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Review

Transcription factors as evolvable biosensors

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Abstract

One of the most prominent features of genetically encoded biosensors (GEBs) is their evolvability—the ability to invent new sensory functions using mutations. Among the GEBs, the transcription factor-based biosensors (TF-biosensors) will be the focus of this review. We will also discuss how this class of sensors can be highly evolvable and how we can exploit it. With an established platform for directed evolution, researchers can create, or evolve, new TF-biosensors. Directed evolution experiments have revealed the TF-biosensors' evolvability, which is based partially on their characteristic physicochemical properties.
1. Introduction

Genetically encoded biosensors (GEBs) are unique among biosensors because they can be genetically “installed” and stably maintained in any cells, natural or artificial\(^1\). While conventional chromatography-based metabolomics can measure the average concentrations of metabolites in the cell lysate, fluorescence-based biosensors enable the real-time monitoring of various metabolites at the single-cell level\(^3,4\). Therefore, fluorescence-based GEBs have the potential for single-cell metabolomics, illuminating the intercellular deviations in the concentrations of targeted metabolites. In addition, they can be used to monitor the subcellular spatiotemporal distribution of metabolites.

There are various types of fluorescence-based GEBs with different mechanisms of action. Forester Resonance Energy Transfer, or FRET, is one of the most widely applied GEBs. In the FRET system, the binding of the target molecules changes the distance between the two chromophores tethered to the sensory unit, thereby indicating the change in energy transfer efficiency\(^5\). In another popular type of fluorescence-based GEBs, the chromophores have been modified such that ligand binding induces changes in the local environment surrounding the chromophores, thereby changing their photophysical properties\(^6\). In either type of GEB, all of the three necessary components for biosensing, recognition, transduction, and reporting functions, are embedded in a single protein molecule, so that ligand binding is directly coupled to reporting. However, the rational design of these stand-alone sensors, in which all sensor components are functioning in concert in a single protein, remains a formidable challenge to protein engineers due to the difficulty in embedding all three functional elements of recognition, transduction, and reporting in a single protein in a coordinated manner.
2. **Transcription factors as biosensors**

The most remarkable feature of GEBs is likely their evolvability. Because they are fully encoded in genetic sequences, they can be subjected to the Darwinian process and evolve into novel biosensors with new functions or properties. By properly taking advantage of the GEBs' evolvability, one can rapidly create novel biosensors in a short period.

Many scientists, including the authors of this review, have been interested in the so-called transcription factors-based biosensors\(^7\) (TF-biosensors). TF-biosensors are especially popular in the synthetic biology community, mainly because any genes can be placed under the control of TF-biosensors. TF-biosensors are also superior to other sensors due to their designability. In this system, the three necessary components of biosensors are physically separated and can be independently engineered.

The TetR/Ptet system\(^8,9\) is a TF-based biosensor for the antibiotics tetracycline (Fig. 1a). TetR is the potent repressor of Ptet, a transcriptional promoter with a specific TetR-binding sequence. When TetR binds its ligand, tetracycline or its analogs, it undergoes structural changes into a low-affinity conformation for Ptet, thereby abolishing TetR's repressor function. When a fluorescent protein such as GFP (green fluorescent protein) is placed under Ptet, the cell harboring this system fluoresces in response to tetracycline in a dose-dependent manner. The resultant engineered cell can be regarded as a whole-cell sensor to tetracycline.

Thus, to make TF-biosensors work in the cell, one must introduce at least three independent genetic components; a gene encoding the TF, target promoter of the TF, and the reporter genes under its control. Although this system appears complicated compared to the single-gene GEBs like FRET sensors, it is believed by many
researchers to be a convenient system for implementing a sensory function to the cell. In the TF-biosensor system, the transfer function of the biosensors depends on each elementary step of the information processing, i.e., target recognition, transduction, and reporting, which can be independently mutated. Therefore, TF-biosensors can be fine-tuned in their signal sensitivity, dynamic ranges, stringency, and signal output strength with minimal engineering or evolutionary efforts.

Another basis for evolvability for the TF-biosensors probably comes from the evolvability of the TF proteins. According to phylogenetic analysis, a diverse set of sensors for different target molecules have emerged from the same ancestors (Fig. 1b). By necessity, nature has casually evolved novel sensors from the same starter motifs. One of our ultimate goals is to fully exploit the TF-biosensors' evolvability to establish a platform for the fast-track, on-demand supply of biosensors for any given target molecules with desired specifications.

