High-Throughput Metabolic Engineering: Advances in Small-Molecule Screening and Selection

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Abstract

Metabolic engineering for the overproduction of high-value small molecules is dependent upon techniques in directed evolution to improve production titers. The majority of small molecules targeted for overproduction are inconspicuous and cannot be readily obtained by screening. We provide a review on the development of high-throughput colorimetric, fluorescent, and growth-coupled screening techniques, enabling inconspicuous small-molecule detection. We first outline constraints on throughput imposed during the standard directed evolution workflow (library construction, transformation, and screening) and establish a screening and selection ladder on the basis of small-molecule assay throughput and sensitivity. An in-depth analysis of demonstrated screening and selection approaches for small-molecule detection is provided. Particular focus is placed on in vivo biosensor-based detection methods that reduce or eliminate in vitro assay manipulations and increase throughput. We conclude by providing our prospectus for the future, focusing on transcription factor-based detection systems as a natural microbial mode of small-molecule detection.

Contents

1. INTRODUCTION: THE CASE FOR SMALL-MOLECULE SCREENS

Natural selection, the force behind the amazing breadth of phenotypic variation in the living world, has long been a source of motivation for the engineering of synthetic biological systems. By mimicking the processes of mutation, recombination, and selection found in nature, directed evolution is used to impart industrial microbes with user-defined phenotypes. Frequently regarded as the First Law of Directed Evolution (1), the central tenet "you get what you screen for" draws attention to the paramount importance in finding an appropriate screen or selection assay to sift through vast libraries of variant hosts. A great body of work has been devoted to development of highly tailored assays specific for detection of single proteins or functions (2).

As applied to metabolic engineering, directed evolution is focused on improving small-molecule biosynthesis. Improving product yields or pathway efficiencies, however, can be a daunting task. Only a small subset of targeted compounds are natural chromophores or fluorophores that can be readily screened for using standard assay techniques. The majority of small-molecule targets for overproduction today do not illicit a conspicuous phenotype. For inconspicuous targets, chromatography-mass spectrometry methods have been the primary mode of detection; although they are nearly ubiquitously applied in small-molecule detection and quantification, these assays are inherently low throughput (3). Screenable library sizes are generally limited to less than 10^3 variants. At this level of throughput, only a paltry number of rational modifications can be introduced into the panoply of host biosynthetic machinery, leaving the majority of sequence space untouched and unexplored.

Today, a discussion of improvements in small-molecule detection assays is set against the backdrop of increased focus in the field on microbial biosynthesis of commodity chemicals and fuels. Microbially produced alcohols, fatty acids, and alkanes are targeted for use as petroleum-derived fuel substitutes (4, 5), aromatics (6), and diols (7); and polyhydroxyalkanoates (8) are being targeted for use as bioplastics. Spurred by an increased demand for renewable, green alternatives to petroleum-derived production routes, microbial production routes are competing economically against entrenched industry stakeholders (9). Product yields and pathway efficiencies demanded from biosynthetic routes are pushing the limits of what can be achieved using existing metabolic engineering technologies. Efforts in the field

Throughput: total number of library variants screened per unit time (experiment or day)

are currently defined by the implementation of a series of rational design-based strategies to modify the host genome and heterologous pathway enzymes to achieve moderate product titers. However, metabolic engineering is a highly complex process, and product yields are dictated by a host of parameters. Biosynthetic pathways comprise multiple native and heterologous catalytic steps; each step is a potential bottleneck when directing carbon flux toward target small-molecule production. Furthermore, the host organism's native genetic network, regulation, and their interaction with the target pathway can all impact product yields. To achieve higher productivities, metabolic engineering must follow the decades-long trail of successes in protein engineering and develop more elegant approaches to high-throughput screening. Directed evolution through random and targeted mutagenesis of the host genome, overexpressed operon(s), and enzyme-encoded genes followed by high-throughput screening or selection is a requisite step in strain development.

In this review, we focus on advances in small-molecule screens using *Escherichia coli* and *Saccharomyces cerevisiae* as model prokaryotic and eukaryotic hosts. These organisms were selected both for their demonstrated application in industrial fermentation processes and because the vast majority of novel screening and selection processes are first demonstrated in these hosts. Given the immense number of mutable genetic elements to be targeted in metabolic pathway evolution, special consideration must be given to library design and sequence coverage. Screening efficacy has been demonstrated to increase in libraries that are maximally diverse, providing evidence for increased library size and mutation rate as methods for improving the diversity in the sample population (10). Technical limitations imposed during library generation, transformation, and screening technologies are addressed below, and we focus in particular on screening and selection as rate-limiting steps in directed evolution efforts for small-molecule overproduction. Throughout our discussion, we highlight novel biosensor-based assays that enable inconspicuous small-molecule detection.

2. STATISTICAL LIMITATIONS ON LIBRARY SIZE AND SEQUENCE SPACE COVERAGE

Any directed evolution experiment, regardless of target, requires a thoughtful analysis of the library size required to gain significant coverage of the targeted sequence space. For good reason, most directed evolution efforts use focused libraries, mutating a relatively small number of preselected positions for saturation mutagenesis. However, even the most straightforward efforts to introduce a small number of substitutions are subject to harsh statistical realities. Simultaneous alteration of n selected positions in a given sequence necessitates the creation and screening of a library of size L, according to Equation 1:

$$L = X^n, \text{ where } X = \begin{Bmatrix} 4 \text{ for, nucleotide substitution} \\ 20 \text{ for, amino acid substitution} \\ 2 \text{ for, genome (binary output)} \end{Bmatrix}. \quad 1.$$

Here, X corresponds to the number of possible genetic elements or states that may be present. The four naturally occurring nucleotides and the 20 naturally occurring amino acids provide the set of states for standard DNA and protein mutagenesis, respectively. The number of states for genome modifications, two, is modeled on deletion and insertion libraries. Although our discussion here has focused predominantly on randomization of existing genetic elements, a similar analysis can be applied to other, equally as important diversity-generating techniques, including insertions (11, 12), deletions (11–13), and recombination (14, 15), among others.

Exhaustive sequence coverage when randomization is not targeted to specific positions, but is instead applied uniformly over the full length of a target sequence, is a difficult prospect. When there exists little-to-no basis for rationalized substitutions using structure elucidation, or otherwise, the researcher may

opt to introduce multiple, random mutations across a sequence of length *K*. The binomial coefficient describes the number of possible variants, *L*, given *N* randomizations with *X* genotypic states. Library sizes now scale according to Equation 2:

$$L = X^n \frac{K!}{N!(K-N)!}. \qquad 2.$$

Exhaustive library coverage for an indicated sequence space differs tremendously between the focused and random mutagenesis approaches. For example, mutation of 2 positions in a 100-amino acid protein (i.e., $X = 20$) yields a library of 400 unique members when the amino acid positions have been preselected and 1.98×10^6 unique members when mutations are randomly incorporated over the length of the entire protein-coding sequence. The four order-of-magnitude difference in library size is of note, but underlying this analysis is the finding that most random mutations have neutral or deleterious effects on protein function (16). Presupposing that some knowledge of protein structure or function can be used to guide a focused mutagenesis strategy, screening efficiency can be dramatically improved. These arguments exemplify why the vast majority of protein engineering efforts possess either a strong screening/selection assay or introduce focused mutations on a small subset of the total sequence space.

Given its small number of genotypic states, statistically speaking, targeted mutagenesis of the genome appears to provide the most straightforward approach. However, a number of caveats must be considered. First, the simplifying assumption was made that genomic elements have only on and off states, a route that ignores the importance of intermediate behaviors. For example, in the case of promoter insertions, induction profiles can range from all-or-none to graded responses (17). Although library size is dependent only on the presence or absence of a promoter at a given position, assay size scales with the number of induction conditions tested. An additional caveat is that relatively little is known about the function of a large fraction of the genomes in experimental and industrial-use host microbes, making prediction of the effects of a targeted mutagenesis strategy difficult. For example, the genome of *E. coli* K-12 MG1655, the most well-studied microbe, contains approximately 4288 annotated protein-coding genes; of these, 19.5% remain of unknown function (18, 19). Without substantial a priori knowledge, the host genome, pathway operons, and its constituent enzymes all remain tenable targets for mutagenesis.

3. TECHNICAL LIMITATIONS IN LIBRARY CONSTRUCTION AND SCREENING

Even though statistical limitations establish a glass ceiling, hindering the exhaustive search of large sequence spaces, technical limitations in library generation and screening are the significant bottlenecks in practice. The workflow for a standard directed evolution assay can be divided into discrete segments: (*a*) in vitro diversity generation, (*b*) transformation and in vivo small-molecule production, and (*c*) screening and selection. Each step can impose significant technical limitations on the efficient exploration of sequence space.

