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Microbes as Biosensors

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Abstract

The ability to detect disease early and deliver precision therapy would be transformative for the treatment of human illnesses. To achieve these goals, biosensors that can pinpoint when and where diseases emerge are needed. Rapid advances in synthetic biology are enabling us to exploit the information-processing abilities of living cells to diagnose disease and then treat it in a controlled fashion. For example, living sensors could be designed to precisely sense disease biomarkers, such as by-products of inflammation, and to respond by delivering targeted therapeutics in situ. Here, we provide an overview of ongoing efforts in microbial biosensor designs, highlight translational opportunities, and discuss challenges for enabling sense-and-respond precision medicines.

Contents

INTRODUCTION

Living cells, including microbes and human cells, carry out their biological functions by sensing and responding to a wide variety of signals. These signals include nutrients, attractants and repellents, environmental stressors, and many other biological stimuli. A complex network of signal transduction pathways within the cell ensures that these signals are relayed to downstream regulators that then trigger responses (33). The natural sensors by which microbes respond to signals can be rewired into new gene networks to build microbial biosensors that detect and report on analytes and other nonchemical stimuli, such as light. For example, cells can be engineered to fluoresce in response to a specific, disease-associated biomolecule or to produce therapeutic compounds. Several microbial biosensors have already progressed to clinical trials (39, 50).

Synthetic biology aims to engineer living organisms, such as microbial biosensors, to execute new functions to solve real-world problems (48). The growing burden of complex, multifactorial human diseases, such as inflammation, cancer, neurodegeneration, and cardiovascular disease, will likely require more sophisticated drugs that can adapt to each patient's specific conditions, thus enabling precision medicine. By designing genetic circuits, a sense-compute-respond paradigm can be introduced into living cells, similar to frameworks used in other engineering fields (93). Through iterative design-build-test-learn cycles, these genetic circuits can be continuously improved to meet specific performance criteria (5). Although biological systems are not naturally modular, synthetic biologists aim to design parts and circuits that can function reliably across many contexts.

Key parts that can be used to compose engineered sense-compute-and-respond genetic circuits include: (*a*) sensors, which are genetically encoded proteins or RNAs that sense chemical or physical inputs from inside or outside the cell and then transduce this information to downstream

parts; (*b*) compute elements, which constitute a genetic circuitry that processes information and then translates it into outputs based on a defined input-output relationship; often, these elements are built as networks of interacting regulatory parts, such as transcription factors and their target promoters; and (*c*) outputs, which can include a wide variety of biological outcomes, such as gene transcription or translation, posttranscriptional modification, metabolic pathway activation, and protein secretion.

The precision medicine paradigm aims to tailor treatments to the individual. Precision medicine requires probing the myriad genetic, environmental, and lifestyle factors that shape a person's response to a particular treatment by objectively determined parameters known as biomarkers. Disease biomarkers, in particular, are molecules that indicate a clinical illness (92). Validated biomarkers enable the classification of patients by their probable disease risk, prognosis, potential to respond to treatment, or a combination thereof.

However, biomarkers have not been found for most diseases and thus need to be discovered and validated. In addition, for many diseases, especially multifactorial ones, a single biomarker may not be sufficient for specific and sensitive diagnosis; thus, a panel of biomarkers may be needed (13). Although affordable omics-based technologies have enabled the rapid identification of putative biomarkers, the validation of biomarkers remains hindered by low statistical power and poor reproducibility of results across multiple cohorts. Multinational efforts to report molecular measurements of patient samples along with their respective medical records could help speed up the process of biomarker validation (98).

One interesting approach that is being evaluated to address these issues is the use of machine learning techniques in combination with metagenomics. For example, Abbas et al. (1) presented an integrative strategy called network-based biomarker discovery (NBBD), consisting of network analysis methods for prioritizing potential biomarkers combined with machine learning techniques for assessing the discriminatory power of prioritized biomarkers. Using a large dataset (657 samples) of metagenomics biopsy samples taken from cases of new-onset pediatric inflammatory bowel disease (IBD), they selected and prioritized biomarkers with the NBBD method and showed that it was equivalent to traditional methods for determining the most highly discriminatory biomarkers from metagenomics data. They examined how the performance of predictive models for IBD diagnosis varied as a function of the size of the data set used for biomarker identification and showed that NBBD was effective in reliably identifying IBD biomarkers, even when the number of samples available for biomarker discovery was small (50 samples) (1).

Once validated biomarkers are available, there is a need for sensors that can respond to these biomarkers and provide timely, precise, and actionable disease information. Sensors can function, for instance, to monitor the progression of a disease continuously or to identify the location of the disease so that therapeutics can be delivered in a targeted fashion. Engineering microbial biosensors is a promising strategy that takes advantage of the intimate interactions between microbes and human cells. Rapid advances in synthetic biology are expanding our ability to exploit the natural information-processing abilities of living bacterial cells. Here, we review microbial circuit design, provide examples of sensing systems, and discuss milestones that highlight the translational potential of microbial biosensors (**Figure 1**). Readers interested in human cell biosensors are kindly referred to References 8, 46, and 96.

MICROBES AS BIOSENSORS: APPLICATIONS IN BIOMEDICINE

Microbial biosensors take advantage of the ability of microbes to adapt and respond to their environment (38). Key performance characteristics that can be achieved by microbial biosensors can make them useful for real-world applications, including robustness, evolvability, high sensitivity

