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Advances in engineering and optimization of transcription factor-based biosensors for plug-and-play small molecule detection

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Transcription factor (TF)-based biosensors have been applied in biotechnology for a variety of functions, including protein engineering, dynamic control, environmental detection, and point-of-care diagnostics. Such biosensors are promising analytical tools due to their wide range of detectable ligands and modular nature. However, designing biosensors tailored for applications of interest with the desired performance parameters, including ligand specificity, remains challenging. Biosensors often require significant engineering and tuning to meet desired specificity, sensitivity, dynamic range, and operating range parameters. Another limitation is the orthogonality of biosensors across hosts, given the role of the cellular context. Here, we describe recent advances and examples in the engineering and optimization of TF-based biosensors for plug-and-play small molecule detection. We highlight novel developments in TF discovery and biosensor design, TF specificity engineering, and biosensor tuning, with emphasis on emerging computational methods.

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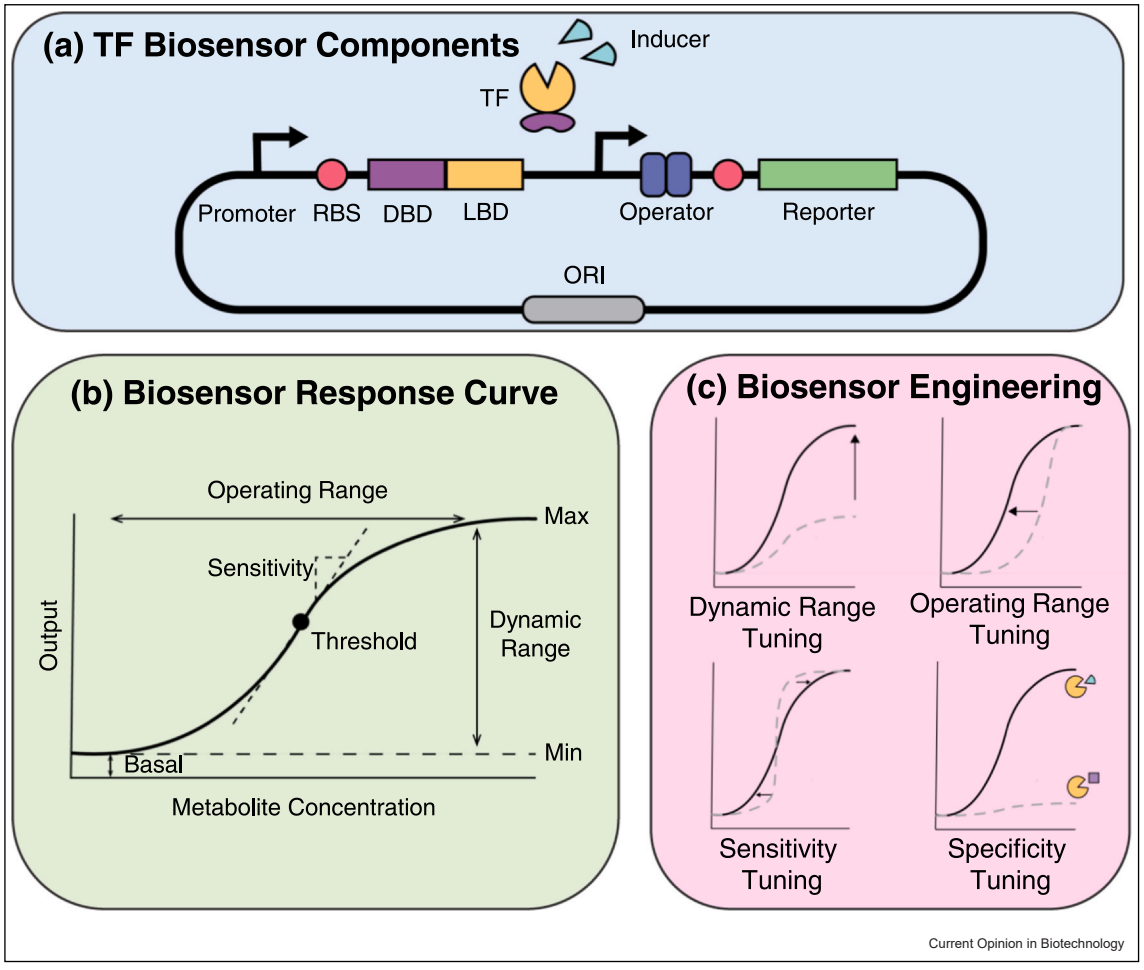
Introduction

