



In vivo biosensors: mechanisms, development, and applications

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Abstract

In vivo biosensors can recognize and respond to specific cellular stimuli. In recent years, biosensors have been increasingly used in metabolic engineering and synthetic biology, because they can be implemented in synthetic circuits to control the expression of reporter genes in response to specific cellular stimuli, such as a certain metabolite or a change in pH. There are many types of natural sensing devices, which can be generally divided into two main categories: protein-based and nucleic acid-based. Both can be obtained either by directly mining from natural genetic components or by engineering the existing genetic components for novel specificity or improved characteristics. A wide range of new technologies have enabled rapid engineering and discovery of new biosensors, which are paving the way for a new era of biotechnological progress. Here, we review recent advances in the design, optimization, and applications of in vivo biosensors in the field of metabolic engineering and synthetic biology.

Keywords Biosensor · Metabolic engineering · Synthetic biology · Metabolite sensing

Abbreviations

ADC	Analog-to-digital converter
ADH	Alcohol dehydrogenase
1,4-BDO	1,4-Butanediol
CAD	Cis-aconitate decarboxylase gene
CCM	Cis,cis-muconic acid
CDA	Cytidine deaminase
2',3'-cGAMP	(2'-5',3'-5') Cyclic guanosine monophosphate-adenosine monophosphate
CE	Capillary electrophoresis
CFP	Cyan fluorescent protein
COMPACTER	Customized optimization of metabolic pathways by combinatorial transcriptional engineering

DFHBI	3,5-Difluoro-4-hydroxybenzylidene imidazolinone
34DHB	3,4-Dihydroxy benzoate
L-DOPA	L-3,4-Dihydroxyphenylalanine
DSRS	Dynamic sensor-regulator system
epPCR	Error-prone PCR
FACS	Fluorescence activated cell sorting
<i>fcy1</i>	Cytosine deaminase gene
FFA	Free fatty acid
FI	Fluorescence intensity
FP	Fluorescent proteins
FPP	Farnesyl pyrophosphate
FREP	Feedback-regulated evolution of phenotype
FRET	Förster resonance energy transfer
GEMM	Genes for the environment, membranes, and motility
GFP	Green fluorescent protein
GlcN6P	Glucosamine 6-phosphate
GlcNAc	N-acetyl glucosamine
<i>glcA</i>	Citrate synthetase gene
GPCR	G-protein-coupled receptors
HHR	Hammerhead ribozyme
Hi-Fi	High-fidelity
HK	Histidine kinase
3-HP	3-Hydroxy propionic acid
IL	Interleukin

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IPTG	Isopropyl-beta-D-thiogalactopyranoside
α -KGDH	α -Ketoglutarate dehydrogenase
LAO	Lysine-binding periplasmic protein
LTTR	LysR-type transcriptional regulator
MAGE	Multiplex automated genome engineering
<i>malQ</i>	Maltase gene
MBP	Metabolite-binding protein
MetN	Methionine-binding protein
MT	Metallothionein
MVA	Mevalonate
NAGK	<i>N</i> -acetyl-L-glutamate kinase
NeuAC	<i>N</i> -acetylneuramine acid
NMM	<i>N</i> -methyl mesoporphyrin IX
OAH	<i>O</i> -acetyl homoserine
OAS	<i>O</i> -acetyl serine
PBP	Periplasmic-binding proteins
PopQC	Population quality control
QS	Quorum sensing
RAGE	RNAi-assisted genome evolution
RBS	Ribosome binding site
ROK	Repressor, open reading frame, kinase
RR	Response regulator
SAM	S-adenosylmethionine
SAH	S-adenosyl-L-homocysteine
SATRE	Sensor-assisted transcriptional regulator engineering
SBA	Streptavidin-binding aptamer
SELEX	Systematic evolution of ligands by exponential enrichment
T6P	Trehalose-6-phosphate
Tc	Tetracycline
TCA	Tricarboxylic acid
TCS	Two-component system
TF	Transcriptional factor
TreR	Trehalose repressor
TRMR	Trackable multiplex recombineering
YFP	Yellow fluorescent protein
YOGE	Yeast oligo-mediated genome engineering
ZF	Zinc finger

Introduction

A biosensor is a biological entity that can be used to report the concentration of a desired compound. The first biosensor was invented to measure glucose in a biological sample using an immobilized glucose oxidase electrode [21]. Since then, numerous biosensors have been constructed in many forms, ranging from electrochemical, fluorescence tagged, nanomaterials, silica or quartz, and genetic constructs with reporter genes [52, 167]. Among them, in vivo biosensors

are designed to enable continuous and real-time monitoring of target metabolites in biological systems, which provide communications into and out of the cell. In vivo biosensors have a number of advantages over other detection methods, which have been summarized in several reviews [27, 52, 167]. Currently, a broad spectrum of metabolites can be detected by in vivo biosensors non-invasively [137, 198]. The emerging in vivo biosensors has begun to be applied in many fields of biotechnology, such as diagnostics, biomedicine, food safety and processing, environmental monitoring, and security. Here, we focus on their applications in the field of metabolic engineering and synthetic biology.

Metabolic engineering enables microbes to produce various valuable products, and many attempts are required before an efficient cell factory is constructed [17]. However, evaluation of microbial phenotypes by the traditional analytical methods such as chromatography and mass spectrometry is slow and cumbersome, which becomes a major rate-limiting step in metabolic engineering. For example, random mutagenesis or genome-scale modifications generate large pools of mutant strains, which are difficult to analyse using the traditional analytical methods. Genetically encoded in vivo biosensors enable rapid and multiplexed phenotypic evaluation, offering the potential for high-throughput screening of novel or improved biocatalysts [32, 125, 137, 151, 198].

Metabolic engineering has greatly benefited from adaptive evolution approaches, which have been used as powerful tools to adapt microorganisms to stress conditions [13] or to improve product formation [5, 183]. Usually, an adaptive evolution approach selects mutants with improved growth and survival. Thus, mutants with improved stress tolerance can be selected [13], and mutants with improved product formation can also be selected if it is a growth-coupled product [5, 41, 183]. When there is no relationship between the desired product and growth, it is hard to use adaptive evolution. In vivo biosensors can sense and respond to desired metabolites, and couple the sensing with a fitness advantage by expressing a gene necessary for survival under selective conditions, expanding adaptive evolution to include phenotypes which are not linked to growth [20, 76, 99].

Another challenge in metabolic engineering is the inefficient use of cellular resources and imbalanced metabolism. For example, excessive expression of a certain pathway gene will burden the host cell and waste cellular resources to produce unnecessary mRNAs and proteins, while uncontrolled or deregulated metabolic pathways may lead to metabolic imbalance, and enzymes or intermediates can accumulate to toxic levels [196]. Furthermore, the balanced distribution of metabolic flux and concentration of key metabolites in a cell are constantly changing depending on the fermentation phases. Synthetic biology is able to design and build novel biomolecular components, networks, and pathways,

and to use these constructs to control and regulate biological systems dynamically [152]. The application of synthetic biology in metabolic engineering can dynamically tune gene expression and intermediate accumulation to proper levels, improving the balance and productivity in microbes [22, 188, 196], but implementation of such control and regulation requires the development of intracellular metabolite sensors.

An exciting application of synthetic biology is focused on imaging or observation of chemicals [8, 23, 74, 135], which is also inspired by the availability of *in vivo* biosensors. The live concentration of metabolites enables metabolic engineers to determine the optimal production condition [135], environmentalists to measure pollutants or toxic compounds [8], or doctors to diagnose disease [23, 74]. Within these applications, new biological systems that do not exist in nature can be designed and built by the assembly of basic genetic parts and modules. Among these genetic parts, *in vivo* biosensors represent a significant contribution [167].

To date, only a few *in vivo* biosensors have been well characterized, which significantly limits their applications. Most *in vivo* biosensors have been designed based on a small number of naturally occurring regulatory systems such as transcription factors [32, 83, 101, 149, 174] and riboswitches [64, 96, 201, 203]. Novel *in vivo* biosensors have also been developed using rational design or random mutagenesis approaches to alter the effector specificities of existing genetic components [16, 19, 25, 65, 134, 159, 160, 185], and we will review their design and optimization strategies in detail in the following sections. Due to limited knowledge, a biosensor for a target product is difficult to generate if an associated transcription factor or riboswitch has not been identified. Advances in systems biology have facilitated the identification of responsive cellular machineries that respond to particular chemical or environmental stresses [22, 93, 123, 147, 158, 192], thus providing an alternative method for biosensor development without the need for isolated transcription factors or riboswitches.

The development of appropriate *in vivo* biosensors mostly relies on several key considerations, such as working range, sensitivity, specificity, and response time. The working range describes the maximum and minimum concentrations of a chemical that the sensor can detect. This range results in a meaningful and accurate output for the sensing system. The desired operational concentration varies by application. In the case of measuring pollutants or toxic compounds, the working range at nanomolar would be ideal, but the working range at millimolar would be more useful in optimizing a pathway to produce a target compound at grams. Several strategies for modulating biosensor operational range have been reported [19, 129]. The sensitivity can be quantified as the ratio of the incremental change in the sensor's output to the incremental change of the measured input. An ideal sensor should have a large and preferably constant sensitivity in

its operating range. Multiple approaches have been reported to increase the sensitivity [101, 150]. The specificity is the extent to which a biosensor is specific for a particular condition or metabolite. A low specificity may cause false and inaccurate readings when applying biosensors. Thus, the need for specific detection and response has to be addressed. Most protein-based biosensors involve the binding of the target molecule to its active site. Such interactions can be extremely specific due to the carefully evolved shape of the active site through natural selection. Another consideration of the biosensor is the response time to a stimulus. *In vivo* biosensors based on protein or nucleic acid act at the transcriptional level or translational level, which, therefore, results in a response time varied from seconds to hours behind actual metabolite changes. In most applications of biosensor, a shorter response time offers substantial advantages such as dynamic regulation [22, 188, 196] and real-time imaging of chemicals [3, 8, 11, 135, 136]. A shorter response time could be obtained using Spinach RNA or a low-molecular-weight protein as the reporter [62, 71, 88].

While biosensors have been reviewed previously [27, 137, 167, 198], this review focuses on the recent advances in the design and engineering of *in vivo* biosensors with increasing numbers of metabolite targets, mechanisms of action, and applications in the past 5 years. Here, two major categories of *in vivo* biosensors are classified based on their diverse mechanisms of sensing, i.e., protein-based and nucleic acid based *in vivo* biosensors. Finally, we discuss future directions to develop and apply *in vivo* biosensors.