3.1. Diversity Generation

Diversity generation stands as perhaps the most robust step in mutant library screening. Methodologies employed to generate in vitro or in vivo genetic diversity are described in the box Methodologies to Generate Sequence or Genetic Diversity. To a first approximation, technologies for diversity generation are agnostic to a genetic element's downstream, in vivo end function. For example, an experimentalist's success in introducing genotypic variability into a protein-coding sequence using error-prone polymerase chain reaction (PCR) is independent of the downstream function of the protein. Disconnect between diversity generation and end function is due, in part, to segregation of in vitro diversity generation from downstream in vivo expression. Creating

genetic diversity in vitro allows for an extremely large number of variants to be created; plasmid libraries on the order of approximately 10^{14} molecules can be tractably prepared, amounting to 1 mg of plasmid DNA (20). For everyday benchtop experiments, however, an upper limit of 10^{12} molecules is more commonly observed.

Until recently, a lack of computational estimates of library sequence diversity, using various methods, left experimentalists to wander through sequence space. Although still seemingly underutilized, in silico approaches to model diversity generation in error-prone PCR, gene shuffling, and oligonucleotide-directed randomization have been exhaustively reviewed (21–23). When coupled with a readily accessible user interface, computational methods can be valuable guides to choose mutagenesis methods satisfying an experimentalist's library size, diversity, and coverage objectives. To this end, a suite of user-friendly diversity analysis software tools have been made readily available online (24). The programs provide a useful statistical analysis of library diversity and sequence space coverage, which can guide library construction and screening when using error-prone PCR, site-directed mutagenesis, and in vitro recombination (10, 25, 26). There still remains an upper limit on the sequence space that can be analyzed by computationally predictive methods; one recently published method for modeling random point mutagenesis establishes an upper limit of analyzing 2000 amino acids or 16,000 nucleotides, and 10^9–10^{10} individual sequences (27). This level of computation power continues to meet or exceed the reasonable number of DNA variants that can be constructed using existing DNA synthesis technologies.

3.2. Transformation Efficiency

The efficiency of introducing variability into the host to gain in vivo functionality provides the next significant bottleneck. The method most commonly employed for both *S. cerevisiae* and *E. coli* library incorporation is nucleotide transformation, our focus here. For *E. coli*, the maximum library size is estimated to be on the order of 10^{12} molecules (28), but libraries on the order of 10^9 transformants are more readily realized at the benchtop scale of everyday experiments. Library sizes in *S. cerevisiae* expression systems are subject to decreased transformation efficiencies, and maximum library sizes are approximately an order of magnitude less than those witnessed for *E. coli*.

Transformation of an in vitro library into the in vivo screening context can entail significant losses in library size, and steps can be taken to circumvent transformation inefficiencies. One option is to generate and screen a library in vitro; the in vitro compartmentalization, mRNA display, and ribosome display

METHODOLOGIES TO GENERATE SEQUENCE OR GENETIC DIVERSITY

Error-prone polymerase chain reaction (PCR) is performed with a low-fidelity polymerase (e.g., with $MnCl_2$, low amounts of template, or with unequal concentrations of nucleotides) to introduce copying errors.

Oligonucleotide-mediated mutagenesis, through PCR, incorporates user-defined nucleotide substitutions.

DNA shuffling involves randomly digested gene libraries, which are rejoined to combine mutations.

Mutator strains exploit deficient DNA repair pathways to generate replication errors in plasmids or genomes (30). Deletions, substitutions, and frameshifts are possible. Inducible mutator strains may also be used that express a dominant-negative mutator under select conditions.

Error-prone DNA polymerase, i.e., a highly inaccurate DNA polymerase I, is used to initiate low-fidelity replication of the target sequence in vivo (31).

Somatic hypermutation is an in vivo approach that makes use of the properties that generate immunoglobulin genes to create sequence diversity in targeted sequences (32).

Multiplex automated genome engineering (MAGE) permits numerous and simultaneous chromosomal changes across a population of cells (34). The bacteriophage λ-Red ssDNA-binding protein β is used to mediate oligonucleotide-based allelic replacement. The strength of MAGE is in the rapid and continuous generation of genetic variants.

Sensitivity: the slope of the assay's response curve, describing the minimal differentiable change in input product concentration between two samples

Dynamic range: the difference in biosensor output signal between the maximum and background states

Linear range of detection: the range of input small-molecule productivities that can be linearly correlated with the output reporter signal

Transfer function: the mathematical relationship between biosensor input and output

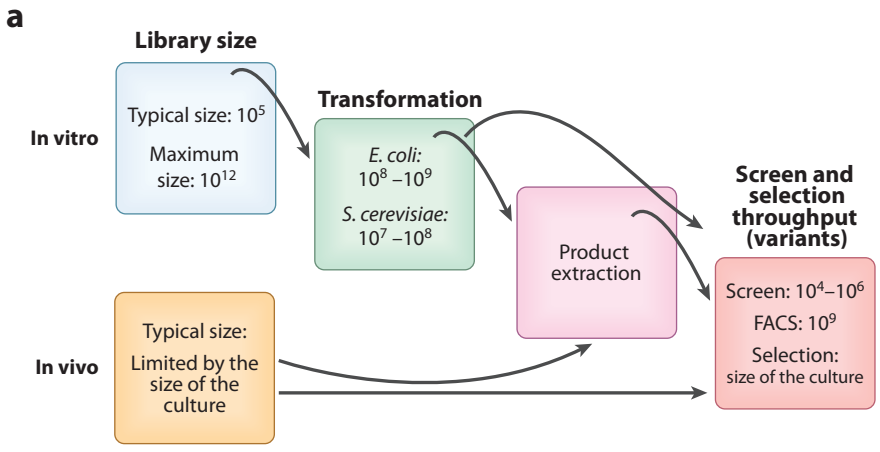
methods have been reviewed elsewhere (29). Commonly used for directed evolution of single proteins, completely in vitro assays are not generally applicable to metabolic engineering for small-molecule production and ignore in vivo biological context and regulation. The second, more commonly employed approach to avoiding library transformation inefficiencies is to develop the library diversity in vivo. Published methods include employing engineered strains of *E. coli* with increased mutation rates (30), an error-prone *E. coli* polymerase I (Pol I) for more targeted diversity generation in plasmids containing a ColE1 origin (31), and somatic hypermutation in mammalian expression hosts (32, 33). The general drawback to in vivo diversity generation is the introduction of untargeted, sometimes genome-wide, mutations. Circumventing this issue, multiplex automated genome engineering (MAGE) (34) uses transformed ssDNA to target individual genomic regions for mutagenesis at high efficiency (>30%).

3.3. Small-Molecule Screening

Last in the directed evolution flowchart stand screening and selection. Technologies for small-molecule screening have long lagged behind those for diversity generation, predominantly owing to a need to independently tailor assay methods for application toward different target small molecules. For this reason, screening and selection processes are the most significant bottleneck in directed evolution efforts for small-molecule detection and quantification.

Although throughput can be generalized for specific screening technologies (**Figure 1**), small-molecule assays are burdened by additional, equally important parameters. Screen and selection strategies are only as good as their sensitivity and selectivity: A desired phenotype that remains undetected will not be captured, regardless of the number of variants analyzed. For this reason, assay sensitivity, dynamic range, and linear range of detection—parameters commonly used to describe transfer functions in genetic circuit design (17, 35–38)—are also apt for the characterization of small-molecule screens and selections (**Figure 2**). While virtually unreported in phenotypic assays described to date, this quantitative framework provides for a minimal set of parameters that enable more accurate comparisons between different assay methodologies. The caveat being that, given the aforementioned molecule-tailored nature of most screens, codified rules regarding screen sensitivity and dynamic range can be ineffectual generalizations.

Foundational work in directed evolution for small-molecule production has been focused on conspicuous targets, those that can be optically detected. Without the need for additional synthetic chemistry or biotransformation, conspicuous products can be accurately measured using standard high-throughput colorimetric and fluorometric assays. In contrast, inconspicuous targets, a class to which the majority of small molecules belong, emit no spectral signature suitable for existing high-throughput screening technologies. Inconspicuous small molecules can be converted into detectable outputs through the activity of biosensors. Biosensors take form as single- or multistep enzymatic pathways, inducible expression systems, and entire host organisms; an accurate description of the correlation between a biosensor's inputs and outputs is provided by its transfer function (**Figure 2**) (38). In the remainder of this review, we provide a discussion of small-molecule screens and selections and present methods for moving up the screening and selection ladder (**Figure 1**). This qualitative metric seeks to incorporate throughput, sensitivity, and dynamic range into a generalized rank of assay strength and molecule applicability. Small-molecule screens exhibiting high-throughput and sensitive analyte detection for a broad spectrum of compounds rank higher than less-specific, lower-throughput assays. In our analysis of the various small-molecule screening methods commonly employed, we highlight the role of biosensor-driven assays that enable inconspicuous small-molecule targets to leapfrog rungs in the ladder.