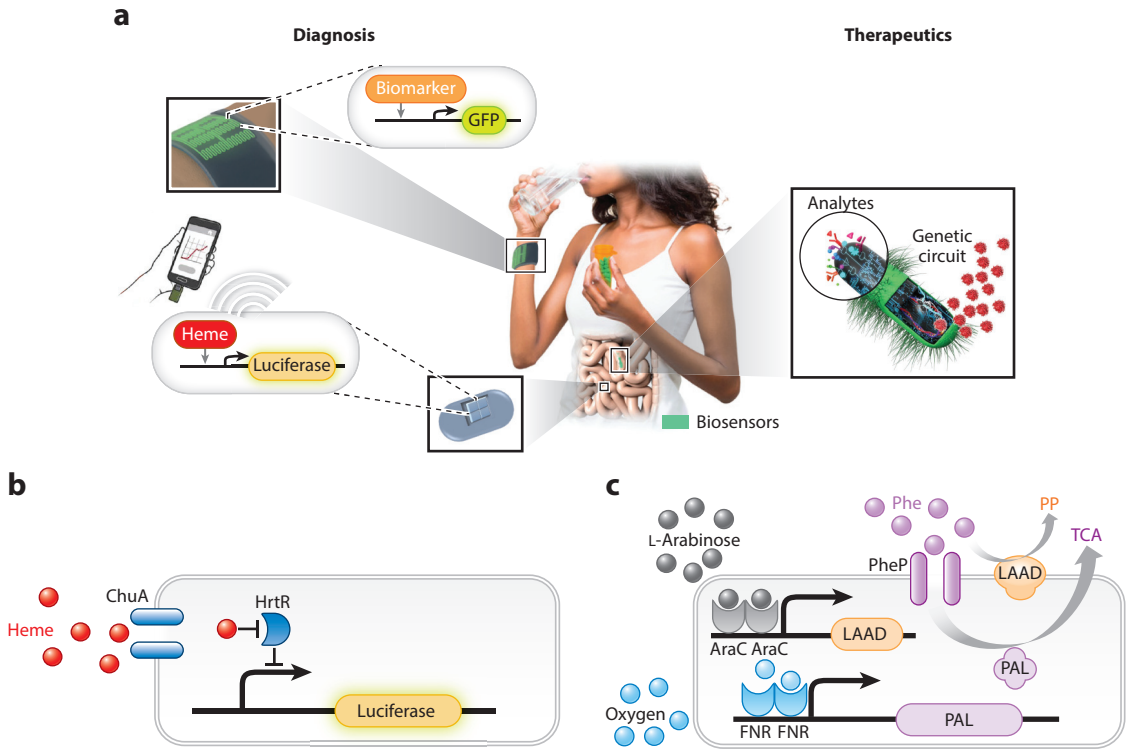

Figure 1

(*a*) Microbial biosensors can be developed for a broad variety of biomedical applications. In one application, an ingestible microbial-electronic device was built to detect bleeding in the mammalian gastrointestinal tract: Probiotic bacteria were engineered to produce bioluminescence in the presence of heme as a proxy for gastrointestinal bleeding. The bacterial biosensors were packaged in a capsule alongside miniaturized luminescence readout electronics, such that bacterial sensing events were communicated to electronics through light, and the data were wirelessly transmitted to an external device. In other applications, living patches can be used for detecting biological threats or biomarkers on the skin. Microbial biosensors can also produce medicines on demand and in situ, avoiding overdosing and systemic exposure to therapeutics. Panel adapted from Reference 38. (*b*) The blood sensor gene circuit. Extracellular heme is internalized through the outer membrane transporter ChuA and interacts with the transcriptional repressor HtrR to allow expression of the bacterial luciferase operon *luxCDABE* (62). Panel adapted from Reference 62. (*c*) A therapeutic microbial gene circuit for phenylketonuria. The synthetic phenylketonuria-therapeutic bacteria, SYNB1618, express PheP, a high-affinity phenylalanine (Phe) transporter that can bring Phe into the cell; Phe ammonia lyase (PAL), which converts Phe into *trans*-cinnamate (TCA); and L-amino acid deaminase (LAAD), a membrane-associated enzyme that converts Phe to phenylpyruvate (PP). Regulation of these components is carried out by anaerobic- and L-arabinose-inducible promoters to enable activation in the mammalian gut for metabolizing Phe (39). Panel adapted from Reference 39. Other abbreviations: FNR, fumarate and nitrate reductase regulator; GFP, green fluorescent protein.

and specificity, continuous sensing, noninvasiveness, and scalability. However, these features are not inherent to microbial systems and may require engineering or evolutionary strategies to be achieved.

Robustness

Microbial biosensors must function reliably in real-world environments, which are often harsh. For example, natural systems that sense biomarkers of inflammation, such as nitric oxide (6), thiosulfate (19), tetrathionate (19, 76), and blood (62), have been engineered into microbial biosensors (**Table 1**). In particular, one of those tetrathionate biosensors was introduced in a commensal

murine *Escherichia coli* strain, which was used to follow the progression of inflammation in mice for over 6 months (77). However, environmental conditions can be detrimental to these living biosensors. For microbial biosensors to work in the gut, they need to survive passage through the gastrointestinal (GI) tract, which includes challenging conditions such as rapid changes to extreme pHs (1.5 to 8.5), proteases, and bile salts. With inflammation in the gut, reactive oxygen species (ROS) and other antimicrobial molecules (71) can create an even more hostile environment.

Cells can be engineered or evolved to enable enhanced robustness in challenging environments. For example, the gene clusters that encode acid-resistance systems (e.g., *AR1*, *AR2*, and *AR3* from *E. coli*) can be introduced to enhance the viability of engineered microbes in the host's stomach, protecting them from the stress of acidic conditions (75). Similarly, evolved osmotolerant *E. coli* mutants, selected under high NaCl stress, can provide protection from high concentration of salts (0.80 M NaCl) during transit through the GI tract (18, 101).

Table 1 Examples of microbial sensors and their applications in biomedicine

Biomarkers	Design principles	Applications
Nitric oxide (6)	The transcriptional activator NorR binds nitric oxide to trigger the permanent activation of a DNA switch that expresses a fluorescent reporter.	Detection of inflammation in the gut
Thiosulfate (19)	The two-component system ThsSR activates the expression of a fluorescent reporter upon thiosulfate detection.	
Tetrathionate (19, 76)	The two-component system TtrSR activates the expression of a fluorescent reporter upon tetrathionate detection, either directly (19) or through a toggle switch (76).	
Heme (62)	Extracellular heme is internalized through the outer membrane transporter ChuA and interacts with the transcriptional repressor HtrR to allow expression of the bacterial luciferase operon *luxCDABE*.	
ROS (65, 80)	The transcriptional activators SoxR and OxyR are activated by the inducers (paraquat and H_2O_2) and trigger the expression of fluorescent reporters either directly (65) or through an analog-to-digital recombinase system (91); an analog compensating system is included in Reference 65.	
Oxygen (39) L-Arabinose	Synthetic phenylketonuria-therapeutic bacteria, SYNB1618, express PheP, a high-affinity Phe transporter that can bring Phe into the cell; Phe ammonia lyase, which converts Phe into *trans*-cinnamate; and L–amino acid deaminase, a membrane-associated enzyme that converts Phe to phenylpyruvate. Regulation of these components is carried out by anaerobic- and L-arabinose-inducible promoters to enable activation in the human gut to metabolize Phe.	A therapeutic microbial gene circuit for phenylketonuria in the gut

(*Continued*)

Table 1 (*Continued*)

Biomarkers	Design principles	Applications
2,4-Diacetylphophloroglucinol (60) Cuminic acid (60) 3OC6-AHL (60) Vanillic acid (60) Isopropyl-β-D-thiogalactoside (60) Anhydrotetracycline HCl (60) L-Arabinose (60) Choline chloride (60) Naringenin (60) 3,4-Dihydroxybenzoic acid (60) Sodium salicylate (60) 3OHC14:1-AHL (60) Acrylic acid (60) Erythromycin (60)	Multiple transcriptional activators were used to optimize cellular resources by simultaneous selection for lower basal expression levels, high dynamic range, increased sensitivity, and low cross talk, allowing complex profiling of multiple small-molecule signals in one cell.	Marionette is a single array of 12 high-performance sensors
Temperature (72)	Two families of transcriptional repressors (TlpA and TcI) provide switch-like control of reporter gene expression at thresholds spanning the biomedically relevant range of 32–46°C.	Host temperature detection for fever monitoring, kill switches, or focus ultrasound therapeutic activation
Cholera autoinducer 1 (59)	CqsS-NisK fusion variants expressed in *Lactococcus lactis* trigger expression of an enzymatic reporter upon binding quorum-sensing signals of *Vibrio cholerae* that is readily detected in fecal samples.	Probiotic-based strategy for cholera detection and treatment
Arabinogalactan (63) Isopropyl-β-D-thiogalactoside (63) Rhamnose (63) Lactose (42)	Diet-inducible promoters (specific for arabinogalactan, IPTG, rhamnose, and lactose) drive the expression of NanoLuc reporter or a recombinase system in commensal *Bacteroides thetaiotaomicron* (63) or *Streptococcus thermophilus* (42).	Diet sensor in the context of a complex microbiota
aTc or arabinose (78)	*Escherichia coli* cells were programmed to respond to the antibiotic aTc or arabinose with a fluorescent reporter. Different output fluorescence patterns were produced depending on whether the cells were exposed to aTc followed by arabinose or an initial input of arabinose followed by aTc.	Scalable biological state machine
Isopropyl-β-D-thiogalactoside (90) Fructose (90) Fucose (90) Ribose (90) Trehalose (90)	Chimeras were built, consisting of the DNA-binding domain of LacI and the ligand-binding domain of repressors that respond to different sugars. Since each chimera can regulate the same promoter, simple transcriptional AND gating is achieved by coexpressing multiple chimeric repressors; the ligand for each chimera must be present to allow downstream transcription to occur.	Multi-input AND gate eliminates the need for ligand-inducible promoters to control each input, simplifying the circuit

Abbreviations: AHL, acyl-homoserine lactone; Phe, phenylalanine; ROS, reactive oxygen species.