Protein-based *in vivo* biosensors

Transcription factor (TF)-based *in vivo* biosensors

In nature, TFs have evolved to regulate gene expression in response to specific metabolites and have been widely used to construct *in vivo* biosensors [98]. These biosensors can offer high sensitivity and dynamic range, where small changes in ligand concentration are amplified through gene expression into large changes in protein abundance. As shown in Fig. 1a, when activated by the target effector molecule, the conformational changes in the activating TF enable it bind to specific DNA sequences in a promoter region and in turn to enhance expression of a reporter gene (e.g., fluorescent reporters, regulatory switches, or selection markers), while the conformational changes in the repressing TF upon effector binding release it from the promoter and allow the expression of the reporter gene. For biosensor design, overexpression of the relevant TF is usually necessary even if it is endogenous to the sensor cells [105, 149]. In general, TF-based biosensors offer a higher potential in their diversity and specificity due to the

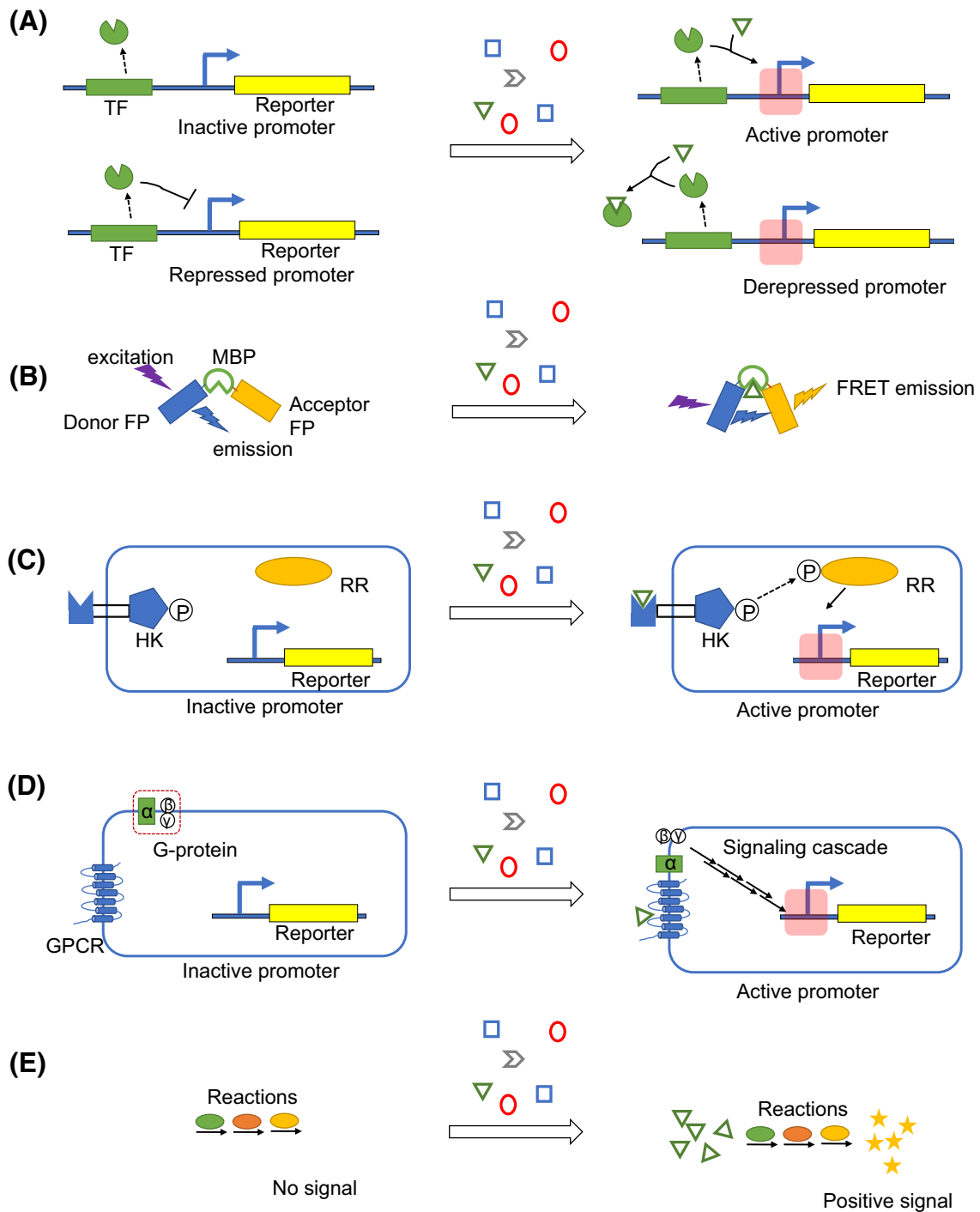


Fig. 1 Schematic of protein-based biosensors. **a** Transcription factor (TF)-based in vivo biosensors; **b** Förster resonance energy transfer (FRET)-based sensors; **c** Two-component system (TCS) biosensor;

d G-protein-coupled receptors' (GPCRs) based sensors; **e** Enzyme-coupled biosensors

versatility and modularity of the functional groups in TFs. Besides, studies have shown that some prokaryotic TFs can also function in eukaryotes [83, 151, 174], further expanding their versatility. TF-based biosensors may have poor orthogonality and high background noise due to potential

uncharacterized interactions between candidate TFs and operator sites [7, 80]. Thus, a successful reconstruction of a TF-based biosensor requires a clear understanding of transcriptional regulation network and protein expression system. Comparative genomics approaches enable in silico

identification of TF-binding sites and regulon reconstruction, such as the LacI family [130] and the ROK (repressor, open reading frame, kinase) family [70].

To date, TFs have been reported to respond to sugars, vitamins, antibiotics, amino acids, secondary metabolites, metal ions, and lipid-related metabolites [98], serving as a large resource of biological components to directly design in vivo biosensors for various metabolites. Bioinformatic approaches and databases have also contributed to the collection, visualization, and analysis of TFs [118], and a Web-based tool has been recently developed to design biosensors by screening for multi-step enzymatic transformations converting non-detectable compounds into detectable ones [28]. Examples for different TF-based biosensors are provided in Table 1. For instance, TFs such as XylR and CebR were known to affect xylose and cello oligosaccharides catabolism, respectively, and a panel of biosensors was generated subsequently to sense xylose [57, 161, 174] and cellobiose [164]. Similarly, NADPH sensors were, respectively, identified in *Escherichia coli* using its native redox sensitive TF SoxR [149] and *Saccharomyces cerevisiae* using its native redox sensitive TF Yap1p [199]. According to this strategy, a variety of metabolite-actuated biosensors have been created, including genetically encoded sensors that respond to L-phenylalanine [94], L-tryptophan [39, 92], lactate [51], acrylate [136], glucarate [136], erythromycin [136], naringenin [102, 136], macrolide [68], *cis,cis*-muconic acid (CCM) [151], 3-hydroxypropionic acid [135, 202], 3,4-dihydroxy benzoate [66], butanol [32], and ferulic acid [97]. Furthermore, several biosensors have been constructed to detect lipid-related metabolites, such as alkane [180], fatty acid/fatty acyl-CoA [162], and malonyl-CoA [83, 186, 188] by re-assembling the natural alkane-responsive TF AlkR, bacterial regulator of fatty acid FadR, and bacterial regulator of fatty acid FapR, respectively. More recent efforts have further engineered natural TFs that can respond to arsenic (e.g., ArsR) [105], zinc (e.g., ZntR) [177], iron–sulfur (e.g., IscR) [50], copper (e.g., MarR) [56], ammonium (e.g., GlnR) [182], and choline (e.g., BetI) [139] into biosensors to measure environmentally and medically relevant concentrations of ions.

The number of known TFs is far lower than the number of metabolites of interest, limiting the application of TF-based biosensors. Hence, expanding the repertoire of compounds detectable by TFs is highly desirable. The alteration of TF effector specificities by various protein engineering approaches to recognize non-natural compounds enhances the availability of sensors with appropriate characteristics. It has already been demonstrated that computational approaches can be used for engineering specificity of TFs relying on a range of strategies that include structure-based predictions [160], metabolite analog searching [197], and

computer-aided design [85]. In synergy with ongoing efforts of biosensor development, computational strategies offer immediate expansion for effectors of a particular TF.

Another potentially generalizable strategy is to create a synthetic TF by fusing a known metabolite-binding protein with a distinct functional domain, whereby the binding of the metabolite of interest brings about a change of activity of the functional domain [20, 43, 165, 194]. In one study, an S-adenosylmethionine biosensor was created by fusing the MetJ repressor to the transcriptional activation domain B42 [165]. The DNA-binding domains of the synthetic TF can be made more programmable using modules with known target DNA sequences such as the zinc finger (ZF) domains. This strategy was applied to generate a novel biosensor conferring maltose-regulated gene expression by fusing a programmable DNA-binding motif (zinc finger module) with a model ligand-binding protein (maltose-binding protein) [194].

Furthermore, random and saturation mutagenesis have shown to be promising in changing the specificity of TF-based biosensors. For example, the approach has been demonstrated in the engineering of the *E. coli* regulatory protein AraC for L-arabinose to activate gene expression in response to triacetic acid lactone [46, 159], or ectoine [16]. In another example, the homology modelling of protein, pobR, which shows high specificity for 4-hydroxy benzoate, led to the identification of key residues for creating 3,4-dihydroxy benzoate response [65]. Through the use of a targeted mutagenesis library, a final mutant was identified with significant induction by 3,4-dihydroxy benzoate. Similarly, variants of qacR were generated by computational protein design and screened for responsiveness to a new targeted effector vanillin [25]. The screen yielded two qacR mutants that respond to vanillin both in vitro and in vivo.