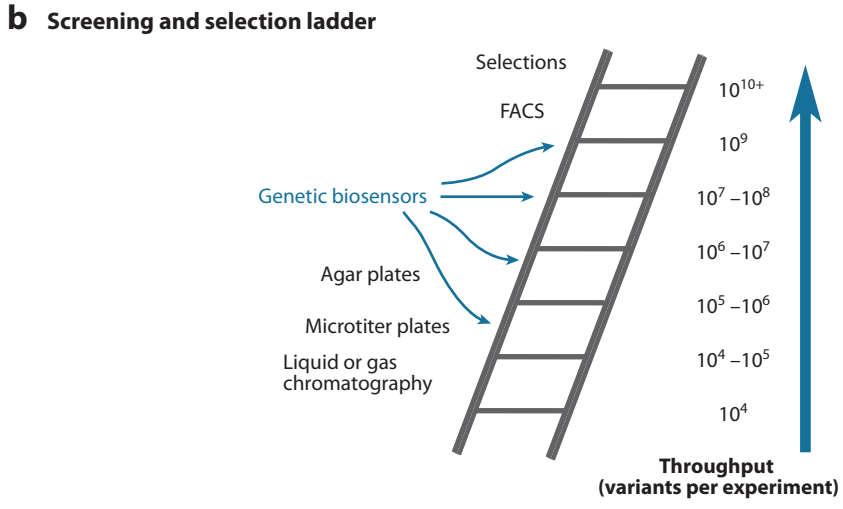


Figure 1

Inefficiencies in library screening. (*a*) The in vitro and in vivo steps associated with standard directed evolution dictate the scope of sequence space that can be explored. Moving between in vitro and in vivo compartments imposes significant losses in library size and diversity. In vivo methods of library generation avoid inefficiencies of transformation and product extraction; however, mutations are randomly inserted across a target sequence and cannot be focused to a few key positions. (*b*) The choice of screening assay will ultimately have the biggest impact on throughput; genetic biosensors converting small-molecule concentration into a readily detectable reporter molecule serve as one method to move away from low-throughput chromatography techniques. Abbreviation: FACS, fluorescence-activated cell sorting.

4. THE SCREENING AND SELECTION LADDER

We present here colorimetric, fluorometric, and growth-complementation assays as the foundation for small-molecule assays in metabolic engineering. Excluded from this review are gas and liquid chromatography and H^1- and C^{13}-based NMR techniques for small-molecule identification and quantification. Although ubiquitous in the field and offering unparalleled accuracy and precision, their extremely low throughput limits their

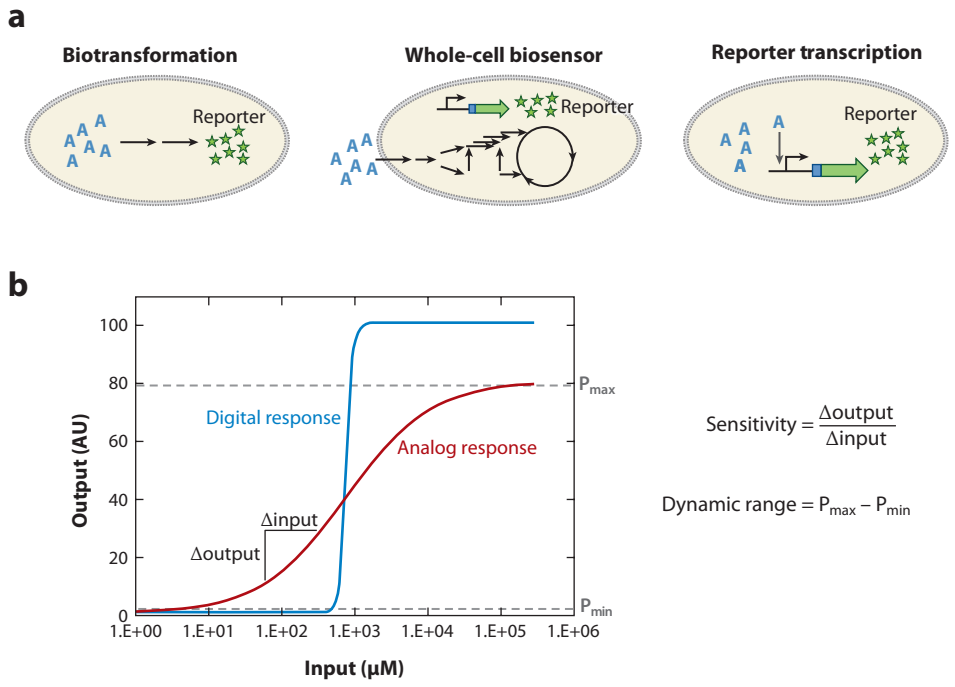


Figure 2

Transfer curves enable a quantitative characterization of performance features. (*a*) Biosynthesis of an inconspicuous small-molecule, A, results in a detectable signal by transformation using a series of biosensor constructs. Small-molecule A can be transformed into a detectable reporter molecule through the action of single- and multistep enzymatic pathways. Whole-cell biosensors couple growth (and a constitutively expressed reporter) with a host microbe's productivity. Lastly, reporter transcription can be achieved using classically regulated promoter systems induced by small-molecule A. (*b*) The correlation between input product concentration and output biosensor response (AU, arbitrary reporter units) provides the biosensor transfer function. Transfer functions provide information on product sensitivity, the linear range of detection, and the detection threshold, which can guide assay design.

application to assays with small sets ($<10^3$) of modifications.

In engineering terms, we define our system as an individual microbe, with the target product being an output. As such, we focus only on whole-cell assays for target small-molecule production. End-product screens detect the desired output (i.e., improved molecule production) and are applicable with directed evolution of any upstream biosynthetic machinery. In this sense, small-molecule screens offer greater versatility and application than screens for intermediate functions. We leave untouched assays that analyze single or intermediate steps in the conversion of a carbon source to product.

For each assay technology, examples of conspicuous small-molecule detection are used to provide a backdrop for development of next-generation, biosensor-driven approaches. Screen throughput, sensitivity, and dynamic range, where available, are highlighted from exemplary studies in the field.

4.1. Colorimetric and Fluorometric Plate-Based Screens

The relative ease of conducting colorimetric and fluorometric assays has established their position as the a posteriori techniques of choice for proof-of-principle experiments in novel mutagenesis and directed evolution (39).

Assay sensitivity, linear range of detection, and, to some extent, throughput will vary depending on the method used and the compound being interrogated. Individual variants are monitored as liquid cultures on microtiter plates or as colonies on solid, agarose media. Photometric assays using microtiter plates are highly robust and provide the distinct advantage over other techniques in being able to broaden the linear range of detection by diluting or concentrating the sample. Assay throughput on microtiter plates is moderate (approximately 10^5 variants per experiment) and is strongly affected by an oft requisite in vitro product extraction step. In comparison, screening colonies on solid media provides increased throughput, and with modern robotics upward of 10^6 variants are screened (40). The increase in throughput comes at the expense of a greatly diminished sensitivity, however, and small differences in intercolony productivities can be overlooked. Thus, the decision between liquid and solid media assays is largely dictated by available equipment and a compromise between throughput and sensitivity.

4.1.1. The upper limit of plate-based screening: carotenoid biosynthesis. Detection of microbially produced photophores provides upper bounds with regard to throughput and sensitivity as no additional chemical transformations are required for product detection. From the expansive body of metabolic engineering work on production of natural photophores, we focus here on lessons learned during directed evolution for improved carotenoid biosynthesis. The carotenoids, including lycopene, β-carotene, and astaxanthin, were recognized early on as high-valued neutraceuticals with remarkable antioxidant properties (41). This feature, coupled with their relative ease in detection, has driven extensive research into establishing microbial production routes and, in large part, has laid the foundation for many aspects of metabolic engineering. To date, lycopene has been the major carotenoid of focus for production in microbial hosts. The majority of titer-oriented research for lycopene has been conducted in *E. coli* and has highlighted a number of the performance features of colorimetric screens. Lycopene production can be monitored colorimetrically by measuring the absorbance at 470 nm following extraction into an organic solvent (42). The assay is highly sensitive, differentiating between submilligram per liter differences in lycopene yields (43) and achieving a level of sensitivity more than adequate for directed evolution. Detracting from the lycopene screen, however, is a requisite organic solvent extraction that drives up assay price while decreasing throughput.

The success of plate-based photometric screening is exemplified by use of the carotenoids in various proof-of-principle experiments in organism and protein engineering. The broad spectrum of colored pigments found in the carotenoid family was utilized to demonstrate strategies in combinatorial biosynthesis (44, 45), a powerful approach wherein promiscuous enzymes serve as molecular scaffolds on which nonnative small molecules are synthesized. Additionally, advances in mRNA-based regulation of pathway flux (46), model and combinatorial-driven methods in genome engineering (47), engineered metabolic control (48), and multiplex genome engineering (34) have been enabled.