Advances in biotechnology, synthetic biology, and metabolic engineering have increased the ability of researchers to harness microorganisms and their genetic components for a variety of applications such as the production of value-added chemicals from renewable resources and the monitoring of environmental pollutants. Such applications require the manipulation of microorganisms and the construction of genetic circuits [1]. Since the turn of the century, rapid advances in biotechnology have led to the emergence of tools such as genetic biosensors that control circuits through actuation (e.g. of fluorescence, selection markers, and dynamic switches) based on binding of ligand molecules [2]. A variety of biosensors have been developed, including aptamers, riboswitches, and transcription factor (TF)-based biosensors [3]. TF-based biosensors are highly modular, orthogonal, well established, and are the focus of this review. TF-based biosensors have proven their use in microbial strain engineering [4], dynamic control [5], protein engineering [6], and environmental pollutant detection [7]. Notably, such biosensors can overcome the analytical bottleneck in screening large libraries of microbial strain and enzyme variants, expediting the design–build–test–learn engineering cycle [1].

TFs are abundant in nature, activating or repressing transcriptional machinery to regulate gene expression in response to ligand binding. TFs are composed of a DNA-binding domain (DBD) specific to a DNA operator sequence to exert control and a ligand-binding domain (LBD) specific to ligand molecules that initiate the control response (Figure 1a). Broadly speaking, TFs exert control by creating conformational changes in the DNA to hinder or improve the access of the RNA polymerase for transcription, by blocking access to the RNA polymerase, or recruiting the RNA polymerase [8]. Creating TF-based biosensors requires well-characterized TF-ligand specificity and the corresponding operator sequences. Biosensors containing TFs are characterized based on performance parameters, including specificity, sensitivity, dynamic range, and operating range (Figure 1b). The modular nature of TFs and the biosensor circuits, allowing for the swapping of LBD and DBD between TFs and the interchange of heterologous genetic circuit components, presents opportunities to engineer and tailor these tools for applications of interest in a plug-and-play manner. Despite the modularity, the limited number of well-characterized

Figure 1



TF-based biosensor components and performance parameters. **(a)** TF-based biosensors center around a TF, which consists of a DBD specific for an operator region and a LBD, which is specific for certain inducers. Other general components of the circuit that can be engineered include the origin of replication (ORI), promoter, and the ribosome-binding site driving expression of the TF and the downstream gene or reporter. The downstream portion also includes the operator specific to the TF. **(b)** Example response curve highlighting the performance parameters. **(c)** Options for altering the biosensor response curve through tuning the dynamic range, operating range, sensitivity, or specificity.

TFs, the lack of systematic biosensor engineering principles and methodologies, and the role of host genetic context limits their application and orthogonality [3]. This review highlights advances in the discovery and development of TF-based biosensors, the engineering of TF specificity, and the tuning of biosensor characteristics for applications in biotechnology, with a particular emphasis on the emerging impact of computational methods. The future of the engineering and application of TF-based biosensors is also discussed.

