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Novel screening methods — biosensors

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Biosensors offer exciting possibilities for improving cells or enzymes as biocatalysts for the synthesis of small molecules. We here review recent progress in the development and the screening applications of transcription-factor-based biosensors. An example is a cofactor-dependent biosensor which provides a generalizable screen for NADPH-dependent enzymes. Another example is the use of a biosensor in combination with recombineering for strain development, thereby expanding the genome engineering techniques to deliver directly bacteria producing small molecules of interest. Biosensor-based techniques in combination with fluorescence-activated cell sorting demonstrate that the gap regarding throughput capabilities of existing methods for the generation of genetic diversity and methods for the subsequent screening can be closed.

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Introduction

In biosensor-based screening strategies, it is not the product of an enzyme or a pathway itself that results in a measurable property, but rather a genetically encoded reporter that provides a discriminating phenotype. If the reporter is *lacZ*, *tetA*, or *gfp*, then product formation can be easily screened by color formation, resistance, or fluorescence instead of laboriously screening for product formation employing chemical methods. There are basically three types of biosensors: (i) constitutively formed reporter-proteins leading to signal generation due to interaction with the analyte — which include the switches based on Förster resonance energy transfer (FRET), (ii) RNA switches where an RNA aptamer controls expression of a reporter gene in response to binding of the analyte, and (iii) systems based on transcriptional regulators (TRs), where interaction of the analyte with a regulatory protein controls expression of the reporter. The different types of sensors naturally involve both advantages and disadvantages. Although FRET sensors can be used for real-time *in vivo* detection of metabolites, they have not been considered for HT screening purposes, since they generate relatively low absolute signals. This is different for RNA switches and one switch has successfully been used for screening libraries for development of a caffeine demethylase. However, difficulties in engineering RNA switches have limited their broader use for screening purposes. Detailed discussions covering a range of aspects on biosensors are available in recent reviews [1,2,3•,4,5]. Most advanced are screening methods using TR-based biosensors, which we present here, focusing on the latest developments in this field.

Biosensors in enzyme screens

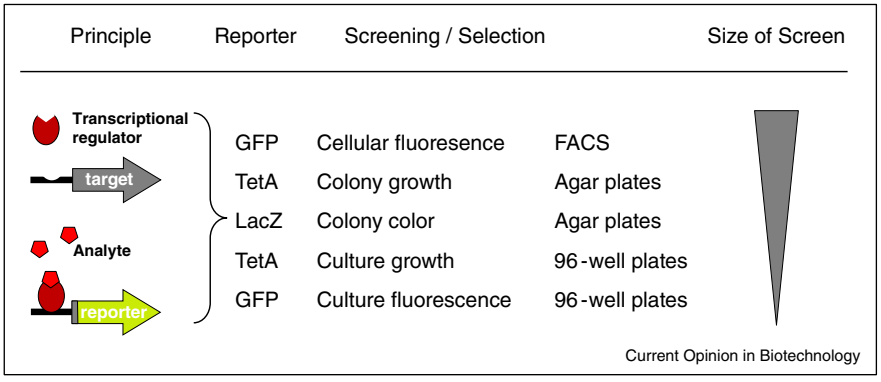
The general principle of TR-systems is illustrated in Figure 1. The screening or selection methodologies include (a) selection of positive clones by colony growth, (b) screening for colony color, (c) screening of cultures for increased growth due to selection, (d) screening of individual clonal cultures using fluorescence as output, and (e) isolation of single cells by fluorescence-activated cell sorting (FACS).

The basic principle of a TR used for screening purposes was demonstrated with NhaR of *Pseudomonas putida* driving the transcription of *tetA* as a function of the benzoate or 2-hydroxybenzoate concentration [6]. It was used to demonstrate selection of benzaldehyde dehydrogenase positive cells on tetracycline-containing agar plates out of 10^5 benzaldehyde dehydrogenase negative cells. Another benzoate-responsive sensor used the TR BenR coupled to control GFP formation [7]. It was applied to screen for benzamidases of an *Escherichia coli* metagenomic library consisting of 96,000 clones. By contrast to other TR-based studies, the library was separately grown and subsequently combined with the sensor-cells in 96-well format to assay for product formation. This enabled identification of 11 different amidases.