4.1.2. In vitro synthetic chemistry and enzyme-coupled assay design. In contrast to the carotenoids, the vast majority of primary and secondary metabolites targeted for overproduction in the laboratory are not natural chromo- or fluorophores. For these molecules, in vitro synthetic and enzyme-coupled catalyses may offer an alternative approach to direct product detection. Using synthetic chemistry, a target molecule-specific chemical moiety reacts with an exogenously added reagent to yield a detectable product. Fluorescent and colorimetric detection of specific chemical moieties and compound classes is an area of great interest in organic synthesis. A wealth of both demonstrated and potential high-throughput assays can be borrowed from the field, including thiols (49), cyclic and linear amines

(50, 51), carboxylic acids (52, 53), alcohols (54), aldehydes (55), and glycosaminoglycans (56), among others (57, 58). Alternatively, indirect methods for product detection can also be employed, such as product-associated changes in pH (59, 60) or coproduction of H_2O_2 (61). The application of synthetic chemistry-based detection to metabolic engineering strategies, however, is not without limitation. Accurate product quantification in synthetic chemistry-based detection schemes may be impeded by a low signal-to-noise ratio, as the majority of moieties being targeted are naturally found at high abundance in the microbial intracellular environment. This issue may be minimized by increasing production titers. Lastly, reagent cost when incorporating in vitro synthetic chemistry may become a significant factor, a problem exacerbated with increasing library size.

When synthetic catalyses are either unavailable or cost prohibitive, or when higher substrate specificity is required to alleviate background noise, enzyme-coupled assays provide another approach. Coupling enzymes are added to the analyte solution to form single- or multistep pathways that yield detectable photophores or utilize traceable cofactors in their catalysis. The distinct advantage in cofactor-dependent assays is the broad range of enzymes and reaction types for which they are necessary; thus, cofactor monitoring can be viewed as a strategy more universally applicable than direct detection methods. Photometric assays have been described for quantification of many enzyme cofactors, ATP and ADP (62–64), reduced and oxidized states of NAD and NADP (65–67), and free coenzyme A (68). When background noise from native, endogenous small-molecule production is not an issue, using coupling enzymes with broad substrate acceptance is an interesting option. For example, substrate promiscuity is a hallmark characteristic of the P450 superfamily (69), and NADH or NADPH consumption in P450-catalyzed reactions is frequently used as an indirect measure for substrate oxidation (70, 71). Similarly, *S*-adenosyl-L-methionine is a cofactor for small-molecule methyltransferases and can be monitored following multienzyme biotransformations to homocysteine or hypoxanthine (49, 72, 73). Although inherently indirect measures (and as such push the boundaries of the rule "you get what you screen for"), the cofactor assays nonetheless are often highly robust, accurate proxies for direct product detection (62, 72, 74).

Design of in vitro small-molecule assays using either synthetic or biocatalyzed transformations requires significant thought with regard to the reagents and enzymes used. Although not a prerequisite, driving a reaction to completion is highly desirable and will facilitate a more accurate back calculation of target small-molecule production titers. For this reason, use of irreversible enzymes without product inhibition is the preferred enzymatic route. The choice of catalyst also determines the reaction conditions. Under the best-case scenario, assays are performed directly in the growth medium without cell removal, product extraction, or pH adjustment; however, optimal reaction conditions always need to be determined experimentally. These considerations become increasingly important as library sizes grow, as each additional in vitro manipulation adds both significant consumable and throughput costs. Lastly, the synthetic reagents or coupling enzymes must also be economically synthesized, purified, or purchased to screen a complete set of library variants.

4.1.3. In vivo biosensor-driven assay design. By shifting from an in vitro, enzyme-coupled product detection regime to an in vivo context, significant advantages can be garnered in terms of both throughput and cost. Extractions, enzyme purifications, and in vitro manipulations are minimized or eliminated. Catalytic biosensors, single and multistep in vivo biosynthetic pathways with inconspicuous substrate inputs and detectable product outputs, have just begun to be explored. Examples to date include assays for intermediates in an engineered *Pseudomonas putida* paraoxon catabolic pathway (75), strictosidine glucosidase-catalyzed transformation of tryptamine analogs (76), and a

tyrosinase-catalyzed transformation of the amino acid L-tyrosine into melanin (77).

Directed evolution of *E. coli* for improved L-tyrosine production exemplifies the role enzyme-based biosensors can assume during inconspicuous small-molecule detection. Tyrosine is a colorless, essential metabolite of great import in the synthesis of a wide range of value-added pharmaceuticals and commodity chemicals, including the morphine alkaloids and *p*-hydroxystyrene (78). Rational engineering strategies, targeting both tyrosine and its intermediates, have been extensively explored and reviewed (79, 80). Concomitant work in the Stephanopoulos lab (77, 81) in both rational engineering and directed evolution of *E. coli* tyrosine biosynthesis enables a thorough comparison of the two approaches. Using strictly rational metabolic engineering, two tyrosine-overproducing strains of *E. coli* were reported (82). The first, T1, incorporated feedback-inhibition-resistant derivatives of pathway enzymes and eliminated native pathway regulation. Building on T1, strain T2 increased the availability of the central metabolite precursors D-erythrose-4-phosphate and phosphoenolpyruvate necessary for tyrosine biosynthesis. Tyrosine production titers in minimal media culture flasks reached 346 ± 26 and 621 ± 26 mg·L^{-1} for strains T1 and T2, respectively.

Before using random mutagenesis to build on their rationally designed strains, the authors developed a pair of high-throughput screening assays. The first, although not biosensor based, is an interesting application of an in vitro synthetic chemistry approach. It has long been known that 1-nitroso-2-naphthol reacts with parasubstituted phenols, such as tyrosine with high specificity, yielding a red-orange product (83). By modifying the published assay methods, the authors developed a high-throughput, microtiter-based fluorometric screen for L-tyrosine (81). This assay was then used to screen a small, combinatorial library of amino acid biosynthetic genes overexpressed in *E. coli* (84). The authors report that their screen accurately differentiates between product concentrations as low as 50 mg·L^{-1} over a linear range of 0.05–0.5 g·L^{-1} tyrosine (81). As with lycopene, throughput is limited to the order of 10^5 variants per experiment owing to requisite use of microtiter plates and in vitro synthetic chemistry.

More recently, Santos & Stephanopoulos (77) describe a catalytic biosensor approach using an in vivo expressed tyrosinase to use melanin as a reporter of tyrosine productivity (**Figure 3**). A black, insoluble pigment, melanin, can be readily assayed in colonies grown on solid media without need for additional in vitro synthetic chemistry or solvent extractions. Starting with a base *E. coli* strain producing 347 mg·L^{-1} tyrosine (similar to the above-mentioned rationally engineered strain T1), the authors constructed a transposon-mediated knockout library of the *E. coli* genome and screened 21,000 colonies on agar plates. Over two five-day rounds of screening, 30 variants were selected for a more rigorous characterization; two mutants were discovered that individually produced 57% and 71% higher L-tyrosine titers than the background strain. The associated chromosomal knockouts occurred in *dnaQ* and *ygdT*, whose gene products are part of the epsilon subunit of PolIII and a hypothetical protein, respectively. Under similar culture conditions, the *yadT* knockout strain exhibited product yields comparable to those measured in the highest producing rationally engineered strains (77, 82). Neither of these genomic knockouts could have been predicted using rational design or flux analysis approaches, a feature that draws attention to the potential value of strong-screening assays. The performance features for this assay were not provided and thus inhibit comparison to their synthetic chemistry-based route; however, visual assessment of colonies is in general a much less sensitive detection method.

4.2. Growth Complementation

Strains auxotrophic for essential small molecules are natural biosensors and have long been used to build strong selection assays (85). These strains provide what is perhaps