Discovery and design of transcription factor biosensors

The construction of a biosensor centers around the selected TF. The TF provides the ligand-binding specificity and actuation capability of the biosensor. A variety of TFs have been characterized and applied in synthetic circuits for the detection of a range of molecules. Well-characterized TFs can be sourced from the literature or from curated databases that have been covered in previous reviews [8]. Despite continued efforts to mine and characterize individual TFs that can be sourced for biosensors, the repertoire of TFs for biosensors is limited to previously characterized TFs or TFs with predicted specificities for DNA and ligand binding, although these options can still serve as natural starting points for molecules of interest that are chemically similar. To address difficulties in mining and characterizing TFs, recent advances in the field have led to high-throughput approaches to characterize TFs that incorporate genomic methods and robotics [9••]. Other methods have bypassed the required TF characterization altogether by strategic implementation of the TF or the construction of chimeric TFs.

Advances in technology and methods have allowed for more high-throughput screening methods for the discovery and characterization of TFs. Substrate-induced Gene Expression (SIGEX) screening allows for the rapid characterization of metagenomic libraries, enabling simultaneous search of a large genomic space by cloning metagenomic fragments in front of a fluorescence gene for fluorescence-activated cell sorting (FACS) when ligands are added [10,11]. Although the SIGEX method requires metagenomic samples and can be limited to only identifying TFs that are close in genetic context to the genes they activate/repress, such techniques allow for large-scale screening of genetic material with the potential to identify novel TFs of interest for further engineering and use. With advancing omics techniques, recent attempts have also identified previously uncharacterized TFs using RNA-seq to discover TFs induced by molecules of interest, leading to the establishment of a biosensor for progesterone [12•]. By incorporating high-throughput technology such as robotics, the efficiency of mining for TFs of interest can also be improved [9••].

Depending on the intended use of the biosensor, the same TF can be strategically implemented for multiple deployments or as proxies for a different molecular target, circumventing the need to characterize a new specific TF for each molecule. For example, Leu3p was used to monitor production of both isobutanol and isopentanol based on different configurations of the biosensor circuit, keeping the specificity for the cognate ligand α-isopropylmalate, a common pathway molecule [6]. The SensiPath tool was developed with the purpose of expanding the possible detectable compounds by identifying enzymatic transformations capable of converting a compound without an identified detector, to a compound with a known biosensor [13]. SensiPath has since been used to develop an indirect sensor for hippuric acid and cocaine through the detection of benzoate by the TF BenR [14]. TFs can also be utilized in conformational or FRET-based sensors where conformational changes of the protein due to ligand binding lead to changes in the fluorescent signals of attached fluorophores, which can then be used for monitoring or detection applications [7,15].

Owing to the modularity of TFs, chimeric TFs can be constructed by switching LBD and DBD domains between different TFs [16,17] or other protein LBDs [18]. Such techniques can take advantage of already-characterized DBDs while shuffling LBDs to expand or uncover potential ligand molecules, reducing the amount of effort needed to fully characterize a TF. Extending this approach from proof of concept to larger libraries would be powerful for screening larger TF libraries.

Most TF-based biosensors so far have been developed using prokaryotic systems, particularly *E. coli*, and are the main focus of the examples in this review. The heterologous plug-and-play use of TF biosensors can be limited between host species due to the differences in transcriptional machinery, especially between prokaryotes and eukaryotes. Non-model TF-based biosensors and eukaryotic biosensors have been reviewed recently [19,20]. For non-model bacteria, universal expression systems incorporating the T7 RNA polymerase can be implemented [21]. Depending on the TF and mechanisms, prokaryotic systems can be implemented in eukaryotic systems with considerations of the placement of the operator [22] or fusion with heterologous activation domains. A recent example of this approach is the combination of prokaryotic xylose-sensing XylR with activation domains VPR and HSF1 for pathway engineering in *Y. lipolytica* [23]. TFs engineered in eukaryotic host organisms can also be transplanted back into a prokaryotic chassis [24]. Such orthogonality of TFs expands the potential uses of biosensors as the host for engineering and host for applications can be different.