Evolvability

Directed evolution is an iterative process that generates genetic diversity, followed by screening for functional gene variants with desired functions (70). Genetic circuits that comprise bacterial sensors can be optimized via the directed evolution of each component, such as promoters (2, 34), protein sensors (60), and gene circuits (2), or at the whole cell level to improve features such as bacterial colonization (18) or stress tolerance (101).

However, this capacity to evolve can also be a disadvantage for the long-term stability of living biosensors, since the taxing of cellular resources to support synthetic genetic circuits can result in compensatory genetic mutations, loss of engineered functions, and impaired growth of the recombinant strain (14) in a host- and environment-dependent manner (32, 64). To limit the burden imposed on the cell, gene expression designs with reduced burden are needed. Measuring the expression of a green fluorescent protein (GFP)-based capacity monitor in *E. coli* enables the assessment of the burden that heterologous gene expression exerts on the cell, and this information can be used to design constructs with less impact on the host (14).

For example, through directed evolution, Meyer et al. (60) developed Marionette, a platform for simultaneously optimizing sensors for complex profiling of multiple small-molecule signals in one cell. Marionette was applied to generate a single array of 12 high-performance sensors that exhibit >100-fold induction with low cross-reactivity. Cellular resources were optimized by simultaneous selection for lower basal expression levels (i.e., low gene expression in the absence of the desired stimulus), high dynamic range, increased sensitivity, and low cross talk. For this optimization, a genetically diversified library of sensors was cloned upstream of an operon containing a mutant of the phenylalanine aminoacyl tRNA-synthetase (PheS) and a thermostable DNA polymerase (DNAP) on a dual-selection plasmid. The PheS cassette enables selection against leakiness in the absence of inducer, while the DNAP cassette enables selection for enhanced sensitivity. After induction, the cells were encapsulated, lysed, and amplified by polymerase chain reaction (PCR) with primers that immediately flank the sensor library using the DNAP expressed by the sensor, such that more amplification was achieved the more DNAP was expressed. In the absence of inducer, leaky transcription of PheS leads to the charging of phenylalanyl tRNA with the noncanonical amino acid 4-chloro-DL-phenylalanine. Adding more of this amino acid increases the stringency of the negative selection against leakiness by making PheS transcription more toxic. For positive selection to enhance the sensitivity to inducer, the sensors responsible for the greatest expression of DNAP are mostly amplified by emulsion PCR. Thus, multiple properties of the sensor response function can be improved through one cycle of negative and positive selection. Chemical specificity can be achieved by adding a potentially cross-reactive inducer during the negative selection, thus selecting against cross-reactive mutants. Chemical antagonism can be selected against by adding a potentially antagonistic inducer to the positive selection, thus selecting for mutants that are less strongly antagonized (60) (**Figure 2**).

Sensitivity and Specificity

Bacteria are naturally equipped with sensors that detect a large number of molecules in their environment (e.g., gut, skin, or mouth) within biologically relevant concentrations [e.g., bacterial sensors can detect tetrathionate (19, 76), nitric oxide (6), thiosulfate (19), and ROS (65, 80) in the micromolar range] (17, 57). For protein biosensors, specific recognition sites within the protein can bind to different small-molecule signals and potentially discriminate between related ones. For example, the bacterial transcription factor NorR can differentiate between nitric oxide and other reactive nitrogen species (6). This sensor allows bacteria to control the expression of cognate nitric oxide reductases that metabolize and detoxify nitric oxide inside the gut.

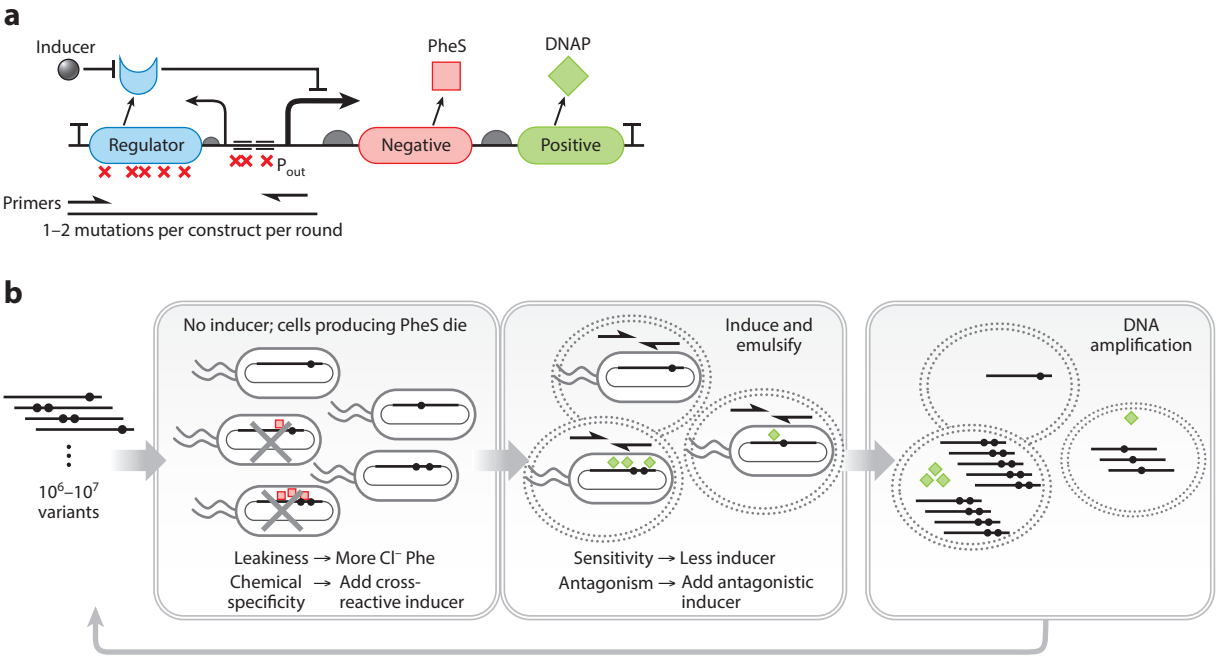

Figure 2

Marionette, a platform for sensor optimization based on a dual selection. (*a*) A genetically diversified library of sensors (regulator, *blue*; targeted degeneracy, *red crosses*) was cloned upstream of an operon containing a mutant of the phenylalanine aminoacyl tRNA-synthetase (PheS; *red square*) and a thermostable DNA polymerase (DNAP; *green diamond*) on a dual-selection plasmid. Transcription from the output promoter (P_{out}) controlled by the inducer molecule determines the expression level of PheS and DNAP. (*b*) A dual-selection scheme enables positive selection and negative selection for optimal circuit performance. The PheS cassette enables selection against leakiness in the absence of inducer, while the DNAP cassette enables selection for enhanced sensitivity. In the absence of inducer, leaky transcription of PheS leads to the charging of phenylalanyl tRNA with the noncanonical amino acid 4-chloro-DL-phenylalanine (Cl^- Phe). Adding more Cl^- Phe increases the stringency of the negative selection against leakiness by making PheS transcription more toxic. For positive selection to enhance the sensitivity to inducer, the sensors responsible for the greatest expression of DNAP are mostly amplified by emulsion PCR. After induction, the cells are encapsulated, lysed, and PCR amplified with primers (*half arrows*) that immediately flank the sensor library using the DNAP expressed by the sensor, such that more amplification is achieved if more DNAP is expressed. Multiple properties of the sensor response function can be improved through one cycle of negative and positive selection (*dots* denote mutations incorporated per round). Chemical specificity can be achieved by adding a potentially cross-reactive inducer during the negative selection, thus selecting against cross-reactive mutants. Chemical antagonism can be selected against by adding a potentially antagonistic inducer to the positive selection, thus selecting for mutants that are less antagonized. Figure adapted from Reference 60.