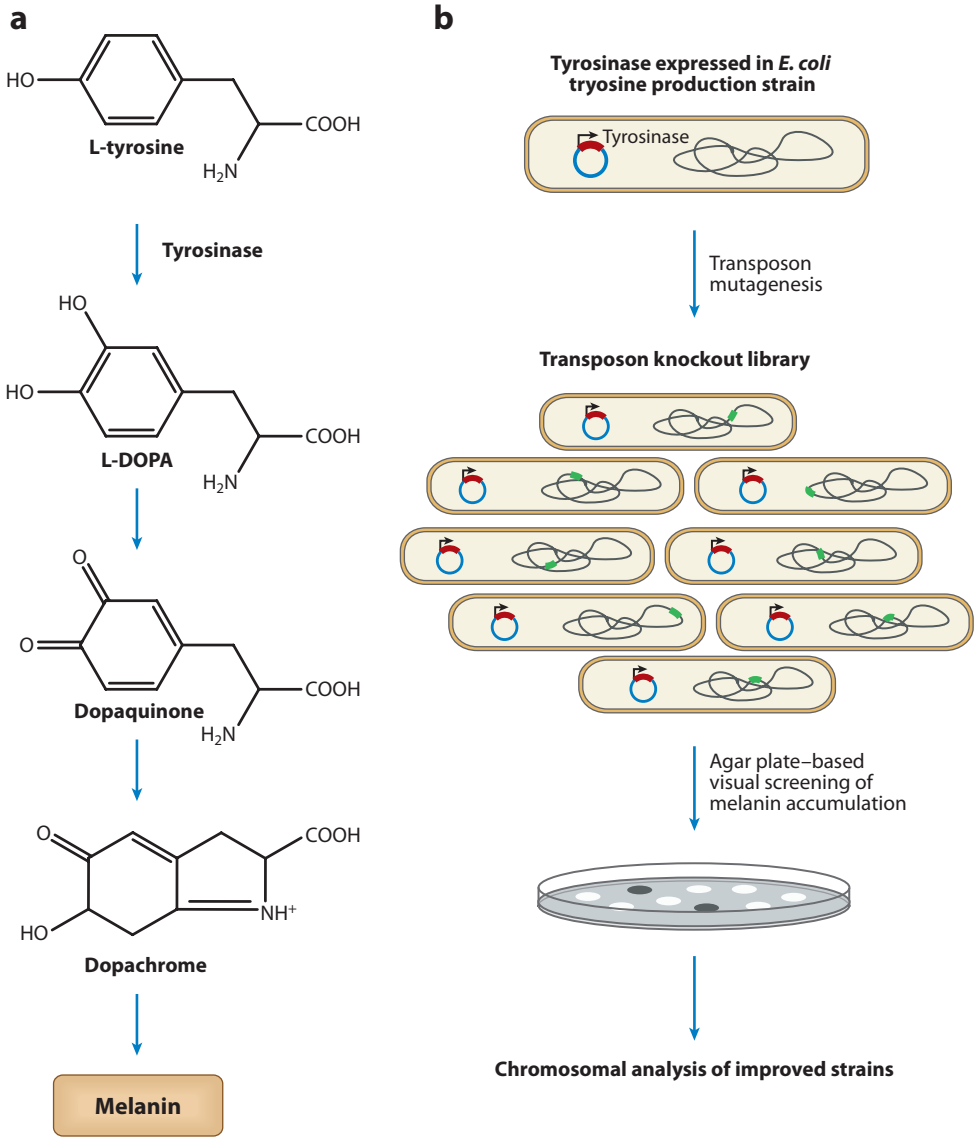


Figure 3

Development of a catalytic biosensor. Santos & Stephanopoulos (77) coupled production of the colorless amino acid L-tryosine to synthesis of the black, diffusible pigment melanin through heterologous expression of a tryosinase in *E. coli*. (*a*) Tyrosinases use molecular oxygen to catalyze the *ortho*-hydroxylation of L-tyrosine to L-DOPA, followed by its oxidation to dopachrome. The generated reactive quinones then polymerize to form melanin. (*b*) Expression of a bacterial tryosinase (*blue plasmid*) enabled a high-throughput screen, as melanin was used to report on the production of L-tyrosine. Agar plate-based screening of a transposon-mediated knockout library identified strains with increased tyrosine production. Analysis of the chromosomal mutations in the improved strains revealed interruptions in two genes, *dnaQ* and *yadT*. Neither gene would have been predicted to impact aromatic amino acid production through rational design or flux analysis strategies. This work illustrates the importance of strong screening assays in uncovering untapped genotypic improvements.

the most readily discernable phenotype, growth, if the auxotrophy is relieved by complementation of lost enzymatic function. Growth-complementation assays have been used extensively to engineer hosts for catabolism of nonnative or nonideal carbon, nitrogen, and phosphate sources (86, 87). Assay development is relatively straightforward: By supplementing the growth medium with a single molecular source of an essential element, only those host variants engineered or evolved for its catabolism survive.

Although highly successful in engineering novel catabolic activities, coupling anabolism to growth has proven to be a more difficult task. Oftentimes, an overproduction phenotype is deleterious to the host strain's reproductive fitness (88, 89), a characteristic easily inferred by depressed growth curves in overproducing strains. To screen for anabolic activity, small-molecule production must complement the auxotrophy and be strongly correlated with the specific growth rate to enrich cultures for high producers.

Auxotrophies of components of amino acid biosynthesis pathways have been successfully utilized in metabolic engineering applications because the pathways are strongly coupled to growth. An early study using growth-complementation knocked out the gene encoding for branched chain amino acid aminotransferase *ilvE*; this strain was then used to evolve an aspartate aminotransferase to recognize branched amino acids (90). Following five rounds of DNA shuffling, the catalytic efficiency for transamination between 2-oxovaline and aspartate was improved by five orders of magnitude. Further selection increased the catalytic efficiency of the final mutant variant by an additional order of magnitude (91). Other examples from amino acid biosynthesis, which used similar strategies, include aminotransferases (92), an alanine racemase (93), and evolution of chorismate mutase (94, 95).

Intermediates in amino acid biosynthesis, keto acids, have been more recently explored in a metabolic engineering context. Atsumi et al. (96) rationally engineered 1-propanol and 1-butanol production in *E. coli* using 2-ketobutyrate derived from threonine degradation. A more direct route to 2-ketobutyrate, however, is the citramalate pathway, identified in *Leptospira interrogans* and *Methanocaldococcus jannaschii* but was not present in *E. coli*. An *E. coli* strain auxotrophic for isoleucine would require flux to be rerouted through a transformed citramalate pathway to restore growth. This strategy was employed in an *E. coli* host for functional expression and directed evolution of a heterologous citramalate biosynthetic pathway leading to 2-ketobutyrate (97). The evolved strain exhibited 9- and 22-fold improvements in 1-propanol and 1-butanol productivities, respectively, over the wild-type citramalate base strain. Furthermore, when similar media conditions and genetic backgrounds were employed, the evolved citramalate pathway provided greater than 35-fold improvement in 1-propanol yield and an eightfold improvement in 1-butanol yield. Given the large number of industrially important compounds that can be derived from amino acid biosynthetic pathways and the success of auxotrophic selection assays for these compounds, there is likely to be continued focus on these assays in the future.

4.2.1. Auxotrophic reporter strains. A more generalized growth-complementation strategy is the use of an auxotrophic reporter strain. Here, a producer strain is engineered for overexpression of the target pathway, and a second reporter strain is constructed that constitutively expresses a detectable market (i.e., green fluorescent protein, GFP) and is auxotrophic for an essential metabolite found in the target pathway (**Figure 2**). When cocultured, the reporter strain's growth—and thus reporter output—is coupled to the metabolite yield achieved by the producer strain. This strategy was successfully employed in the development of a whole-cell biosensor for mevalonate (**Figure 4**) (98). A 10% difference in mevalonate production could be discerned between liquid cell cultures with 95% confidence. When utilized in a spray-on technique for solid media screens, however, the assay was only effective in distinguishing