Förster resonance energy transfer (FRET)-based biosensors

Förster resonance energy transfer (FRET)-based genetically encoded biosensors are also used to sense in vivo metabolites. Usually, FRET sensors comprise an internal metabolite-binding protein (MBP) flanked by a pair of donor and acceptor fluorescent proteins (FPs) (Fig. 1b). When MBP binds the metabolite of interest, there is a conformational change to alter the intramolecular distance of the donor and acceptor FPs, where the emission from the donor FP excites the acceptor FP, giving a measurable radiometric index of a metabolite concentration. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) are often used as the pair of donor and acceptor FPs. Compared to TF-based biosensors, FRET sensors have a faster signal response by avoiding the time lag of TF-based biosensors between the induction of gene expression and the signal. Due to this ability to monitor molecules in real time, as shown in Table 1,

Table 1 List of natural and engineered sensors developed and applied mentioned in this review

Sensing elements	Target metabolites	Type of sensor	Host organism	References
Dynamic regulation				
TyrR	Tyrosine	TF	<i>E. coli</i>	[20]
FapR	Malonyl-CoA	TF	<i>E. coli</i>	[90, 186, 188]
FapR	Malonyl-CoA	TF	<i>S. cerevisiae</i>	[24]
FadR	Fatty acids	TF	<i>E. coli</i>	[196]
FadR	Fatty acids	TF	<i>S. cerevisiae</i>	[162]
LuxR	Cell density	TF	<i>E. coli</i>	[91, 153]
EsaRI70 V	Cell density	TF	<i>E. coli</i>	[54]
XylR	Xylose	TF	<i>S. cerevisiae</i>	[57]
GlnR	Ammonium	TF	<i>E. coli</i> , <i>Pseudomonas putida</i> , and <i>Synechocystis</i> sp.	[182]
BetI	Choline	TF	<i>E. coli</i>	[139]
TDAG8	pH	GPCR	HEK293 cells, CHO-K1 cells, hMSC-TERT cells	[6]
OREG	Eugenol	GPCR	Hana3A cells, HEK-293T cells	[114]
OR5P3	Coumarin	GPCR	Hana3A cells, HEK-293T cells	[114]
OR1A1	Dihydrojasnone	GPCR	Hana3A cells, HEK-293T cells	[114]
OR129-1	Acetophenone	GPCR	Hana3A cells, HEK-293T cells	[114]
Lysine riboswitch	Lysine	Riboswitch	<i>C. glutamicum</i>	[201]
A synthetic lysine-ON riboswitch	Lysine	Riboswitch	<i>C. glutamicum</i>	[200]
P _{FUS1}	Cell density	DNA	<i>S. cerevisiae</i>	[178]
P _{ybrL} , P _{gadE} , P _{rstA}	Farnesyl pyrophosphate	DNA	<i>E. coli</i>	[22]
P _{ERG1} , P _{ERG2} , P _{ERG3} , P _{ERG11}	Ergosterol	DNA	<i>S. cerevisiae</i>	[195]
P _{HXT1}	Glucose	DNA	<i>S. cerevisiae</i>	[184]
P _{gas}	pH	DNA	<i>A. niger</i>	[192]
P _{SUC2}	Sucrose	DNA	<i>S. cerevisiae</i>	[179]
Improving the titer and productivity of a product				
Lrp	Valine	TF	<i>C. glutamicum</i>	[99]
BmoR	1-Butanol	TF	<i>E. coli</i>	[32]
LysR	<i>cis,cis</i> -Muconic acid or Naringenin	TF	<i>S. cerevisiae</i>	[151]
The mutated AraC	Mevalonate	TF	<i>M. extorquens</i>	[84]
TtgR	Naringenin	TF	<i>E. coli</i>	[129]
FadR	Fatty acids	TF	<i>E. coli</i>	[181]
TyrR	Phenylalanine	TF	<i>E. coli</i>	[94]
AraC	Ectoine	TF	<i>E. coli</i>	[16]
TnaC	Tryptophan	TF	<i>E. coli</i>	[92]
DIG ₀	Digoxin and progesterone	TF	<i>S. cerevisiae</i> , K562 cells, <i>Arabidopsis thaliana</i>	[43]
Lactate oxidase	Lactate	Enzyme-coupled	<i>E. coli</i>	[171]
Fumarate reductase and peroxidase	Succinic acid	Enzyme-coupled	<i>E. coli</i>	[157]
Naringenin riboswitch	Naringenin	Riboswitch	<i>E. coli</i>	[185]
Theophylline riboswitch	Theophylline	Riboswitch	<i>E. coli</i>	[37]
<i>N</i> -acetylneuramine riboswitch	<i>N</i> -acetylneuramine	Riboswitch	<i>E. coli</i>	[191]
Lysine riboswitch	Lysine	Riboswitch	<i>E. coli</i>	[190]
Flavin mononucleotide riboswitch	Flavin mononucleotide	Riboswitch	<i>B. subtilis</i>	[106]
Glucosamine 6-phosphate riboswitch	Glucosamine 6-phosphate	Riboswitch	<i>S. cerevisiae</i>	[77]

Table 1 (continued)

Sensing elements	Target metabolites	Type of sensor	Host organism	References
Promoter pA	Lysine	DNA	<i>E. coli</i>	[176]
P _{YGP1}	Low pH	DNA	<i>S. cerevisiae</i>	[128]
P _{cysJ} , P _{cysH}	Threonine	DNA	<i>E. coli</i>	[93]
P _{mtt}	Phenylalanine	DNA	<i>E. coli</i>	[100]
Improving substrate utilization and production of key precursors				
XylR	Xylose	TF	<i>S. cerevisiae</i>	[161, 174]
FerC	Substituted cinnamic acid-based compounds	TF	<i>E. coli</i>	[97]
FapR	Malonyl-CoA	TF	<i>S. cerevisiae</i>	[83]
TnaC	Tryptophan	TF	<i>E. coli</i>	[39]
5-HT ₄	Serotonin	GPCR	<i>S. cerevisiae</i>	[38]
Pyranose oxidase	Xylose	Enzyme-coupled	<i>S. cerevisiae</i>	[171]
Isolation and improvement of desired enzymes				
MetJ	S-adenosylmethionine	TF	<i>S. cerevisiae</i>	[165]
DmpR	Phenol	TF	<i>E. coli</i>	[18]
LysG	Arginine	TF	<i>C. glutamicum</i>	[142]
SoxR	NADPH	TF	<i>E. coli</i>	[149]
PcaU	3,4-dihydroxy benzoate	TF	<i>E. coli</i>	[66]
AraC	Triacetic acid lactones	TF	<i>E. coli</i>	[159]
α -Ketoglutarate dehydrogenase	Free CoA-SH	Enzyme-coupled	<i>S. cerevisiae</i>	[86]
DOPA dioxygenase	L-3,4-dihydroxyphenylalanine	Enzyme-coupled	<i>S. cerevisiae</i>	[29]
Lysine riboswitch	Lysine	Riboswitch	<i>E. coli</i>	[173]
In vivo monitoring				
CdaR	Glucarate	TF	<i>E. coli</i>	[136]
AcuR	Acrylate	TF	<i>E. coli</i>	[136]
MphR	Erythromycin	TF	<i>E. coli</i>	[136]
TtgR	Naringenin	TF	<i>E. coli</i>	[136]
Lrp	Valine	TF	<i>C. glutamicum</i>	[115]
AcuR	Acrylate (3-hydroxypropionate)	TF (Enzyme-coupled)	<i>E. coli</i>	[135]
ZntR	Zinc	TF	<i>E. coli</i>	[177]
LldR	Lactate	TF	<i>E. coli</i>	[51]
CebR	Cellobiose	TF	<i>Streptomyces</i>	[164]
IscR	Iron-sulfur	TF	<i>E. coli</i>	[50]
FNR protein	Oxygen	TF	<i>Synechocystis</i> sp.	[63]
Fur	Iron	TF	<i>E. coli</i>	[146]
ArsR	Arsenic	TF	<i>E. coli</i>	[105]
PobR	3,4-Dihydroxybenzoate	TF	<i>E. coli</i>	[65]
BenR	Cocaine	TF	<i>E. coli</i>	[85]
DmpR	Parathion	TF	<i>E. coli</i>	[85]
BenR	Hippuric acid	TF	<i>E. coli</i>	[85]
NarL	Nitroglycerin	TF	<i>E. coli</i>	[85]
Yap1p	NADPH	TF	<i>S. cerevisiae</i>	[199]
LysR	3-Hydroxypropionic acid	TF	<i>Pseudomonas denitrificans</i>	[202]
MphR	Macrolide	TF	<i>E. coli</i>	[68]
AlkR	Alkane	TF	<i>E. coli</i>	[180]
MarR	Copper	TF	<i>E. coli</i>	[56]
LacI	Fucose	TF	<i>E. coli</i>	[160]
LacI	Gentiobiose	TF	<i>E. coli</i>	[160]
LacI	Lactitol	TF	<i>E. coli</i>	[160]
LacI	Sucralose	TF	<i>E. coli</i>	[160]

Table 1 (continued)

Sensing elements	Target metabolites	Type of sensor	Host organism	References
ChnR	Lactam	TF	<i>E. coli</i>	[197]
Maltose-binding protein	Maltose	TF	<i>E. coli</i>	[194]
QacR	Vanillin	TF	<i>E. coli</i>	[25]
LAO-BP	Lysine	FRET	<i>C. glutamicum</i>	[154]
LldR	Lactate	FRET	Astrocytes, HEK cells and T98G glioma cells	[103]
TreR	Trehalose-6-phosphate	FRET	<i>E. coli</i> and <i>S. cerevisiae</i>	[124]
PdhR	Pyruvate	FRET	HEK293 cells	[140]
MetN	Methionine	FRET	<i>E. coli</i> and <i>S. cerevisiae</i>	[111]
LivK	Leucine	FRET	<i>E. coli</i> and <i>S. cerevisiae</i>	[110]
Lysine-binding periplasmic protein	Lysine	FRET	<i>E. coli</i> and <i>S. cerevisiae</i>	[3]
Leucine-binding periplasmic protein	Leucine	FRET	<i>E. coli</i>	[73]
A complex containing Cys ₂ His ₂ -binding pocket	Zinc	FRET	HeLa, HEK293T, MCF-7 and TamR cells	[58]
AmTrac and MepTrac	Ammonium	FRET	<i>S. cerevisiae</i>	[26]
Yap1	Redox	FRET	<i>S. cerevisiae</i>	[120]
HydHG	Zinc or lead	TCS	<i>E. coli</i>	[67, 126]
DcuS/EnvZ	Fumarate	TCS	<i>E. coli</i>	[48]
MalK/EnvZ	Malate	TCS	<i>E. coli</i>	[49]
FlhS/EnvZ	Methanol	TCS	<i>E. coli</i>	[145]
AauS/EnvZ	Acidic amino acids	TCS	<i>E. coli</i>	[132]
OR1G1 and GPR40	Medium-chain fatty acid	GPCR	<i>S. cerevisiae</i>	[113]
CysR and MetX	Sulfur	Enzyme-coupled	<i>C. glutamicum</i>	[59]
Cobalamin riboswitch	Cobalamin	Riboswitch	<i>E. coli</i>	[203]
Thiamine 5'-pyrophosphate riboswitch	Thiamine 5'-pyrophosphate	Riboswitch	<i>E. coli</i>	[193]
S-adenosyl-L-homocysteine riboswitch	S-adenosyl-L-homocysteine	Riboswitch	<i>E. coli</i>	[155]
Theophylline riboswitch	Theophylline	Riboswitch	<i>E. coli</i>	[4]
Tetramethylrosamine riboswitch	Tetramethylrosamine	Riboswitch	<i>E. coli</i>	[4]
Fluoride riboswitch	Fluoride	Riboswitch	<i>E. coli</i>	[4]
Dopamine riboswitch	Dopamine	Riboswitch	<i>E. coli</i>	[4]
Thyroxine riboswitch	Thyroxine	Riboswitch	<i>E. coli</i>	[4]
2,4-Dinitrotoluene riboswitch	2,4-Dinitrotoluene	Riboswitch	<i>E. coli</i>	[4]
NiCo riboswitch	Ni ²⁺ and Co ²⁺	Riboswitch	<i>Clostridium scindens</i>	[47]
Bisphenol A riboswitch	Bisphenol A	Riboswitch	<i>E. coli</i>	[204]
Theophylline riboswitch	Theophylline	Riboswitch	<i>Synechococcus elongatus</i> PCC 7942, <i>Leptolyngbya</i> sp. strain BL0902, <i>Anabaena</i> sp. strain PCC 7120, and <i>Synechocystis</i> sp. strain WHSyn	[96]
Theophylline riboswitch	Theophylline	Riboswitch	<i>Synechocystis</i> sp. PCC 6803	[119]
Theophylline riboswitch	Theophylline	Riboswitch	<i>E. coli</i>	[169]
A synthetic neomycin aptamer	Neomycin	Riboswitch	<i>S. cerevisiae</i>	[72]
GEMM-I riboswitch	Cyclic di-GMP and cyclic AMP-GMP	Riboswitch	<i>E. coli</i>	[71, 175]
GEMM-II riboswitch	(2'-5',3'-5') Cyclic guanosine monophosphate-adenosine monophosphate	Riboswitch	<i>E. coli</i>	[10]
Naringenin riboswitch	Naringenin	Riboswitch	<i>E. coli</i>	[64]