A further example of the use of a TR-based screen for a specific enzyme is use of an AraC variant of *E. coli* responsive to triacetic acid lactone (TAL) and coupled to LacZ as reporter [8]. TAL is the product of 2-pyrone synthase activity of *Gerbera hybrida* and an intermediate of polyketide synthesis. Two rounds of mutagenesis and visual screening on plates for colony color yielded a variant conferring ~20-fold increased TAL production due to an increased catalytic efficiency of the 2-pyrone synthase.

From classical screening approaches *p*-nitrophenol-generating substrates for glycosidases, lipases, chitinases, and

Figure 1



In transcriptional-regulator based sensors, the target gene is replaced with a reporter. The type of reporter determines which screening or selection strategy is possible. Strategies differ with respect to the possible size of the screen and thus the throughput of variants per experiment.

other enzymes are known, enabling activity detection by spectrophotometry. Using the dimethylphenol regulator DmpR of *P. putida* recognizing *p*-nitrophenol and phenol generation of fluorescence by *E. coli* due to the presence of such enzymes could be demonstrated [9]. A FACS screen of a metagenomic library with phenyl phosphate as substrate yielded a new alkaline phosphatase.

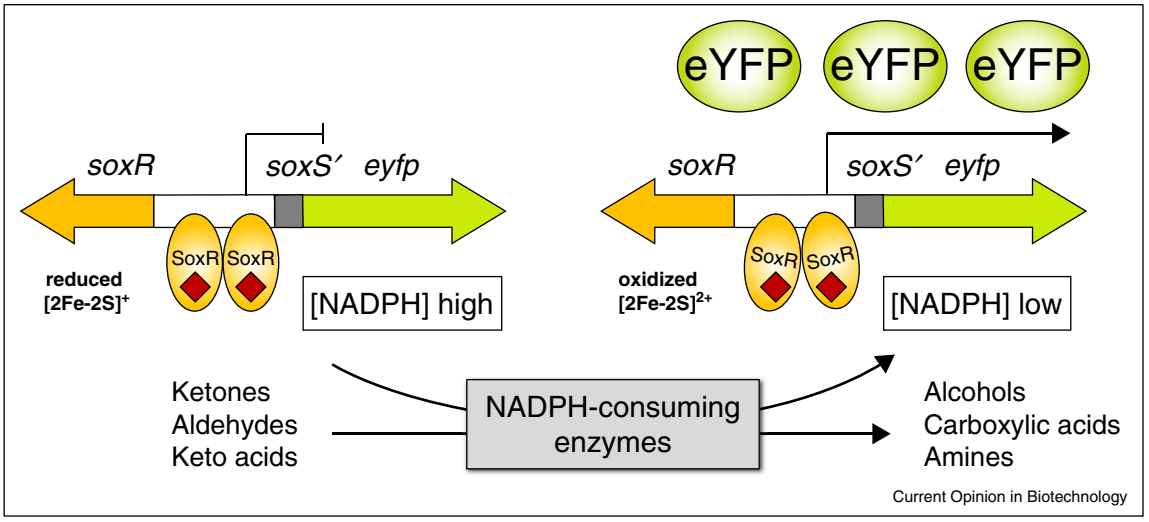
The full potential of biosensors giving cells different optical properties is only exploited in combination with FACS, as was recently shown by engineering the N-acetyl-L-glutamate kinase ArgB of *Corynebacterium glutamicum* [10]. ArgB is the flux controlling enzyme at the start of the long L-arginine biosynthesis pathway. Allosteric inhibition of its activity by elevated L-arginine concentrations prevents increased L-arginine synthesis, as required in industry [11]. The key to HT-screening is the TR LysG of *C. glutamicum* which recognizes basic amino acids and naturally serves to drive their export at high toxic cytosolic concentrations. This is necessary when cells are growing in the presence of peptides [12]. An *argB* mutant library was introduced into *C. glutamicum* carrying the sensor consisting of LysG coupled to *eyfp* [13••]. About 22^8 cells of the mutant library were screened via FACS, and 96 cells exhibiting increased fluorescence were selected. Verifications confirmed that 41 clonal cultures accumulated up to 18 mM L-arginine and that this is due to *argB* muteins which are no longer controlled in their activity by L-arginine. Based on the unspecificity of LysG, collections of the key enzymes of the L-lysine and L-histidine biosynthesis pathways were also isolated with abolished inhibition of the endproduct of the respective pathway.

