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Research review paper

Enabling tools for high-throughput detection of metabolites: Metabolic engineering and directed evolution applications

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ABSTRACT

Within the Design-Build-Test Cycle for strain engineering, rapid product detection and selection strategies remain challenging and limit overall throughput. Here we summarize a wide variety of modalities that transduce chemical concentrations into easily measured absorbance, luminescence, and fluorescence signals. Specifically, we cover protein-based biosensors (including transcription factors), nucleic acid-based biosensors, coupled enzyme reactions, bioorthogonal chemistry, and fluorescent and chromogenic dyes and substrates as modalities for detection. We focus on the use of these methods for strain engineering and enzyme discovery and conclude with remarks on the current and future state of biosensor development for application in the metabolic engineering field.

1. Introduction

Metabolic Engineering efforts seek to rewire organisms for the industrially-relevant production of value-added products from renewable feedstocks (Liu et al., 2013). In doing so, the product may be a native, endogenous metabolite or the result of de novo biosynthesis through the use of heterologous enzymes and pathways. Either way, the great power of this approach is the ever-expanding chemical diversity inherent in and reachable from cellular metabolism. However, the intrinsic complexity of biological systems imposes long and costly Design-Build-Test (DBT) cycle during research and development. Fortunately, recent breakthroughs are beginning to change this picture. Specifically, advances in the adjacent fields of DNA sequencing and synthesis (Esvelt and Wang, 2013), directed and in vivo evolution (Crook et al., 2016; Esvelt et al., 2011; Ravikumar et al., 2014; Wang et al., 2009), targeted transcriptional regulation (Bikard et al., 2013; Deaner and Alper, 2017; Gilbert et al., 2013; Mali et al., 2013a; Perez-Pinera et al., 2013; Qi et al., 2013), and precision genome editing (Cong et al., 2013; DiCarlo et al., 2013; Jiang et al., 2013; Jinek et al., 2013; Mali et al., 2013b) enable more rapid prototyping of cell factories. However, these advances have clearly exposed the Test phase as the rate limiting step in the development process. To reach parity, the Test phase needs to match the high and increasing throughput of strain engineering and synthetic biology. However, unlike DNA, RNA, and protein detections that rely on complementarity and/or a finite set of building-blocks (like amino acids and nucleotides), metabolites display a very wide chemical diversity. As a result, many applications rely on either “one-off” screens and selections or spectrometric analysis. Thus, substantial improvements in the areas of metabolite sensing are required to achieve high throughput in a way that is not just brute-force replication of slow technologies (i.e. requiring chromatography). The field of metabolic sensing and high throughput detection is a highly explored area. This review serves to compile and highlight the diverse modalities used to construct biosensors for high-throughput metabolic detection and provide examples of their application in high throughput strain engineering workflows.

2. Platforms for high throughput screening

To maximally harness the power of large DNA and evolution libraries (often 10^7–10^9 variants/week), equally high throughput platforms are required for assessing the phenotype of interest. The easiest and least equipment-dependent method used is to link the desired phenotype with a growth-based selection (Dragosits and Mattanovich, 2013). However, this approach inherently selects solely for growth rate enhancement, a complex and non-specific trait that is often counterproductive to high level biosynthesis of the desired product. For many cases, growth-based selections are either not optimal or impossible due to the required coupling of growth and production via endogenous or synthetic routes. In such cases, traditional methods for small molecule

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quantification are used including high pressure liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), and well-plate based spectrophotometric assays. However, these approaches generally cannot achieve the throughput needed to effectively and efficiently screen very large libraries (Dietrich et al., 2010). Massive parallelization, automation, and miniaturization of well-plate assays has partially bridged this gap (especially for screening pharmacologically active compounds) (Janzen and Bernasconi, 2002), but wide deployment often demands high capital costs and specialized skills.

In contrast to selections and traditional approaches, biosensors enable high throughput quantification by transducing the concentration of the target molecule into an easily measurable signal such as fluorescence, luminescence, or absorbance. In this regard, screening for product concentration can leverage single cell, high throughput analysis techniques such as fluorescence activated cell sorting (FACS) (Mattanovich and Borth, 2006) or droplet-based microfluidic sorting (Agresti et al., 2010). While there is great power in such an approach, sensing metabolites is notoriously challenging. Unlike nucleic acid detection and sequencing technologies based on relatively predictable thermodynamic binding between complementary bases (Aebersold and Mann, 2003), a generalizable technique to detect wide varieties of small molecules at very high throughput is not available. Specifically, the microbial metabolome and resulting chemical space span nearly every measurable molecular property (Fernie and Stitt, 2012; Fernie et al., 2004). This reality has led to a proliferation of different biosensor modalities and approaches that are each tailored to specific classes of molecules. The remainder of this review focuses on describing these various sensing modalities (along with their advantages and disadvantages) and their applications in the area of metabolite overproduction. We conclude with a summary of the state-of-the-art and perspectives for moving forward.

3. Modalities for metabolite sensing

3.1. Intrinsic properties of chemicals

In particular applications, the product itself serves as a scalable biosensor. Specifically, the chemical nature of absorbance or fluorescence of product molecules can be used as an inherent screening technique (Fig. 1). Numerous metabolic engineering products have chromphores or fluorophores. As examples, flavonoids and carotenoids are natural pigments that display a wide range of colors, spanning from red to violet (Tanaka et al., 2008; Verpoorte and Alfermann, 2000). Molecules such as β-carotene, apigenin, anthocyanin, are colored in red-orange, yellow, and blue, respectively, and these compounds can be thus be selected for via simple colorimetric assays. Several other products have innate fluorescent capability, with the capacity to absorb light or electromagnetic radiation of a particular wavelength and then emit a signal at a different wavelength. As examples, riboflavin, betaxanthin, and camptothecin exhibit excitation/emission at 450/518, 470/510, and 370/434 nm, respectively (Chandrakuntal et al., 2006; Gandia-Herrero et al., 2005; Loh and Ahmed, 1990). Table 1 summarizes instances and some applications where intrinsic spectral properties of target molecules have been used for engineering or evolution. As an example, metabolic engineering efforts have focused on the production of lycopene, and important antioxidant (Seren et al., 2008). The intrinsic property of this red pigment allows for rapid strain identification. This trait was explored in conjunction with global stoichiometric modeling and transposon-mediated mutagenesis to identify 8.5- and 2-fold increases in titers over wild type and engineered parental strains (Alper et al., 2005). Likewise, to improve $_{L-}$tyrosine production in *E. coli*, global transcription machinery engineering (gTME) was employed to perturb gene expression and strains were selected on the basis of melanin accumulation (a brown pigment) on plates. The result was a strain with a 114% increase in tyrosine production (Santos and Stephanopoulos, 2008b; Santos et al., 2012). While these applications were of relative ease due to inherently detectable product attributes, most molecules of interest do not possess such intrinsic detection capacity.

3.2. Protein-based biosensors

The proteome responds to and detects metabolites naturally using a variety of modes (Fernie and Stitt, 2012) which makes proteins a natural choice for the molecular recognition of many small molecules of interest to metabolic engineering and synthetic biology. Moreover, proteins and their constituent domains can be rewired and modularized to establish biosensors. These protein-based biosensors can be imported into a wide variety of host organisms for in vivo application, and sometimes can be purified for effective in vitro function as well (de los Santos et al., 2016; Li et al., 2017). Among the many different classes of protein-based biosensors available, this review will focus on the most common types, including transcription factors, engineered fluorescent proteins, and G protein-coupled receptors.

3.2.1. Transcription factors

Transcription factors (TFs) are proteins that can often natively react to stimuli such as small molecules and then transduce an easily

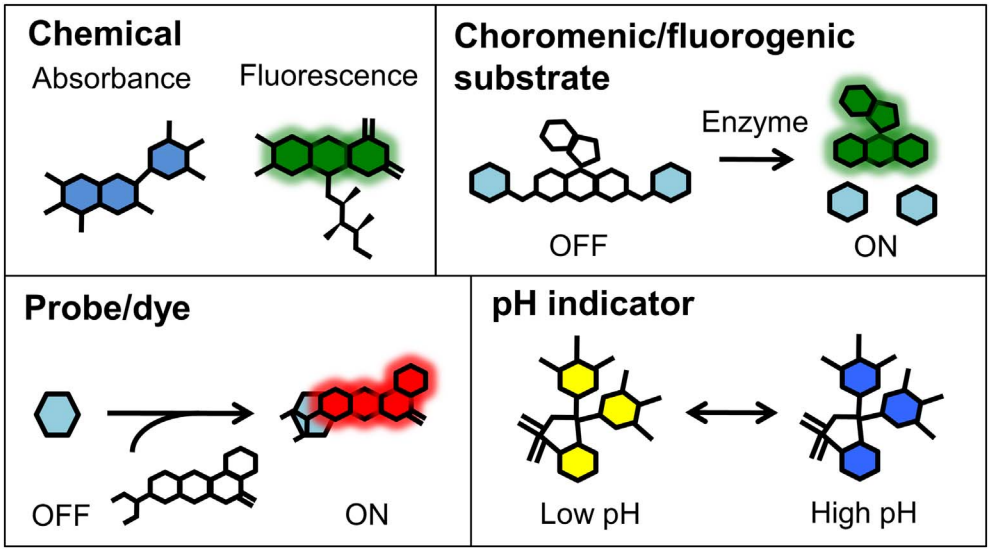


Fig. 1. Chromogenic and fluorogenic compounds, and mechanisms of activation. The physicochemical properties can be activated by enzymatic hydrolysis, chemical interactions, and pH change. Abbreviation: Enz., enzyme.

Table 1
Molecules with intrinsic chromogenic/fluorogenic property and highlighted applications for strain engineering.

Target molecule	Modality class	Chromogenic/fluorogenic property	Biosensor host	Reference	Highlighted applications
β-carotene	Intrinsic property	Absorbance		(Tanaka et al., 2008)	–
Apigenin	Intrinsic property	Absorbance		(Tanaka et al., 2008)	–
Anthocyanin	Intrinsic property	Absorbance		(Tanaka et al., 2008)	–
Melanin	Intrinsic property	Absorbance	*Escherichia coli*	(Santos and Stephanopoulos, 2008b)	Used as the reporter for high throughput screening of tyrosine production in *E. coli* (Santos et al., 2012)
Lycopene	Intrinsic property	Absorbance	*Escherichia coli*	(Alper et al., 2005)	Screened for computation-guided and transposon-mediated knockout for lycopene production, and improved 8.5-fold increase in titer over wild type (Alper et al., 2005)
Betanidin	Intrinsic property	Absorbance	*Saccharomyces cerevisiae*	(DeLoache et al., 2015)	Used as the reporter for DOPA oxidase activity (DeLoache et al., 2015)
Betaxanthin	Intrinsic property	Fluorescence	*Saccharomyces cerevisiae*	(Gandia-Herrero et al., 2005)	Used as the reporter for directed evolution of tyrosine hydroxylae (DeLoache et al., 2015)
Camptothecin	Intrinsic property	Fluorescence		(Loh and Ahmed, 1990)	–
Riboflavin	Intrinsic property	Fluorescence		(Chandrakuntal et al., 2006)	–

measurable transcriptional response through a reporter gene (Fig. 2). Thus, TFs are one of the most common and valuable biosensor types developed for high throughput screening (Mahr and Frunzke, 2016). While most commonly used as a genetically-encoded, in vivo biosensor, it is also possible to employ these elements in vitro (de los Santos et al., 2016; Li et al., 2017). TFs have natively evolved for selective response to a wide range of environmental ligands by modulating the activity of their cognate promoter(s) (Cuthbertson and Nodwell, 2013). More recent efforts have further engineered these components to respond to new, non-natural ligands of interest to biotechnology. In particular, a wide-ranging array of examples for different TF-based biosensors are provided in Table 2. This list shows a diverse range for TF-based detection, including but not limited to acids, alcohols, sugars, amino acids, aromatics, antibiotics, flavonoids, fatty acids, and CoA compounds. Due to the large and continuously growing number of TF-based biosensors now available, this review will not cover all of the TF-based biosensor designs that are included in Table 2. Instead, we focus on discussing efforts to create TF biosensors for new effector molecules, the construction of hybrid or fusion TF biosensors, and in vitro use of TFs for screening. In addition, we highlight some advances in the application of TF-based biosensors for strain and enzyme engineering applications.

3.2.1.1. TFs with altered specificity. Many desired products for metabolic engineering are either non-native metabolites or metabolites for which a cognate TF does not exist. In such cases, random mutagenesis or computational protein design can potentially be used alter the selectivity and specificity of mutant TFs to new ligand(s) of interest (Combs et al., 2013; de los Santos et al., 2016; Jha et al., 2015; Tang and Cirino, 2011; Taylor et al., 2016). However, TF redesign for biosensing applications is often difficult due to allosteric communication that links ligand-binding domain(s) and the downstream DNA binding and transcriptional regulation of the reporter (Taylor et al., 2016). Even with the knowledge of an established crystal structure, a priori determination of key residues or domains affecting this allostery is challenging (Suel et al., 2003). Despite these challenges, there have been many successes toward re-engineering novel TF specificity profiles that we highlight here.