Table 1 (continued)

Sensing elements	Target metabolites	Type of sensor	Host organism	References
P_{cusC} , P_{pcoE}	Copper	DNA	<i>E. coli</i>	[61]
$P_{YDL167C-T3}$ or $P_{YIL104C-T4}$	1-Butanol	DNA	<i>S. cerevisiae</i>	[147]
CdMT promoter	Cadmium	DNA	<i>T. thermophila</i>	[2]
P_{yhljX}	1,4-Butanediol	DNA	<i>E. coli</i>	[158]

FRET sensors have been widely applied in the studies of monitoring intracellular metabolite dynamics, or protein interaction and signal transduction [112]. However, FRET sensors are not used in screening due to their relatively low absolute signals, quantified as emission ratios of FRET acceptor over donor.

Over the years, FRET sensors have been developed to sense a broad range of metabolites, such as sugars [124], amino acids [3, 73, 110, 111, 154], pyruvate [140], lactate [103], and ions [26, 58], and stresses in the environment, such as redox status [120]. The key to constructing FRET sensors is the identification of appropriate MBPs that bind the target products. The reported vast number of metabolite-binding protein scaffolds in the presence and absence of the targets, such as TFs, periplasmic-binding proteins (PBPs), membrane proteins, and other types of ligand-sensing domain, can serve as important resources. For example, bacterial transcriptional proteins can be used as MBPs. A pyruvate sensor named Pyronic was constructed by flanking transcriptional regulator PdhR from *E. coli* with the FPs mTFP and Venus, at the N-terminus and C-terminus, respectively [140]. Exposure of Pyronic to increasing concentrations of pyruvate showed that it can respond to pyruvate between 10 μ M to 1 mM. This enabled high temporal resolution of pyruvate at single cell level in live mammalian cells. Similarly, the lactate-binding TF LldR fused between mTFP and Venus at N- and C-termini, named as Laconic, was capable of quantifying lactate levels from 1 to 10 mM [103]. Beyond TFs, periplasmic-binding proteins have also been used to engineer FRET biosensors [53]. LivK, a bacterial periplasmic-binding protein, was sandwiched by CFP and YFP and expressed in *E. coli* and yeast, which allowed the quantification of leucine between 8 μ M and 1 mM [110]. In a similar way, a lysine sensor was recently designed and developed using the lysine-binding periplasmic protein (LAO) from *Salmonella enterica* serovar typhimurium LT2 strain as the binding element for the FRET sensor [3]. The developed sensor was found to be specific to lysine and can work in bacterial as well as yeast cell. Furthermore, membrane proteins also contributed the construction of FRET sensors. For example, ammonium transceptors, which are membrane proteins that possess both solute transport and receptor-like signaling activities, were applied to ammonium sensing [26]. Addition of ammonium to yeast cells

expressing the sensors triggered concentration-dependent fluorescence intensity (FI) changes.

Two-component system (TCS) biosensor

TCSs allow microbes to sense environmental changes or target metabolites. The previous studies have demonstrated that TCS could be designed and engineered as in vivo biosensors for different applications [131]. Typically, a TCS system includes a histidine kinase (HK) and a response regulator (RR) (Fig. 1c). The presence of environmental changes or target metabolites induces autophosphorylation of HK, followed by phosphoryl transfer to the RR. The phosphorylated RR further regulates the expression of a reporter gene. HK is a transmembrane protein. The C-terminal autokinase domain of HK is usually in the cytosol, and its N-terminal domain for stimulus sensing could be localized in the extracytoplasmic compartment (periplasm, inner or outer membrane, or even extracellular space). The localization of the N-terminal sensor domain enables the TCS sensor to sense metabolites in different cytoplasm or extracellular space, which is not possible for many other sensors if the metabolite is membrane-impermeable. Examples are provided in Table 1.

The HK domains of natural TCS, which have a variety of signal recognition capabilities, could be adopted to construct a broad range of TCS sensors. In *E. coli*, an HydHG (also known as ZraSR) TCS was found to have a response to zinc and lead [81]. The HydHG TCS was subsequently engineered to respond to different zinc concentrations, with the threshold level reduced via the introduction of a self-activation loop and elevated by genomic integration [126]. Likewise, the same TCS also contributed to the development of a lead sensing and removal system [67].

It is reported that the chimeric HK strategy can be employed for the development of powerful TCS sensors, in which the sensory domain (N-terminal) and catalytic domain (C-terminal) from different HKs are combined. Because the EnvZ–ompR complex in *E. coli* is a well-studied TCS that is widely distributed in bacteria, the sensory and catalytic domains of EnvZ are commonly used in domain swapping strategies. For example, *E. coli*, which does not have the methanol sensing apparatus, was engineered to sense methanol by employing chimeric HK strategy [145]. The TCS biosensor was constructed by fusing the methanol sensing

domain of *Paracoccus denitrificans* FlhS with the catalytic domain of *E. coli* EnvZ. The constructed chimeric FlhS/EnvZ TCS enabled the cell to sense methanol by the expression of *ompC* and GFP (green fluorescent protein) gene regulated by *ompC* promoter. Similarly, a chimeric DcuS/EnvZ (DcuSZ) TCS was constructed by fusing the sensor HK of DcuS with the cytoplasmic catalytic domain of EnvZ [48]. The output signals produced by the chimeric DcuSZ TCS were enough to detect fumarate concentration quantitatively. Another demonstration of the chimeric strategy is the development of malate TCS sensor based on chimeric MalK/EnvZ [49], which was created by fusing the sensor domain of MalK (*Bacillus subtilis*) to the catalytic domain of EnvZ. In the same manner, a chimeric AauS/EnvZ TCS was built by integration of the sensing domain of AauS (*Pseudomonas putida*) with the catalytic domain of EnvZ to control the expression of the *ompC* gene in response to acidic amino acids [132].

G-protein-coupled receptors (GPCRs)-based biosensors

The GPCRs' system (also known as seven transmembrane receptors, 7TMRs) is a class of transmembrane receptors that transduces various extracellular signals into intracellular responses, making itself an attractive candidate for engineering and development of in vivo biosensors [109]. As shown in Fig. 1d, when GPCRs are activated by extracellular signals, the GPCR would couple to a G-protein composed of α -, β -, and γ -subunits, dissociating the heterodimer of β - and γ -subunits from the α -subunit and activating downstream effectors. Similar to other protein-based biosensors, the versatile nature of GPCRs makes them broadly useful for metabolite sensing (Table 1). However, GPCR-based biosensors are usually limited to extracellular sensing.

A recent study was reported to construct medium-chain fatty acid biosensors using GPCRs known to bind fatty acids in mammalian (OR1G1 and GPR40) [113]. One of the sensors detected even-chain C8–C12 fatty acids with dynamic range up to 17-fold increase after activation. Introduction of a synthetic response unit gave a further improvement in the response to decanoic acid using the GPCR OR1G1. Later, the same group swapped OR1G1 with GPCRs known to bind serotonin, and kept the rest of the sensor cell intact, which resulted in a serotonin sensor [38]. Among the six known human serotonin GPCRs, 5-HT₄-based sensor performed better, and the sensor was also demonstrated to work in the 96-well plate format with reliable statistical parameters. In another example, using the odorant receptors specific for the volatile fragrance compounds, a series of GPCR-based sensors were constructed to sense the volatile fragrance compounds eugenol, coumarin, dihydrojasnone, and acetophenone [114], upon which the authors designed a synthetic

multi-layered gaseous-fragrance-programmable analog-to-digital converter (ADC). Similarly, a GPCR-based pH sensor was created to sense pH value with the help of the human proton-activated cell-surface receptor TDAG8 [6]. Besides organic acids, this sensor could also be adjusted by CO₂ that shifts the CO₂-bicarbonate balance toward hydrogen ions.

For GPCRs, the structure-based drug design of receptors received limited success until recently due to the low progress in determination of GPCR crystal structures [69]. Currently, the high-resolution crystal structures of GPCRs enabled the ligand discovery for several pharmaceutically relevant receptors [75, 79]. However, the structure-based approach has not been applied in the construction of GPCR biosensors.

Enzyme-coupled biosensors

Another kind of protein-based in vivo biosensor relies on enzyme-coupled reactions. This biosensor takes advantage of enzyme-catalysed reactions that can convert a target metabolite into detectable chromogenic and fluorogenic substances (Fig. 1e). The development of this sensor requires a priori knowledge of relevant reactions. Examples of these biosensors can be found in Table 1.

In some cases, the required reactions can be accomplished in one-step. For example, an enzyme-coupled biosensor for L-3,4-dihydroxyphenylalanine (L-DOPA) was developed by converting L-DOPA into the yellow, fluorescent pigment betaxanthin with the help of DOPA dioxygenase [29]. Using this sensor, a tyrosine hydroxylase with 2.8-fold improved activity was identified, which was able to further improve the yields of L-DOPA. In another study, a biosensor for free CoA-SH was developed through a coupled reaction by monitoring NADH production with α -ketoglutarate dehydrogenase (α -KGDH), which can be quantified by absorbance at 340 nm [86].