Biosensors for NADPH and NADH-dependent enzymes

A recently developed TR-based FACS screen is particularly interesting. It monitors the NADPH demand of the *E. coli* cell and is consequently a generalizable system enabling screening for high NADPH use by enzymes [14•]. It also circumvents the problem that for each new enzyme to be screened a new assay has to be developed. It is based on the [2Fe–2S] cluster-containing TR SoxR of *E. coli*, which activates expression of *soxS* in the oxidized but not in the reduced state (Figure 2). As SoxR is retained in the cell in its reduced state by NADPH-dependent reductases, an increased NADPH demand counteracts SoxR reduction and increases *soxS* expression. Fusion of *eyfp* with *soxS* and use of an NADPH-dependent alcohol dehydrogenase, which reduces methyl acetoacetate to (R)-methyl 3-hydroxybutyrate results in correlation of cellular fluorescence with enzyme activity. The NADPH-responsiveness of the sensor was successfully used to screen a dehydrogenase library for variants showing improved activity with the substrate 4-methyl-2-pentanone.

A biosensor responding to increased NADH availability was developed for *Saccharomyces cerevisiae* [15]. In this case the promoter region controlling GPD2 expression was fused to yEGFP3. Of relevance is the fact that under anaerobiosis high expression and activity of the GPD2-encoded glycerol-3-phosphate dehydrogenase are needed, since the NADH-coupled reduction of dihydroxyacetone phosphate and its further conversion to glycerol is required as a redox sink. Using a set of heterologous xylose reductases having different selectivities for NADH, the responsiveness of the system to different NADH demand could be demonstrated, so that the xylose reductase with the highest selectivity for NADH displayed the lowest fluorescence. This example demonstrates that even without detailed knowledge on the TFs involved in expression control successful signaling systems can be build, as long as the transcriptional control elements are provided from the native host. The disadvantage of the GPD2 based system is that cells with high

Figure 2



The NADPH biosensor based on the transcriptional regulator SoxR of *E. coli*. At high NADPH-consumption the capacity of the cell to maintain SoxR in its reduced state is limited and oxidized SoxR drives soxS transcription. Coupled to eYFP formation, this sensor provides a means to screen for increased activity of NADPH-consuming enzymes.

enzyme activity and high NADH consumption have low fluorescence. Since the screening for cells with increased signal output against a background yields less false positives than screening for cells with reduced signal output against a background, a further development of the NADH-sensor may be required to make full use of its potential for high-throughput screening.

Biosensors in synthetic biology

In the examples described above biosensors were used to improve a single protein. However, in synthetic biology frequently whole cells and entire pathways are used and have to be improved. This may require the adjustment of a variety of plasmid-encoded functions as well as functions of the host chromosome. An example where the ribosome-binding site was targeted is the use of an AraC variant of *E. coli* responsive to mevalonate and an *E. coli* strain enabling mevalonate synthesis by three plasmid-encoded genes. Libraries of variant ribosome binding sites in front of a hydroxymethylglutaryl-CoA reductase were successfully screened by colony color for increased mevalonate formation [16]. Another biosensor took advantage of the TF BmoR of *Thauera butanivorans* for coupling butanol production in *E. coli* to increased tetracycline resistance [17]. A limited 96-well-plate screen for variant ribosome binding sites yielded a pathway variant with 35% increased butanol specific productivity. Furthermore, two biosensors were developed for *E. coli* possibly suitable for the screening of genes involved in flavonoid biosynthesis [18]. One sensor used the TR FdeR from *Herbaspirillum seropedicae* which activates expression of its target gene in presence of naringenin. The other used QdoR from *Bacillus subtilis* which upon interaction with quercetin and kaempferol leads to derepression of target genes.