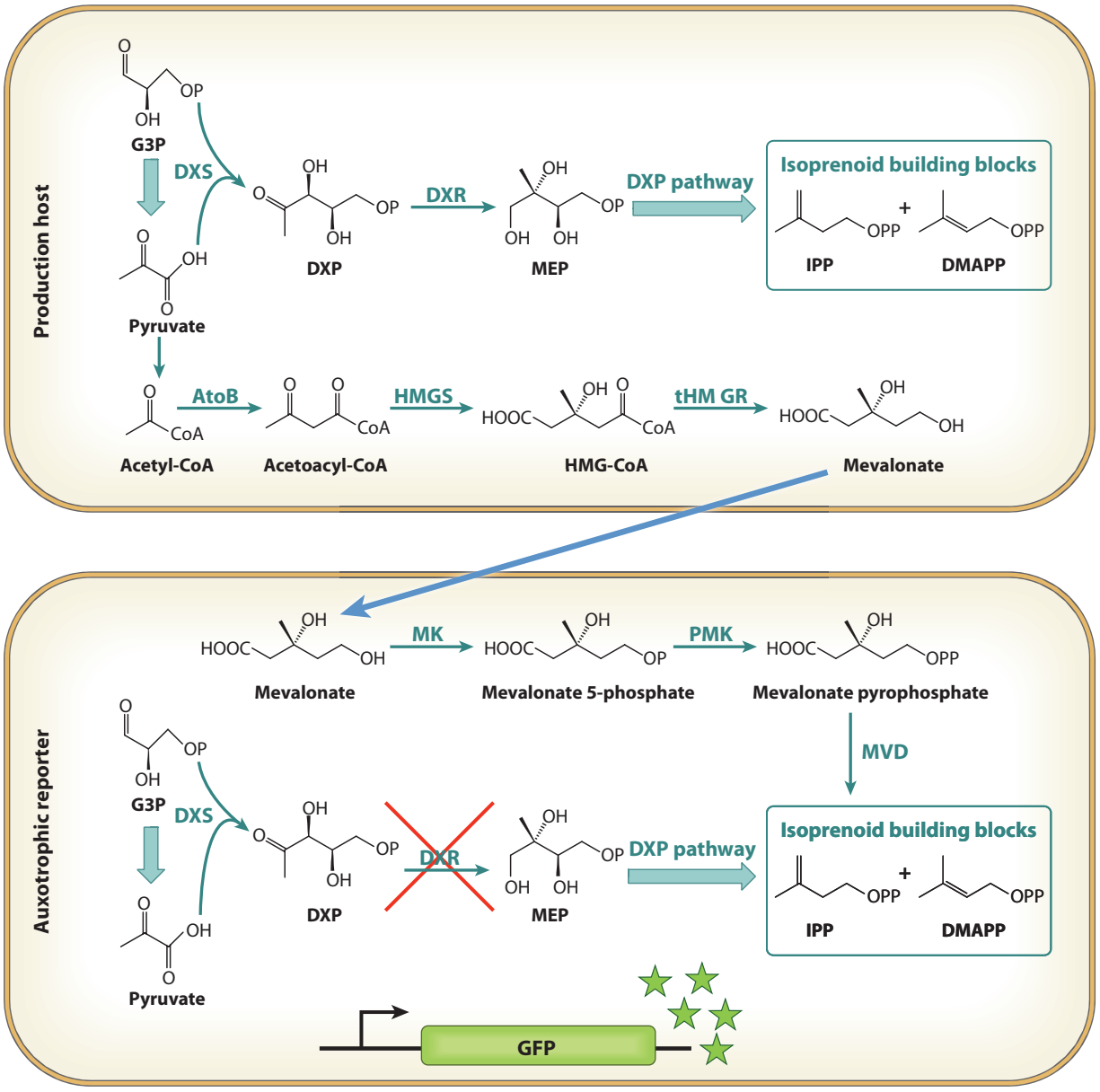


Figure 4

Development of a mevalonate biosensor. The isoprene subunits isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are essential for *E. coli* growth. Natively, IPP and DMAPP are produced in *E. coli* through the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway; however, expression of a heterologous mevalonate pathway provides an alternative route. By concomitantly knocking out the DXP pathway while overexpressing the enzymes necessary for mevalonate utilization, a mevalonate biosensor was constructed (98). The mevalonate biosensor also contained a constitutively expressed gene for green fluorescent protein (GFP) to provide a measurable readout of cell growth on solid media plates. Mevalonate was supplied by a production host containing genes necessary for transformation of acetyl-CoA to mevalonate. Abbreviations: AtoB, acetoacetyl-CoA thiolase; DMAPP, dimethylallyl diphosphate; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXS, 1-deoxyxylulose-5-phosphate synthase; G3P, glyceraldehyde-3-phosphate; HMGS, HMG-CoA synthase; IPP, isopentenyl diphosphate; MEP, 2-C-methyl-D-erythritol-4-phosphate; MK, mevalonate kinase; MVD, mevalonate pyrophosphate decarboxylase; PMK, phosphomevalonate kinase; tHMGR, truncated HMG-CoA reductase.

mevalonate producers from nonproducers (98); a finding that again highlights the higher sensitivity observed in liquid versus solid media plate-based assays.

In general, auxotrophic strains provide a unique, high-throughput approach for the screening or selection of small-molecule overproducing strains. However, there remain some significant drawbacks when considering their broad-scale adoption. First, the essential small molecule must be native to the host or reporter microbe; thus, growth complementation is more suitable to building platform production strains for metabolite precursors than exotic secondary metabolites. Second, the dynamic range in growth-complementation assays can be limited. From a protein engineering perspective, even slightly functional biocatalysts can be sufficient to restore cell growth; in practice, this places a glass ceiling on the small-molecule product yields that can be accurately screened or selected. Addressing this challenge, user-defined transcription and enzyme degradation tags have been explored as a means of increasing selective pressures and assay dynamic range (95). Other readily accessible approaches for fine-tuning protein expression levels include decreasing plasmid copy number, modifying RNA stability, and modifying translation initiation efficiency (99).

4.3. Fluorescence-Activated Cell Sorting-Based Screening

Near the top of the phenotypic screening and selection ladder stands fluorescence-activated cell sorting (FACS). Through rapid analysis of size and fluorescence measurements of single cells, this technique possesses nearly ideal high-throughput screening characteristics (100). Libraries are commonly first passed through a primary FACS screen, thereby enriching the population between 80- to 5000-fold for the 0.5% to 1.0% cells exhibiting the highest measured fluorescence (101–105). Library sizes between 10^8 and 10^9 variants have been readily screened in assays for modified protein specificity (101, 106), achieving the realistic 10^9 variant cutoff incurred owing to low microbial transformation efficiencies (28). Those cells surviving the primary screen are subjected to a secondary screen to provide phenotypic confirmation. In practice, between 10 and 10^3 clones enriched by the primary FACS screen are analyzed by gas chromatography-mass spectrometry (GC-MS) for identification or quantification (107). Although FACS is ultra high throughput, a drawback is overlapping fluorescence profiles, and aberrant fluorescence can lead to a high rate of false positives during screening. This arises from the distribution of fluorescence values found in a population when single cells are analyzed, a problem not witnessed in plate-based screens because of population averaging (**Figure 5**). For this reason, FACS-based screens are used to enrich for a target population and are then followed by secondary, orthogonal screens to provide a more accurate validation.

FACS: fluorescence-activated cell sorting

4.3.1. Direct and coupled-fluorescence detection. Direct detection of fluorescent proteins and metabolites using FACS is a straightforward application and has been well explored in both protein and metabolic engineering. In metabolic engineering, naturally fluorescent metabolites can be directly screened so long as they are intracellular and so long as their emission spectra are not masked by that of the native host. Again, the carotenoids have served as model small molecules for isolation of hyperproducing yeast strains. The inherent fluorescent property of astaxanthin was used to recover yeast strains at high sensitivity, capturing productivity improvements as low as 260 $\mu g \cdot g^{-1}$ dry cell weight (108, 109). A recent effort to improve astaxanthin production from *Xanthophyllomyces dendrorhous* achieved a 3.8-fold production improvement and reported fivefold improvement in screening efficiency as compared to plate-based methods (110). Beyond the carotenoids, FACS screening has been restricted to a small number of natural and synthetically coupled fluorophores, including gramicidin S (111), polyhydroxyalkanoates

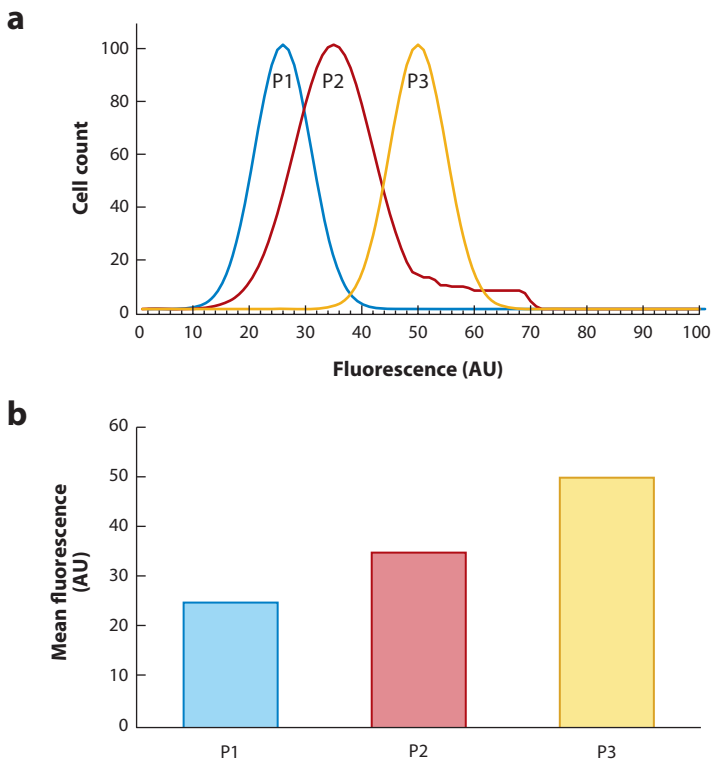


Figure 5

Population distributions can inhibit efficient cell sorting. (*a*) Representative data collected from fluorescence-activated cell sorting (FACS) and (*b*) fluorometer analyses of output reporter fluorescence highlight the difficulties in efficient screening. We examine the hypothetical strains P1, P2, and P3, displaying different levels of target small-molecule production. Although the mean fluorescence for all cell populations does not change between fluorometer and FACS instruments, population distributions inhibit efficient cell sorting by FACS. Overlapping population profiles (separating P2 from P1 and P3) and aberrant fluorescence (the right-hand tail on P2) can decrease enrichment efficiencies. Lower-throughput, orthogonal secondary screens must be used to further purify the target population. Abbreviations: P1, -2, -3, hypothetical populations.

(112), poly(β-hydroxybutyrate) (113), and lipids (114, 115).