In other cases, a cascade of reactions was required to form a detectable molecule. As an example, xylose or lactate can be detected via enzyme-coupled biosensors. It was shown that pyranose oxidase and lactate oxidase would oxidize xylose and lactate, respectively, with concomitant production of byproduct, hydrogen peroxide (H₂O₂). The H₂O₂ byproduct was subsequently used to oxidize Amplex UltraRed by horseradish peroxidase, leading to the generation of the detectable, fluorescent compound resorufin [171]. In principle, the assay described in this work allowed for use of any oxidase enzyme, which could be considered as a generalized method to construct enzyme-coupled biosensors for other products of interest. In a similar example, an enzyme-coupled biosensor for succinic acid was established using fumarate reductase and peroxidase [157]. In this reaction, fumarate reductase can catalyse succinic acid to fumarate

and produce H_2O_2 , which can be further detected by the mentioned peroxidase-dependent fluorescent assay.

To create enzyme-coupled biosensors, there are also several cases combining the use of enzymatic and chemical reactions. Recently, an enzymatic assay for cytidine was developed by combining cytidine deaminase (CDA) and the indophenol method [33]. CDA catalysed the cleavage of cytidine to uridine and NH_3 , the latter of which can be accurately determined using the indophenol method. The sensor enabled a sensitive and convenient assay for cytidine detection, which was used to screen cytidine producing strains. The strategy was also adopted by the same group to construct a biosensor for adenosine [34].

Nucleic acid-based biosensors

RNA-based biosensors

Nucleic acid-based sensors work as an alternative to protein-based biosensing systems. Compared to protein-based sensors, nucleic acid-based *in vivo* biosensors are usually specific to a limited number of metabolites, but show advantages such as faster response time and decreased metabolic burden on cells [89]. Furthermore, nucleic acid-based biosensors can, in principle, be combined as tandem copies, leading to a tighter regulation of gene expression.

There are two popular kinds of nucleic acid-based biosensors, which are based on RNA or DNA. In general, RNA riboswitch is a genetic control element of a messenger RNA molecule that binds a small molecule, resulting in a change in production of the protein encoded by the mRNA. RNA riboswitch can selectively bind to various metabolites (via RNA aptamer domains) and change its own structure, which thereby modulates the transcription of a measurable read-out such as fluorescence, enzyme activity, and cell growth (Fig. 2a). Aptazyme is a variation of the RNA riboswitch, in which the output domain is a hammerhead ribozyme whose activity depends on aptamer binding status where ligand binding induces the RNA to cleave itself (Fig. 2a). RNA riboswitches have been engineered as *in vivo* biosensors responsive to target molecules of interest [137, 198]. Currently, as shown in Table 1, most of the RNA-based biosensors have been reported in bacterial systems, and few could be found in yeast, due to the fact that the majority of riboswitches discovered in bacteria are non-functional in yeast.

Aptamers are the sensing elements of the riboswitch construct. Metabolite-binding RNA aptamers targeting small molecules of interest can be mined from natural metabolite-regulation systems. For example, the lysine riboswitch is one natural riboswitch. Based on it, genetic RNA biosensors were constructed to monitor changes in the intracellular concentration of lysine [173, 190, 200, 201]. Furthermore, the

construction of theophylline sensors using the well-characterized theophylline aptamer as the sensor element has also been demonstrated [37, 96, 119, 169]. Ribozymes have been developed as *in vivo* biosensors for *N*-acetylneuraminic acid [191] and glucosamine 6-phosphate [77], while the Spinach RNA aptamer, which was described to emit green fluorescence on binding with 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) [122], was applied in the development of thiamine 5'-pyrophosphate, [193] and S-adenosyl-L-homocysteine [155] biosensors. These examples will be further elaborated in the application section. On the other hand, it was reported that riboswitches were found for specific heavy metal ions using comparative sequence analysis to search bacterial genomes and metagenomic sequences [47]. This study finally demonstrated and validated the discovery of riboswitch RNAs to sense Ni^{2+} and Co^{2+} . In yeast, an RNA sensor was established in response to neomycin by attaching a synthetic neomycin aptamer to the catalytic core of the type III hammerhead ribozyme (HHR) from *Schistosoma mansoni* [72]. These innovations enabled the development of neomycin-dependent RNA modules that switch gene expression up to 25-fold. It can also be used as a general strategy to create novel yeast synthetic riboswitches by combining an *in vitro* selected synthetic aptamer to the catalytic core of ribozyme such as HHR.

Metabolite-binding RNA aptamers can also be generated towards a ligand of choice by screening a pool of synthetic aptamer libraries. In particular, in the past 20 years, the systematic evolution of ligands by exponential enrichment (SELEX) and its enhancements, such as High-Fidelity (Hi-Fi) SELEX, semi-automated SELEX, capillary electrophoresis (CE) SELEX, capture-SELEX, and branched SELEX, have yielded new aptamers that respond to a plethora of compounds [55]. The traditional SELEX can select aptamers using iterative cycles of binding, partition, and amplification. In a recent example, a single-stranded RNA was isolated that can respond to naringenin from combinatorially prepared nucleic acid libraries using SELEX [64, 185]. The isolated riboswitch contributed to the construction of a naringenin sensor, which displayed an increased fluorescent signal generation after activation upon binding with naringenin. In another study, a new platform, Hi-Fi SELEX, was reported that introduced novel fixed-region blocking elements to improve the functional diversity of the starting library [121]. Then, the improvement of Hi-Fi SELEX to aptamer selections was demonstrated using human α -thrombin as the target. Besides, to shorten the time of selection in SELEX, capillary electrophoresis (CE) was reported to be more efficient for the separation of bound and unbound DNA/RNA. CE-SELEX has been reported to select DNA aptamers for large-molecule targets, such as cancer biomarker HE4 protein [36], and small-molecule targets, such as *N*-methyl mesoporphyrin IX (NMM) [189].

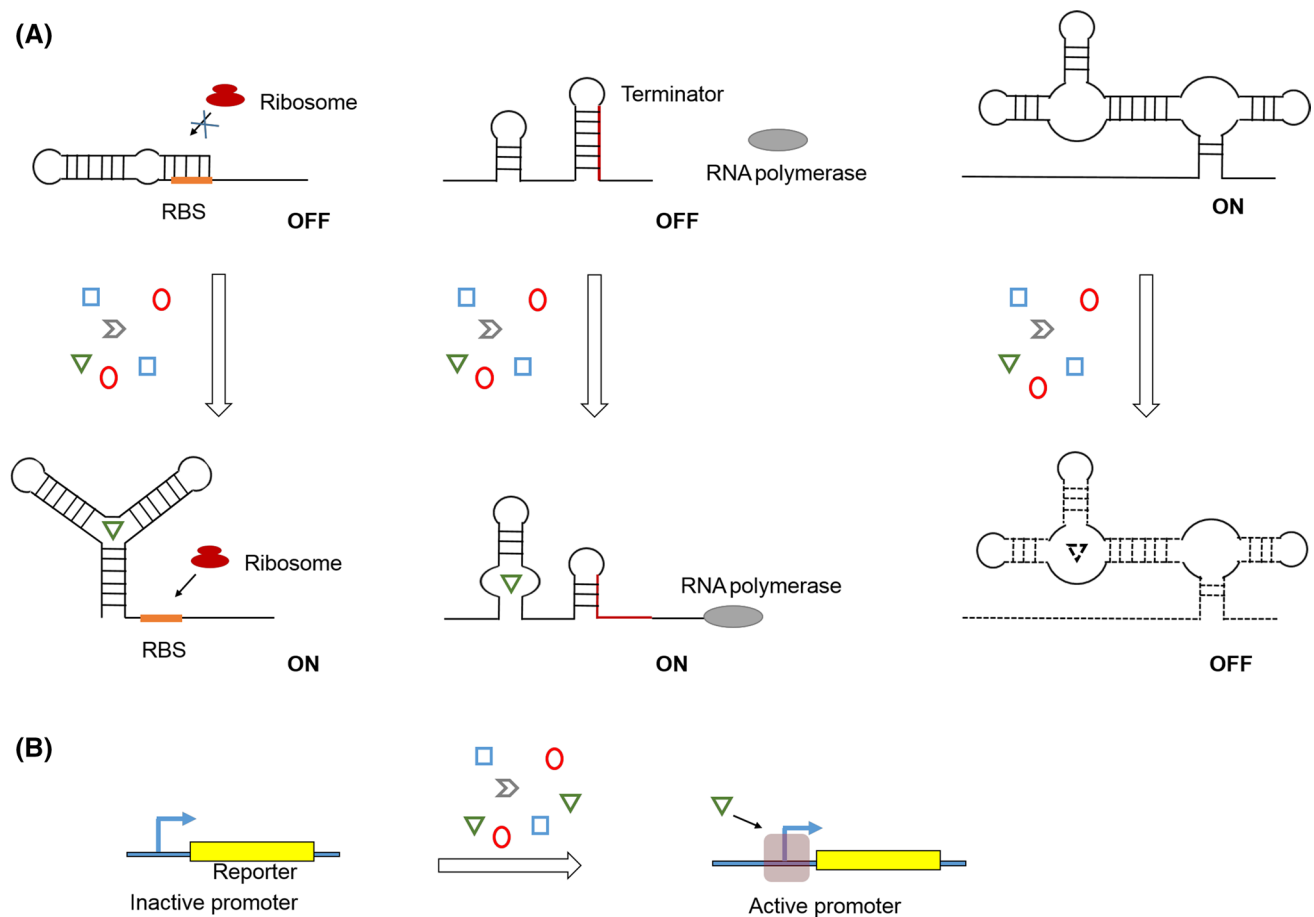


Fig. 2 Schematic of nucleic acid-based sensors. **a** RNA-based biosensors. Left: binding ligand results in changes in mRNA secondary structure and renders the ribosome binding site (RBS) inaccessible to ribosome; Middle: in the absence of ligand, the intrinsic terminator stops transcription, while binding ligand eliminates the terminator

structure; Right: binding ligand activates the hammerhead ribozyme and triggers self-cleavage, which subsequently leads to mRNA degradation. **b** DNA-based biosensors. Binding of target metabolites to the promoter enables the expression of reporter gene

These studies demonstrated that CE-SELEX reduced the selection time and the number of cycles. Later, a fraction collection approach was developed in capillary electrophoresis SELEX (FCE-SELEX) for the partition of a bound DNA-target complex [95]. By integrating fraction collection with a facile oil seal method, in a single round of selection, a streptavidin-binding aptamer (SBA) was generated. There are also other emerged solutions to detect the binding of a target molecule to an RNA. In two recent studies, it was demonstrated that select riboswitch readout domains from the existing riboswitches, called expression platforms, can be engineered using simple design rules to host natural or synthetic aptamers to create novel chimeric RNAs that regulate transcription both in vitro and in vivo [14, 15]. In another example, the aptamer domain of a known switch that regulates expression of the marker TetA linked to GFP was replaced with 40 random bases [204], and then, the library was transformed into *E. coli*. The dualistic nature of TetA allowed the application of dual genetic selection

to identify riboswitches that expressed TetA-GFP only in response to bisphenol A. Furthermore, structure information could be used to help the screening. For example, structure-guided chemical genetic screening of the natural adenine-binding *add* A aptamer was performed, and two additional ligands [pyrimido(4,5-d)pyrimidine-2,4-diamine and 2-aminopyrimido(4,5-d)pyrimidin-4(3H)-one] were identified that bound to the aptamer with high affinity, exhibiting improved in vivo gene-induction properties and reduced cellular toxicity [134]. The same group also proposed targeted mutagenesis of native riboswitches to change their specificity toward rationally designed synthetic ligand analogs [133]. In parallel, a novel bioinformatics-guided selection and screening strategy was used to rapidly isolate aptamers against two different targets: the human interleukin (IL)-10 and human 4-1BB receptors [82].