Aromatic compounds are common analytes of interest both due to their presence as environmental pollutants and as intermediates in metabolic pathways for valuable products such as muconic acid (Curran et al., 2013). Many well-studied TFs that recognize aromatic compounds are native to *Pseudomonas* and *Acinetobacter* species, two organisms that natively degrade xenobiotic compounds (van der Meer et al., 1992). For example, XylS and XylR are well-studied transcription factors that are natively responsible for regulation of the TOL pathway for toluene metabolism in *Pseudomonas putida*. Moreover, these TFs have served as templates for effector specificity modifications. In an early example, Ramos et al. placed a tetracycline resistance gene (Tc^r) under control of the cognate promoter for the benzoate-responsive *Pseudomonas putida* XylS in *E. coli* (Ramos et al., 1986). Using both growth selection on tetracycline-containing along with ethyl methanesulfonate (EMS) chemical mutagenesis, a panel of novel XylS mutants responsive to alternative inducers were successfully isolated. Subsequently, these mutant XylS were used to construct a LacZ biosensor module for detecting a wide range of benzoate derivatives. Several mutants yielded induction ratios of 10–30 in the presence of hydroxylated, alkylated, methoxylated, or halogenated, non-native benzoates (Ramos et al., 1990; Ramos et al., 1986). Finally, one mutant was used to broaden the catabolic range of *Pseudomonas* WR216 to include 3,5-dichlorobenzoate, a non-native substrate for the strain (Ramos et al., 1986). A similar mutagenic strategy involving *N*-methyl-N′-nitro-N-nitrosoguanidine led to a mutant of the toluene-responsive *Pseudomonas putida* XylR that could stimulate LacZ production in the presence of a novel effector, m-nitrotoluene (Delgado and Ramos, 1994).

Beyond random mutagenesis, the techniques of DNA shuffling and

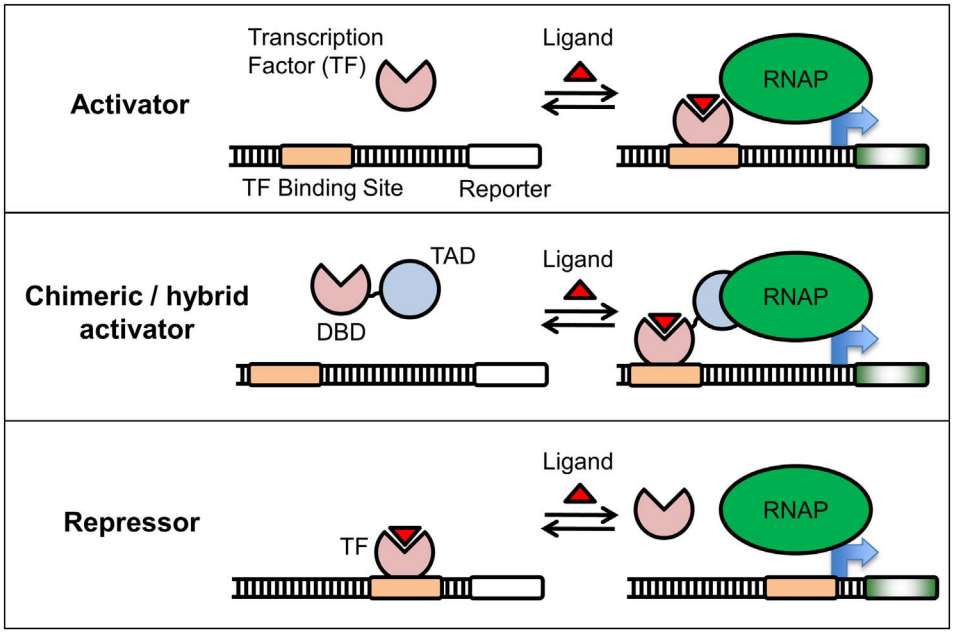


Fig. 2. Transcription factor-based biosensors. Activators: upon ligand binding, the transcription factor (TF) binds to the TF binding site (or operator) within the cognate promoter and activates reporter gene expression by recruiting transcriptional machinery and RNA polymerase (RNAP). Chimeric/hybrid activator: upon ligand binding to the effector domain, the DNA binding domain (DBD) of the chimeric or hybrid protein binds to the operator site within the cognate promoter. The fused transcriptional activating domain (TAD) then activates reporter gene expression by recruiting transcriptional machinery and RNA polymerase (RNAP). Repressor: upon ligand binding, the repressor unbinds from its operator site, allowing the transcriptional machinery and RNA polymerase (RNAP) to engage with the promoter region and drive reporter gene expression.

error-prone PCR (EP-PCR) have generated XylR mutants responsive to different isomers of nitrotoluene (Garmendia et al., 2001), methylbenzyl alcohol (Skärfstad et al., 2000), and 2,4 dinitrotoluene (DNT) (Galvao et al., 2007). Similar approaches were also conducted with the *Pseudomonas* LysR-type transcriptional regulator (LTTR) NahR to develop mutants responsive to the non-native inducers of benzoate, chlorobenzoate, and salicylamide (Cebolla et al., 1997). Likewise, the *Pseudomonas putida* phenol-responsive TF DmpR has also been mutated by EP-PCR to detect phenol and several synthetic substituted phenols that are recognized as priority pollutants by the U.S. Environmental Protection Agency (EPA) with induction ratios reaching 12–56 times that of WT DmpR (Wise and Kuske, 2000). More recently, the natively 4-hydroxybenzoate responsive TF PobR from *Acinetobacter* sp. ADP1 has been engineered using Rosetta computational modeling and design (Das and Baker, 2008; Jha et al., 2015). By homology modeling, ab initio prediction of protein structure led to the identification of key residues for creating 3,4-hydroxybenzoate response. Through the use of a targeted mutagenesis library, a final mutant was identified with significant induction by 3,4-hydroxybenzoate. Together, these examples suggest that these TFs are particularly adaptable for the detection of new ligands that share a general aromatic structure.

Beyond aromatic targets, the *E. coli* sugar-responsive TFs AraC and LacI have also been explored as biosensors. Initially the sugar responsiveness of AraC was altered to recognize D-arabinose instead of the native L-arabinose ligand using saturation mutagenesis of key residues in the binding pocket (Tang et al., 2008). Through a series of positive and negative selections, Tang et al. isolated two AraC variants that respond to D-arabinose but not L-arabinose. A similar process of positive and negative selection has broadened the molecular recognition capabilities of AraC even further, yielding biosensors for the industrially important compounds mevalonate (Tang and Cirino, 2011), triacetic acid lactone (Tang et al., 2013), and ectoine (Chen et al., 2015). Likewise, the allolactose and isopropyl β-D-1-thiogalactopyranoside (IPTG) responsive TF LacI (Lewis et al., 1996) has recently been computationally redesigned and mutated to recognize four new non-native inducers (Taylor et al., 2016). This investigation employed a three-pronged approach to library design and generation, including Rosetta computation-guided mutagenesis of most of the allolactose binding pocket, tiled saturation mutagenesis for all LacI single residue substitutions at sites outside the binding pocket, and random EP-PCR mutagenesis. Resulting mutant libraries were pre-screened for retained ability to repress a TolC reporter (a porin that allows entry of toxic colicin E1) by growth enrichment in the absence of inducers but in the presence of colicin E1. The enriched LacI libraries were then positively screened by FACS for their ability to activate production of GFP, resulting in variants of LacI that were responsive to fucose, gentiobiose, lactitol and sucralose (Taylor et al., 2016). In this instance, no active counter-screening was performed against activation by IPTG, so nearly all mutants retained their ability to respond to IPTG, a phenotype that was reduced for some designs upon shuffling of mutations for activity maturation. Interestingly, random EP-PCR and screening was sufficient to identify LacI mutants responsive to fucose and lactitol, but TFs responsive to sucralose were only isolated using computationally-aided designs. Furthermore, computationally generated mutants for fucose and lactitol exhibited an approximately 1.5–2.1 times higher maximum induction relative to mutants generated from EP-PCR.

Native TF repressors such as the prokaryotic tetracycline-responsive repressor TetR are commonly used for tight transcriptional regulation in both bacteria (Lutz and Bujard, 1997) and eukaryotic cells (Blau and Rossi, 1999). Based on the crystal structure (Hinrichs et al., 1994), Scholz et al. were able to shift ligand recognition from the native tetracycline to the related derivative 4-de(dimethylamino)-6-deoxy-6-demethyl-tetracycline (referred to as cmt3) (Scholz et al., 2003). This shift was accomplished by linking the cognate promoter to LacZ and employing multiple rounds of directed evolution and selection via DNA shuffling, in vitro recombination, and random oligonucleotide mutagenesis to ultimately obtain a mutant with 20,000-fold improved specificity for cmt3 (relative to tetracycline). In a similar fashion, structure-guided randomization of two residues yielded a TetR variant that responds to 4-de(dimethylamino)anhydrotetracycline and displays a 200-fold increase in specificity (Henssler et al., 2004). Beyond TetR, the *Staphylococcus aureus* QacR has been explored as a repressor that can bind a diverse set of cationic lipophilic ligands (Grkovic et al., 2003). Based on this observation and the availability of a crystal structure (Schumacher et al., 2001), de los Santos et al. employed the computational protein design tool Phoenix Match (Lassila et al., 2006) to model potential binding sites for vanillin (de los Santos et al., 2016). Designed QacR mutants were then tested in *E. coli* for induction by vanillin, and two variants were found to be significantly induced by vanillin. These mutants contained 6–11 residue changes, a leap in sequence space that necessitates computational design (de los Santos et al., 2016).

Table 2
Transcription factor-based biosensors and highlighted applications for strain engineering. Abbreviations: GFP, green fluorescent protein; YFP, yellow fluorescent protein; RFP, red fluorescent protein; ALPHA, amplified luminescent proximity homogeneous assay; TF, transcription factor; DBD, DNA binding domain; TAD, transcriptional activation domain; TFBS, transcription factor binding site; FREP, feedback-regulated evolution of phenotype; DSG, dosage sensitive gene.

Target molecule	Modality class	Sensor/Reporter	Biosensor host	Sensor development	Highlighted applications
Methylating Agents (e.g., methyl iodide, methylnitronitrosoguanidine, methyl methanesulfonate, dimethyl sulfate, ethyl methanesulfonate)	TF (Fusion protein)	Ada DBD fused to Gal4 TAD/GFP (Ada from *E. coli*, Gal4 from *S. cerevisiae*)	*Saccharomyces cerevisiae*	(Moser et al., 2013)	Biosensor fluorescence shown to correlate with activity of methyl halide transferases expressed in yeast strains (Moser et al., 2013)
S-adenosylmethionine (SAM)	TF (Fusion protein)	MetJ fused to B42 TAD/YFP, LEU2, or HIS3 (MetJ and B42 from *E. coli*)	*Saccharomyces cerevisiae*	(Umeyama et al., 2013)	Screened yeast genomic library and successfully identified a novel multi-copy enhancer of SAM levels (GAL11) (Umeyama et al., 2013)
Isopentenyl diphosphate (IPP)	TF (Fusion protein)	Gal4 DBD fused to IPP Enzyme + Gal4 TAD fused to IPP Enzyme/YFP (Gal4 from *S. cerevisiae*)	*Saccharomyces cerevisiae*	(Chou and Keasling, 2013)	–
Isopentenyl diphosphate (IPP)	TF (Fusion protein)	AraC DBD fused to IPP Enzyme/MutD5 and RFP (AraC from *E. coli*)	*Escherichia coli*	(Chou and Keasling, 2013)	*E. coli* strains with 3 to 17-fold increases in lycopene production generated via FREP
Tyrosine	TF	TyrR/MutD5 and RFP (TyrR from *E. coli*)	*Escherichia coli*	(Chou and Keasling, 2013)	*E. coli* strains with up to 5-fold increase in tyrosine production generated using FREP
D-Arabinose	TF (Engineered)	AraC/GFP (AraC from *E. coli*)	*Escherichia coli*	Mutagenesis of AraC to select for D-arabinose responsive variant (Tang et al., 2008)	–
Triacetic acid lactone (TAL)	TF (Engineered)	AraC/GFP (AraC from *E. coli*)	*Escherichia coli*	Mutagenesis of AraC to select for TAL-responsive variant (Tang et al., 2013)	Improved TAL synthase catalytic efficiency (k_{cat}/K_m) by 19-fold in *E. coli* (Tang et al., 2013)
Mevalonate	TF (Engineered)	AraC/GFP, AraC/LacZ (AraC from *E. coli*)	*Escherichia coli*	Mutagenesis of AraC to select for mevalonate-responsive variant (Tang and Cirino, 2011)	3–4 Fold improvement in *E. coli* mevalonate production from screening a randomized ribosomal binding site (RBS) library upstream of soluble HMG−CoA reductase (Tang and Cirino, 2011) Library of transcriptional regulator (QscR) mutants was screened, and identified mutant resulted in 60% increase in mevalonate production from *Methylobacterium extorquens* AM1 (Liang et al., 2017)
Ectoine	TF (Engineered)	AraC/GFP (AraC from *E. coli*)	*Escherichia coli*	Mutagenesis of AraC to select for ectoine-responsive variant (Chen et al., 2015)	Identified mutant EctB aminotransferase that improved ectoine production 3.3-fold in *E. coli* (Chen et al., 2015)
Fucose	TF (Engineered)	LacI/GFP or TolC (LacI from *E. coli*)	*Escherichia coli*	(Taylor et al., 2016)	–
Gentiobiose	TF (Engineered)	LacI/GFP or TolC (LacI from *E. coli*)	*Escherichia coli*	(Taylor et al., 2016)	–
Lactitol	TF (Engineered)	LacI/GFP or TolC (LacI from *E. coli*)	*Escherichia coli*	(Taylor et al., 2016)	–
Sucralose	TF (Engineered)	LacI/GFP or TolC (LacI from *E. coli*)	*Escherichia coli*	(Taylor et al., 2016)	–
Vanillin	TF (Engineered)	QacR/(QacR from *Staphylococcus aureus*)	*Escherichia coli*	(de los Santos et al., 2016)	–
3,4 Dihydroxy benzoate (3,4DHB)	TF (Engineered)	PobR/GFP (PobR from *Acinetobacter baylyi* ADP1)	*Escherichia coli*	Rosetta model-guided mutagenesis to select for 3,4DHB induction (Jha et al., 2015)	–
Benzoate and substituted benzoates (chlorinated)	Mutant TF	NahR/LacZ (NahR from *Pseudomonas putida*)	*Escherichia coli*	(Cebolla et al., 1997)	–
Salicylamide	Mutant TF	NahR/LacZ (NahR from *Pseudomonas putida*)	*Escherichia coli*	(Cebolla et al., 1997)	–
Benzoate	Mutant TF	NahR/LacZ or TetA (NahR from *Pseudomonas putida*)	*Escherichia coli*	(van Sint Fiet et al., 2006)	Isolation of benzaldehyde dehydrogenase (XylC) expressing (i.e. benzoate-producing) *E. coli* biocatalyst cells from a one part per million mixture with non-catalyst cells (van Sint Fiet et al., 2006)
Nitrotoluene	Mutant TF (chemical	XylR/LacZ (XylR from *Pseudomonas putida*)	*Escherichia coli*	(Delgado and Ramos, 1994)	–