4.3.2. Physiology-associated fluorescence detection. Another route for FACS-based screening is to assess those changes to cellular physiology induced by small-molecule production or cell stress. In this indirect method, fluorescent dyes act as indicators for changes in intracellular pH or viability (116). Following this strategy, strains of *S. cerevisiae* capable of improved lactic acid tolerance and production have been isolated from a mutant library, using both ethidium bromide staining and a pH-dependent fluorescent probe (117). The fluorescent probe used, cSNARF-4F, exhibits a shift in the spectral emission depending on intracellular pH; the ratio of the two local maxima indicate the pH (118). Because the assay is physiology dependent and not product dependent, it can be extrapolated toward detection of a range of microbially synthesized weak organic acids and bases.

The approach of screening for a downstream effect of molecule overproduction, and not for the small molecule itself, may be a versatile strategy given that entire classes of molecules can produce similar changes in physiology. For instance, FACS-based screens could prove effective in cases of product-induced oxidative stress, a common side effect of P450 overexpression (119), using in vivo fluorescent probes for reactive oxygen species (120, 121). Similarly, membrane fluidity (122), intracellular magnesium (123), and intracellular calcium (124) can also be quantified using fluorescent dyes. Despite the straightforwardness of this screening approach, it is hampered by the dearth of available in vivo reporter probes. In addition, it remains unknown whether a strong, reproducible correlation can be drawn between small-molecule biosynthesis and a microbe's physiological state over a large set of molecule classes.

4.3.3. Biosensor-driven fluorophore production. Genetic biosensors can take inconspicuous small-molecule inputs and output one of several available fluorescent proteins as reporters. In general, methods described to date have focused on transcriptional read through from product-encoding operons as a proxy for end function. One interesting approach has been construction of genetic traps, screens used to isolate genetic elements from metagenomic libraries that encode for user-desired phenotypes. SIGREX, or substrate-induced gene-expression screening, uses a fluorescent reporter housed behind a multiple cloning site

into which a metagenomic library is inserted (125, 126). When a small-molecule substrate is exogenously added, only clones harboring metagenomic inserts whose transcription is activated by the substrate small molecule will express the downstream fluorescent reporter. A second, negative screen under uninduced conditions can remove variants with constitutive fluorescence. For the insert to produce a fluorescent signal, it must encode for a responsive transcription factor-promoter system. The promoter must also be oriented in the correct direction to transcribe the downstream reporter gene. The success of this method hinges on the overarching assumption that the requisite transcription factor is housed proximal to its cognate promoter controlling GFP expression (126), a guilt-by-association strategy that is by no means a genotypic rule (127).

User-selected biosensors can also be applied for isolation of anabolic operons from metagenomic libraries encoding for biosynthesis of biosensor-responsive small molecules (128). This was demonstrated using the well-characterized acyl-homoserine lactone-responsive LuxR- P_{luxI} genetic actuator isolated from *Vibrio fischeri* (129). By placing GFP under control of P_{luxI}, a metagenomic library was transformed into *E. coli* and screened for novel inhibitors of the quorum-sensing response. Approximately 10,000 colonies survived the preliminary FACS assay by inhibiting GFP production in the presence of exogenously added acyl-homoserine lactone. Only a meager 0.2%, or two colonies, produced confirmable inhibition of transcription from P_{luxI}, a finding that draws light toward the high rate of false positives when using FACS for phenotypic characterization. A similar approach was recently used to screen for alkane biosynthesis and transport in engineered strains of *E. coli* using the AlkR transcription factor isolated from *Acinetobacter* (130). In cases where a target small molecule lacks no known cognate transcription factor, FACS-coupled directed evolution of the transcription factor's effector recognition domain can realize minor perturbations in ligand specificity (131). Nearly all genetic biosensors described to date have been used to obtain an all-or-none fluorescent response profile, which is well suited for gene discovery but poorly suited for mechanistic studies where intermediate behaviors provide information equally as important as the final product. As discussed in detail below, however, genetic biosensors providing a linear response profile can have also been characterized and can be used to elucidate intermediate behaviors.

5. FUTURE DIRECTIONS: TRANSCRIPTION FACTOR-BASED SMALL-MOLECULE SCREENING

The screening strategies described above have demonstrated success in identifying proteins or strains capable of increased small-molecule production titers. Many small molecules, however, are not natively chromophoric, fluorescent, or essential for growth. Furthermore, only a limited few can be readily transformed into compounds possessing these properties. Thus, a universal high-throughput screening platform for small molecules remains out of reach. In spite of that, to expand our existing repertoire of detection techniques, we can turn to nature, the apt experimentalist, and borrow from the plethora of molecular devices evolved for small-molecule recognition.

Nature has evolved RNA, DNA, and protein devices for the binding of small molecules and for activation of a downstream transcriptional response. Some molecular devices, including the aptamer (132) and FRET biosensors (133), can be engineered for small-molecule detection and deserve further exploration. One strategy deserving special attention is the use of transcription factors, proteins that regulate a promoter's transcriptional output in response to a small-molecule ligand, to report on in vivo small-molecule production. Bioengineers have long used transcription factors to construct whole-cell biosensors for the detection of environmental small-molecule pollutants (134). However, this same approach has remained

largely untranslated toward library screening and directed evolution purposes.

5.1. Biosensor Response Characterization

Screening methods utilizing transcription factors possess many characteristics ideal for developing small-molecule screens and selections. The reporter gene housed downstream of an inducible promoter can be user selected to encode for colorimetric, fluorescent, or growth-coupled responses. Additionally, because the transcription factor-promoter pair is responsible for direct, in vivo detection of the target analyte, the need for downstream synthetic chemistry or in vitro manipulation is eliminated.

The first task when designing a screen is to understand the relationship between small-molecule input and reporter protein outputs. This process is greatly facilitated in transcription factor–based detection technologies by continued work in genetic circuit design. In part, this is due to a trend in the synthetic biology community toward better characterization of standardized hosts and biological parts (17, 135, 136). For example, a thorough characterization of the frequently used LuxR-P_{LuxR} expression system for *E. coli* provides a solid framework for biosensor design and characterization (37). The mathematical description of an expression system's transfer function can elucidate the conditions under which a biosensor can provide robust, reproducible function. As with optical detection techniques, biosensor transfer functions are characterized by their dynamic range, sensitivity, small-molecule specificity, and range of linear analyte detection (**Figure 2**) (38). All of the relevant parameters can be readily calculated using existing models that describe a range of regulatory schemes (137–139).

Even in *E. coli*, unfortunately, standardized promoter characterization remains scant to date and remains virtually nonexistent for yeast expression systems. We provide here a retrospective analysis of published response curves and provide estimates for a minimum set of governing parameters for many of the most commonly used biosensor-ligand induction systems (**Table 1**). Shown are the sensitivity, dynamic range, and linear detection range for a number of promoter-induction systems when fit using the Hill equation. Few of the small-molecule ligands for which these systems were designed to detect are industrially relevant; thus, their cognate biosensors are unlikely to be used for small-molecule assay design. However, we can still glean useful information on generalized trends that will facilitate biosensor-based assay design.

From this data there emerge a number of key trends that can help guide more efficient biosensor design. Perhaps the most important performance feature describing a transfer function is the apparent Hill coefficient, n_{app}. By definition n_{app} describes the assay sensitivity, but information on the linear range of detection, the detection threshold, and the dynamic range can be inferred. For example, a large n_{app} indicates a high slope within the linear portion of the transfer curve and a sensitive assay, but it is also associated with a small-linear induction window, a large dynamic range, and a low detection threshold. In electric and genetic circuit design, a large n_{app} describes digital-like behavior. The tight regulatory control displayed by digital logic gates has made them a staple for in vivo genetic circuit design; the P_{Tet}-TetR and P_{LuxR}-LuxR systems, in particular, are archetypal model systems providing robust, user-defined behaviors (140–143). Analog-like behavior is also witnessed in our selection of expression systems. For example, the P_{BAD} and P_{PRO} systems surveyed here have low sensitivity and a broad window for which the output response is linearly correlated to the input concentration. In general, analog-type responses are more in line with established screening assays for small-molecule detection. Along a similar vein, analog logic gates are suitable for use in directed evolution assays, where phenotypic improvements are typically small and incremental.