Currently, only a limited number of RNA aptamers have transitioned into effective and widely used intracellular biosensors, and many do not reliably function in a

cellular context. To solve this problem, it was proposed that recurrent structural folds found in natural RNA aptamers and small nucleolytic ribozymes can be reprogrammed to host a broad spectrum of small molecule-binding sites while preserving the robust folding and highly stable architectural properties of the parent [127]. Using scaffolds derived from two different riboswitch aptamer domains and a ribozyme, a diverse set of aptamers were obtained to recognize 5-hydroxytryptophan, serotonin, and dopamine. Recently, computational tools were developed to rationally design riboswitches using the existing aptamers [55, 169]. A statistical thermodynamic model was developed to predict the sequence–structure–function relationship for translation-regulating riboswitches [4]. Using the model, automated computational design of 62 synthetic riboswitches was carried out using six different RNA aptamers to sense diverse chemicals (theophylline, tetramethylrosamine, fluoride, dopamine, thyroxine, and 2,4-dinitrotoluene). Moreover, the development of fluorescent biosensors that exhibited a response to (2'-5',3'-5') cyclic guanosine monophosphate-adenosine monophosphate (2',3'-cGAMP) was reported [10]. The biosensor was designed by making rational mutations to natural riboswitch aptamers that recognize the related molecule 3',3'-cyclic di-GMP. In another example, a fluorescent biosensor for c-di-GMP was first generated by fusing a natural GEMM-I (genes for the environment, membranes, and motility) riboswitch aptamer to Spinach [71]. Then, rational mutations to the ligand-binding pocket guided by structure were exploited to alter specificity of the RNA-based biosensor to c-AMP-GMP. Later, the same group constructed a series of improved biosensors that were up to 450% brighter and 13 times faster, which together enabled detection of c-di-GMP from picomolar to micromolar concentrations [175].

DNA-based biosensors

DNA can also serve to detect molecules *in vivo*. In nature, cells are evolved with responsive promoters to sense stress or metabolites. Like other sensors, DNA-based sensors are also comprised of different genetic elements: a sensing element (metabolite-responsive promoters) and a reporter gene (Fig. 2b). Identification of the appropriate promoter is usually considered as the most crucial step. Examples for different DNA-based sensors are provided in Table 1.

Currently, many inducible promoters have been reported for the inducer molecule, such as isopropyl-beta-D-thiogalactopyranoside (IPTG) [1], arabinose [116], lactose [87], and tetracycline (Tc)-regulated gene expression system [107]. Meanwhile, many promoters that respond to target molecules could be mined from the published literature. The devices lysGE, pN, and pA from *C. glutamicum* ATCC13032 were reported to be capable of responding to lysine [9]. When these devices were transferred to *E. coli*,

the promoter pA responded to the change of extracellular lysine concentration [176]. Metallothioneins (MTs) are metal-binding proteins involved in cellular protection from metal toxicity. After investigating promoters that drove the expression of MTs, the CdMT promoter was found to have a good response to cadmium ions [2]. In another example, by mining literature, sucrose responsive promoters were identified from selected candidates which were repressed during growth on glucose, and upregulated during growth on sucrose [179].

When intermediate metabolites accumulate to toxic levels, one of the native stress responses is the change of promoter activity, which allowed the identification of novel metabolite-responsive promoters. For example, promoters that responded to farnesyl pyrophosphate (FPP) were identified by whole-genome transcriptome analysis in *E. coli* [22]. In *Aspergillus niger*, a low pH-inducible promoter, P_{gas}, was also identified by transcriptional analysis [192]. The strength of P_{gas} was independent of acid type and acid ion concentration, showing dependence on pH only. Similarly, application of transcriptome analysis was also applied to identify responsive promoters to 1-butanol [147] and 1,4-butanediol (1,4-BDO) [158]. Moreover, novel threonine promoters were discovered upon the proteomic analysis of *E. coli* in response to extracellular threonine [93]. In parallel, a phenylalanine-responsive promoter was screened from a promoter library [100].

Rational design and engineering of responsive promoters were also reported. Recently, a set of promoters that were inducible under acidic conditions were selected from literature searches and data mining [128]. By engineered the upstream activating sequence, the *YGPI* promoter was successfully engineered for improved performance at low pH. The engineering strategy outlined for the *YGPI* promoter was subsequently used to build low pH induction into native promoters lacking this response.

Applications of *in vivo* biosensors in synthetic biology and metabolic engineering

In addition to the examples described above, there are many advanced applications of the *in vivo* biosensors, such as dynamic regulation of pathways, optimizing the product titers, enhancing substrate utilization and precursor availability, isolation and improvement of desired enzymes, as well as *in vivo* monitoring of the target compounds (Table 1). We will discuss these applications in detail below. Applications in each category will be broadly grouped according to biosensor types.

Dynamic regulation

Balanced utilization of cellular resources, in particular the control of metabolic intermediate fluxes and biosynthetic pathway genes, is important for the production of value-added chemicals in microorganisms. Despite recent advances in synthetic biology, most control systems for heterologous pathways still rely on constitutive or inducible promoters, for which the outputs are optimized for a particular condition and are static in nature, unable to respond to growth and environmental changes [22]. Moreover, these static control systems may not be suitable when piecing together a complicated pathway with biosynthetic modules with mismatched input/output levels, or when there is a need to minimize the accumulation of potentially toxic intermediates [60]. In contrast, dynamic regulation enables microorganisms to adapt to changes in their internal or external conditions. A dynamic regulation system consists of a sensing component, which can detect the metabolite of interest or physiological state, and a regulator component, which converts the sensor signal to into a transcriptional signal, often resulting in the upregulation or downregulation of a key pathway gene [196]. The ability to autonomously regulate gene expression and, consequently, metabolic flux through the biosynthetic pathways in response to its environment enables cells to utilize resources efficiently and improve productivity, and also minimize the need for chemical inducers and human intervention of fermentations. Dynamic regulation has been hailed as a new frontier in metabolic engineering [12, 104, 166]. Since the one of the first demonstrations of the dynamic flux control to improve yield and productivity of a heterologous pathways by Farmer and Liao in 2000 [40], the number of dynamic regulation systems has grown, particularly in the past 5 years. Here, we present recent applications of riboswitch-, transcription factor-, and promoter-based biosensors.

Lysine-responsive riboswitches from *E. coli* and *B. subtilis* were used to control competing but essential metabolic by-pathways of lysine biosynthesis in *Corynebacterium glutamicum* [201]. Citrate synthetase (*gltA*) gene encoding the first enzyme of the tricarboxylic acid (TCA) cycle was placed under the control of the lysine-OFF riboswitches, which repress translation initiation in the presence of lysine, thus repressing TCA cycle activity. This allowed metabolic fluxes to be directed towards lysine biosynthesis instead of cell growth, and enhanced lysine yield by up to 63% in the resulting LPECRS strain. To expand the *C. glutamicum* engineering toolbox and explore the potential synergistic effects of a combined ON–OFF control, a lysine-ON riboswitch was developed by screening a riboswitch library with randomized sequences

between the lysine aptamer and the ribosomal-binding site using the *tetA*-based dual selection system [200]. A lysine-ON riboswitch, which upregulated the expression of the downstream gene by more than 18-fold in the presence of 0.1 mM lysine, was identified from the screening. The lysine-ON riboswitch was then introduced into the LPECRS strain [201] to control the expression of *lysE*, a gene encoding a lysine transporter protein. The lysine production yield of the resulting strain with the dual lysine-OFF and -ON riboswitches was enhanced by 21% over the parent LPECRS strain.

Chou et al. developed the feedback-regulated evolution of phenotype (FREP) system that regulated the mutation rate of the cell population inversely to the target metabolite concentration [20]. Metabolite sensors were assembled from natural or synthetic metabolite-responsive TF to gauge the concentration of the target metabolite and regulate the mutator *mutD5* expression. Under low metabolite concentration, mutation rate was increased to generate greater diversity in the cell population. Conversely, mutation rate was decreased in the presence of high metabolite concentration, thereby fixing the genotype of the cell population to the high production phenotype. As proof-of-concept, FREP was used to evolve *E. coli* strains for increased production of tyrosine and isoprenoid.

Malonyl-CoA is an important building block for a variety of biofuels, fine chemicals, and pharmaceuticals, and is the rate-limiting precursor in the chain elongation reaction during the synthesis of these compounds [42, 45, 187]. Hence, optimizing metabolic flux control through the malonyl-CoA node has also been the subject of numerous microbial cell factory engineering efforts. Central to the development of malonyl-CoA-responsive biosensors for regulating the malonyl-CoA pathways is the transcription factor, FapR, from *B. subtilis* [144]. FapR binds to its DNA operator sequence in the absence of malonyl-CoA to repress the downstream gene, and upon binding of malonyl-CoA, changes its conformation and releases from the operator sequence to allow transcription of the downstream gene [143]. FapR and its cognate operator sequence were transplanted into *E. coli* to build a malonyl-CoA sensor [188], and the sensor was applied in a feedback regulatory circuit to regulate fatty acid synthetic pathway in *E. coli*, improving the productivity and titer by 33 and 34%, respectively [90]. Discovery of the P_{GAP} promoter in *E. coli*, which is positively regulated by FapR in the absence of malonyl-CoA, enabled the creation of a feed-forward regulatory circuit that activates fatty acid synthesis in the presence of high malonyl-CoA concentrations [186]. When implemented together with the feedback circuit, the malonyl-CoA regulatory system improved fatty acid synthesis by more than twofold compared to the unregulated *E. coli* strain. In a hierarchical dynamic control system developed by David et al. for improved production of 3-hydroxy

propionic acid (3-HP) in *S. cerevisiae* [24], malonyl-CoA was channeled to fatty acid synthesis in the growth phase by subjecting the fatty acid synthase gene to the control of the glucose-inducible P_{HXTI} promoter. In the fermentation stage, the fatty acid synthesis will be downregulated due to glucose limitation, and the malonyl-CoA accumulated will be directed towards 3-HP production by FapR which upregulates the expression of the malonyl-CoA reductase gene.