(continued on next page)

Table 2 *(continued)*

Target molecule	Modality class	Sensor/Reporter	Biosensor host	Sensor development	Highlighted applications
	mutagenesis)				
2 − /4-Methylbenzyl alcohol	Mutant TF	XylR-DmpR/Luciferase (XylR/DmpR from *Pseudomonas putida*)	*Pseudomonas putida*	(Skärfstad et al., 2000)	–
Nitrotoluene	Mutant TF	XylR/LacZ (XylR from *Pseudomonas putida*)	*Pseudomonas putida*	(Garmendia et al., 2001)	–
2,4 Dinitrotoluene (DNT)	Mutant TF	XylR/LacZ (XylR from *Pseudomonas putida*)	*Pseudomonas putida*	(Galvao et al., 2007)	–
Substituted benzoates (hydroxy, methyl, ethyl, methoxy, halogenated)	Mutant TF	XylS/Tc^r or LacZ (XylS from *Pseudomonas putida*)	*Escherichia coli*	(Ramos et al., 1986)	Introduction of dominant *xylS*352 mutant allele into *Pseudomonas* WR216, enabled growth on 3,5-dichlorobenzoate, a non-native substrate for the strain (Ramos et al., 1986)
Substituted benzoates (hydroxy, methyl, ethyl, methoxy, halogenated)	Mutant TF	XylS/Tc^r or LacZ (XylS from *Pseudomonas putida*)	*Escherichia coli*	(Ramos et al., 1990)	–
Substituted phenols (methyl, nitro, chloro)	Mutant TF	DmpR/LacZ (DmpR from *Pseudomonas putida*)	*Escherichia coli*	(Wise and Kuske, 2000)	–
BTX (benzene, toluene, xylenes)	TF	XylR/Luciferase (XylR from *Pseudomonas putida*)	*Escherichia coli*	(Willardson et al., 1998)	Testing of field–collected soil samples from BTX contaminated sites (Willardson et al., 1998)
Cis,cis-muconate (muconic acid)	TF	BenM/GFP (BenM from *Acinetobacter baylyi* ADP1)	*Saccharomyces cerevisiae*	(Skjoedt et al., 2016)	Selected improved muconic acid producing yeast strain (multiple integrations of key enzymes) from 6-member strain pool (Skjoedt et al., 2016)
Benzamide (indirect, via metabolic conversion to benzoate)	TF	BenR/GFP fusion (BenR from *Pseudomonas putida*)	*Escherichia coli*	(Uchiyama and Miyazaki, 2010)	Screened a 96,000 member metagenomic library and identified 3 novel enzymes with activity toward benzamide (Uchiyama and Miyazaki, 2010)
Malonyl-CoA	TF	FapR/RFP (FapR from *Bacillus subtilis*)	*Saccharomyces cerevisiae*	(Li et al., 2015)	Screened a genomic library and identified 2 novel gene targets for increasing malonyl-CoA and 3-hydroxypropionic acid in *S. cerevisiae* (Li et al., 2015)
Malonyl-CoA	TF	FapR/GFP or Luciferase (FapR from *Bacillus subtilis*)	COS-1 cell line	(Ellis and Wolfgang, 2012)	Screened a human kinase library in COS-1 cells and identified several targets involved in regulation of cellular malonyl-CoA concentration (Ellis and Wolfgang, 2012)
Malonyl-CoA	TF	FapR/GFP (FapR from *Bacillus subtilis*)	*Escherichia coli*	(Xu et al., 2014b)	–
Malonyl-CoA	TF	FapR/GFP (FapR from *Bacillus subtilis*)	*Escherichia coli*	(Xu et al., 2014a)	Implemented dynamic pathway control to increase fatty acid production in *E. coli* (Xu et al., 2014a)
Malonyl-CoA	TF	FapR/RFP (FapR from *Bacillus subtilis*)	*Escherichia coli*	(Liu et al., 2015a, 2015b)	Utilized feedback regulation to increase fatty acid production in *E. coli* (Liu et al., 2015a, 2015b)
Methylating agents (e.g., methyl iodide, methylnitronitrosoguanidine, methyl methanesulfonate, dimethyl sulfate, ethyl methanesulfonate)	TF	Ada/GFP (endogenous *E. coli* Ada)	*Escherichia coli*	(Moser et al., 2013)	Detection of fumigant methyl iodide in soil samples (Moser et al., 2013)
Naringenin	TF	FdeR/GFP (FdeR from *Herbaspirillum seropedicae*)	*Saccharomyces cerevisiae*	(Skjoedt et al., 2016)	Selected improved naringenin producing yeast strain (multiple integrations of key enzymes) from 4-member strain pool (Skjoedt et al., 2016)
3,4 Dihydroxy benzoate (3,4DHB)	TF	PcaU/GFP (PcaU from *Acinetobacter baylyi* ADP1)	*Escherichia coli*	(Jha et al., 2014)	Isolation of 3,4 dihydroxy benzoate producing *E. coli* cells from a one part per million mixture with non-producers (Jha et al., 2014)
Lactams (ε-caprolactam, δ-valerolactam, and	TF	ChnR/RFP (ChnR from *Acinetobacter* sp.	*Escherichia coli*	(Zhang et al., 2017)	–

(continued on next page)

Table 2 *(continued)*

Target molecule	Modality class	Sensor/Reporter	Biosensor host	Sensor development	Highlighted applications
butyrolactam)		NCIMB9871)			
Xylose	TF	XylR/GFP (XylR from *Tetragenococcus halophile*, *Clostridium difficile*, or *Lactobacillus pentosus*)	*Saccharomyces cerevisiae*	(Teo and Chang, 2015)	–
Acrylate	TF	AcuR/GFP (AcuR from *Rhodobacter sphaeroides*)	*Escherichia coli*	(Rogers et al., 2015)	–
Glucarate	TF	CdaR/GFP (from *E. coli*)	*Escherichia coli*	(Rogers et al., 2015)	Enrichment for high glucarate producing enzymes from 4-member pool in *E. coli* (Rogers et al., 2015)
Erythromycin	TF	MphR/GFP (MphR from macrolide resistant strain of *E. coli*)	*Escherichia coli*	(Rogers et al., 2015)	–
Naringenin	TF	TtgR/GFP (from *Pseudomonas putida)*	*Escherichia coli*	(Rogers et al., 2015)	–
Phenol	TF	TtgR/GFP (from *Pseudomonas putida)*	*Escherichia coli*	(Rogers et al., 2015)	–
3-Hydroxypropionate (indirect, via metabolic conversion to 2-methylcitrate)	TF	PrpR/GFP (PrpR from *Salmonella enterica*; system also required propionyl-CoA synthase (pcs) from *Chloroflexus aurantiacus* and *E. coli* 2-methylcitrate synthase (prpC)	*Escherichia coli*	(Rogers and Church, 2016)	–
Cis, cis-muconate (muconic acid)	TF	BenM/GFP (BenM from *Acinetobacter baylyi* ADP1)	*Escherichia coli*	(Rogers and Church, 2016)	–
3-Hydroxypropionate (indirect, via metabolic conversion to acrylate)	TF	AcuR/GFP (AcuR from *Rhodobacter sphaeroides;* system also required truncated propionyl-CoA synthase (pcs) from *Chloroflexus aurantiacus* and acrylyl-CoA hydrolase (ach) from *Acinetobacter baylyi*)	*Escherichia coli*	(Rogers and Church, 2016)	–
NADPH/$NADP^+$	TF	Yap1/GFP or DSGs (endogenous *Saccharomyces cerevisiae* Yap1)	*Saccharomyces cerevisiae*	(Zhang et al., 2016)	FACS enrichment for strains with higher NADPH/$NADP^+$ redox states using DSG sensor-selector scheme (Zhang et al., 2016)
NADPH/$NADP^+$	TF	SoxR/YFP (endogenous *E. coli* SoxR)	*Escherichia coli*	(Siedler et al., 2014)	Screening of a mutant NADPH-dependent alcohol dehydrogenase library yielded improved enzyme activity toward new substrate 4-methyl-2-pentanone (Siedler et al., 2014)
Organophosphates (indirect, via metabolic release of 4-nitrophenol)	TF	DmpR/RFP (DmpR from *Pseudomonas* sp. CF600)	*Escherichia coli*	Mutagenesis of DmpR to alter sensitivity for indirect detection of organophosphorous compunds through 4-nitrophenol intermediate (Chong and Ching, 2016)	Colorimetric sensing of toxic environmental contaminant parathion with whole-cell *E. coli* biosensor (Chong and Ching, 2016)
Vanillin	Mutant TF	QacR/GFP (QacR from *Staphylococcus aureus*)	In vitro cell-free transcription-translation (*E. coli* lysate)	(de los Santos et al., 2016)	–
Uric acid	His-tagged TF + Biotinylated TFBS	HucR/ALPHA (HucR from *Deinococcus radiodurans*)	In vitro binding assay (ALPHA)	(Li et al., 2017)	–
Oxytetracycline	His-tagged TF + Biotinylated TFBS	OtrR/ALPHA (OtrR from *Streptomyces rimosus*)	In vitro binding assay (ALPHA)	(Li et al., 2017)	–
Tyrosine	TF	ARO9/YFP or KanMX (ARO9 from *S. cerevisiae*)	*Saccharomyces cerevisiae*	(Leavitt et al., 2016)	Improved tyrosine-derived heterologous pathway (muconic acid biosynthesis) titer up to 3-fold (Leavitt et al., 2017)
Branched chain amino acids (L-valine, L-leucine, L-isoleucine)	TF	Lrp/YFP (endogenous Lrp from *Corynebacterium glutamicum*)	*Corynebacterium glutamicum*	(Mustafi et al., 2012)	Screening of mutagenesis libraries derived from wild type non-producing *C. glutamicum* parent strain to select for L-valine, L-leucine, and L-isoleucine production (Mustafi et al.,

(continued on next page)

Table 2 *(continued)*

Target molecule	Modality class	Sensor/Reporter	Biosensor host	Sensor development	Highlighted applications
					2012). Evolution of a *C. glutamicum* strain with increased growth rate, increased L-valine production, and significantly reduced byproduct production (Mahr et al., 2015)
Methionine	TF	Lrp/YFP (endogenous Lrp from *Corynebacterium glutamicum*)	*Corynebacterium glutamicum*	(Mustafi et al., 2012)	–
L-lysine, L-arginine, L-histidine	TF	LysG/YFP (endogenous LysG from *Corynebacterium glutamicum*)	*Corynebacterium glutamicum*	(Binder et al., 2012)	Enhanced L-lysine production from *Corynebacterium glutamicum* by up to 185-fold by sorting chemical mutagenesis library (Binder et al., 2012) Screening of error-prone mutant enzyme libraries relieved feedback inhibition at key enzymatic steps, resulting in significantly improved L-lysine, L-arginine, and L-histidine production(Schendzielorz et al., 2014)
L-arginine	TF	ArgP/YFP (endogenous ArgP from *E. coli*)	*Escherichia coli*	(Binder et al., 2012)	–
L-serine	TF	NCgl0581/YFP (endogenous NCgl0581 from *Corynebacterium glutamicum*)	*Corynebacterium glutamicum*	(Binder et al., 2012)	–
O-acetyl-L-serine	TF	CysR/YFP (endogenous CysR from *Corynebacterium glutamicum*)	*Corynebacterium glutamicum*	(Binder et al., 2012)	–

Finally, the LuxR TF from *Vibrio fischeri* serves a native role in microbial quorum sensing and responds to the quorum signaling molecule oxohexanoyl-homoserine lactone (HSL). By linking this promoter system to GFP, Collins et al. successfully isolated eight mutants from an EP-PCR library that showed significantly increased sensitivity to octanoyl-HSL, a weak inducer of WT LuxR (Collins et al., 2005). These same variants were also responsive to pentanoyl-HSL, tetradecanoyl-HSL, and oxohexanoyl-HSL ligand, suggesting a broadening of tolerated effectors instead of a true shift in specificity to novel inducers. Nevertheless, this study provides yet another example of successful reprogramming of a TF-based biosensor to respond to new inducers.