The biosensor based on the TF LysG of *C. glutamicum* (see above) is not only responsive to L-arginine and L-histidine but also to L-lysine. This latter amino acid is produced on a million-ton scale with *C. glutamicum* and *E. coli* [19], and new chromosomal mutations are required to further improve the production properties. Using the L-lysine-responsiveness of LysG a library of 10^7 genomic mutants of the wild type of *C. glutamicum* obtained by undirected mutagenesis was screened by FACS yielding more than 100 improved L-lysine producers [13••]. Whole genome sequencing of several clones that do not harbor mutations in genes previously known to contribute to L-lysine synthesis identified a novel cellular function of the host chromosome for strain development. Introduction of the identified mutation *murE*-G81E into an existing L-lysine producer increased L-lysine accumulation by 15%. In a similar study the TR Lrp of *C. glutamicum* was used to successfully screen for genomic mutants synthesizing increased concentrations of the branched-chain amino acids or L-methionine [20].

It should be mentioned that in addition to offer new screening opportunities, TR-based biosensors find various additional applications in synthetic biology. They can be also used for the online monitoring of metabolite production by live cell imaging using microfluidic lab-on-a-chip devices [21], and they represent suitable tools for the monitoring of population dynamics [22]. Furthermore,

they might be useful in cultures for dynamic pathway control to prevent the accumulation of toxic intermediates, as done for biodiesel production with *E. coli* and control of genes involved in fatty acid synthesis by the TR FadR [23].

A perfect alliance: biosensors meet recombineering

The power of an efficient sensor-based screen also opens up fascinating possibilities in combination with genome engineering techniques for the HT generation of microbial producers at the single-cell level (Figure 3). Most current genome engineering techniques only permit the introduction of one single mutation at a time into the chromosome [24]. For each mutation this usually requires the synthesis of a specific plasmid, which is a cumbersome procedure. By contrast, single-strand DNA recombineering operates with easily accessible oligonucleotides, but still requires a visible or selectable phenotype [25]. The establishment of recombineering in *C. glutamicum* and its fusion with the aforementioned LysG-based L-lysine sensor enabled screening via FACS of L-lysine producing mutants directly from the recombineering assay [26••]. Even more exciting, using a mixture of 19 different oligonucleotides to target one codon in the chromosome, in one experiment a set of mutants was isolated with 12 different amino acid exchanges all leading to different L-lysine production titers. Thus by this procedure genetic diversity and selection for new producers was performed in one single step.

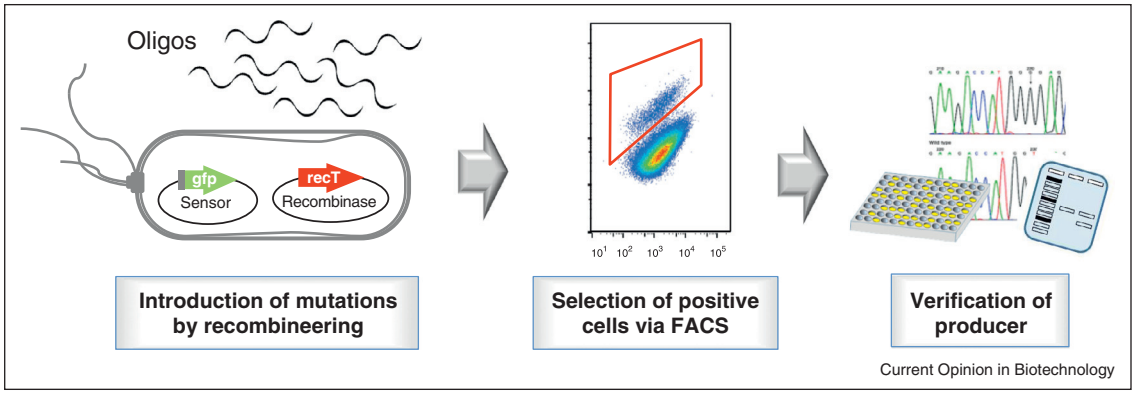
Access to biosensors and suitability

The field of TR-based biosensors useful for screening purposes is developing rapidly. Although in this review we have only given detailed consideration to selected biosensors, a range of such biosensors is already available (Table 1). The key to success is certainly the TR itself and requirements include its availability, its specificity, and its dynamic range. Since biosensors operate with entire cells, also the permeability of substrates and products through the membrane has to be considered.