A dynamic sensor-regulator system (DSRS) based on the fatty acid/acyl-CoA-responsive TF, FadR, was developed to regulate the expression of genes involved in the production of fatty acid-based products [196]. The FadR sensor was used to regulate the pyruvate decarboxylase and alcohol dehydrogenase genes for ethanol production, as well as the acyl-CoA synthase and wax-ester synthase genes in response to fatty acid accumulation, achieving 1.5 g/L of fatty acid ethyl ester titer and a threefold improvement in theoretical yield in *E. coli* over the non-dynamically regulated strain, as well as increased strain stability.

The competition for cellular resources between native and heterologous pathways in a production host often results in poor cell growth and productivity [78]. Other than using glucose-responsive promoters such as P_{HXTI} as sensors to initiate the redirection of metabolic flux after the growth phase, quorum sensing (QS) systems, which enable bacterial cells to detect local cell population density via extracellular signaling molecules and regulate gene expression in a coordinated manner in response [108], have emerged as an attractive method of separating growth from production phases in industrial microorganisms. In recent years, QS circuits have been applied in the dynamic redirection of metabolic flux from the central metabolic pathways in the growth phase to heterologous pathways for the production of isopropanol and 1,4-butanediol in *E. coli* [91, 153], and *para*-hydroxybenzoic acid in *S. cerevisiae* [178]. A tunable pathway-independent genetic control module based on the Esa QS system from *Pantoea stewartii* was engineered to regulate target genes in *E. coli*. In this system, the trigger to redirect glycolytic flux into heterologous pathways can be optimized for individual systems by controlling the strength of the promoter-ribosomal-binding site driving the expression of 3-oxohexanoylhomoserine lactone synthase, EsaI [54]. This module was applied to improve *myo*-inositol titers by 5.5-fold, and increase titers of glucaric acid and shikimate from undetectable to more than 0.8 g/L and 100 mg/L, respectively.

Farnesyl diphosphate (FPP) is a common precursor for mevalonate pathway-based isoprenoids, which includes high-value compounds such as drugs (artemisin and taxol), flavors and fragrances, and advanced biofuels. However, FPP accumulation is toxic to common production hosts such as *E. coli* and *S. cerevisiae*. Hence, maintaining a high metabolic flux through the FPP node while keeping

its intracellular concentration low is important to developing industrial isoprenoid production strains. Creating a dynamic control system to achieve this goal would require a biosensor that can detect and regulate the pathway flux through the FPP node. To address the lack of known natural sensors for FPP, Dahl et al. applied whole-genome transcript arrays to identify promoters that respond to the accumulation of toxic FPP, and applied the promoters to regulate the production of FPP in the isoprenoid biosynthetic pathway in *E. coli* [22]. Consequently, amorphaadiene production in the dynamically regulated *E. coli* strain was increased twofold over production strains with inducible or constitutive promoters. This strategy circumvents the need for known sensors for metabolites of interest, of which there are very limited numbers, and it also eliminated the need for expensive inducers. In another study targeting the branch point at the FPP node, a set of ergosterol-repressed promoters from the yeast native ergosterol biosynthetic pathway were used to downregulate the expression of *ERG9* when ergosterol was in excess [195]. This feedback regulation of *ERG9* enhanced the metabolic flux towards the production of a non-native isoprenoid, amorphadiene, and improved its titer by up to fivefold in yeast. Using glucose-responsive promoters as sensors, Xie et al. developed a sequential control strategy to dynamically control the downstream, upstream, and competitive pathways from the FPP node of the carotenoid production yeast strain [184]. In this system, a glucose-repressed-modified GAL regulation system was used to regulate *tHMG1*, the rate-limiting gene of the upstream mevalonate (MVA) pathway, and the beta-carotene biosynthetic pathway, while the *ERG9* gene, encoding squalene synthase which commits FPP to the competing ergosterol biosynthetic pathway was placed under the control of a glucose-inducible promoter, P_{HXTI} . In the growth stage, the ergosterol synthetic pathway, which is essential for cell viability, was expressed under high glucose concentration to sustain cell growth. In the second stage, under glucose limitation, *tHMG* and the beta-carotene pathway genes were induced, while the squalene pathway was downregulated, maximizing carotenoid production. Subsequently, a titre of 1165 mg/L carotenoids was achieved using this inducer-free strategy in a fed-batch fermentation.

In other promoter-based dynamic regulation applications, a low pH-inducible promoter, P_{gas} , was identified using transcriptome analysis for the dynamic control of *Aspergillus niger* for the itaconic acid production [192]. Placing the *cis*-aconitate decarboxylase (*CAD*) gene under the control of the low pH-inducible promoter, P_{gas} , enhanced itaconic acid titer in *A. niger* by more than fivefold compared to the strain with *CAD* placed under a strong constitutive promoter, P_{gpdA} .

Improving product titer and productivity

Naturally occurring organisms are seldom optimized to produce industrially relevant molecules, and extensive modifications to the cells are often required to achieve economically viable production. To this end, directed genome evolution approaches, such as multiplex automated genome engineering (MAGE) [172], trackable multiplex recombineering (TRMR) [141], yeast oligo-mediated genome engineering (YOGI) [31], the heritable recombination system [138], and RNAi-assisted genome evolution (RAGE) [148], are highly efficient for strain development, while combinatorial pathway optimization methods such as customized optimization of metabolic pathways by combinatorial transcriptional engineering (COMPACTER) [35] have been shown to optimize the expression of pathway genes. However, our ability to create the genetic diversity using these methods far outstrips the throughput of current analytical methods. Biosensors provide an alternative to bridge this gap by converting hard-to-quantify phenotypes, for example accumulation of non-colorimetric small molecules, to easily measurable parameters such as fluorescence and growth, thereby providing a quantifiable link between genotype and phenotype amenable for high-throughput assays. Here, we present some of the recent applications of the various forms of biosensors to address the high-throughput screening bottleneck.

Driven by an L-valine responsive sensor based on the Lrp transcription regulator from *C. glutamicum*, the L-valine production in *C. glutamicum* was improved by 25%, and byproduct formation was reduced by more than threefold through adaptive laboratory evolution [99]. The alcohol-responsive BmoR transcriptional activator from *Pseudomonas butanov*a was engineered to a 1-butanol biosensor regulating *tetA* expression, and enabled the isolation of an *E. coli* strain with 35% improvement in 1-butanol-specific productivity through optimization of pathway enzyme expression [32].

Using a multi-parametric screening approach, a functional design for biosensors based on LysR-type transcriptional regulators (LTTRs) in *S. cerevisiae* was developed [151]. Notably, this approach enabled the direct transplantation of small molecule-binding prokaryotic transcriptional activators and their cognate DNA operators into *S. cerevisiae* to create metabolite biosensors without the need for further engineering. As proof-of-principle, the CCM and naringenin biosensors developed by this approach were applied for in vivo screening of cells, and successfully identified strains with improved product titers.

Liang et al. developed a strategy termed sensor-assisted transcriptional regulator engineering (SATRE) based on

the mevalonate-responsive biosensor to improve acetyl-CoA flux from methanol to mevalonate in the methylotrophic cell factory, *Methylobacterium extorquens* AM1 [84]. Two million variants from a QscR transcriptional regulator library were screened in one round by flow cytometry using the mevalonate biosensor-based system, yielding a mutant strain (Q49) with 60% increase in mevalonate concentration, and capable of producing 2.67 g/L of mevalonate in a fed-batch fermentation.

To eliminate the amplification of unproductive cheater populations during selection process, a toggled selection scheme was developed in *E. coli* based on the *tolC* selector gene regulated by a naringenin-responsive TF, TtgR [129]. The selection scheme switches between positive selection using sodium dodecyl-sulfate resistance conferred to the host by the activation of *tolC* expression in a naringenin overproducer and negative selection using colicin E1 to eliminate any evolutionary escapees with constitutive *tolC* expression. In four rounds of evolution targeting the expression of key pathway genes using MAGE [172], naringenin production from glucose in *E. coli* was increased by 36-fold to reach 61 mg/L.

In addition to screening pathway optimization libraries, biosensors can also be applied to maintain high productivity in fermentation. By putting an auxotrophic selection marker under the control free fatty acid (FFA)-responsive transcription factor, FadR, an isogenic *E. coli* population is continuously enriched for non-genetic variants with enhanced FFA production when grown under selection pressure [181]. When this strategy, termed in vivo population quality control (PopQC), was applied to a fed-batch fermentation of an FFA overproducing strain, a titer of 21.5 g/L, which was fourfold enhanced over the non-selective fermentation condition, was achieved.

A naringenin-responsive riboswitch based on a novel synthetic aptamer was developed to activate GFP expression upon naringenin binding [185]. This riboswitch biosensor successfully identified naringenin overproducing *E. coli* strains in co-culture through flow cytometry-based high-throughput screening. The theophylline riboswitch [30] regulating the tetracycline resistance gene, *tetA*, was used to facilitate the selection of a combinatorial library consisting of differing promoters, RBS, origins of replication, and chaperone proteins influencing the expression of the caffeine demethylase enzyme, which converts caffeine to theophylline, for improved theophylline production in *E. coli* [37]. The negative Ni^{2+} -selection system based on a self-cleaving NeuAc (*N*-acetylneuraminic acid)-responsive ribozyme regulating the *tetA* gene was used to select for improved NeuAc overproducing *E. coli* strains from combinatorial RBS and randomly mutagenized NeuAc synthase gene libraries [191]. This selection system improved the titer of the NeuAc biosynthetic pathway, which was previously

recalcitrant to rational metabolic engineering, by up to 34%, with the final strain achieving a titer of 8.31 g/L NeuAc. Using a similar Ni^{2+} -selection, a lysine riboswitch which represses the expression of the downstream *tetA* gene upon binding lysine was used to screen the promoter libraries of the phosphoenolpyruvate carboxylase for improved lysine producing *E. coli* strains in the selection media, yielding a variant with significantly improved lysine titer (0.6 g/L) [190]. Based on a putative *E. coli* FMN riboswitch, Meyer et al. engineered an FMN-responsive ribozyme, which self-cleaves in the presence of FMN to activate the expression of GFP, to screen for *B. subtilis* variants with improved vitamin B12 production [106]. In this workflow, engineered *B. subtilis* cells, which convert cellobiose to vitamin B2, were co-confined with *E. coli* sensor cells harboring the FMN ribozyme sensors in nanolitre reactors. Vitamin B2 produced by *B. subtilis* will be taken up by *E. coli* sensor cells and converted to FMN, which then activates GFP fluorescence. Using *E. coli* sensor cells and fluorescence-activated cell sorting (FACS), *B. subtilis* variants with elevated cellobiose-to-vitamin B12 conversion efficiency were effectively isolated from a genome shuffling library.