3.2.1.2. Hybrid/chimeric TFs. Despite the broad range of molecules detectable with natural or redesigned TFs, a significant portion of chemical space is still not readily covered. To further broaden the scope of TF-based biosensors, the molecular recognition and transcriptional regulation domains can be synthetically merged into a single, novel biosensor to produce a hybrid TF. Despite not always being applicable, there are many advantages to constructing such chimeric TFs. Specifically, hybrid TFs enable the use of a prokaryotic ligand recognition domain along with a DNA binding module and transcriptional regulator suitable for use in fungal, plant, or mammalian cell systems. As an interesting example of this approach, the natural ability of *E. coli* to sense strong methylating agents was transferred into *S. cerevisiae* by fusing the N-terminal domain of the DNA methylation sensing Ada TF to the yeast Gal4 trans-activator (Moser et al., 2013). When combined with an engineered yeast promoter containing 8 Ada operators driving GFP, the chimeric Ada-Gal4 TF enabled fluorescence detection of methyl iodide (MeI) at levels as low as 28 μM in *S. cerevisiae*. A similar approach has also enabled the high throughput detection of S-adenosylmethionine (SAM) in *S. cerevisiae* using the *E. coli* SAM-responsive TF MetJ (Umeyama et al., 2013). In this example, fusion of MetJ to the B42 transcriptional activation domain yielded a hybrid TF that was capable of SAM-dependent expression from a yeast promoter containing an inserted MetJ operator site.

Extending upon this theme, fusions of dimer-forming enzymes (specific for the inducer of interest) to DNA binding domains (DBD) and/or transcriptional activation domains (TAD) are an effective way to establish hybrid TFs. As an example, Chou and Keasling have applied this approach to create chimeric TFs that can be used as biosensors of isopentenyl diphosphate (IPP), a compound for which there are currently no known TFs (Chou and Keasling, 2013). When IPP isomerase (Idi) was fused to the DBD of AraC, reporter expression from P_{BAD} in *E. coli* was reduced in the presence of increasing IPP concentrations. It was postulated that IPP-induced dimerization of Idi reduced the affinity of the chimeric TF for AraC DNA binding sites in the promoter, thus reducing reporter expression in the presence of sufficient IPP. A similar design was then recapitulated in yeast using Erg20p fused to the Gal4 DBD and the Gal4 TAD. In this case, slight activation (approximately twofold over baseline) was achieved using the Gal10 promoter in response to IPP. This strategy could potentially be optimized further and may be an option for detecting target molecules for which biosensors do not readily exist.

To further extend the potential of chimeric or hybrid TFs, the DNA binding domains can be more modular and programmable, as in the use of zinc finger (ZF) domains that have known target DNA sequences (Kang and Kim, 2000). Likewise, custom ZF proteins can be programmed to create a binding site analogous to the operator for a natural transcription factor (Beerli and Barbas, 2002). In this regard, fusing a ligand-responsive domain and a transcriptional modulator to the ZF protein can create a nearly synthetic TF-sensor. By way of example, Beerli et al. coupled the hormone receptor ligand domains for mifepristone (RU486), 4-hydroxytamoxifen (4-OHT), and ponasterone A to a customized zinc finger protein to create biosensors that modulate luciferase expression (Beerli et al., 2000). A similar approach has also

been developed by substituting the ZF DNA-binding domain for a programmable transcription activator-like effector (TALE) (Mercer, 2014). In a different design, the DNA-binding domain of zinc finger transcription factors was mutated to create a cavity that resulted in loss of the DNA-binding function (Lin et al., 2003). A variety of small molecule heterocyclic ligands, particularly indoles, were then shown to bind into this cavity and reconstitute DNA binding and subsequent reporter gene activation. While the breadth of inducer-binding modules that have been used in this manner is not wide, synthetic hybrid TFs have potential for use as biosensors if an appropriate ligand-binding domain can be discovered or engineered.

3.2.1.3. In vitro TF methods. Most of the TF-based biosensors described to date have been genetically encoded and implemented in vivo, however, there are also viable in vitro methods for exploiting the specificity of TF binding to the cognate TF binding site (TFBS). In an example of this approach, de los Santos et al. utilized an in vitro transcription/translation (TX-TL) system based on *E. coli* cell lysate to prototype biosensors (de los Santos et al., 2016). This approach consisted of introducing two plasmids into the TX-TL cell lysate: one encoding the QacR transcription factor and one with its cognate promoter (containing QacR TFBS) driving a GFP reporter. TX-TL reactions were then assayed for GFP activity in the presence of exogenous vanillin to select for biosensor designs that could recognize this alternative inducer. This approach is mechanistically similar to the in vivo action of TF-based biosensors, and final designs also functioned in vivo in *E. coli*.

The amplified luminescent proximity homogeneous assay (ALPHA) format has been utilized in 384-well and 1536-well plate based high-throughput screening for pharmaceutically active molecules (Yasgar et al., 2016). Similar to Förster resonance energy transfer (FRET), the ALPHA signal is transduced by molecular proximity. In contrast to FRET, ALPHA relies on a time-resolved fluorescence signal propagated from the interaction of functionalized donor and acceptor beads. For use as a TF-based biosensor system, Li et al. attached a biotinylated TFBS to a streptavidin donor bead and a His-tagged TF to a nickel ligand acceptor bead (Li et al., 2017). The TF examples (HucR and OtrR) are repressors that bind with high affinity to their TFBS in the absence of inducing ligand, forming a bridge to bring donor and acceptor beads and generate a baseline ALPHA signal. In the presence of the appropriate inducer molecule (uric acid or oxytetracycline, respectively), the tested repressors dissociated from their TFBS, resulting in a concentration dependent reduction in ALPHA signal. This approach could potentially be applied to a wide variety of TFs to enable high-throughput in vitro deployment of TF-based screening.

3.2.1.4. Applications of TF-based biosensors. The aromatic amino acid pathway is used to produce precursors for a variety of industrially important compounds (Curran et al., 2013), and the development of TF-based biosensors has enabled wide-ranging applications and strategies for strain engineering toward overproduction of these target molecules. In *S. cerevisiae*, a tyrosine-responsive TF-based biosensor module based on the endogenous TF Aro9p was recently developed (Leavitt et al., 2016) and linked to a KanMX gene for product selection using an EMS-induced adaptive laboratory evolution approach to increase the heterologous production of the polymer precursor muconic acid by nearly 3-fold in yeast (Leavitt et al., 2017). To evolve increased tyrosine production in *E. coli*, TyrR was used as a sensor to regulate mutD5 mutator (the proofreading component of DNA polymerase III) and RFP activity in a process called feedback-regulated evolution of phenotype (FREP) (Chou and Keasling, 2013). As the level of tyrosine production in this scheme increases due to accumulated mutations, TyrR represses mutator and RFP expression to reduce mutation rate and “lock in” beneficial mutations. By isolating low RFP colonies, *E. coli* strains with up to a 5-fold increase in tyrosine production were successfully generated using FREP.

In addition to aromatic amino acids, some amino acids with hydrophobic side chains (e.g., L-valine, L-leucine, L-isoleucine, L-methionine) or positively charged side chains (e.g., L-lysine, L-arginine, L-histidine) have value as metabolic engineering targets due to their nutritional value or use as chemical building blocks (Mustafi et al., 2012). These amino acids have been produced in large-scale, industrial quantities using *Corynebacterium glutamicum* for many years, but TF-based biosensors have recently enabled further advances in production. For example, the *C. glutamicum* transcription factor Lrp has been used to enable high-throughput FACS screening of chemically mutagenized strains to select for millimolar (mM) level production of L-valine, L-leucine, and L-isoleucine in a situation where the parental wild type strain does not produce these branched chain amino acids (Mustafi et al., 2012). The same Lrp-based sensor was later used to evolve a *C. glutamicum* strain with increased growth rate, increased L-valine production, and significantly reduced byproduct production (Mahr et al., 2015). One of the individual key mutations identified by this approach demonstrated ability to increase L-valine by nearly 100%, and another key mutation was shown to inhibit undesirable L-alanine byproduct production. A LysG-based LTTR biosensor has also been used to select for up to 185-fold improved lysine production from a chemically mutagenized *C. glutamicum* strain pool (Binder et al., 2012). This screen also identified a novel G81E mutation in *murE* (encoding UDP-*N*-acetylmuramyl-tripeptide synthetase) that increased lysine accumulation up to 20-fold in some strain backgrounds. The same biosensor has since been applied to enzyme engineering in addition to strain engineering, resulting in the isolation of mutant alleles for *C. glutamicum argB, lysC*, and *hisG* that released feedback inhibition (Schendzielorz et al., 2014). The elimination of feedback inhibition in these key amino acid biosynthesis enzymes increased production from 0.4 to 34.9 mM, 1.2 to 44.9 mM, and 0.0 to 17.3 mM for arginine, lysine, and histidine, respectively.

Similar to amino acids, the mevalonate pathway has also attracted considerable attention for metabolic engineering due to the diverse range of value-added downstream compounds, including polymer precursors, biofuels and pharmaceuticals (Martin et al., 2003). Using the previously discussed mevalonate-responsive mutant AraC biosensor, Tang and Cirino were able to screen a randomized ribosomal binding site (RBS) library for increased translation of soluble HMG−CoA reductase and a resulting 3–4 fold improvement in mevalonate production in *E. coli* (Tang and Cirino, 2011). The same mevalonate biosensor has also more recently been utilized to screen a library of transcriptional regulator (QscR) mutants in the methylotrophic *Methylobacterium extorquens*, resulting in a 60% increase in mevalonate production (Liang et al., 2017). Downstream of mevalonate, biosensors have also been applied for increasing the production of the key pathway intermediate isopentenyl diphosphate (IPP). Using the previously discussed FREP scheme, a 3-fold increase in *E. coli* lycopene production has been achieved through biosensor-enabled evolution of elevated cellular IPP levels (Chou and Keasling, 2013). While lycopene itself is a brightly colored product that can be screened in high throughput through colorimetric means, this method could be employed to improve production of other less easily detected IPP-derived products as well.