However, not all natural sensors are ready for real-world applications, since their original functions may not match the desired use cases. Strategies for improving their sensitivity and specificity include directed evolution and rational engineering of the protein components themselves or the introduction of circuit-level designs, such as digitalizing, amplifying, and multiplexing signal detection (17), which is discussed in the section titled Using Synthetic Biology to Engineer Computation Circuits. To demonstrate specificity, many potential off-target molecules need to be tested.

For example, unwanted cross talk between different signals can confound the interpretation of the responses of microbial biosensors (19, 72). The TtrSR two-component system (TCS) from *Salmonella enterica* Typhimurium (36, 73), which responds to tetrathionate, is composed of the TtrS kinase sensor and the TtrR response regulator, which controls downstream transcription via the ttrB promoter (P_{ttrB}). The global regulator FNR (fumarate and nitrate reductase regulator)

is required for transcription from P_{ttrB} and is repressed by O_2. Thus, using P_{ttrB} as a readout for tetrathionate sensing is potentially confounded by O_2 levels due to FNR regulation. This cross-regulation could compromise the performance of TtrSR-based tetrathionate sensing in the gut, where O_2 levels may vary depending on their proximity to the epithelial mucosal boundary. To avoid this cross-repression, Daeffler et al. (19) computationally identified a novel tetrathionate sensor from the marine bacterium *Shewanella baltica*, which bypasses the FNR system. By expressing this sensor in *E. coli*, they provided an alternative tetrathionate sensor with improved performance sensing in the gut. The authors demonstrated the specificity of the sensor by showing that it has selectivity for tetrathionate over other terminal electron acceptors.

Orthogonality

Here, we define orthogonality as the lack of interference between one gene circuit component and another when they are put together in a cell, or between one gene circuit component and a component of the endogenous cellular biology. Orthogonality is not a given in biological systems, especially when multiple signaling networks interoperate within environments that are not physically segregated. Thus, orthogonality is a crucial element of design when using multiple sensors in the same cell (17, 60, 63). For example, for thermal biosensors, temperature-dependent transcriptional repressors have been shown to be less susceptible to cross talk compared to microbial heat shock promoters, which can be activated not only by temperature changes but also by other chemical stressors (22, 72).

Unanticipated cross talk between different pathways may interfere with the proposed functioning of engineered gene networks. To make signal engineering more predictable, Schmidl et al. (83) rewired bacterial TCSs by modular DNA-binding domain swapping, achieving robust signaling even in heterologous hosts. These authors showed that the two largest families of response regulator DNA-binding domains (OmpR/PhoB and NarL/FixJ families) can be interchanged, enabling the corresponding TCSs to be rewired to synthetic output promoters. This remarkable flexibility has been exploited to eliminate cross-regulation, unsilence a gram-negative TCS in a gram-positive host, and engineer a system that can achieve an increase in activation of over 1,300-fold (83).

In addition to choosing or designing better sensor parts, synthetic gene networks can be engineered to compensate for cross talk. For example, a common approach is to insulate signal transduction pathways by identifying and minimizing responsible interactions between the genetic components. Alternatively, one can introduce new gene network connections to cancel out cross talk at the network level. For example, we recently showed that ROS-responsive gene circuits in *E. coli* can exhibit concentration-dependent cross talk with noncognate ROS molecules. After quantitatively mapping the degree of cross talk, we designed gene circuits that introduced compensatory cross talk at the gene network level, essentially cancelling out the noncognate ROS contribution to the output and improving the specificity of the overall circuit to the cognate ROS molecule (**Figure 3**) (65).

Continuous Sensing

Microbial biosensors can continuously monitor their environments without the need for interrupted or periodic sampling. Sensing biomarkers in situ as they are produced could aid in elucidating underlying mechanisms of disease, studying disease dynamics (57), and improving disease management. For example, an ingestible microbio-electronic device was developed to detect blood in the GI tract (62). Specifically, probiotic *E. coli* Nissle 1917 (EcN) was engineered as a microbial