Engineering of transcription factor specificity

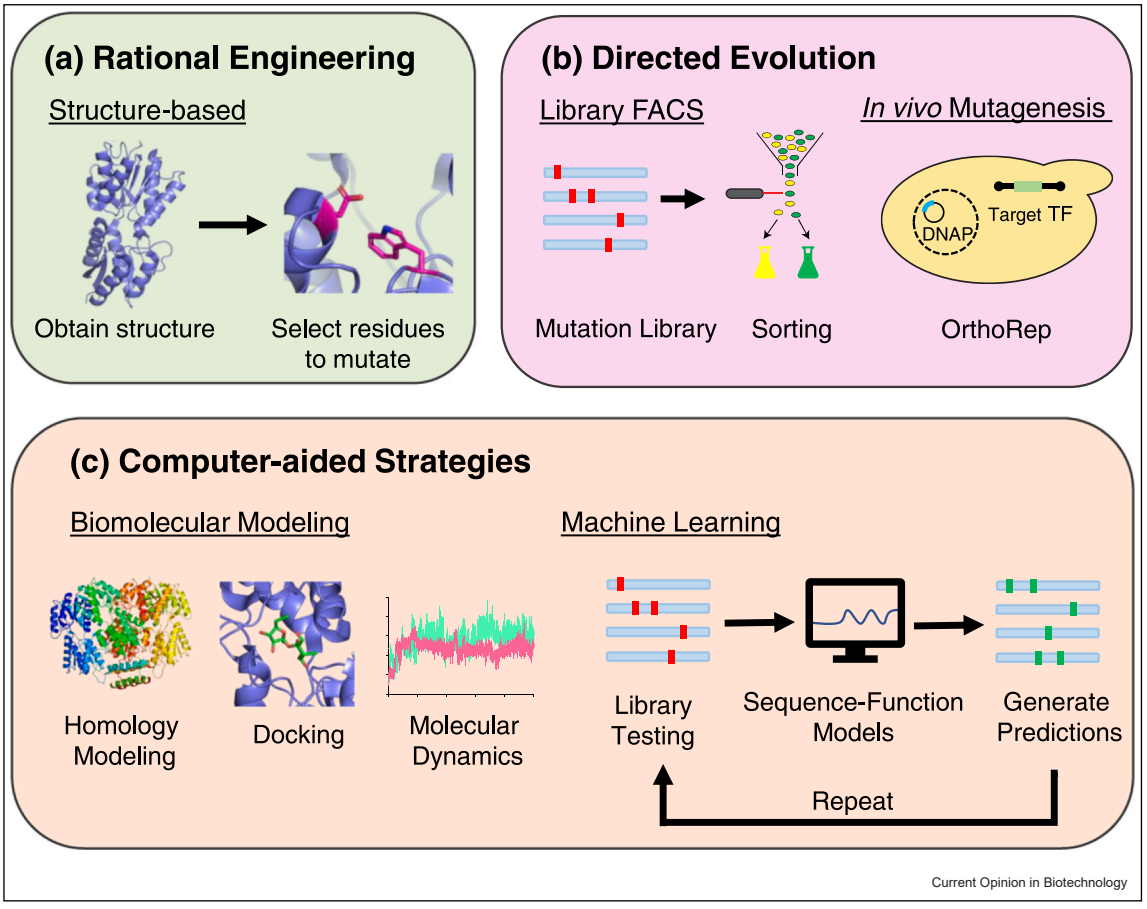
The most critical biosensor parameter is the ligand specificity. The specificity is the main limitation to the creation of novel biosensor circuits. When a suitable natural TF cannot be identified with the required ligand specificity or the need for a specific TF cannot be circumvented with methods discussed in the previous section, the specificity can be engineered. TFs are modular and highly evolvable, with the potential to achieve most desired performance parameters, especially specificity through protein design. Recent examples of TF specificity engineering include rational engineering, directed evolution, and computer-aided methods.