Due to their roles as chemical building blocks, implication in environmental pollution, and ease of detection using TFs, a variety of applications have been described for TF-based biosensing of aromatic compounds and their catabolic products. Screening for aromatic compound production using TFs is also particularly promising due to the relatively high plasticity of these TFs and large number of resulting known mutants (discussed above) with specificity for new effectors. For example, a *Pseudomonas putida* TF mutant (NahR) with altered ligand specificity for benzoate has been used to enrich for benzaldehyde dehydrogenase expressing *E. coli* whole-cell biocatalysts from a one in one million mixture with non-catalyst cells (van Sint Fiet et al., 2006). TF-based biosensors for aromatics have also been applied to rapidly screen large metagenomics libraries and identify candidate enzymes for

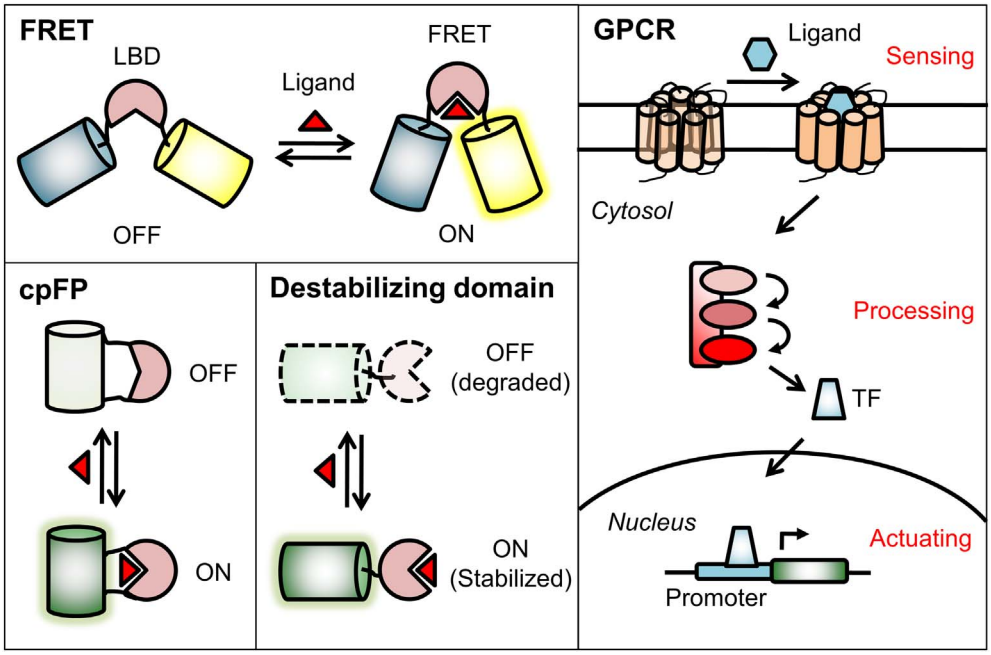


Fig. 3. Fluorescence protein- and GPCR-based biosensors. FRET: the efficiency is enhanced in the presence of the ligand. cpFP: fluorescence is turned on when the ligand is bound. Destabilizing domain: fluorescence of the destabilizing domain (DD)-fluorescence protein (FP) is preserved via binding of the ligand to DD, preventing the DD-FP fusion from protein degradation. GPCR: expression of the reporter is informed through the signaling cascade initiated by GPCR-ligand interaction. Abbreviations: FRET, Förster resonance energy transfer; cpGFP, circularly permuted GFP; GPCR, G-protein coupled receptor; TF, transcription factor.

bioconversions. For example, a benzoate-responsive BenR biosensor (from *P. putida*) was used to screen a 96,000 member metagenomics library for amidase enzymes, leading to the identification of three novel enzymes with specific activity toward the substrate benzamide (Uchiyama and Miyazaki, 2010).

3.2.2. *Fluorescent proteins (FRET, circular permuted FP, and destabilizing domains)*

Moving beyond TFs, another class of proteins, fluorescent proteins (FPs), can be extended to detect metabolites via introduction of ligand-binding domains. In this section, we consider three modes of FP sensors (Fig. 3)—Förster resonance energy transfer (FRET) (Heim and Tsien, 1996), circularly permuted (Baird et al., 1999), and destabilizing domain-coupled (Feng et al., 2015). A list of FP-based biosensors is summarized in Table 3.

Proteins often change conformation upon ligand binding (Teague, 2003). This structural change can be exploited as a sensing modality for applications such as FRET-based and circularly permuted FP biosensors. The FRET-based biosensor comprises an internal ligand-binding domain (LBD) flanked by a donor and an acceptor FP (Frommer et al., 2009; Heim and Tsien, 1996). Binding of the metabolite to the LBD induces conformational change, leading to a change in the intramolecular distance of the donor and acceptor FPs, thereby resulting in altered FRET efficiency. Over the years, a palette of FRET-based biosensors has been developed, which target a variety of metabolites and cofactors (Li et al., 2006).

In a similar fashion to FRET-sensors, it is possible to transduce the conformational change of ligand binding through a single FP. In this case, the protein is circularly permuted, in which the N- and C-termini of the protein are connected with a short peptide linker. Translation thus begins with an internal amino acid (Baird et al., 1999). Such circularly permuted FPs (cpFPs) have an internal ligand binding domain (LBD) that establishes a ligand-induced conformation change (Nagai et al., 2001). Recently, a transposase-assisted domain insertion method was exploited in vitro to generate a random domain-insertion library, which significantly accelerated the speed of diversifying insertion variants and minimized the need for structural information to guide rational design of insertions (Nadler et al., 2016). This approach was then applied to engineer a sensor for trehalose.

As an alternative approach, biosensors have been established through the concept of a destabilizing domain (DD) (Feng et al., 2015). In this approach, the stability of the LBD can be evolved to be ligand dependent with binding preventing protein degradation, thus transforming the LBD to DD (Banaszynski et al., 2006; Miyazaki et al., 2012). In this regard, the chemical controllable manner of DD is reversible and dose dependent (Banaszynski et al., 2006). As an example of this approach, computationally-designed LBDs linked together with yeast enhanced GFPs (yEGFPs) were mutagenized for propensity toward protein degradation, and the biosensors were developed to detect digoxin and progesterone (Feng et al., 2015). These efforts demonstrate the possibility of small protein domains to enable fluorescent proteins to serve as biosensors directly.

3.2.3. *G-protein-coupled receptors*

As a final set of protein-based biosensors, G-protein coupled receptors (GPCRs) serve as a natural starting point. These cell membrane proteins detect a diverse set of extracellular small molecules, including odorants (Dryer and Berghard, 1999), metabolites (Dryer and Berghard, 1999), and biogenic amines (Liberles, 2015). The signal from binding is transmitted via intracellular signaling pathways, which then triggers corresponding transcription factors and promoters (Fig. 3) (Bardwell, 2005). Synthetic signal transduction pathways enabled by swapping protein domains has expanded this approach in mammals and yeast (Kiel et al., 2010; Lienert et al., 2014; Muller et al., 2017). Such modularity opens up great opportunities for biosensor development (Mukherjee et al., 2015) and drug discovery (Lappano and Maggiolini, 2011). As examples, extracellular fatty acid concentrations are sensed by mammalian GPCRs (OR1G1 and GPR40) targeting fatty acids, and the resulting signal is transmitted through mating pathway components consisting of Ste. proteins and Fus3. Finally, GFP is expressed with the synthetic transcription factor based on Gal4 DNA binding and activation domains, and its cognate synthetic promoter (Mukherjee et al., 2015). In a similar approach, although the intent was not for high throughput screening, the biosensors have been developed to detect molecules including eugenol, coumarin, dihydrojasmone, and acetophenone (Muller et al., 2017). The GPCR-based biosensors discussed above are summarized in Table 3.

3.3. *Nucleic acid-based biosensors*

While proteins are rather complex and have evolved to interact with the metabolome, nucleic acids can also serve as biosensors despite their

Table 3
Fluorescence protein- and GPCR-based biosensors and highlighted applications for strain engineering.

Target molecule	Modality class	Sensor/reporter	Biosensor host	Sensor development	Highlighted applications
Lactate	FP (FRET)	LldR/mTFP & Venus (from E. coli)	Mammalian (HEK293)	(San Martin et al., 2013)	–
Lysine	FP (FRET)	LAO/CFP & YFP (from *Salmonella enterica* serovar trphimurium LT2)	*Escherichia coli* and *Saccharomyces cerevisiae*	(Ameen et al., 2016)	–
Citrate	FP (FRET)	CitA/CFP & Venus (from *Klebsiella pneumoniae*)	*Escherichia coli*	(Ewald et al., 2011)	–
Leucine	FP (FRET)	LivK/CFP & YFP (from *E. coli*)	*Escherichia coli*	(Mohsin et al., 2013)	–
Glutamate	FP (FRET)	jbeJ/CFP & Venus (from *E. coli*)	Rat brain slice	(Dulla et al., 2008)	–
Glucose	FP (FRET)	MglB/CFP & YFP (from *E. coli*)	Mammalian (HEK293)	(Hou et al., 2011)	–
Maltose	FP (FRET)	MBP/ECFP & EYFP (from *E. coli*)	*Saccharomyces cerevisiae*	(Fehr et al., 2002)	–
Methionine	FP (FRET)	MetN/CFP & YFP (from *E. coli*)	*Saccharomyces cerevisiae*	(Mohsin and Ahmad, 2014)	–
Glutamine	FP (FRET)	glnH/mTFP1 & venus (from *E. coli*)	Mammalian (COS-7)	(Gruenwald et al., 2012)	–
Inositol 1,4,5-triphosphate	FP (cp)	rIP3R3/ECFP & EYFP (from rat)	Mammalian (SH-SY5Y)	(Tanimura et al., 2004)	–
ATP/ADP ratio	FP (cp)	GlnK1/Venus (from *M. jannaschii*)	Mammalian (HEK293)	(Berg et al., 2009)	–
NADH	FP (cp)	Rex/YFP (from *Bacillus subtilits*)	Mammalian (293FT)	(Zhao et al., 2011)	–
Maltose	FP (cp)	MBP/GFP (from *E. coli*)	*Escherichia coli*	(Nadler et al., 2016)	–
2-oxogluatarate	FP (cp)	GAF/YFP (from *Azotobacter vinelandii*)	*Escherichia coli*	(Zhang and Ye, 2014)	–
Digoxin	FP (DD)	DIG_0/EGFP	*Saccharomyces cerevisiae*	(Feng et al., 2015)	–
Progesterone	FP (DD)	PRO_0/EGFP (from human)	*Saccharomyces cerevisiae*	(Feng et al., 2015)	–
Medium-chain fatty acids (C8, C10, & C12)	GPCR	OR1G1/GFP (from human)	*Saccharomyces cerevisiae*	(Mukherjee et al., 2015)	–
Eugenol	GPCR	OREG/mCherry (from mouse)	Mammalian (Hana3 A)	(Muller et al., 2017)	–
Coumarin	GPCR	OR5P3/mCherry (from human)	Mammalian (Hana3 A)	(Muller et al., 2017)	–
Dihydrojasmone	GPCR	OR1A1/mCherry (from human)	Mammalian (Hana3 A)	(Muller et al., 2017)	–
Acetophenone	GPCR	OR129–1/mCherry (from mouse)	Mammalian (Hana3 A)	(Muller et al., 2017)	–

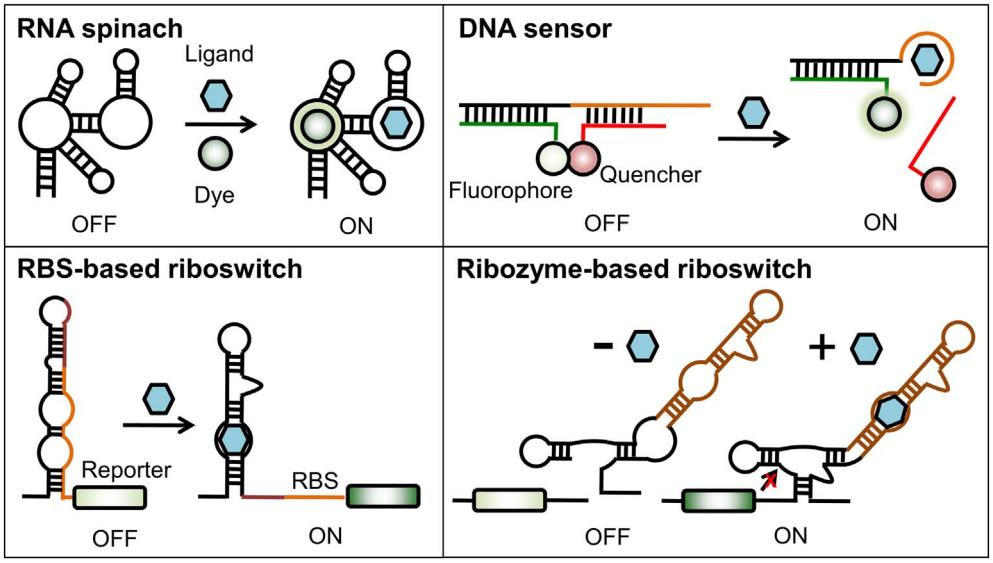


Fig. 4. Nucleic acid-based biosensors. RNA spinach: the fluorescence is activated when the ligand and dye are bound. The structure switching-based DNA biosensor: it is lighted on upon exclusion of ssDNA-quencher by ligand binding in the initial tripartite structure. RBS-based riboswitch: translation of the reporter encoded is activated by exposing RBS for ribosome access when the ligand is bound. Ribozyme-based riboswitch: the ribozyme activity is disrupted if the ligand is entrapped. Abbreviations: ssDNA, single-stranded DNA; RBS, ribosome binding site.

"simpler" biochemical language of four natural nucleotides. The mechanism-of-action of these modalities takes advantage of structural changes of the nucleic acid that result from direct binding of sequence and ligands. In this section, we describe RNA riboswitches, RNA spinach, and structure switching-based DNA biosensors that are able to detect and respond to an analyte (Fig. 4). Applications for sensing via this modality are also discussed.

3.3.1. RNA riboswitches

RNA riboswitches can sense various metabolites (via RNA aptamer domains) and respond to such stimuli through modifying expression and translation (Serganov and Nudler, 2013). Likewise, synthetic RNA riboswitches can alter transcriptional, translational, and posttranslational processes. For more details, a comprehensive review is available (Chang et al., 2012). Here, we focus on the use of ribosome binding site (RBS) and ribozyme-based biosensors and their utility for metabolite screening and strain engineering (Fig. 4 and Table 4).