3. Probing the evolvability of TF-biosensors

The TFs' natural evolvability is not necessarily ensured in the laboratory. The attempt to synthetically explore it has been ongoing for more than 40 years. The first systematic mutational analysis of TF was conducted for the gene encoding LacI, the sensory protein regulating the lactose operon in *Escherichia coli*. In 1990, Kleina and Miller systematically explored effectively 1,600 different amino-acid substitutions resulting from the combinatorial expression of 141 amber mutants in 13 amber-suppressor strains within the gene encoding LacI. Many amino-acid substitutions were neutral, resulting in no apparent change in transcriptional behavior, or deleterious with impaired function. However, a population of amino-acid replacements were found to result in enhanced repression, implying the increased affinity of LacI protein to *lacO*, its targeted sequence.
Interestingly, Kleina and Miller identified at least two mutations inverting the response pattern of LacI to its ligand (ligand-induced de-pression to ligand-induced repression). Indeed, LacI mutants with irregular response curves have been known for 50 years\textsuperscript{12,13}. Later, many more mutations were shown to confer an inverted response to lacI\textsuperscript{14,15}. The exact mechanism for this functional reversal is unclear, but a similar functional reversal of the ligand response upon mutations has been reported for various other TF-sensors\textsuperscript{16–22}.

The functional plasticity of TFs has also been examined by the chimeragenesis of related TFs. It has been repeatedly confirmed that the DNA binding domains and ligand-binding domains of the TFs can be combined into novel functional biosensors with composite functions\textsuperscript{23–27}. For instance, an in-frame fusion of DNA binding domain from TetR and ligand-binding domain of MphR yielded a new TF that controlled Ptet by erythromycin, the inducer molecule of MphR\textsuperscript{27}. Thus, created chimera sensors can maintain allosteric coupling between ligand binding and DNA binding despite the mismatched interfaces. It was unclear how ligand-binding events in one TF can be correctly translated to the changes in the affinity for the operator of the other TF. Still, this remarkable feature allows engineers to create a new ligand-to-promoter relationship rapidly by mixing and matching.

4. Evolutionary platforms for TF-biosensors

TFs and TF-biosensors are highly evolvable. In addition, TFs are probably one of the most tractable targets for directed evolutionists. Irrespective of the different target molecules and the different dynamics between ligands and targeted responses, all TF-sensors ultimately turn gene transcription on or off. Because the output states of the sensor variants are visible via cellular fluorescence, it is easy to screen for sensor
variants with the desired behavior. Moreover, molecular genetics tools enable the direct coupling of sensor properties with the survival rate of their host cells. For example, the on-state of the TFs can be selected using positive selectors or markers, while the off-state can be selected using negative-selectors.

In the early 2000s, Yokobayashi et al. started developing the high-throughput screening/selection systems$^{28-30}$ suitable for engineering TF-biosensors. Among the developed systems, the system adopting TetA$^{29}$ is particularly favored by synthetic biologists. TetA, a multi-drug exporter, protects the host cell from antibiotics such as tetracycline. Therefore, in the presence of tetracycline, the TF variants in the pool that are in the on-state can be selectively enriched. TetA can also be used as an off-state selector. Because TetA is an antiporter, it imports toxic transition metal ions, such as Ni$^{2+}$, thus sensitizing host cells to these ions. TetA can be used to select against or eliminate the TF-biosensor variants at on-state. This dual functionality of selectors is especially advantageous for directed evolution experiments that require the consecutive operation of on and off-selections.

We have developed another selection system where a nucleoside kinase is used for both the on and off-states of the genetic switches and sensors$^{31}$. This system is unique, particularly in the selection mechanism for off-state selection. Instead of using biochemically toxic genes, such as metal sensitizers$^{29}$ or degradation enzymes, we adopted an artificial nucleoside called dP that could be highly mutagenic only when phosphorylated$^{32,33}$ (Fig. 2). The expression of the tailored dP-kinase eliminates the cells' capability to grow in as short as minutes. In theory, it is notoriously difficult to invent resistance against this error catastrophe$^{34}$, i.e., informational breakdown. We found no selection escape even in 10$^9$ cells. By fusing this dP-kinase with aminoglycoside-phosphotransferase$^{35}$, we have established the selection system where
both the on and off-selection could be completed in several minutes. Because all procedures are completed only with liquid handling, researchers can now conduct dozens of different directed evolution projects simultaneously. Furthermore, the duplication of dP-kinase further increases the selection fidelity, achieving a $10^{11}$-fold enrichment of the variants to be selected. With this level of fidelity, our selection system can be regarded to be virtually free from false positives. Lastly, we demonstrated that chromosomal gene insertion could be conducted with multiple consecutive rounds without isolating or verifying the clones\textsuperscript{36}.