Rational engineering and directed evolution

Although not available for all TFs, crystal structures enable rational or semi-rational engineering and are especially useful to design for molecules similar in structure to the natural ligands (Figure 2a). Specific sites can be selected to create a mutational library to screen [25]. This method was used to alter LacI affinity from lactose to lactulose, although the specificity change was incomplete even with negative selection on FACS [26]. In another example, PcaV was evolved toward a range of aromatic aldehydes and concurrently to become unresponsive to the cognate ligand protocatechuic acid [27].

TF mutant libraries are an effective method to identify TF variants with altered characteristics. A round of positive and negative sorting of a TF library produced by error-prone polymerase chain reaction (PCR) can yield TF variants with changes of ligand specificity, dynamic range, operational range, and even inversion-of-function, which can all be further refined by additional experiments [24]. FACS-based approaches can also be combined with selection methods to take advantage of the utility of FACS to

Figure 2



Methods for engineering TF specificity. **(a)** Rational engineering uses available structures to select residues to mutate. **(b)** Directed evolution methods include performing one or more rounds of FACS on a mutation library or *in vivo* mutagenesis methods such as OrthoRep. **(c)** Computer-aided strategies for specificity engineering can include biomolecular modeling through a combination of homology modeling, docking, and/or molecular dynamics. Developments in protein engineering expand the potential of applying machine learning for future TFs. PDB 4RZT is used as an example protein structure [31].

select highly responsive sensors, and selection's ability to stringently ensure tight repression [28]. Such high-throughput directed evolution strategies can sample a larger mutational sequence space and yield specificity for chemically different ligands without a specific understanding of the TF structure compared with more rational methods (Figure 2b). Directed evolution's ability to search through a more extensive mutational landscape can also be combined with semi-rational methods to target specific critical residues, uncovering residues inside and outside of the binding pocket that impact TF specificity and characteristics [25]. More recently, methods of continuous directed evolution allow for *in vivo* mutant generation. The orthogonal DNA replication (OrthoRep) system, which utilizes a highly error-prone DNA polymerase to mutate user-defined genes beyond natural genomic mutation rates, was used to alter specificity and the dynamic/operating ranges of BenM through multiple rounds of passaging and selection [29].

Often, the engineering of specificity may actually broaden the inducer profile, requiring careful implementation of counterselection or design [30,31]. However, the expansion of specificity can be used as an advantage to first evolve for a more relaxed ligand specificity in order to serve as a starting point to generate more specific biosensors for a range of different compounds [32•]. Some TFs such as the macrolide-sensing MphR are known to have a broad inducer profile, which can be used as a starting point to be tailored to specific ligands through mutagenesis [33].

Regardless of the method used, generating libraries for testing also allows for the potential to gather large amounts of data. More data can contribute to efforts in the design–build–test–learn cycle to better assess the design constraints and gain insight into sequence-function relationships. Collected samples can be analyzed through next-generation sequencing (NGS) to elucidate

Table 1

Select examples of TF biosensor performance parameter optimization.

TF name	Compound	Improved parameter	Targeted component	Reference
BenM	Cis,cis-muconic acid	Dynamic range, operating range	TF	[24]
CamR	Bicyclic monoterpenes	Dynamic range	TF, reporter promoter, reporter RBS, and operator	[30]
MphR	Erythromycin	Sensitivity	TF RBS	[33]
CgmR	Putrescine	Dynamic range, sensitivity	TF, TF promoter, and reporter	[48]
LacI	IPTG	Basal, dynamic range, and threshold	Operator	[54]
AraC	Arabinose	Dynamic range	Reporter promoter	[58]
FdeR	Naringenin	Sensitivity	Plasmid copy number	[59]
CdaR	Glucaric acid	Dynamic range	TF RBS, reporter RBS	[63]

the full scope of the tested mutational space [34] or to characterize larger libraries [35••]. Such efforts can provide insights beyond the top-performing variants that are typically selected in screening efforts.