The RBS-based RNA riboswitch functions through a ligand-responsive RNA aptamer in the 5′ untranslated region (5'UTR) that influences translation rate (Desai and Gallivan, 2004; Kotter et al., 2009). For bacteria, ligand binding can promote translation via RBS release whereas in eukaryotes, ligand binding can create RNA secondary structure that inhibits translation by blocking ribosome binding or scanning. Using this approach, RNA aptamers responsive to thiamine pyrophosphate (TPP) were linked with GFP or RFP to develop fluorescent sensors in *E. coli* (Nomura and Yokobayashi, 2007).

Table 4
Nucleic acid-based biosensors and highlighted applications for strain engineering.

Target molecule	Modality class	Sensor/reporter	Biosensor hos4t	Sensor development	Highlighted applications
Theophylline	RNA riboswitch (RBS)	RNA aptamer/DsRed	*Escherichia coli*	(Lynch and Gallivan, 2009)	–
Theophylline	RNA riboswitch (ribozyme)	RNA aptamer/GFP	*Saccharomyces cerevisiae*	(Michener and Smolke, 2012)	Directed evolution of bacterial P450 monooxygenase, and improve 33-fold increase in activity (Michener and Smolke, 2012)
Thiamine pyrophosphate	RNA riboswitch (RBS)	RNA aptamer/GFPuv	*Escherichia coli*	(Muranaka et al., 2009)	–
Atrazine	RNA riboswitch (RBS)	RNA aptamer/β-galactosidase	*Escherichia coli*	(Sinha et al., 2010)	–
Xanthine	RNA riboswitch (ribozyme)	RNA aptamer/GFP	*Saccharomyces cerevisiae*	(Win and Smolke, 2007)	Detection of xanthine biosynthesis in yeast (Win and Smolke, 2007)
Neomycin	RNA riboswitch (ribozyme)	RNA aptamer/GFP	*Saccharomyces cerevisiae*	(Townshend et al., 2015)	–
Tetracycline	RNA riboswitch (ribozyme)	RNA aptamer/GFP	*Saccharomyces cerevisiae*	(Townshend et al., 2015)	–
Adenosine	RNA spinach	RNA aptamer/fluorophore		(Paige et al., 2012)	–
Guanine	RNA spinach	RNA aptamer/fluorophore		(Paige et al., 2012)	–
ADP	RNA spinach	RNA aptamer/fluorophore		(Paige et al., 2012)	–
GTP	RNA spinach	RNA aptamer/fluorophore		(Paige et al., 2012)	–
SAM	RNA spinach	RNA aptamer/fluorophore	*Escherichia coli*	(Paige et al., 2012)	–
Cyclic di-GMP	RNA spinach	RNA aptamer/fluorophore		(Kellenberger and Hammond, 2015)	–
Thiamine pyrophosphate	RNA spinach	RNA aptamer/fluorophore		(You et al., 2015)	–
Pyrithiamine pyrophosphate	RNA spinach	RNA aptamer/fluorophore		(You et al., 2015)	–
5-Hydroxytrptophan	RNA Broccoli (spinach variant)	RNA aptamer/fluorophore	*Escherichia coli*	(Porter et al., 2017)	–
Serotonin	RNA Broccoli (spinach variant)	RNA aptamer/fluorophore	*Escherichia coli*	(Porter et al., 2017)	–
Dopamine	RNA Broccoli (spinach variant)	RNA aptamer/fluorophore	*Escherichia coli*	(Porter et al., 2017)	–
ATP	DNA biosensor	DNA aptamer/fluorophore		(Nutiu and Li, 2003)	–
GTP	DNA biosensor	DNA aptamer/fluorophore		(Nutiu and Li, 2005)	–
Glucosamine 6-phosphate (GlcN6P)	RNA riboswitch (ribozyme)	RNA aptamer/FCY1	*Saccharomyces cerevisiae*	(Lee and Oh, 2015)	Screened mutated GFA in *S. cerevisiae* and discovered a mutant that improved over 5-fold GlcN6P production (Lee and Oh, 2015)

Ribozyme-based RNA riboswitch consists of two components, cis-acting hammerhead ribozyme (regulatory domain) and RNA aptamer (sensor domain). The binding of ligand to the sensor domain controls catalytic activity of ribozyme due to strand-displacement, leading to alteration of stability of the RNA molecule and functional translation. The design was used to develop biosensors to detect theophylline, xanthine, and glucosamine 6-phosphate (GlcN6P) (Lee and Oh, 2015; Win and Smolke, 2007). These RNA sensors have been used to screen enzymes from directed evolution with improved activities. For examples, yCDM1 is an enzyme variant of bacterial P450 monooxygenase, which demethylates caffeine to theophylline with poor activity. The theophylline biosensor (enabled by ribozyme-based RNA) was used for enzyme evolution in yeast. In this case, binding of theophylline to RNA riboswitch deactivates the ribozyme activity; as a consequence, GFP reporter is expressed due to successful translation. To evolve the enzyme, EP-PCR was employed to mutagenize the gene and the biosensor was used for high throughput screening with FACS. A mutant version, yCDM8, was obtained showing 33-fold increase in enzyme activity and 22-fold increase in selectivity, a ratio comparing production of theophylline to paraxanthine (the side product) (Michener and Smolke, 2012). In contrast with this mechanism, the binding of ligand to ribozyme-based RNA riboswitch can also activate the catalytic activity. In the other case, *glmS* ribozyme was encoded to 3'UTR of *FYC1*, which converts fluorocytosine to toxic fluorouracil. Under low GlcN6P concentration, the ribozyme-inserted FYC1 mRNA remains intact, which ensures functional expression of the enzyme, leading to cell death. Upon GlcN6P concentration is increased, the binding of ligand to the ribozyme enables the cleavage of the mRNA, resulting in reduction of protein expression due to 3′ exonuclease attack and rescue cell viability. Such RNA riboswtich enabled suicide program was used to screen two enzymes involved in GlcN6P biosynthesis pathway, glutamine-fructose-6-phosphate transaminase (GFA). To mutagenize GFA, EP-PCR was utilized to create a mutant library. Assisted by the growth associated screening method, a mutant GFA1-m12 was identified with over 5-fold increase when the variant was expressed in *S. cerevisiae* (Lee and Oh, 2015).

3.3.2. RNA Spinach

As an alternative, non-transcript dependent RNA sensor, the RNA Spinach molecule is capable of sensing intra- and extra-cellular small molecules and reporting concentrations with fluorescent output (Paige et al., 2012; Paige et al., 2011). RNA Spinach is composed of scaffold (dye binding), transducer, and ligand binding module. The small molecule is first bound by the ligand-binding module, and fluorescence occurs once the dye is bound to RNA-ligand complex. This biosensor design is theoretically modular and generalizable, as the ligand binding module can be functionally swapped with any pre-defined or identified RNA aptamer (Strack and Jaffrey, 2013). As many RNA aptamers have been well documented (McKeague and Derosa, 2012; Pfeiffer and Mayer, 2016; Ruscito and DeRosa, 2016), the plug-and-play attribute enables broad biosensor development. In this regard, many RNA Spinach biosensors have been developed to detect small molecules and cofactors including molecules such as 5-hydroxytrptophan (5HTP), ADP, GTP, and thiamine pyrophosphate, which are summarized in Table 4. An additional benefit of RNA Spinach is the reported linear fluorescent response to analyte binding (Paige et al., 2012). When these biosensors are applied in vivo, distribution and dynamics of metabolites and RNA can be monitored and tracked (Guet et al., 2015; You et al., 2015). This type of sensor is theoretically capable of applications of high-throughput metabolite detection.

Evolving RNA sequences to bind analytes in vitro is the common way to create RNA aptamers for molecule detection. However, importing such RNA aptamers to function in cells is often problematic as folding of RNA in intracellular environment is poor. This technical challenge was addressed by employing natural RNA ribozymes and riboswitch as structural scaffolds for in vivo function. In the study of Porter et al., guanine riboswitch (GR) from *Bacillus subtilis* was evolved to recognize 5HTP, serotonin, and dopamine by in vitro selection. By integrating the derived analyte recognition riboswitch with RNA Broccoli (RNA spinach variant) and tRNA bridged by a communication module, the developed biosensors were functional in *E. coli* (Porter et al., 2017).

3.3.3. DNA biosensors

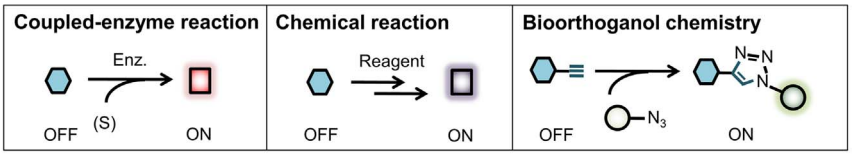
DNA can also serve to detect small molecules. DNA biosensors require three pieces—one that contains stem and DNA aptamer, a second short oligo, complementary to the stem sequence, that is modified with a fluorophore (FDNA), and a third short oligo that is complementary to the DNA aptamer that is modified with a fluorescence quencher (QDNA). The three DNA oligos form a tripartite structure, in which the fluorophore and quencher are designed to be located in close proximity, resulting in low fluorescence of the sensor. In the presence of the target molecule, QDNA is expelled giving rise to enhancement of fluorescence intensity (Fig. 4) (Nutiu and Li, 2003, 2004). Similar to RNA aptamers, extensive reviews of DNA aptamers are provided elsewhere (McKeague and Derosa, 2012; Pfeiffer and Mayer, 2016; Ruscito and DeRosa, 2016), which provides a niche for biosensor development. In this regard, DNA biosensors have been developed to detect ATP and GTP (Table 4) (Nutiu and Li, 2003, 2005).

3.4. Coupled enzyme reactions

Molecular detection modalities describes so far either relied on intrinsic molecular traits or a binding event with the analyte to create a detectable signal. We next turn to a series of technologies that rely on chemical/enzymatic reactions to establish a detectable signal. In particular, enzymes can be used to convert metabolite concentration into chromogenic and fluorogenic substances that can be detected (Fig. 5). This technology has long been used to assay for individual chemical concentrations in food product and supernatants through the absorbance of species like NAD(P)H (Kleyn, 1985; Pomeranz, 2013) Transducing metabolite levels into a detectable signal can require either one-step enzymatic reactions or a cascade of reactions. Detailed considerations regarding conditions for these enzyme couplings including buffers, pH, temperature, ions, etc., have been reviewed previously (Acker and Auld, 2014; Bisswanger, 2014). Here, we focus on several enzymatic reactions that connect metabolic products to chromogenic or fluorogenic derivatives. Coupled enzyme assays that detect a series of small molecules and their applications in metabolic engineering field are summarized in Table 5.

In some cases, single enzyme steps can convert metabolites of interest into a chromogenic or fluorescent substrate. As examples, pigmented melanin is biosynthesized by tyrosinase activity on tyrosine (Santos and Stephanopoulos, 2008b). Fluorescent betaxanthin is generated by conversion of $_{L}$-DOPA via DOPA dioxygenase activity (DeLoache et al., 2015). This activity was then used to engineer tyrosine hydroxylase and resulted in a 2.8-fold improved activity using this method (DeLoache et al., 2015). In most cases, additional substrates and cofactors are needed to form a detectable molecule. Fig. 5b & c highlight a few coupled-enzyme reactions, their byproducts, and paths toward color and fluorescence development. As examples, long-chain alcohol (a molecule of interest for biofuels applications) can be detected via an enzyme-coupled reaction. The alcohols are converted by long-chain alcohol oxidase with concomitant production of byproduct, hydrogen peroxide (H_2O_2) (Vanhanen et al., 2000). By coupling horseradish peroxidase (HRP) activity, the H_2O_2 byproduct is used to oxidize Amplex UltraRed, leading to the generation of the detectable, fluorescent compound resorufin (Wang et al., 2014). A similar cascade can be used for other products of interest. Morphine concentration can be quantified with fluorescence by pairing morphine dehydrogenase (Bauer et al., 1999) and diaphorase (Zhu et al., 2009), in which the NADH generated from the first enzyme is utilized by the second to

(a)



(b)

Enzyme subclass	Byproduct	Example
Oxidase	H_2O_2	Long chain alcohol oxidase
Phosphoribosyl transferase, CoA ligase, synthase	PPi	Taxadiene synthase
Dehydrogenase	NAD(P)H	Morphine dehydrogenase
Acyl transferase and hydrolase	CoA	Alcohol acetyl/acyltransferase
CoA transferase and decarboxylase	Acetyl-CoA	Malonyl-CoA decarboxylase
Methyltransferase	AdoHcy	Resveratrol O-methyltransferase
Kinase	ADP	Mevalonate kinase

(c)