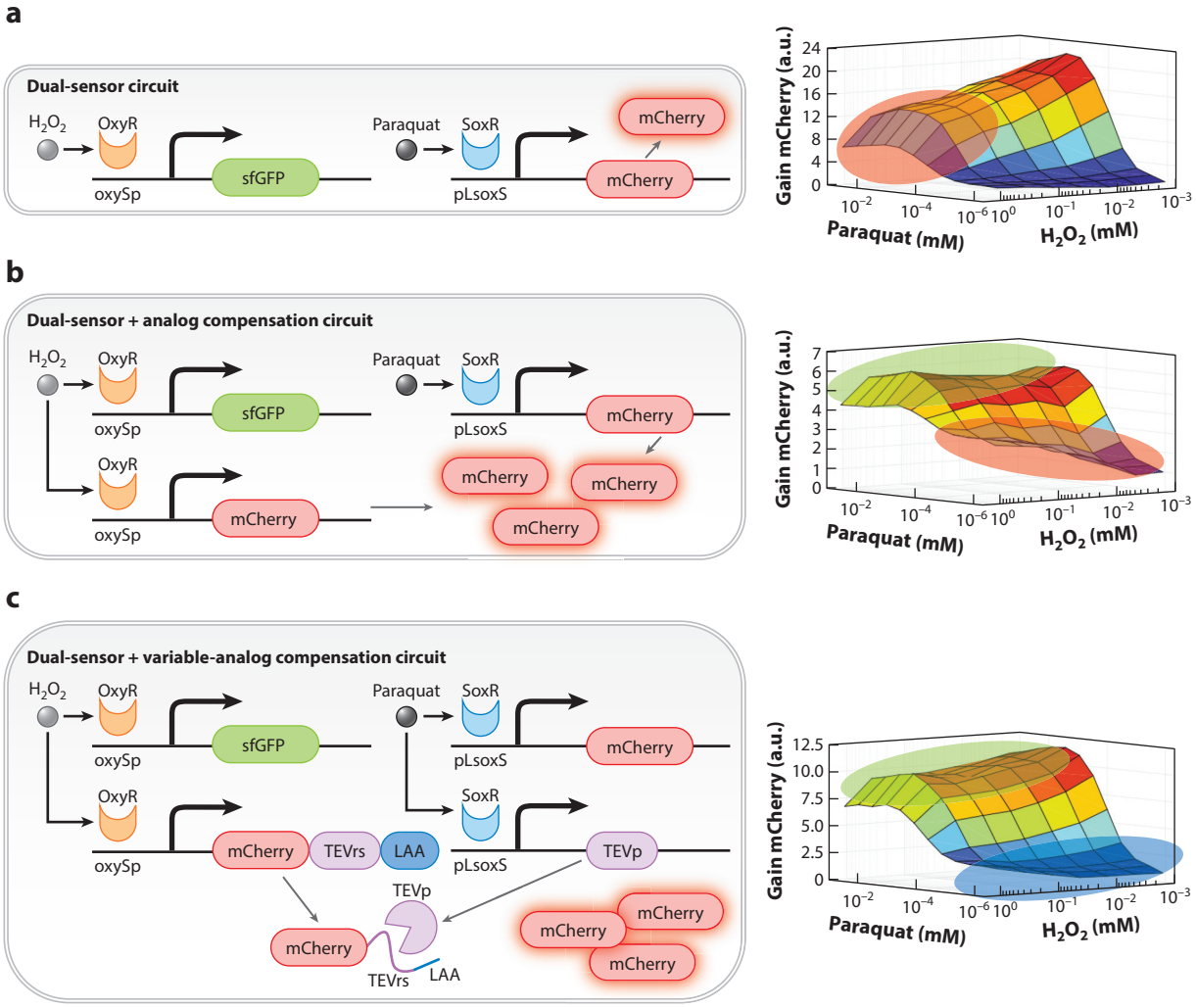

Figure 3

Synthetic network-level cross talk compensation. (*a*) A simple dual-sensor circuit that suffers from cross talk. SoxR and OxyR are constitutively expressed, and once activated by the inducers (paraquat and H_2O_2, respectively), these transcription factors activate mCherry expression from P_{LsoxS} and superfolder green fluorescent protein (sfGFP) expression from oxySp, respectively. The mCherry output (*glowing red shape*) from the dual-sensor circuit is mostly dependent on paraquat concentration, but there is considerable cross talk at high H_2O_2 concentration (*graph on the right*, *red shape*). (*b*) The dual-sensor circuit with analog compensation. An extra copy of mCherry controlled by oxySp was added to the dual-sensor circuit so that the total mCherry level is controlled by both oxySp and P_{LsoxS} promoter activity. The mCherry output now shows reduced H_2O_2 cross talk at high paraquat concentrations (*graph on the right*, *green shape*) but increased cross talk at low paraquat concentrations (*graph on the right*, *red shape*). (*c*) The dual-sensor circuit with variable-analog compensation. An oxySp promoter was added to express mCherry fused to Tobacco Etch Virus recognition sequence (TEVrs) and a Leu-Ala-Ala degradation tag, as well as the TEV protease (*TEVp*) gene controlled by P_{LsoxS}. TEVp posttranslationally cleaves the Leu-Ala-Ala degradation sequence from the oxySp-expressed mCherry protein at the TEVrs site, stabilizing the mCherry protein. The mCherry output from this circuit shows substantially reduced H_2O_2 cross talk at high paraquat concentrations compared to the original dual-sensor circuit (*graph on the right*, *green shape*) without considerably increasing cross talk at low paraquat concentrations (*graph on the right*, *blue shape*) (65). Figure adapted from Reference 65.

biosensor to produce bioluminescence in the presence of heme, once imported into the cell. The biosensor was packaged in a capsule with miniaturized electronics that transmitted the detected luminescence signal as information wirelessly to an external device (**Figure 1*a*,*b***). This platform detected blood in the porcine stomach.

One important caveat is that microbial biosensors that rely on gene expression may have slower signal transduction than enzyme-based detection methods, although the former have the advantage that they can be coupled to biological responses. Another challenge with gene-expression-based sensors is how to build them to detect extracellular analytes that do not penetrate into cells. To address this problem, Chang et al. (15) developed a bacterial transmembrane receptor using single-domain antibodies for extracellular ligand detection. These receptors consist of a ligand-induced dimerization domain fused to a single-domain antibody in the periplasm and a monomeric DNA-binding domain inside the cell. In the presence of the stimulus, the chimeric receptor undergoes ligand-induced dimerization and activates downstream reporter gene expression. The authors successfully expressed a synthetic alpha-rep (helicoidal repeat protein) binder recognizing GFP and found that it could bind recombinant enhanced GFP in *E. coli* cells with a deficient outer membrane. The transmembrane receptor principle may be general enough to be used in other bacterial hosts, such as gram positives, that do not have an outer membrane. In that case, the receptor sensing domain would be directly exposed on the cell surface and accessible to extracellular ligands (15).

Noninvasiveness

Given their micron-scale size, microbial biosensors can be readily deployed in difficult-to-access sites of the body (39). Even in remote areas [e.g., the small intestine (39) or the center of a tumor, as has been reported for *Salmonella* bacteria naturally infecting solid tumors (81, 97) and EcN as a living biotherapeutic for the treatment of cancer (53)], microbial biosensors can detect biologically relevant labile compounds that would be otherwise metabolized, absorbed by the host, or inactivated in biological samples (serum, urine, or feces) (82). However, choosing bacterial chassis that are well adapted to the environment of interest is important for ensuring the accessibility and function of bacterial sensors. For example, microbiome studies often use antibiotics to clear out the native host microbiome to enable exogenously delivered bacteria to engraft, since exogenous bacteria are often unable to compete with the endogenous flora. However, this is not ideal if one wishes to use microbial biosensors to study the native host microbiome. An alternative is to directly engineer bacteria that are already well adapted to the microbiome of interest and then introduce these bacteria into the host, or to use bacteriophages that deliver gene circuits directly into bacteria already residing in the specific environment of interest. These last approaches may be of special interest in the search of new cancer therapies. Recent research that characterized the tumor microbiome has shown that each tumor type (breast, lung, ovary, pancreas, melanoma, bone, and brain tumors and the normal surrounding tissues were studied) has a distinct composition of bacteria, residing intracellularly either in cancer or immune cells, with potential consequences in the patient's response to immunotherapy (67).

Scalability

The manufacturing of microbial biosensors is potentially cost-effective because microbial cells can self-renew and can be scaled up in industrial processes. These features can drive down the cost of deployment into real-world environments, which may be especially important for applications in the developing world. For example, Mao et al. (59) used a probiotic-based strategy to detect cholera

and trigger therapeutic functionality in an approach that could be deployed in the developing world. Specifically, these authors engineered *Lactococcus lactis* to detect quorum-sensing signals produced by *Vibrio cholerae* in the gut and then trigger the expression of an enzymatic reporter that is readily detected in fecal samples. The authors used a nitrocefin-based β-lactamase assay to generate a visible color change from yellow to red as the output. However, the potential for horizontal gene transfer of the β-lactamase gene to pathogenic bacteria restricts the use of this specific construct in the field. Therefore, a food-grade enzymatic reporter is needed that is not degraded in the human gut and can be detected with a cost-effective substrate to enable its use in low-resource settings.

By further engineering these probiotics to produce lactic acid, Mao et al. (59) showed that the probiotic bacteria could reduce the intestinal *V. cholerae* burden and improve the survival of infected infant mice. Preventive dietary interventions with this technology could help block cholera progression and provide community-level surveillance of cholera cases in populations at risk of outbreaks (59). In addition to horizontal gene transfer, challenges posed by the self-renewing ability of microbial biosensors include the potential need for biocontainment due to regulatory demands as well as the possibility of genetic drift over time (40).