Computer-aided strategies

Developments in software and algorithms have enabled the testing of mutations *in silico* and the design of *de novo* proteins, expediting engineering workflows and drastically decreasing the wet-lab experimental resources and efforts required to characterize mutations (Figure 2c).

When suitable crystal structures are available, docking can assess the binding pocket and be used to successfully mutate residues based on biochemical knowledge to alter interactions, creating biosensors specific for a variety of molecules based on the same TF [36,37]. Docking analysis can also be used to gain a better understanding of sequence-function relationships for insights into the impact of mutations on specificity [27,38]. In the place of available crystal structures, homology modeling can also be used to successfully generate targets for mutations [32•]. A combination of homology modeling and docking was used to identify critical residues to target for the engineering of BenR and XylS with experimental methods [39]. Such workflows could be extended to higher-throughput docking experiments, requiring less *a priori* knowledge and judgments of the interactions of the binding pocket. In addition, the introduction of AlphaFold2 to public use will accelerate the computational modeling-based engineering of TFs using predicted protein structures [40]. A caveat worth noting is that the dynamic and allosteric nature of TFs in terms of binding-induced conformational changes test the capabilities of the current version of AlphaFold2 and other homology-modeling tools. Biomolecular modeling methods can be combined such as using molecular dynamics to accommodate flexible active sites and to gain further insights into TF–ligand interactions, allowing for mechanistic insights that can be used to design mutant TFs [41] or provide guidance on engineering future TFs [32•,42].

Incorporating computational design with Rosetta has been shown to be more successful than conventional methods such as error-prone PCR, likely from being able to effectively target more mutations [31]. Rosetta as a suite of tools can be used for comparative modeling and docking to design libraries, without the need for solved structures or structures in complex with a known ligand [43,44].

An emerging paradigm within protein engineering is the incorporation of machine learning to engineer proteins with improved characteristics beyond what can be found by other methods [45]. Such examples signify the importance of following developments in the field of protein engineering to apply as methods for TF engineering. Future work may include the *de novo* design of TFs based on developments in *de novo* protein design [46].

Tuning of biosensor characteristics

Since organisms naturally tightly regulate their transcriptional machinery, TF-based biosensors containing heterologous components are often not well-optimized for applications of interest. The plug-and-play nature of TF-based biosensors due to the variety of required genetic components provides many opportunities for fine-tuning the response curve. Tuning biosensors by modifying circuit components allows for the same TF to be used for different applications with needs for distinct performance parameters (Table 1). Such tuning can be achieved rationally through manual selection of components or the incorporation of computational modeling. There are also additional considerations for successful implementation of TF-based biosensors beyond the shape of the response curve, such as crosstalk.

Engineering biosensor circuit components

Owing to the modular nature of TF-based biosensor circuits, it is possible to engineer biosensors with desirable performance parameters (Figure 1c). For example, by changing the TF affinity for its operator through mutational promoter libraries, variants were generated with different gene expression levels, dynamic ranges, and ligand sensitivities [47]. While such performance parameters can be altered through engineering of the TF itself, it is harder to predict or model these parameters due to constraints for maintaining TF allostery

and differences between TFs and TF families limiting generalizability.

When it comes to characterization of the biosensor response curve, performance parameters of interest include the dynamic range, operating range, and sensitivity [8]. The dynamic range is defined as the difference between the maximum and minimum value of the output such as fluorescence, often expressed as a signal-to-noise ratio or fold activation between the basal level and maximum expression. Among other methods, dynamic range can be engineered with modifications in the reporter fluorescent gene [48] or altering the location of the binding site [49]. Careful tuning can limit the impact of leaky expression, or a response output even when no ligand is present, on the dynamic range [8].