Fortunately, known TRs are available detecting various small molecules, and new TRs can be identified and their function inferred on the basis of sequence studies. An abundant source of new TRs might be the metagenome, as inspired by substrate-induced gene expression known as SIGEX [27•]. In SIGEX, metagenomic fragments are cloned in front of *gfp* and the resulting library is screened with FACS after the addition of substrate. The rationale behind SIGEX is the fact that catabolic operons are frequently induced by their substrate or an intermediate and that regulatory elements are often situated in close proximity to catabolic genes. Using a *gfp*-trap vector and FACS, a selection of cells for analyte responsiveness might yield the TR required.

Access to TRs with new specificity is also possible by engineering approaches. Indeed, the field of altering TR specificities has a long tradition, in particular in developing TRs recognizing benzene derivatives [28]. The reason is that bacterial cultures with the appropriate constructs fused to *gfp* have been developed for the detection of environmental small-molecule pollutants. Currently many efforts using TR-based sensors in HT screens have focused on evolving TR specificities [28,29]. As shown for AraC of *E. coli*, which originally detected arabinose, a variant was isolated via FACS which responds to mevalonate and which enabled screening of cells with increased mevalonate synthesis [16]. Another AraC variant responding to triacetic acid lactone was additionally made [8]. Also promoters of TR-based sensors have been developed by FACS screens, like the *pcaU* promoter of *Acinetobacter* interacting with PcaU which recognizes 3,4 dihydroxy benzoate [30]. Screening of a promoter library in *E. coli* resulted in promoter variants with an up to 10-fold increased response to 3,4 dihydroxy benzoate. The requirements with respect to specificity depends on the

Figure 3



Generating diversity and selection of producers in one step. Genetic modification is performed with recombinant proficient cells using synthetic oligonucleotides, followed by FACS enrichment and selection, with a final characterization and verification step for producers.

Table 1

Overview of biosensors applied in screenings and characteristics of screens.