Aptamers are often applied as biosensors to regulate gene expression in prokaryotes by exposing the RBS of the regulated gene in the presence of the target ligand, hence upregulating expression [30, 117, 156, 163, 168, 191]. However, it is difficult to replicate this strategy in *S. cerevisiae* because of the difference in the eukaryotic translation initiation mechanism. To address this issue, a self-cleaving, glucosamine 6-phosphate (GlcN6P)-responsive ribozyme *glmS* was integrated into the 3'-untranslated region of the cytosine deaminase (*fcy1*) gene in *S. cerevisiae* to create a suicide riboswitch for evolutionary engineering [77]. The presence of GlcN6P activates *glmS* self-cleavage and destabilizes the *fcy1* mRNA, making the *S. cerevisiae* host less susceptible to the toxic conversion of fluorocytosine to fluorouracil by FCY1. Using this selection system, improved mutants of the glutamine-fructose-6-phosphate transaminase and haloacid dehalogenase-like phosphatase enzymes in the *N*-acetyl glucosamine (GlcNAc) production pathway were identified, and an improved GlcNAc producer strain capable of producing more than 3 mg GlcNAc/g dry cell weight was isolated.

A biosensor composed of a lysine-responsive promoter regulating the expression of GFP was used to select for randomly mutagenized lysine producing *E. coli* strains using FACS, yielding a mutant strain with 21% titer improvement [176]. Liu et al. engineered a selection circuit to optimize L-tryptophan production [92]. In this circuit, the L-Trp biosensor, based on the nascent leader peptide, *tnaC*, of the *tnaCAB* operon, regulates the expression of the maltase (*malQ*) gene, and sustains the growth of *E. coli* strains which overproduce L-Trp when maltose is introduced as the sole carbon source. This method was successfully used to

enrich for L-Trp overproducers from site-specific saturation mutagenesis libraries to genome-wide random mutagenesis libraries.

Improving substrate utilization and production of key precursors

In addition to facilitating direct enhancement of product titer, biosensors have also been applied to improve the substrate utilization and concentration of key precursors in biosynthetic pathways. To improve xylose utilization in *S. cerevisiae*, a xylose-responsive gene circuit was engineered in *S. cerevisiae* based on the xylose binding repressor, XylR, from *Staphylococcus xylosus* [174]. Using FACS-based screening, a sugar transporter HXT14 mutant with 6.5-fold improvement in xylose transportation was identified from a random mutagenesis library. Using the FerC repressor from *Sphingobium* sp. SYK-6, a biosensor that detects substituted cinnamic acid CoA-esters was developed in *E. coli* [97]. The biosensor can detect 13 different substituted cinnamic acid-based compounds, which are characteristic products from the enzymatic degradation of lignin, and was applied to screen for efficient feruloyl esterases against different biomass sources.

A biosensor based on the FapR TF enabled the identification of two gene targets from a genome-wide cDNA library that improved intracellular malonyl-CoA concentration in *S. cerevisiae* and subsequently enhanced 3-HP titer by 120% [83]. L-Trp is the key intermediate in the biosynthesis deoxyviolacein, a useful natural product with a variety of bioactivities. Fang et al. applied the TnaC-based L-Trp biosensor to sequentially improve the upstream module for the production of the L-Trp in *E. coli* through combinatorial optimization of pathway RBS, and then the downstream module for the conversion of L-Trp to deoxyviolacein through the random mutagenesis of VioB, the rate-limiting enzyme [39]. Using this strategy, termed intermediate-sensor-assisted push-pull strategy, the L-Trp titer from the upstream module was first enhanced by about 100-fold. When coupled with the engineered downstream module, the resultant strain showed a 4.4-fold improvement in deoxyviolacein production with a titer of 1.92 g/L, representing the highest deoxyviolacein production reported.

Isolation and improvement of desired enzymes

As biosensors can be coupled to the expression of fluorescent or selection markers, they have been employed as high-throughput screening or selection assays to identify key genes or directed evolution of single enzymes. The synthetic

gene circuit based on the S-adenosylmethionine (SAM)-responsive MetJ transcriptional repressor of *E. coli* enabled the screening of a genomic library to identify the *GAL11* gene as a novel multicopy enhancer of SAM levels in *S. cerevisiae* [165]. Based on a phenol-responsive TF, DmpR, a genetic circuit capable of detecting a phenol compound was developed to facilitate the rapid discovery of novel enzymes from metagenomic libraries that can act on synthetic phenyl substrates to produce phenol [18].

Isolation of improved *N*-acetyl-L-glutamate kinase (NAGK) mutants with more than 20-fold less sensitivity toward L-arginine inhibition, which in turn improved the *C. glutamicum* production of L-arginine, was achieved using the pSenLys-Spc biosensor, based on the LysG TF [142], while an NADPH-responsive biosensor based on the transcriptional regulator SoxR was engineered to facilitate the high-throughput screening of the NADPH-dependent *Lactobacillus brevis* alcohol dehydrogenase (ADH) variant library for improved activity on the 4-methyl-2-pentanone substrate [149]. In one round of FACS screening, 10^6 ADH variants from error-prone PCR (epPCR) and active site saturation mutagenesis libraries were screened to yield a variant with 36% improved activity. A single cell sorting system based on the 3,4-dihydroxy benzoate (34DHB)-responsive *pcaU* transcription factor from *Acinetobacter* sp. ADP1 was used to screen for dehydroshikimate dehydratase enzymes with improved activity on dehydroshikimate to produce 34DHB [66]. A chimeric aspartate kinase was evolved through random mutagenesis followed by Ni^{2+} -selection using a high-throughput synthetic RNA device which comprises of a lysine-sensing riboswitch regulating the *tetA* selection module [173].

In vivo monitoring

The ability to observe the real-time formation of metabolites in production strains is desirable as it allows engineers to rapidly evaluate strain phenotypes as well as effects of process parameters [135]. To this end, various biosensors have been developed to monitor the production of value-added chemicals. Using a diacid-responsive transcriptional activator, CdaR, a biosensor for the real-time monitoring of glucarate biosynthesis was developed, and this biosensor was subsequently applied to identify superior enzyme variants [136], while an Lrp-based biosensor was developed for the live cell imaging of L-valine production to monitor single cell productivity and study the phenotypic pattern of microbial production strains [115]. As no natural TF for 3-HP exists, Rogers and Church developed a strategy to quantify 3-HP concentration by intra-cellularly converting 3-HP to acrylate, which can be measured by the *acuR* transcription factor-based biosensor [135]. Using this method, process

parameters leading to 23-fold higher production of 3HP than previously reported was identified. Using a cobalamin ribosensor engineered from a putative riboswitch in *Propionibacterium freudenreichii* spp. *shermanii* DSM 20270, a sensitive detection method for vitamin B12 content in fermented foods was developed [203].

A genetically encoded sensor, termed Spinach riboswitch, based on the fusion of natural riboswitches with the Spinach aptamer which binds the DFHBI fluorophore enabled the live imaging of dynamic changes in intracellular TPP concentrations in individual *E. coli* cells [193]. Using an inverted fusion design strategy, an S-adenosyl-L-homocysteine (SAH) riboswitch was coupled with a modified Spinach2 aptamer to generate an RNA-based fluorescent SAH biosensor capable of monitoring in vivo methylthioadenosine nucleosidase activity, which has potential applications in evaluation of inhibitor drug efficacies [155]. Exploiting the naturally occurring AdoCbl-responsive riboswitch from *E. coli*, intracellular sensors of coenzyme B12 were engineered to examine the synthesis and the import of coenzyme B12 in *E. coli*, generating data pertinent to improving the understanding of vitamin B12 biology [44].

FRET-based biosensors are non-invasive and have short response time, making them good candidates for real-time monitoring of cellular processes. An FRET-based biosensor for monitoring trehalose-6-phosphate (T6P) was developed from the fusion of the trehalose repressor (TreR) from *E. coli* with eCFP and Venus [124]. This sensor enabled the in vivo monitoring of T6P dynamics in *E. coli* and *S. cerevisiae*, which is virtually impossible for conventional metabolite analytical methods due to their invasiveness and low sensitivity, and it can potentially be applied to evaluate trehalose pathway-targeting drugs against pathogenic nematodes. In another example, an FRET-based methionine biosensor based on methionine binding protein (MetN) from *E. coli* between CFP and YFP was developed for the in vivo imaging of methionine flux [111].

In other applications, using the CysR transcription factor from *C. glutamicum*, which is activated by *O*-acetyl serine (OAS) and *O*-acetyl homoserine (OAH)—intermediates of sulfur containing amino acids L-cysteine and L-methionine synthesis, a biosensor was developed to allow the study of consequences of sulfur limitation in single cells such as in pathogens under the conditions that may exist in their hosts [59]. To develop a low cost and equipment-free monitoring method for micronutrient zinc in blood, Watstein et al. developed a whole-cell biosensor based on a pair of zinc-responsive TFs, Zur, a transcriptional repressor, and ZntR, a transcriptional activator [177] in response to different extracellular zinc concentrations, the whole-cell biosensor can produce different colored pigments (violacein, lycopene, and β -carotene) as qualitative indication zinc levels. A QS-based amplifier based on

metal ion-induced promoters was developed to improve the performance of environmental metal ion biosensing [61].

Perspectives and concluding remarks

Advances in genome sequencing and synthetic biology have greatly enhanced our ability to identify, build, modify, and apply in vivo biosensors. Over the last few years, applications of in vivo biosensors have moved from simple metabolite sensing and reporting to more complex genetic systems, such as dynamic and multi-layered genetic circuits, offering more advanced tools for engineering biosynthetic pathways and whole cells.

To accelerate the development of biosensors, it is critical to expand the repertoire of detectable metabolites and products. Currently, there are only a limited number of biosensors compared to the vast number of small molecules of interest. Genomic and transcriptomic analysis can help researchers to uncover more TF- or promoter-based in vivo biosensors, while advances in protein engineering as well as computational design can help researchers to better tailor the specificity of TFs. In addition, methods to study genome-wide RNA secondary structures [170] can help researchers to identify novel RNA-based sensors.

With the increasing complexity of biochemical production strategies enabled by synthetic biology, there is a greater need for standardized and streamlined engineering methods, as demonstrated by Gupta et al. [54]. Another implication of more complex genetic designs is that biosynthetic pathways will become increasingly modularized to enable parallel optimization, which presents opportunities for the application of biosensors to dynamically regulate flux between modules [60]. Hence, better control over the dynamic ranges of in vivo biosensors is needed to match the output and input of adjacent modules. This can be achieved with the development of more standardized genetic parts as well as protein engineering.

The challenges presented here are non-trivial, but offer great opportunities to advance the field of in vivo biosensors. Going forward, we anticipate increasing adoption of in vivo biosensors in metabolic engineering and synthetic biology for the optimization of strain development, and the dynamic regulation of biosynthetic pathways.

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