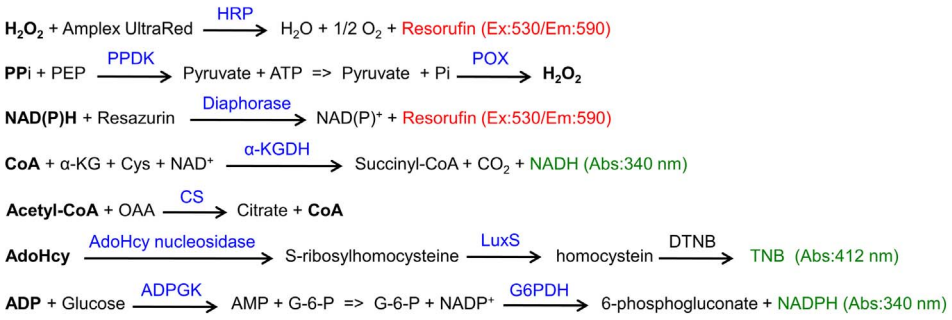


Fig. 5. Reaction-based chromogenic or fluorogenic modalities. (a) Absorbance and fluorescence for analytes can be achieved by coupled-enzyme reactions, chemical reactions, and bioorthoganol chemistry. (b) Enzyme subclasses, examples, and byproducts they commonly produced. (c) Reaction designs of coupled-enzyme assays that detect byproducts produced from enzymes reacting to analytes of interest. Fluorogenic and chromogenic detection of PPi and acetyl-CoA can be linked to H_2O_2 and CoA assays, respectively. Abbreviations: Enz., enzyme; HRP, horseradish peroxidase; PPDK, pyruvate phosphate dikinase; POX, peroxidase; α-KGDH, α-ketoglutarate dehydrogenase; CS, citrate synthase; AdoHcy, s-adenosyl-L-homocysteine; LuxS, S-ribosylhomocysteine lyase; ADPGK, ADP-dependent glucokinase; G6PDH, glucose-6-phosphate dehydrogenase; Ex/Em, excitation/emission; Abs, absorbance.

produce resorufin. Resveratrol concentration can be determined with absorbance by coupling resveratrol *O*-methyltransferase (Schmidlin et al., 2008), s-Adenosyl-L-homocysteine (AdoHcy) nucleosidase, S-ribosylhomocysteine lyase (LuxS), and Ellman's reagent (DTNB) to ultimately produce the colored compound TNB (Hendricks et al., 2004).

A similar scheme can be used for enzyme discovery and engineering. To rapidly screen alcohol-O-acyltransferase (AATase) activity toward ester biosynthesis from different organisms, a coupled-enzyme assay was developed and used to determine kinetics, in which AATase from tomato was discovered with high activity toward C4 to C10 acyl-CoA when ethanol was used as the co-substrate (Lin et al., 2016). To find high adenosine producers, *E. coli* was mutagenized with a laser and adenosine concentration was assayed colorimetrically with a combination of enzyme and chemical reactions (Dong et al., 2015b). The screen yielded four high producing mutants with a top production of 1.2 mM. For the engineering of β-glucosidase, HRP was displayed on the yeast cell surface and both substrates and fluorogenic reagents were co-encapsulated in microdroplets for analysis of enzyme activity. After incubation, a handful of enzyme mutants derived from EP-PCR mutagenesis exhibited more than 10-fold enhancement in activity (Agresti et al., 2010).

The use of coupled-enzyme systems in conjunction with microdroplet systems can establish many routes for rapid small molecule detection. As an example of this, Wang et al. used a coupled-enzyme assay to assess xylose concentration in microfluidic droplets to screen for improved xylose utilization by *S. cerevisiae* (Wang et al., 2014). The result was a strain that had undergone adaptive evolution with multiple copies of *xylA*. Subsequently, this same approach was used to enhance lactate production. In a final instance, a microdroplet-enabled FACS based screening strategy was also developed for improving cellulase activity. In this study, Ostafe et al. used double emulsions to encapsulate yeast cells expressing cellulase in microfluidic droplets (Ostafe et al., 2013). Glucose that was released by cellulose activity on carboxymethylcellulose substrate was detected via a hexose oxidase-based coupled enzyme assay and enrichment of a positive population was demonstrated based on droplet fluorescence.

3.5. Chemical reactions and bioorthogonal chemistry

In the section above, we discussed enzymes as the driving force between converting a nonchromogenic molecule into a detectable species. However, prior to prevalence of recombinant DNA technology, such conversions were only performed chemically (Fig. 5a). In this regard, metabolites of interest can react with chemical reagents to create a molecule that is easily detectable via absorbance. Though chemicals for color development are off the shelf, one might be cautious the quality of chemicals used as reactions are sometimes sensitive to impurities (Lobs et al., 2016). Nevertheless, we discuss a few such reactions that target various molecules including esters, alcohols, aldehydes, ketones, fatty acids, and tyrosine, which are included in Table 5.

Esters (Hill, 1946) and amides (Bergmann, 1952) react with hydroxylamine in alkaline solution to form hydroxamic acid which can be turned into a red ferric hydroxamate complex subsequent to addition of ferric iron to the solution. As an example, ethyl acetate produced from various sugar carbon sources in *K. marxianus* strains can be determined in microtiter plates via a chemical reaction-based colorimetric assay (Lobs et al., 2016). Alcohol can be converted into a violet-colored complex via oxidization with potassium dichromate-sulfuric acid reagent followed by *s*-Diphenylcarbazide reaction (Williams and Reese, 1950). Aldehydes and ketones react with 2,4-dinitrophneylhydrazine to form yellow-orange colored 2,4-dinitrophenylhydrazone in acetic solution (Bartos and Pesez, 1979). Tyrosine and to tyramine react with 1-nitroso-2-naphthol in nitric acid solution to form red-colored derivatives (Udenfriend and Cooper, 1952). As an example, the assay was used to quantify tyrosine, a pathway flux surrogate for muconic acid production. Combining adaptive laboratory evolution (ALE) and EMS mutagenesis, a strain was generated with 3-fold increase in titer of the composite muconic acid pathway compared to the parental engineered strain (Leavitt et al., 2017). Many of these traditional chemical approaches are used for quick assays of supernatants and are amenable to automation platforms.

Beyond chemical reactions, target molecules (including proteins, lipids, and glycans) can be labeled with fluorescent probes via bioorthogonal chemical reactions (click chemistry) (Bertozzi, 2011; Li and Chen, 2016). The chemical conjugation is enabled by cycloaddition of

Table 5
Reaction-based biosensing modalities and highlighted applications for strain engineering.

Target molecule	Modality class	Enzyme or reagent/Reporter (chromogenic/ fluorogenic property)	Biosensor host	Assay development	Highlighted applications
Carboxymethylcellulose	Coupled-enzyme reaction	HOx & VbrPOx/3-carboxy-7-hydroxycoumarin (fluorescence)	*Saccharomyces cerevisiae*	(Ostafe et al., 2013)	Enrichment of yeast cells with high cellulase activity (Ostafe et al., 2013)
CoA	Coupled-enzyme reaction	α-KG DeH/NADH (absorbance)	*Saccharomyces cerevisiae*	(Lin et al., 2016)	Screened AATase activity in *S. cerevisiae*, and identified ATF from tomato with high activity toward C4 to C10 acyl-CoA (Lin et al., 2016)
L-DOPA	Coupled-enzyme reaction	DOD/Betaxanthin (fluorescence) & betanidin (absorbance)	*Saccharomyces cerevisiae*	Overexpression of DOD to derive fluorogenic or chromogenic compounds (DeLoache et al., 2015)	Directed evolution of tyrosine hydroxylase. 2.8-fold enhanced activity was achieved (DeLoache et al., 2015)
Hydrogen peroxidae	Coupled-enzyme reaction	HRP/Resorufin (absorbance)	*Saccharomyces cerevisiae*	(Agresti et al., 2010)	Directed evolution of HRP with yeast surface display. More than 10-fold improvement in activity (Agresti et al., 2010).
Xylose	Coupled-enzyme reaction	Pyranose oxidase and HRP/Resorufin (absorbance)	*Saccharomyces cerevisiae*	(Wang et al., 2014)	Enrichment of yeast strains with high xylose consumption (Wang et al., 2014)
Lactate	Coupled-enzyme reaction	Lactate oxidase/Resorufin (fluorescence)	*Saccharomyces cerevisiae*	(Wang et al., 2014)	Enrichment of yeast strains with high lactate production (Wang et al., 2014)
Tyrosine	Coupled-enzyme reaction	Tyrosinase/Melanin (absorbance)	*Escherichia coli*	Expression of tyrosinase in *E. coli* (Santos and Stephanopoulos, 2008b)	Perturbed global gene expression with gTME, screened for high tyrosine production through melanin accumulation, and gained 114% increase (Santos and Stephanopoulos, 2008a; Santos et al., 2012)
Adenosine	Coupled-enzyme and chemical reaction	ADA and the chemical reagent/Diazonium salt (absorbance)	*Escherichia coli*	(Dong et al., 2015b)	Screened laser-mutagenized *E. coli* and identified a mutant with production of 1.2 mM (Dong et al., 2015b).
Ethanol	Coupled-enzyme reaction	ADH/MTT formazan (absorbance)		(Zanon et al., 2007)	–
Octanol & decanol	Coupled-enzyme reaction	ADH & luciferase/Light (luminescence)		(Minak-Bernero et al., 2004)	–
Octanal & decanal	Coupled-enzyme & chemical reactions	ADH & Schiff reagent/Purple color pigment (absorbance)		(Minak-Bernero et al., 2004)	–
Alkanes	Coupled-enzyme reaction	Alkane hydroxylase, ADH, & luciferase/Light (luminescence)		(Minak-Bernero et al., 2004)	–
Cytidine	Coupled-enzyme & chemical reactions	CDA & the chemical reagent/Indophenol (absorbance)		(Dong et al., 2015a)	–
Methionine	Coupled-enzyme reaction	AdoMetS, PPDK, POX, & HRP/Chromogen or resorufin (absorbance or fluorescence)		(Kameya et al., 2014)	–
Pyruvate	Coupled-enzyme reaction	POX & HRP/Chromogen or resorufin (absorbance or fluorescence)		(Kameya et al., 2014)	–
Arginine	Coupled-enzyme reaction	ADI, ASS, PPDK, POX, & HRP/Chromogen (absorbance)		(Kameya and Asano, 2014)	–
Citrulline	Coupled-enzyme reaction	ASS, PPDK, POX, & HRP/Chromogen (absorbance)		(Kameya and Asano, 2014)	–
Succinic acid	Coupled-enzyme reaction	FRD & Peroxidase/Chromogen or resorufin (absorbance or fluorescence)		(Sun et al., 2013)	–
Triglyceride	Coupled-enzyme reaction	Lipase, glycerol kinase, oxidase, & peroxidase/ Chromogen (absorbance)		(Fossati and Prencipe, 1982)	–
Fatty acids	Chemical reaction	The chemical reagent/chromogen		(Mackenzie et al., 1967)	–
Malonyl-CoA	Coupled-enzyme reaction	MCAT & KDH/NADH (absorbance)		(Molnos et al., 2003)	–
Ascorbate	Chemical reaction	The chemical reagent/Chromogen (absorbance)		(Vislisel et al., 2007)	–
Lactate	Coupled-enzyme reaction	Lactate-cytochrome *c* oxidoreductase/Formazan (absobance)		(Smutok et al., 2013)	–
Cholesterol	Coupled-enzyme reaction	Esterase, oxidase, HRP/Resorufin (fluorescence)		(Robinet et al., 2010)	–
Esters (ethyl acetate, ethyl butyrate, isoamyl acetate, ethyl hexanoate, and	Chemical reaction	Hydroxylamine and ferric ion/Ferric hydroxamate complex (absorbance	*Kluyveromyces marxianus*	(Hill, 1946; Lobs et al., 2016)	Demonstration of C5, C6, and C12 sugar utilization and ethyl acetate production of *K. marxianus* (Lobs

(continued on next page)

Table 5 *(continued)*

Target molecule	Modality class	Enzyme or reagent/Reporter (chromogenic/fluorogenic property)	Biosensor host	Assay development	Highlighted applications
ethyl octanoate)					et al., 2016)
Sugar alcohols	Chemical reaction	The chemical reagent/3,5-diacetyl-1,4-dehydrolutidine (absorbance)	*Pichia anomala*	(Zhang et al., 2015)	Screened yeast undergone genome shuffling, and acquired 32% increase in titer over parental strain (Zhang et al., 2015).
Fatty acids	Chemical reaction	The chemical reagent/ferric hydroxamate complex (absorbance)		(Hill, 1946)	–
Alcohol	Chemical reaction	Potassium dichromate-sulfuric acid reagent and *s*-Diphenylcarbazide reaction/Violet chemical complex (absorbance)		(Williams and Reese, 1950)	–
Aldehydes	Chemical reaction	2,4-Dinitrophneylhydrazine/formazoan (absorbance) & 9-substituted decahydroacridine-1,8-dione (fluorescence)		(Bartos and Pesez, 1979)	–
Ketones	Chemical reaction	2,4-Dinitrophneylhydrazine/2,4-dinitrophenylhydrazone (absorbance)		(Bartos and Pesez, 1979)	–
Tyrosine	Chemical reaction	1-Nitroso-2-naphthnol/Red color chromogen (absorbance)		(Udenfriend and Cooper, 1952)	Quantified tyrosine as a surrogate of pathway flux for muconic acid production in *S. cerevisiae* (Leavitt et al., 2017)
Hexanoic acid alkyne	Bioorthogonal chemsitry	Click chemistry/Fluorogenic probe (fluorescence)		(Zhu et al., 2016)	Directed evolution of JamB, and improved enzyme activity in 20-fold (Zhu et al., 2016)

azide-modified fluorescent probes to alkyne-bearing biomolecules, thus enabling a fluorescent detection (Fig. 5a). For example, the insulin receptor can be visualized when an alkyne-containing, non-canonical amino acid is incorporated into the receptor and this group reacts with azide-conjugated dye (Nikic et al., 2015). Just recently, this bioorthogonal chemistry has been applied to detect metabolites for metabolic engineering application to improve alkyne-containing natural products (Zhu et al., 2015; Zhu et al., 2016). Indeed, numerous metabolites including fatty acids and natural products contain acetylene or polyacetylenes (Minto and Blacklock, 2008). These products can be detected via the reaction of azido fluorogenic probes via click chemistry in culture medium (Zhu et al., 2016). Finally, to increase the activity of a membrane-bound bifunctional desturase/acetylenase (JamB) toward hexanoic acid, the gene was evolved using a fluorogenic assay (based on click chemistry) in microtiter plates to achieve a 20-fold increase in activity (Zhu et al., 2016) (Table 5). These examples demonstrate the potential of chemical reactions to aid in the detection of desired metabolites.