Analyte	Sensor, source	Host	Out put	Format	Characteristics	Reference
Benzoate	BenR, *P. putida*	*E. coli*	GFP	96-MTP	Amidases were screened from a metagenome library generating benzoate from added benzamide according to culture fluorescence.	[7]
Benzoate, 2-hydroxybenzoate	NahR*, *P. putida*	*E. coli*	TetR	Colony growth	Demonstration of selection of benzaldehyde dehydrogenase positive cells generating benzoate from added benzaldehyde.	[6]
L-Arginine	LysG, *C. glutamicum*	*C. glutamicum*	eYFP	FACS	Isolation of key enzymes of L-lysine, L-arginine, and L-histidine synthesis no longer inhibited by the endproduct of the respective pathway.	[10]
Theophylline	Synthetic RNA-aptamer	*S. cerevisiae*	yEGFP	96-MTP FACS	An engineered RNA switch was used to screen libraries of a caffeine demethylase for variants with increased enzyme activity and selectivity.	[5]
NADPH	SoxR, *E. coli*	*E. coli*	eYFP	FACS	Libraries of an NADPH-dependent alcohol dehydrogenase were screened for variants with increased activity towards 4-methyl-2-pentanone reduction.	[14•]
NADH	#*S. cerevisiae*	*S. cerevisiae*	yEGFP3	FACS	Demonstration that xylose reductases with different selectivities for NADH cause different fluorescence.	[15]
L-Lysine	LysG, *C. glutamicum*	*C. glutamicum*	eYFP	FACS	Libraries of genomic mutants were screened for increased L-lysine production and new loci leading to increased production were identified.	[13••]
L-Valine	Lrp, *C. glutamicum*	*C. glutamicum*	GFP	FACS	Libraries of genomic mutants were screened for increased production of branched-chain amino acids and L-methionine.	[20]
Mevalonate	AraC*, *E. coli*	*E. coli*	LacZ	Colony color	RBS libraries in front of a hydroxymethylglutaryl-CoA reductase gene were screened for increased mevalonate formation.	[16]
Triacetic acid lactone (TAL)	AraC*, *E. coli*	*E. coli*	LacZ	Colony color	Libraries of a 2-pyrone synthase were screened for variants with increased catalytic efficiency for TAL formation.	[8]
Butanol	BmoR, *Thauera butanivorans*	*E. coli*	TetR-GFP	96-MTP	Demonstration of selection of an RBS library in front of a 2-keto acid decarboxylase for improved butanol formation from added oxopentanoate.	[17]
Adipate	PcaR, *P. putida*	*E. coli*	TetR	Culture growth	Demonstration of adipate dependence.	[17]
Succinate	DcuS, *E. coli*	*E. coli*	TetR	Culture growth	Demonstration of succinate dependence.	[17]
Malonyl-CoA	FapR, *B. subtilis*	*E. coli*	eGFP	96-MTP	Demonstration of malonyl-CoA dependence.	[35]
Malonyl-CoA	FapR, *B. subtilis*	Mammalian cell line	D2eGFP	96-MTP	Use of a biosensor for a small-scale expression screen to discover novel kinases regulating fatty acid metabolism.	[33]
3,4 dihydroxy benzoate	PcaU, *Acinetobacter*	*E. coli*	GFP	FACS	Demonstration of selection of dehydroshikimate dehydratase positive cells from cellular-synthesized dehydroshikimate.	[30]
Naringenin	FdeR, *Herbaspirillum seropedicae*	*E. coli*	CFP	FACS	Demonstration of naringenin responsiveness.	[18]
Kaempferol and quercetin	QdoR, *B. subtilis*	*E. coli*	GFP	FACS	Demonstration of kaempferol responsiveness due to added dehydrokampferol and different activities of a flavonol synthase from *Arabidopsis thaliana*.	[18]
p-Nitrophenol	DmpR*, *P. putida*	*E. coli*	eGFP	FACS	Detection of *p*-nitrophenol liberation by various enzyme activities, and isolation of a phosphatase from a metagenomic library via FACS.	[9]

Transcriptional regulators marked by a * are mutated variants. 96-MTP are 96-well microtiter plates. # In this case the TF(s) involved in regulation is unspecified but present in the host.

type of experiment. For instance, in screens for an improved enzyme the added substrate will allow the formation of only one product and strict specificity is not required. This is different when a number of products can be formed, as is the case with whole cells converting glucose to an amino acid. In this case, specificity may be of relevance as found with an Lrp-based screen of genomic mutants of *C. glutamicum* resulting in overproducers of the branched-chain amino acids and of L-methionine as well [20].

In addition to specificity, the relationship between biosensor input and output also determines its applicability. Important features are low background noise and a large dynamic range [31]. Desirable are reporter systems for use in biocatalyst screens that respond to the analyte in the μM-mM concentration range. The decisive property is the cytosolic concentration of the analyte, which is an interplay of diffusion, active import, and active export [13••,32]. In the limited number of screens applied so far in synthetic biology, the products are formed in the mM range. No information is yet available on the application of biosensors in the presence of high production titers.

Outlook

The potential of TR-based screening strategies is enormous, and possible applications go far beyond the field of microbial product formation. For example, using a codon-optimized FapR from *B. subtilis* it was possible to screen a human kinase library in mammalian cells to identify a novel kinase as a metabolic regulator that affects human fatty acid metabolism [33]. The biological recognition of products by biosensors and their use at the single cell level enables the full use of ultra-high throughput screens. Availability of such screens is necessary since selected and more or less directed alterations of enzymes or cells cannot cope with the complexity of biocatalysts, and thus do not enable the full use of biocatalysts [34]. It can be expected that in coming years reporter-based strategies will help to accelerate random approaches, in order to obtain increased activity, selectivity, and productivity of cells and enzymes.

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