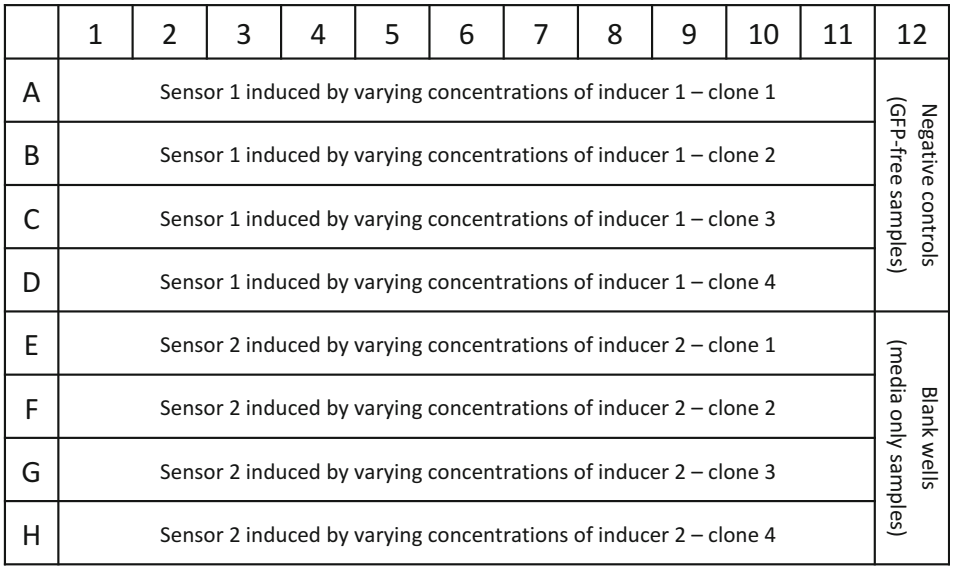

Fig. 6 Exemplar experimental setup for characterizing cellular sensor response. An exemplar 96-well plate layout showing two different cellular sensors to be characterized in response to varying ligand inducers with four biological repeats each

fluorescence and optical density reading each well every 20 min. Alternatively, a plate shaker can be used when more than one plate are used at the same time. In this case, a snapshot-reading can be performed 4–6 h post induction (depending on the strain/media used and the circuit tested).

3.4 Analyzing the Assay Results

1. Since dynamic monitoring data were obtained for a cellular sensor in response to varying concentrations of a target ligand, we generally select the 5 or 6 h data post initial induction for subsequent analysis when the cell growth are at the transition from exponential to stationary phases. The first step in the analysis of the assay results is to subtract the background from both the optical density and the fluorescence readings. This can be done by subtracting the value from the well containing only the media (blank wells).
2. The second step is to normalize the measurement result. It is obvious that the more cells are present in the culture the higher the fluorescent measurement will be. To normalize fluorescence reading, the blank-corrected fluorescence will be divided by the blank corrected optical density (*see* **Note 5**).
3. Finally, since the host cells have auto-fluorescent background, we need to subtract this value from the normalized ratio we obtained. To do so, we simply subtract the mean ratio of the negative control samples from the ratios of the cognate sensor culture samples.

4. The obtained sensor outputs can be plotted against different concentrations of the cognate sensor inducers used to obtain the sensor dosage response curve.
5. Curve fitting will then be applied to the above obtained dosage response curve to obtain the standard measurement curve that may be used for the assay of future unknown samples [4].

4 Notes

1. Gain of fluorescence readings: As the plate reader has a maximum threshold, the gain should be adjusted to the experimental conditions. Otherwise, the reading can saturate and it will not be possible to characterize the dynamic range of the output. On the other hand, if the gain is too low, the detection level of the biosensor cannot be accurately and reliably estimated. To obtain sufficient output signal dynamic range, we set our gain at 1200 for the green fluorescent protein and at 2000 when we read the red fluorescent protein. The highest gain of the BMG Labtech FLUOStar plate reader we used in this work is 4095 and it has been suggested by the manufacturer that gain beyond 2800 may significantly amplify the background electronic signal noise.
2. Generally there are two options for the sensor design: One uses only an endogenous promoter (e.g., P_{zraP} and P_{zntA} shown in Fig. 2c, d) that is usually coupled to the host signalling network. The other utilizes both the transcription factor receptor and the cognate regulatory promoter that are organized into a single architecture as shown in Fig. 2a, b. For the second sensor design, generally a constitutive promoter is used to express the receptor protein while an efficient terminator is incorporated between the receptor gene and the cognate regulatory promoter to prevent any transcriptional read through.
3. To obtain robust reproducible results, always restreak sensor strains from glycerol stocks on fresh media plates such that the physiological state of the sensor strain is predictable and guaranteed. Avoid inoculation directly from old plates or glycerol stocks. To minimize the variation caused by different media batches, we suggest using growth media that is prepared following the same media recipe and autoclaving protocol.
4. Minimal media: When a minimal medium is used, one can set the initial optical density of the culture to a higher level. We usually set the optical density at $OD_{600} = 0.05$. The purpose is to compensate the slower growth in this kind of media and ensure enough cells will be produced for the characterization.

5. Fluctuation of OD_{600} readings: Due to potential clumping and aggregation of cells growing in plate wells, the optical density reading can sometimes fluctuate between adjacent cycles of readings in the plate reader. If abnormal absorbance readings are seen in a cycle, we recommend not using such readings since using them to calculate the ratio of fluorescence per OD_{600} will lead to misleading value.

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