The operating range is defined as the range of ligand concentrations that can be detected by the biosensor. The operating range can also be reported as the half-maximal or threshold, the metabolite concentration where the output is equal to half of the dynamic range. Operating range should be carefully tuned toward the range of ligand concentrations necessary for the desired application through methods such as modifying the expression level of the TF [50].

While the dynamic range indicates the change of response to concentrations of the ligand and the operating range indicates the range of detectable ligand concentrations, the sensitivity indicates how much the output changes with increases of the ligand concentration. In other words, this would approximate the slope of the dose–response curve. The sensitivity impacts how easily different concentrations of the ligand can be distinguished and characterizes whether the biosensor is more digital-like or analog-like, which can be used to implement a stringent response threshold before readout of the response [51•].

Directed evolution strategies can be implemented in multiple rounds with different selection conditions to simultaneously target optimization of several parameters and achieve lower background, higher dynamic range, and increased sensitivity [52]. Previous literature has elucidated antagonistic relationships between different properties of biosensors, so trade-offs must be considered during the tuning process. For example, the sensitivity was reduced to improve operating range through engineering of the TF XylR [53]. While this may appear less useful, it can be desirable based on the necessary application and the range required to be detected, moving from a digital response to an extended analog response. Consideration of trade-offs increases the importance of tuning for the circumstances of the application. The many considerations of biosensor response and application necessitate more generalizable and systematic design rules.

Modeling-based strategies

Tuning biosensors by rational strategies can require many rounds of engineering, adding more time and resources even before biosensors can be tested for the desired application. Recently, a strategic approach incorporating statistical modeling achieved efficient navigation of the optimization space and furthered the understanding of the impact of different circuit components to develop design rules [51•]. Another study modeled the well-characterized Lac system to identify constraints for biosensor design and control of performance parameters such as the dynamic range and response threshold [54]. Such efforts elucidate the impacts of individual circuit components on the performance parameters and the trade-offs [55]. Modeling can also be used to decouple the modular biosensor, considering the detector and effector modules as distinct modules [56]. In other implementations, models have been used to predict resource competition effects, which has the potential to aid the development of multi-logic gates or more complicated cascades [57]. Developments in biosensor modeling will enable more predictive design and uses of biosensors in contexts even outside of the circumstances used for development and testing.

Current modeling-based strategies are often more explanative rather than predictive, relying on extensive characterization to create the model before explaining biosensor tuning effects. Thermodynamic models [58], phenomenological models [54], and Hill-based mathematical formulism [50,59] all require characterization and model fitting. Simple Hill formula allows applications to fairly uncharacterized systems [59]. Through modeling with the Hill framework, the role of plasmid copy number on the threshold was investigated, showing that the presence of more binding sites and available TFs allows for more operator binding with less inducer, reducing the threshold [59]. However, this only functions to a certain extent as copy numbers can prove overly taxing on the host by diverting cellular resources.

More standardization of biosensor construction and an understanding of the impacts of circuit components on performance parameters will simplify the design of future biosensors. The incorporation of higher-throughput screening to develop design rules for both activators and repressors advances the field toward the development of generalizable frameworks [60]. Such efforts to enable creation of models for more systematic tuning are already being undertaken and will prove valuable in the efforts to expedite the design–build–test–learn cycle [61].

Biosensor tuning can be aided by tools such as machine learning, which has been implemented to better predict promoter strength to reduce the workload of characterization [62•] or predict dynamic range based on a ribosome-binding site (RBS) library [63]. Machine learning also elucidated the impact of the location of the core motif and the flanking sequences of the core motif of the operator sequence [64]. Integration of machine learning methods also drastically improved the time to obtain results from a biosensor, which is critical for applications requiring on-site monitoring [65]. A recent novel workflow integrated biosensor tuning using a barcoded combinatorial component library with FACS, NGS, and machine learning for large-scale genotype-to-phenotype prediction and also successful prediction of the performance of uncharacterized samples [66••]. Extending these efforts to consider other biosensor components and the interplay of the components on the parameters will allow for *a priori* prediction of components of the biosensor and allow less extensively characterized parts to be employed. Such tools in the future may be able to provide sequence-to-function information, further characterizing biological components. Current efforts toward generalizable, systematic, and predictable biosensor tuning can be built on by increasing incorporation of high-throughput construction and characterization such as through robotics and NGS, and obtaining more predictive power through machine learning [67].