USING SYNTHETIC BIOLOGY TO CONSTRUCT SENSORS

Cells can be engineered with chemical sensors to detect small molecules or macromolecules and produce a measurable signal whose magnitude is proportional to the concentration of the analyte. Chemical microbial biosensors have been built for environmental monitoring of heavy metals (11, 41, 44, 74, 85, 87, 99), organic compounds for metabolic engineering (7, 55, 102, 103), and many small molecules of clinical relevance (6, 19, 49, 60, 76). In addition to chemical sensors, microbial biosensors can be designed to sense light (28, 54, 94), temperature, and even pressure (24).

Several approaches are being taken to construct microbial biosensors that detect a broader variety of clinically relevant analytes. High-throughput techniques have the potential to identify new candidates for sensing by genome mining or by pairing metabolomic screens with streamlined strategies for biosensor discovery (61). For example, TCS sensors can be mined from genome databases based on structural similarities. The novel tetrathionate sensor from *S. baltica* described above was found during a bioinformatics search for TCSs containing a sensor kinase with a periplasmic sensing domain similar to that of the *E. coli* phosphonate-binding protein PhnD. PhnD is involved in the active transport of alkylphosphonates across the inner membrane, which are chemically similar to tetrathionate. This is a reasonable strategy to search for putative tetrathionate sensors, since the previously characterized tetrathionate sensor TtrS from *S.* Typhimurium also binds tetrathionate through a PhnD-like binding domain. Ultimately, this mined system was experimentally validated through functional expression and characterization in heterologous bacterial hosts (19).

Further bioengineering of biosensors can enable improvements in sensor performance. Hybrid proteins can be constructed with domains from multiple sources so as to redirect signal transduction pathways, to change substrate specificity, to introduce orthogonality with other sensors, and to extend sensing to new stimuli. For example, modular transcription factors from the LacI/GalR family have been used to create chimeras to expand the variety of inducers and the construction of logic gates triggered by multiple inputs. Each chimera consists of the DNA-binding domain of LacI and the ligand-binding domain of repressors that respond to five different sugars (isopropyl-β-D-thiogalactoside, fructose, fucose, ribose, and trehalose). As each chimera regulates the same promoter, simple transcriptional AND gating is achieved by coexpressing multiple chimeric

repressors; the ligand for each chimera must be present to allow downstream transcription to occur. Because ligand control works directly at the level of each transcription factor, this multi-input AND gate eliminates the need for ligand-inducible promoters to control each input, simplifying the circuit (90). To increase the possible combinations of functionally orthogonal transcriptional repressors, point mutations were introduced into the DNA-binding domain to regulate an additional promoter, P_{TAN}, expanding the possibilities of assembling a variety of chimeras.

In another interesting example, Mao et al. (59) fused the cholera autoinducer 1 (CAI-1)-binding domain of the *V. cholerae* natural receptor (CqsS) with the histidine kinase domain of NisK from *L. lactis* to enable *L. lactis* to detect quorum-sensing signals produced by *V. cholerae* (CAI-1). Gene expression constructs for CqsS-NisK fusion variants were screened with randomized ribosome binding sites to identify those that had a strong response to CAI-1. The success of this strategy is based on the modularity of histidine kinase receptors (59).

Finally, biosensors can be discovered through in vivo screens by using a synthetic memory circuit. Specifically, Naydich et al. (66) built a library of biosensors and used them to drive the expression of a memory element in a commensal gut *E. coli* strain. This engineered strain library was then introduced into mice with intestinal inflammation and healthy controls. The memory circuit enabled post hoc selection of strains with in vivo activation of a given sensor construct in response to inflammation. This platform could be applied to screen for sensors in other disease-specific models (66).

USING SYNTHETIC BIOLOGY TO ENGINEER COMPUTATION CIRCUITS

Computation is an important part of microbial biosensors because it enables multiple bits of information to be integrated and then translated into desired outputs (91). Synthetic gene circuits are typically built by engineering gene regulatory networks that receive inputs from sensors via regulatory molecules, such as transcription factors and their target promoters, such that these modules interact with each other in a multifactorial fashion. Ideally, biological parts could be readily composed together to create higher-order circuits independent of genetic, organismal, or environmental contexts (3). This method of composition would allow for the optimization and characterization of parts, followed by their scalable assembly for any application of interest.

However, in reality, biological parts are not totally modular and may instead depend on host-dependent or circuit-dependent factors, many of which are not yet well understood. Thus, high-throughput strategies for assembling and characterizing libraries of gene circuits composed of many alternative configurations are needed. Massively parallel DNA synthesis, automation strategies, and computational design tools such as Cello (68), j5 (37), or iBioSim (100) have enabled faster and larger-scale approaches over time. Ultimately, the generation of large datasets from these experiments will create opportunities for machine learning to derive quantitative characterization of parts and their associated interdependencies, thus leading to more highly automated and more accurate design algorithms.

Below, we discuss several types of useful computation circuits, including open-loop, feedforward, and feedback systems, and memory devices.

Open-Loop, Feedforward, and Feedback Circuits

Synthetic gene networks can have multiple architectural configurations. For example, in simple open-loop circuits, the input signal drives computational circuits and leads to an output (or outputs) in a linear, cascading fashion; in a feedforward circuit, the input affects the output through

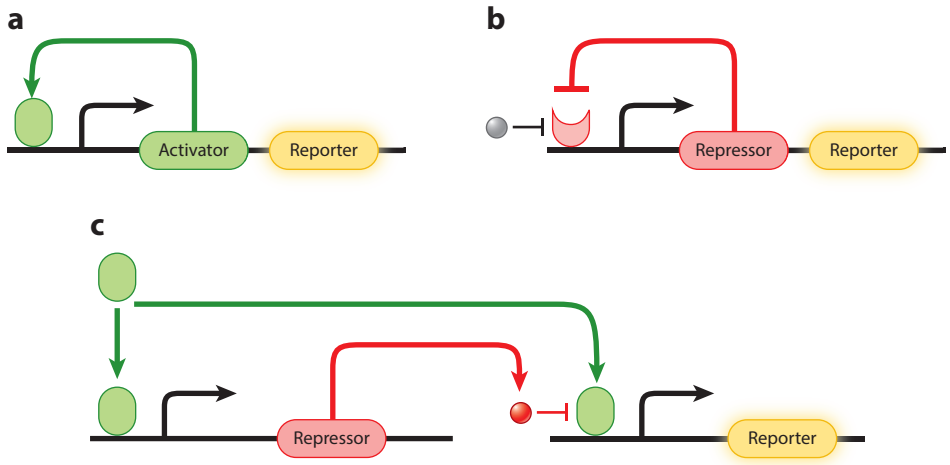

Figure 4

Synthetic gene networks can have multiple architectural configurations. (*a*) Positive autoregulation occurs when a gene is activated by its own product (20). (*b*) Negative autoregulation occurs when a gene represses its own expression (79). (*c*) A feedforward loop occurs when an input simultaneously activates an output and a repressor of the output (43).

multiple paths, including indirect nodes; and in a feedback circuit, the output is fed back into the input and can have negative or positive effects (**Figure 4**).