Although broad-reaching conclusions remain elusive, evolutionary arguments support some general trends seen among the transfer

Table 1 Transfer function–derived performance parameters for common induction systems

Induction system	Ligand	Sensitivity (n_{app})	Dynamic range (fold increase)	Linear detection range	Reference
$P_{LtetO-1}$-TetR	Anhydrotetracycline	10.43	2535	≈0–4.5 nM	149
P_{TETO7}-rtTA[a]	Doxycycline	1.12	28	0.01–2.5 μM	144
P_{TETO2}-rtTA[a]	Doxycycline	0.96	15	0.05–11 μM	144
P_{TETO1}-rtTA[a]	Doxycycline	0.70	15	0.25–35 μM	144
$P_{LlacO-1}$-LacR	Isopropyl β-D-1-thiogalacto-pyranoside (IPTG)	1.14	620	0.01–1 mM	149
P_{BAD}-AraC	Arabinose	0.43	5.5	0.08–20 mM	150
P_{prpB}-PrpR	Propionate	2.25	6.5	0.2–12.6 mM	150
P_{Trc}-lacI	Isopropyl β-D-1-thiogalacto-pyranoside (IPTG)	0.52	5	4–6.3 μM	150
P_{LuxR}-LuxR	3-Oxohexanoyl-L-homoserine lactone ($3OC_6HSL$)	1.6	500	0.5–10 nM	37
P_{pu}-XylR	Toluene	2.3	16	50–300 μM	151
P_{pu}-XylR	Benzene	3.2	9.8	—	151
P_{pu}-XylR	4-Xylene	2.0	26	—	151
P_{pu}-XylR	O-xylene	0.79	20.3 ± 1.6	0.05–5 mM	146
P_{po},-XylR	O-xylene	[b]	3151 ± 156	0.1–10 mM	146
P_{ps}-XylR	O-xylene	1.41	32.5 ± 6.1	0.1–5 mM	146

[a]The O# in P_{TETO} indicates the number of operator sites included in the promoter design.
[b]Curve could not be accurately fit using the provided data points, however an $n_{app} > 10$ is estimated.

functions between various expression systems. Evolution has tailored the regulation of each promoter to function in a specific environmental context. Digital behavior is more often witnessed in genetic systems evolved for antibiotic, quorum sensing, and sugar utilization. In contrast, analog behavior is more typical of nonideal carbon source utilization. Of course, when the system is removed from its original genetic context (i.e., removing a transcription factor from control by its native promoter), these observations may not hold. Transcriptional logic gates have proven to be readily engineered genetic parts, and transfer functions can be altered for more digital- or analog-like profiles, as needed for the application at hand. A shift toward a more digital response was observed by increasing the number of operator sites on a doxycycline-induced P_{Tet} promoter (144), which manifested as an increase in n_{app}, a lower detection threshold, and an increase in dynamic range (**Table 1**). Similarly, for transcription factors exhibiting a poor binding profile to the target ligand, directed protein evolution has been demonstrated to increase ligand specificity and biosensor sensitivity (145). Perhaps more often than realized, response profiles can be quickly adjusted without the need for laborious protein or promoter engineering. Poor ligand-transcription factor binding is often a characteristic of an analog response, suggesting transcription factors with promiscuous ligand binding could serve as ideal analog devices using weak inducers. Lastly, in the case of the transcription factor XylR, just moving between one of its three native promoter systems dramatically changed the response profiles in an o-xylene-induced system (146). Whether analog or digital, both types of behaviors can be either pilfered from nature's abundant stock or engineered from preexisting, standardized biological parts.

5.2. Applications Using Digital and Analog Biosensor Response Profiles

Digital and analog biosensor response modes are both equally important for in vivo small-molecule assays; digital behavior has demonstrated a value for construction of genetic traps, whereas analog behavior is well poised for applications in directed evolution. Mohn et al. (147) describe both digital and analog transcription factor–based biosensors in their detection of the biotransformation of linade into 1,2,4-trichlorobenzene. The authors first construct their biosensor using an evolved mutant variant of the σ^{54}-dependent transcription factor XylR, which is sensitive to 1,2,4-trichlorobenzene but not to lindane. Using this system, three reporter strains were created: two *P. putida* strains harboring luciferase or LacZ reporters and an *E. coli* strain harboring a LacZ fusion protein reporter. To achieve a strong digital response, the biosensor's dynamic range was increased by eliminating sources of background noise caused by leaky transcription from their promoter. In the luciferase reporter strain, the authors couple their evolved XylR protein with the *Po* promoter, which exhibits a substantially lower level of leaky expression as compared to the more commonly used *Pu* promoter (146, 148). In the *E. coli* reporter strain, LacZ was fused to XylU, the first native gene product downstream of the *Pu* promoter. Strains harboring the fused reporter construct were reported to exhibit background expression levels below the detectable limit of a β-galactosidase assay (147). When cotransformed with a lindane dehydrochlorinase from *Sphingomonas paucimobilis* UT26, the strains provided proof-of-concept demonstration of digital biosensor response using both colorimetric and growth-complementation assays. Although not employed for purposes of directed evolution, the *E. coli* biosensor system yielded a near-linear relationship between output β-galactosidase activity and concentration of exogenously added lindane. Linearity was held over a two orders-of-magnitude concentration range, providing strong evidence this strain could have a use as an in vivo screen for improved lindane or 1,2,4-trichlorobenzene biosynthesis.

6. CONCLUSIONS

Metabolic engineers require high-throughput screens to facilitate the directed evolution of microbial strains for small-molecule overproduction. The sheer number of variables that can be modified within either the genome and heterologous pathway are astounding; furthermore, biosynthetic pathways comprise multiple enzymes subject to regulation, which is too often poorly understood. Without detailed experimental evidence, there is little hope for rational mutagenesis strategies to substantially improve small-molecule yields and pathway efficiencies.

When coupled with a high-throughput screen, random mutagenesis can be a powerful method to explore sequence space with little-to-no a priori knowledge of the subject's structure or function. The walk through sequence space, however, is limited by the underlying statistics behind library generation and screening assay throughput. For the vast majority of small-molecule targets, screening throughput stands as the rate-limiting step, with low-throughput liquid and gas chromatography being the modus operandi in the field today. Recent innovations are enabling experimentalists to "climb the screening and selection ladder," the underlying principle being the design of biosensors to transform inconspicuous small molecules into more readily detectable reporters.

SUMMARY POINTS

1. A continued focus in metabolic engineering on directed evolution for small-molecule-producing microbes demands improved high-throughput screening technologies; whole-cell small-molecule screens and selections address this issue.

2. An exhaustive search of sequence space is limited by choice of mutagenesis target, library construction method, and screening technology. When sufficient a priori knowledge is available, a targeted mutagenesis strategy can substantially reduce the required library size.
3. In vitro manipulations (transformation and screening) are the most significant bottlenecks restricting assay throughput. When possible, assays should be designed to minimize the number of in vitro manipulations.
4. Transfer functions serve as important guides in the design of biosensor-based screening techniques. The mathematical relationship between biosensor input and output describes key performance features: dynamic range, sensitivity, and linear range of detection.
5. Colorimetric and fluorescence plate-based screens are the most well-explored screening assays; throughput is moderate (10^5–10^{6+} variants), and sensitivity can vary from low (colony analysis on solid media plates) to high (product extraction and concentration). These methods are supported by a wealth of experience in small-molecule labeling from synthetic chemistry literature.
6. Growth-coupled assays can be constructed by utilizing strains auxotrophic for essential metabolites in pathways required for target small-molecule biosynthesis. Throughput can be extremely high—limited only be the size of the culture and mutagenesis technique—when selections are employed. Assay sensitivity varies but can be low if only a moderate level of activity is required to restore growth.
7. FACS-based screens provide the highest throughput and sensitivity. Up to 10^9 cells can be screened per experiment with single-cell resolution. FACS screening is limited by the requirement for in vivo localization of a fluorophore.
8. Transcription factor-based screens can be used to create whole-cell biosensors coupled to colorimetric, fluorescent, and growth-coupled detection assays. Progress in this area is ongoing and builds on biological parts characterization and circuit design in the synthetic biology community.

FUTURE ISSUES

1. Can in vitro compartmentalization and phage display technologies be adapted to be more readily applicable to small-molecule screening?
2. Can in silico enzyme design be coupled to existing high-throughput screening approaches to breach the current limits on the maximum screenable library size?
3. In our directed evolution flow diagram, throughput losses are incurred when in vitro steps are added. Are there in vivo methods for the simultaneous selection and mutagenesis of targeted genetic elements (i.e., accelerated evolution)?
4. Currently, mutagenesis of genomic targets remains more difficult than mutagenesis of plasmid-borne targets. Are there approaches for the simultaneous modification (both up- and downregulation) of multiple genomic targets?

5. A more rigorous standard in small-molecule assay characterization needs to be designed and applied. What additional performance features need to be included? Can elements from the high-throughput screening of small-molecule targets for pharmaceutical development be incorporated?
6. As production titers are improved, product-induced toxicity is becoming a reoccurring theme. In addition to standard selection assays for improved tolerance, can existing themes in small-molecule detection be adapted to transporter engineering?
7. Implementation of transcription factor–derived biosensors will require a greater understanding of our ability to modify transfer curves. To what extent can analog and digital behaviors be engineered? Is there modularity within transcription factor families that can be used to screen for undiscovered ligand receptor domains?

DISCLOSURE STATEMENT

J.D.K. has financial interests in Amyris and LS9. J.A.D. and A.E.M are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Errata

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