3.6. Dyes, probes, and chromogenic substrates

Finally, we turn to a final class of detection that comprises the use of exogenous fluorescent dyes and probes, chromogenic substrates and other indicators. These molecules can be added to supernatants and cultures to enable visualization and detection of metabolites. Small molecules including celluolose, acids, and triglycerides that can be detected by this class is included in Table 6.

Perhaps one of the most commonly used fluorescent dyes in metabolic engineering applications is Nile Red (Liu et al., 2015a; Terashima et al., 2015) (Fig.1). While the fluorescence of this dye is extremely low in water and organic solvents, it becomes significantly enhanced with surrounded by hydrophobic molecules. As a result, Nile Red has been used to detect triglycerides (TAG)(Blazeck et al., 2014), cholesterol in lipid droplets (LDs)(Rumin et al., 2015), and even polymers such as polyhydroxybutyrate (PHB)(Tyo et al., 2006). Based on its propensity to bind neutral lipids, Nile Red has been used in the metabolic engineering of many oleochemicals. For example, to boost lipid accumulation in the host *Yarrowia lipolytica*, lipid content from chemically mutagenized libraries was screened via Nile Red after a primary screen by physical property—buoyancy—of the compounds. Through the iterative mutagenesis and Nile Red/Floating Cell screening processes, a strain E26 carrying uga2 mutation was evolved, with 76% conversation of theoretical maximum yield, 87% lipid content, and 0.89 g/L/h instantaneous specific productivity (Liu et al., 2015a). This dye has also been used to screen random insertional mutagenesis libraries in the green algae, *Chlamydomonas reinhardtii*. Through the enrichment of high producers by FACS, at least six distinct strains were created with improved production (Terashima et al., 2015; Zhang et al., 2014). Additional product-binding dyes (beyond Nile Red) have been used in metabolic engineering applications. For example, to enhance the biosynthesis of hyaluronic acid in *E. coli*, gTME libraries (Alper et al., 2006) were screened on the basis of colony morphology (translucent colonies) and subsequently via alcian blue staining for product to achieve a titer of 561.4 mg/L, a 38% increase to the control strain (Yu et al., 2008).

The use of chromogenic or fluorescent substrates (i.e. a detectable moiety covalently linked to a substrate) for enzymes has been used as another way to enable high-throughput analysis of function (especially for enzyme evolution experiments). In this scheme, the physicochemical property of the moiety is quenched when it is bound to the substrate and activated upon cleavage activity by the enzyme. As such, enzymatic activity can be readily monitored with absorbance or fluorescence readouts without applying complex analytic methods (Maruthamuthu et al., 2016; Najah et al., 2014) (Fig. 1). For example, to identify enzymes degrading cellulose and hemicellulose, metagenomic libraries were prepared from soil microbial communities in

Table 6
Chromogenic/fluorogenic chemical-based biosensing modalities and highlighted applications for strain engineering.

Target molecule	Modality class	Reporter (chromogenic/fluorogenic property)	Biosensor host	Sensor development	Highlighted applications
Cellulose and hemicellulose	Chromogenic substrate	Indole (absorbance)	*Escherichia coli*		Screened metagenomic libraries, and discovered β-galactosidase and β-xylosidase (Maruthamuthu et al., 2016)
Cellobiose	Fluorogenic substrate	6,8-Difluoro-7-hydroxycoumarin-4-methanesulfonate (fluorescence)			Identified of bacteria from soil that have good cellobiohydrolase and endogluconase activities (Najah et al., 2014).
Dicaprylate	Fluorogenic substrate	Fluorescein (fluorescence)	*Escherichia coli*		Screened metagenomic library and found a gene encoding esterase (Hosokawa et al., 2015)
di-(β-D-glucopyranoside)	Fluorogenic substrate	Fluorescein (fluorescence)	*Escherichia coli*		Sequence-function mapping of Bgl3 (Romero et al., 2015)
Hyaluronic acid	Probe/dye	Alcian blue (absorbance)	*Escherichia coli*		Perturbed global gene expression with gTME, screened hyaluronic acid production with alcian blue, and obtained 38% increase in titer (Yu et al., 2008)
Triglyceride	Probe/dye	Nile red (fluorescence)	*Yarrowia lipolytica* & *Chlamydomonas reinhardtii*		Screened lipid accumulation of EMS mutagenized yeast and random insertional mutagenesis of algae (Liu et al., 2015a, 2015b; Terashima et al., 2015).
Ketoacids	pH indicator	Bromophenol blue (absorbance)	*Escherichia coli*		Protein engineering of KIVD for high specificity toward C8 ketoacid (Mak et al., 2015)
Lactic acid	pH indicator	cSNARF-4F AM (fluorescence)	*Saccharomyces cerevisiae*	(Valli et al., 2006)	–
α-Ketoglutaric acid	pH indicator	Bromocresol green & quinaldine (absorbance)	*Yarrowia lipolytica*	(Zeng et al., 2015)	Screened α-ketoglutaric acid production in *Y. lipolytica* (Zeng et al., 2015)

which multiple chromogenic substrates were fed to the host for color development of colonies, leading to the discovery of thermostable β-galactosidase and β-xylosidase enzymes (Maruthamuthu et al., 2016). A similar approach was conducted to discover enzymes that hydrolyze ester bond from a metagenomics library through the use of *E. coli* in droplet-based microfluidics using a fluorogenic substrate (Hosokawa et al., 2015). To bioprospect for microbes with enhanced cellulose hydrolysis, cellulose activity was assayed using fluorogenic substrates in droplets. After sorting, the enriched bacterial population, mostly consisting of *Gammaproteobacteria* and *Bacillales*, showed 17- and 7-fold enhancement of cellobiohydrolase and endogluconase activities, respectively (Najah et al., 2014).

The use of pH indicator dyes can be used to detect more crude changes in cell and supernatant composition (Han and Burgess, 2010; Ramamoorthy et al., 2009) (Fig. 1). Albeit not a direct measurement of metabolites, the monitoring of pH change can be useful to detect for the production of acids. In the study of Zeng et al. (Zeng et al., 2015), production of α-ketoglutaric acid (α-KG) from *Y. lipolytica* is implied by extracellular pH change. Six chromogenic pH indicators were evaluated and two, bromocresol green and quinaldine, were used for high throughput screening on agar plates and 96-well plates, respectively. Mutagenized by atmospheric and room temperature plasma (ARTP), a strain was evolved and showed 4-fold improved α-KG production. Finally, to improve substrate specificity of ketoisovalerate decarboxylase (KIVD) toward long-chain ketoacids, critical active site residues accommodating the substrate predicted computationally were mutagenized and activity was analyzed based on color change from bromophenol blue pH dye on microtiter plates. The resulting variant exhibited a higher specificity toward C8 than C3, C5, and isoC5 ketoacid and was applied for long-chain alcohol production (Mak et al., 2015). These findings demonstrate the use of various dyes and chromogenic probes to detect metabolites and enzymatic function.

3.7. Performance parameters and considerations

Although a wide variety of sensing modalities have been developed as biosensors, performance parameters such as specificity, sensitivity, linear range of detection, signal-to-noise (S/N) ratio, and dynamic range need to be carefully considered for each application. The potential promiscuous activity of biosensors and coupled-enzyme reactions requires As some biosensors and coupled-enzyme reactions show promiscuous activity, meticulous testing with a series of similar analytes with similarity (e.g., in functional groups or molecular shape/structure). to the target product is often required if the microbial host produces related endogenous metabolites or highly similar precursors. If sufficient specificity can be achieved, then the quality and efficacy of the sensing modalities can be partially characterized by S/N ratio and dynamic range across the desired or expected range of product concentrations to be detected. Unfortunately, these parameters are not always well-documented in the literature and are often inconsistent in unit presentation and even basic definition. This lack of standardization in characterizing the performance of biosensors (Dietrich et al., 2010) significantly hampers direct comparison of performance and suitability based on information available in the published literature. This challenge becomes particularly clear when examining historical trends over time and across the many different biosensor sub-fields and journals, as conventions and expectations for reporting biosensor performance have varied substantially.

Comparisons of biosensor performance are made significantly easier when overall transfer function data, describing the mathematical correlation between analyte concentration and biosensor signal, is provided in addition to calculated or inferred performance parameters. However, transfer function data is often represented only by plots embedded in static figures, thus preventing retrospective analysis of consistent performance parameters if they are not already calculated and reported in a standardized format at the time of publication. As

more sensing modalities are being developed and applied, reporting of raw transfer function values with well-defined units could thus provide more reliable insight into the functional usefulness of biosensors and allow more facile comparisons across modalities for a particular molecule of interest.

4. Conclusions and perspectives

Developing efficient cell factories is a time-consuming, expensive, and tedious process that is often encumbered at the test phase due to the low throughput of analytical methods relative to strain or variant generation. This review highlights the various modes and advances in detection of small molecules and enzyme functions. To enable high-throughput, automated detection, biosensors need to rapidly transduce analyte concentration into an easily measurable absorbance or (more ideally) fluorescence signal. Using these signal generation modes, it is possible to create rapid, high throughput assays in microwell plates or even microfluidic droplets. As discussed, current efforts in the deployment of biosensors for metabolic engineering are highly focused on TF-based detection. While this modality is generalizable and portable (among prokaryotic hosts), there are challenges in transferring this sensing function across to eukaryotes. Nevertheless, recent successes such as the detection of *cis, cis*-muconic acid and naringenin in *S. cerevisiae* brings forward the prospect of transplanting prokaryotic transcriptional machinery into eukaryotes (Skjoedt et al., 2016). Other protein-based detection methods such as GPCRs also have strong potential, but still retain a basic requirement: developing a genetic signaling circuit that links product concentration to a detectable output signal. In this regard, future developments interfacing biosensors (both in vivo and in vitro) with droplet-based microfluidics will be critical for enabling high throughput screening.

In similar ways, the development of RNA-based technology (especially RNA riboswitch-based biosensors) in vivo is not trivial across hosts, as intracellular environments and subcellular compartmentalization in eukaryotes may affect RNA stability and function. As alternatives, in vitro RNA biosensors (such as RNA Spinach-linked aptamers) may better blend the plug-and-play nature of nucleic acid biosensors with the ease of portability across a variety of hosts and target molecules. Once again, the coupling of microfluidic platforms with these technologies can increase throughput and enable novel modes of high throughput detection.

The use of stains, dyes and chromogenic substrates has also seen success, but is often limited in scope by available chemistries and biological compatibilities. However, these (typically) benign and orthogonal substrates can be a facile way to detect improved pathways and enzymes if suitable chemistry exists or can be designed. For many of these dyes, the signal is also retained intracellularly, thus allowing for straightforward FACS-based detection and sorting.

Collectively, this review highlights the various modalities for transducing a metabolite level or enzyme activity into a detectable signal. Despite advances in bioprospecting, directed evolution, and computational de novo design, nearly all of these sensors rely on a “lock-and-key” type model. Thus, the inherent requirement for highly specific and selective binding limits the overall scope of detectable molecules. Going forward, an ultimate screening solution to high-throughput metabolic engineering might be the advent of high throughput mass spectroscopy coupled with microdroplet systems. In this regard, using a technology that can generically sense thousands of molecules would bypass the need to find individualized solutions. Nevertheless, selections will still require and expanding toolkit of sensing modalities.

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