As a simple example of an open-loop circuit, Mimee et al. (63) engineered the commensal bacteria *Bacteroides thetaiotaomicron* as a diet sensor in the context of a complex microbiota. Specific-pathogen-free mice were colonized with an engineered *B. thetaiotaomicron* strain containing a NanoLuc reporter driven by an arabinogalactan (AG)-inducible promoter. Within a day of adding AG to the drinking water, luciferase activity in fecal pellets increased by 75-fold. Following removal of the inducer from the water, luciferase activity rapidly returned to baseline, demonstrating the tight temporal control of gene expression, which was dependent on AG. The expression of NanoLuc did not affect the in vivo fitness of the engineered bacteria compared to that of uninduced controls.

A type of feedforward circuit is the incoherent type-1 feedforward loop (I1-FFL), in which an input simultaneously activates an output as well as a repressor of the output. The I1-FFL is a prevalent network motif in transcriptional networks found in nature. For example, in the *gal* system from *E. coli*, this motif accelerates the response time of the *gal* operons, *galETK* and *galP*, upon a cAMP signal (43, 58). One application of the I1-FFL is to use it as a pulse generator: The concentration of the output has a strong, transient response to a change in the input, which is then dampened to a new steady state through the delayed action of the inhibitor. I1-FFL also enables perfect adaptation, which means the new steady-state concentration of the output is the same as it was before the stimulus. As an example of a pulse-generating network, Basu et al. (10) created a synthetic multicellular bacterial system for coordinating behavior in cell communities, in which receiver cells exhibit transient gene expression in response to a long-lasting signal from neighboring sender cells. The engineered sender cells synthesize an acyl-homoserine lactone inducer, which freely diffuses to spatially proximate receiver cells. The receiver cells contain a feedforward motif that, in response to the acyl-homoserine lactone stimulus, exhibits an initial excitation followed by subsequent delayed inhibition of the output, which in this case was GFP expression.

The circuit can even differentiate between various rates of increase in stimulus levels, enabling a spatiotemporal behavior in which cells respond transiently to communication from nearby cells but ignore communication from cells that are farther away (10).

Following a similar strategy, Barger et al. (9) built an incoherent feedforward loop circuit that reduced unwanted cross talk between two stress-responsive promoters (P_{katG} and P_{recA}) to develop an environmental stress-responsive system with increased specificity. They first built a synthetic gene circuit for each promoter to check for specificity. However, they found that whereas the katG-based bacterial biosensor was highly specific to H_2O_2, the recA-based bacterial biosensor had no specificity for either H_2O_2 or nalidixic acid (NA). Therefore, they built a cross talk-compensating circuit that consisted of two parallel antagonistic paths of an AND operation, in which they used the recA promoter so that the output would be activated by either H_2O_2 or NA and, at the same time, used katG to repress the output induced by H_2O_2 only. The AND gate was constructed by splitting the *luxCDABE* operon so that the luciferase reporter could be produced only if all the parts of this operon were expressed. While the first input of the AND gate (luxCDE) was expressed directly from P_{recA}, the second input (luxAB) was regulated by P_{katG} via a repressor, which led to a delay in its response. If the time delay was synchronized with the kinetics of the NA response, two clearly separated peaks could be resolved when both NA and H_2O_2 were present in the culture (9).

Positive autoregulatory feedback, when a gene is activated by its own product, can lead to increased steady-state levels of expressed gene (amplification), faster response kinetics, and increased sensitivity to exogenous inducers or autoinducers. In addition, positive feedback can be designed to expand the input dynamic range of a circuit. For example, Daniel et al. (20) built a positive-feedback component located on a low-copy-number plasmid in which the P_{BAD} promoter regulates the transcription of the AraC transcriptional activator, which is induced by arabinose to activate the P_{BAD} promoter, thus increasing the generation of transcription factors. This positive loop reduces saturation between inducer and transcription factor. In addition, the authors added a shunt component located on a high-copy-number plasmid comprised of promoters that bind the AraC transcription factor, effectively redirecting some of them away from the low-copy-number plasmid and reducing saturation between transcription factors and DNA. When both components were combined, this circuit had an input dynamic range of more than three orders of magnitude. The wide dynamic range behavior of the circuit was especially striking when compared with the narrow dynamic range of the open-loop control (AraC–GFP expression from a P_{lacO} promoter instead of P_{BAD}), which had a shunt motif but no positive-feedback motif (20).

Negative autoregulatory feedback can be introduced by using transcription factors that negatively regulate their own transcription (79). One advantage of negative feedback is that it reduces cell-to-cell fluctuations in the steady-state level of the transcription factor. It can also speed up responses by reducing rise time (the time it takes the output to rise from its minimum to its maximum value). For example, in a synthetic circuit in which the tet promoter controls the production of its repressor, TetR fused to GFP, negative autoregulation reduces rise time to about one-fifth of the rise time of the equivalent circuit (with the same steady state) without negative autoregulation. (79). As another example, a double-negative feedback loop has been used to implement the toggle switch, a synthetic memory circuit with several applications as a diagnostic tool, described in detail below.

Memory Circuits

For diagnostic applications, memory circuits that can remember the history of exposure to desired signals are useful for probing environments in which real-time readouts are inconvenient. For

example, microbial biosensors with memory can be used to sense disease biomarkers in the gut and subsequently report on this information when they are recovered in feces. Toggle switches (49, 76) and recombinases (63) have been used in memory circuits.

For example, Kotula et al. (49) designed a memory system based on the cI/cro toggle switch found in bacteriophage lambda. This switch uses two mutually repressing transcription factors, cI and cro, which sets up a system in which only one of the two transcription factors is expressed at a time until a signal flips the switch. The utility of the toggle switch as a diagnostic tool was demonstrated by building bacteria that remembered exposure to anhydrotetracycline (aTc) (49) and tetrathionate (76) in the gut and were able to report this information after being recovered in feces.

Recombinases have also been used to implement long-term memory circuits (91). Recombinases recognize specific sequences of DNA delineated by recombinase-recognition sites and can invert them or cut them out, leaving long-lasting changes in DNA that serve as memories of past events and can be used to create digital counters in living cells (29). For example, recombinase memory has been used to record the presence of lactose (42) and rhamnose (63) within the gut. Recombinases can also be used to implement circuits that perform Boolean logic (91) and integrated logic function of multiple signals (80). Finally, recombinase circuits can be tuned to convert analog signals [e.g., concentrations of an environmental signal such as ROS (80)] to a digital switching event, thus enabling analog-to-digital computation systems (91) and memory devices (26).

To design more complex circuits with recombinases that can respond to the order in which biological events occur, Roquet et al. (78) created scalable biological state machines. As a proof of concept, *E. coli* cells were programmed to respond to the antibiotic aTc or arabinose. The researchers designed a circuit that produced different output fluorescence patterns depending on whether the cells were exposed to aTc followed by arabinose or an initial input of arabinose followed by aTc. This was achieved by designing orthogonal recombinase-recognition sites and then interlacing them into DNA so that different input orders would generate distinct DNA recombination events. They tested this approach with a circuit encoding green, red, and blue fluorescent proteins and expressing different combinations of the proteins for each identity and order of two inputs. For example, when cells carrying this circuit were exposed to arabinose as the first input and then to aTc, they fluoresced red and green, whereas cells that received aTc and then arabinose fluoresced green and blue. Such order-sensing microbial biosensors make it possible to study important temporal information. For example, microbial biosensors could respond appropriately to disease progression by releasing a therapeutic molecule at each stage in response to different biomarker permutations. By tracking cellular events that occur in a particular order, environmental sensors can be created to store complex histories or program cellular trajectories.