Additional considerations for biosensor tuning

In application, there are considerations beyond the shape of the response curve. When applying biosensors to screen for improved enzyme variants in a single pool of cells, crosstalk can be a concern due to false-positive cells obtaining molecules that diffuse out of higher-producing cells. A prescreen for active variants can reduce the rounds of FACS required, or the sensitivity can be reduced to avoid the presence of false-positive cells, as such cells likely have lower intracellular concentrations of the ligand [68]. When applying selection using antibiotics, cheater cells can survive without producing the molecule of interest by evolving to survive the selection process through mutations in the selection system rather than toward the goal of higher target molecule synthesis. Such concerns increase the importance of negative and positive selection, which can be toggled between [69].

When implemented *in vivo*, TF-based biosensors require that molecules be able to permeate the cell membrane to be detected, limiting the potential molecule based on the host. In certain contexts, this limit can be overcome with strategies such as expression of transporters [51•] or cellular sequestration that can be used to trap molecules such as erythromycin A using a cellular-phosphorylation strategy, reducing diffusion out of the cells and improving sensitivity [70]. Efflux pumps have also been shown to impact the response curve of genetic circuits [71] and mediate toxicity of target compounds [72].

The future of transcription factor-based biosensors and emerging applications

In recent years, technical advances incorporating high-throughput experimental techniques and computational methods including machine learning have expanded the understanding of the biosensor discovery, design, and tuning process. Such advances have improved the engineering of biosensor specificity and characteristics, and expanded the possible applications of TF-based biosensors. TF-based biosensors can even go beyond requiring a chemical ligand with AraC being engineered to respond to light through the addition of a light-inducible dimerization domain, enabling optogenetic experiments [73]. Cell-free systems also extend the potential applications of biosensors and allow for rapid testing and development of biosensors, improvements in biosafety, and detection of toxic compounds or compounds difficult to test in most host organisms [74,75]. Notably, cell-free systems can overcome the issues of porting heterologous components to different host organisms. Cell-free systems can additionally be utilized at point-of-care or at home [12•,14]. Recently, biosensors have also been demonstrated in wearable form, potentially expanding to novel applications such as high-risk work with chemical or biological threats [76].

Synthetic biology works toward the goal of plug-and-play synthetic circuits and efficient workflows. Incorporation of automation for experiments and machine learning for data analysis can expedite the applications of biosensors in areas such as screening for improved chemical production strains [77]. Methods for the discovery and characterization of biosensor components, including TFs for applications and molecules of interest, generate additional useable components for future work. The need for extensive characterization can also be circumvented through methods such as *de novo* engineering of promoters to expand the potential use of TFs from the metagenome without native promoter characterization [64]. Expansion to the set of available components also enables the creation of orthogonal collections, combinatorial logic gates, and complex circuits, expanding the potential to exert cellular control [78].

To advance the use and applications of TF-based biosensors, the field requires concerted efforts to develop generalizable, systematic, and predictable workflows for the discovery, engineering, and tuning of TF-based biosensors tailored toward specific applications. Such efforts must include the generation of biosensor component libraries for plug-and-play utilization with

consideration of host orthogonality. In particular, biosensor libraries for community use and investigation would expedite the construction of TF-based biosensors. To realize these goals, holistic computational tools must be developed to consider a variety of tuning strategies, and technological advances such as robotics and machine learning must be included to expedite the entire biosensor discovery and tuning process. Addressing these issues will further advance the utilization of TF-based biosensors.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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