CRISPR-Cas9-Based Memory

As biosensing applications become more complex, memory architectures with minimized fitness effects and extended recording capacities are needed. Genetically encoded DNA writing devices offer an alternative approach to recombinases and toggle switches, enabling targeted, dynamic, and recurring modifications of DNA in living cells (27). These modifications can take the form of targeted insertions, deletions, inversions, or base substitution mutations that can serve as distinct DNA memory states, information that can be retrieved by sequencing, turning ON or OFF reporter expression, or measuring signal intensity (25). On the basis of the mutational outcomes, these devices can be classified as precise writers (those that use well-defined mutations) or pseudorandom writers (those that use stochastic mutations).

In the precise writing mode, a base editor, such as a cytidine (47) or an adenosine (30) deaminase domain fused to dead Cas9, is addressed to a desired target site by the expression of a complementary guide RNA, generating C→T or A→G substitutions, respectively, in the vicinity of the target site (25, 95).

As an example of the pseudorandom writing mode, the Cas1-Cas2 writing system can be used to record the temporal order of multiple signals in a CRISPR array composition. The system uses the Cas1-Cas2 protein complex, which naturally mediates spacer acquisition in the CRISPR bacterial immune system, and integrates short (approximately 20 to 30 base pairs), single-stranded DNA fragments from an intracellular pool into a preexisting CRISPR array, resulting in extension of the array over time. New spacers are added to the leader-proximal site, so the chronological order of spacer addition events is preserved within the array configuration. The intracellular single-stranded DNA pool can originate from various sources, including multiple template plasmids with tunable copy numbers that can be dynamically modulated (88). By placing the expression of the Cas1-Cas2 cassette under the control of signal-inducible promoters into *E. coli* cell populations, Shipman et al. (89) demonstrated that the signal intensity and duration can be inferred from array extensions and that the temporal order of the addition of the oligonucleotide pools can be inferred from the array composition.

USING SYNTHETIC BIOLOGY TO TRANSLATE SENSING INTO OUTPUTS

The outputs of microbial biosensors can be reporters or therapeutic payloads. For example, diagnostic readouts could be the expression of colorimetric, fluorescent, or bioluminescent reporter genes. It is possible to measure the signals released by diagnostic bacteria directly, in devices [e.g., embedded in skin patches and gloves (56)], or in complex biological samples [e.g., urine (21, 39) or feces (76)]. A problem with light-based reporters is that light can be scattered as it passes through tissues, leading to poor resolution. To address this issue, genetically encoded gas vesicles (86) from bacteria and archaea have been developed as reporters to enable ultrasound detection of gene expression in deep tissues (12, 51). Alternatively, recombinase-based or CRISPR-based sensors that mutate DNA sequences directly can be read out through quantitative PCR-based methods (78) at the expense of requiring downstream processing.

Finally, the ability to drive controlled therapeutic production as the output from microbial biosensors has the potential to enable in situ therapeutics that respond to clinically relevant biomarkers. These living drugs could provide personalized and targeted medicines to address complex diseases, such as metabolic disorders, inflammatory diseases, and cancer. Bacterial biosensors can produce medicines on demand in a localized fashion (i.e., only during active disease and at precise sites in the body), thus reducing systemic exposure (**Figure 1*a***). For example, EcN was engineered into an in vivo gut-activated therapeutic microbe to treat phenylketonuria, a metabolic disorder that causes phenylalanine (Phe) to accumulate in the body, leading to neurotoxicity. Specifically, EcN was modified to produce enzymes that metabolize Phe only in the hypoxic conditions of the gut (**Figure 1*c***) (39). This strain reduced blood Phe concentration by 38% in treated compared with untreated phenylketonuria mice. In healthy cynomolgus monkeys, the engineered bacteria prevented increases in serum Phe levels after a high-protein diet. Clinical trials are being performed to study these engineered bacteria in human patients. There are multiple other examples of living bacterial therapeutics; for a detailed list of completed, active, and proposed clinical trials, please see References 40 and 77.

In the future, feedback circuits coupled with microbial biosensors that measure disease severity or therapeutic payloads could be used to autoregulate drug dosing to achieve maximal safety and

efficacy. Furthermore, gene circuits could be designed to deliver multiple therapeutics at defined levels and orders, rather than just single ones.

CHASSIS SELECTION

The chassis is the organism that houses the DNA-encoding gene circuits and enables execution of the programs. Choosing a suitable chassis for the application of interest is an important part of the bacterial biosensor design process.

One important question when selecting a chassis is whether colonization is required for the intended goal. If colonization is desired, then using a chassis that naturally localizes and survives in the desired environment is helpful. For example, members of the *Bacteroidaceae* family are abundant in the colon, whereas those of the *Lactobacillaceae* family are also found in the small intestine (23, 35). Thus, one may wish to choose a microbial chassis appropriate for the particular characteristics of the site to be targeted (16, 52). However, many bacteria are still difficult to genetically engineer, so engineering of these chassis may require new transformation methods and gene regulation parts (63). Instead of picking a member of the native flora to use as chassis, circuits in bacteria can be engineered to enhance their ability to colonize a specific environment—for example, by introducing genes that enable the catabolism of new food sources (45).

If long-term colonization is not necessary (e.g., in situations in which repeated dosing of transient bacteria is sufficient for disease surveillance or to increase efficacy), then noncolonizing bacteria that do not perturb the native microbiome may be preferable. EcN is considered safe, as it does not reach high numbers when the native gut microbiota is intact (84) and it survives passage through the GI tract (39).

Beyond the use of microbial biosensors inside humans, living cells can be integrated into living functional materials that exert their effects on humans or in the environment. For example, microbial biosensors can be integrated into biocompatible polymers that provide nutrients and water and then detect multiple chemicals, including biological threats (31, 56) or glucose, for point-of-care diagnosis (17) (**Figure 1*a***). Future challenges include determining how to maintain the viability and functionality of these living materials over long periods of time and how to ensure their robustness to environmental changes.

Furthermore, microbial biosensors can be integrated into biomaterials that are grown from simple cultures. For example, Gilbert et al. (31) created a synthetic symbiotic culture of bacteria and yeast that generates a bacterial cellulose matrix that hosts yeast. These yeasts could be engineered to sense analytes and trigger useful functions, such as degrading β-lactam antibiotics and estrogen hormones, which can contaminate wastewater streams. Other microbial biosensors might be used to detect pathogens (69) or biomarkers, including peptides (4).

FUTURE PERSPECTIVES

The emerging field of synthetic biology offers a potential means to address major challenges in human health, from prevention to diagnosis to treatment. Although microbial biosensors offer several benefits compared to other sensing technologies, microbial biosensor engineering is still in its infancy, and advancements of biosensors into clinical settings will be important to identify areas needing improvement in terms of their performance and safety. Important bottlenecks impeding the deployment of these technologies into the real world lie in the lack of well-validated disease biomarkers and hurdles for clinical translation. As the underlying design-build-test-learn synthetic biology cycle is advancing at a rapid pace, it will be important for regulators, industrialists, and clinicians to help accelerate the testing of these concepts in clinical and industrial settings.

DISCLOSURE STATEMENT

T.K.L. is a cofounder of Senti Biosciences, Synlogic, Engine Biosciences, Tango Therapeutics, Corvium, BiomX, Eligo Bioscience, Bota Bio, and Avendasora. T.K.L. also holds financial interests in Nest.Bio, AmpliPhi, IndieBio, Medicus Tek, Quark Biosciences, Personal Genomics, Thryve, Lexent Bio, MitoLab, Vulcan, and Serotiny.

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