5. Seeing is believing

With the directed evolution platforms, researchers are exploring the remarkable evolvability of the TFs. Novel prize laureate Frances H. Arnold and her colleagues have examined LuxR, the activator-type homoserine lactone, by random mutagenesis and screening for mutants with altered ligand specificity. They found that LuxR mutants frequently became responsive to new molecules. Additional mutations in the gene encoding LuxR quickly eliminate LuxR’s response to the original ligands\textsuperscript{37,38}. Variants with 1–3 amino-acid differences could exhibit complete orthogonality relative to each other. We independently tried to artificially divert LuxR into a pair of variants independently sensing two different homoserine lactones with small different side chains (3OC6-HSL and 3OC8-HSL). Directed evolution experiment revealed that the three amino-acid difference was necessary to acquire an orthogonal set of LuxR variants that sensed similar but different ligands\textsuperscript{39}.

The evolvability of TFs in target-specificity has been thoroughly explored by a series of works from the Cirino’s lab. They created the variant library of AraC, the L-arabinose-responsible TF, by simultaneously introducing random mutations in 4 or 5
of its 7 ligand-binding residues. The subsequent on or off-selection in the absence or presence of various chemicals yielded AraC variants that could detect D-arabinose\textsuperscript{40}, mevalonate\textsuperscript{41}, triacetic acid lactone\textsuperscript{42}, as well as salicylic acid and vanillin\textsuperscript{43}. We have recently re-examined AraC's evolvability in ligand specificity to determine the minimal evolutionary steps, i.e., mutations. For instance, the salicylic acid sensors can be obtained by as little as three amino-acid substitutions on AraC (Kimura, unpublished data).

Besides the ease of TFs in acquiring new sensory functions, directed evolution experiments demonstrated the remarkable functional plasticity of TFs in terms of signaling sensitivity\textsuperscript{39}, stringency (reduced leakiness)\textsuperscript{31}, signaling output strength\textsuperscript{22}, and high signal-to-noise ratio\textsuperscript{44}. With the maturation of the directed evolution platforms for TF-biosensors, more than several or even dozens of novel sensors can be simultaneously created\textsuperscript{45}.

6. Ligand addiction as the source of evolvability.

The apparent evolvability of TF-biosensors may be partially ascribed to its instability. Contrary to the generally-accepted notion that stability confers evolvability to proteins\textsuperscript{46–48}, moderate instability is beneficial to evolve functions when it comes to biosensors or molecular switches.

On the course of evolving various TF-biosensors in the lab, we noticed many TF mutations confer a high signal-to-noise (S/N) ratio with surprisingly high frequency. In some cases, as much as 10%–20% of the mutant pool exhibited a much higher S/N ratio than their parents without functional selection\textsuperscript{44}. With the extraordinary frequency and destabilizing nature of the mutations found in the variants with a high S/N ratio, we concluded that the gain in signal came from the mechanism depicted in Fig. 3. Due to
the destabilizing effect of the accumulated mutations, these TF variants cannot fold correctly by themselves. Interaction with their ligands significantly stabilizes the TF variants (by $\Delta G_{\text{BIND}} = -RT \ln K_{\text{assoc}}$), thereby restoring their functional structure. This way, TFs are “addicted” to ligand binding for its folding and function. The ligand acts as a molecular chaperon of the TF variants, in contrast to it acting as the allosteric effector for the parent TF.

As was discussed above, it is surprisingly easy to confer binary switch-like behavior to TF-sensors, simply by moderate de-stabilization. Notably, it would take many more evolutionary steps, i.e., engineering efforts, to widen the functional gaps or difference in binding affinity between the two states of a TF. Darwinian evolution is the blind process that employs the most accessible molecular answers. The “ligand-addicted folding” could be the prevailing mode of functional switching of proteins. There are a handful of available full-length crystal structures of TFs. A group of LuxR family is known to be unfolded in the absence of their ligands. One of them, TraR, was shown to be purifiable only in the presence of its ligand. Previous studies infer that some of the TF variants with inverted response behaviors could be those undergoing a ligand-induced folding noting “in some cases, these repressors seems to be stabilized by binding to the inducers”.

The memorable success in the elucidation of genetic switches has attracted the attention of many molecular biologists and later protein engineers. Many of the novel TFs have been designed by rational, mechanism-based approaches. Directed evolution experiments have uncovered many mutations that confer new functions to TF-biosensors or improve their specifications in sensitivity, selectivity, signal-to-noise ratio, and so on. Notably, only a handful of them can be satisfactorily explained using simple allosteric model where molecular machines transit from one rigid form to
another. The physicochemical properties of the TFs' base material, polypeptides, should be considered in addition to the mechanical aspects of TFs to fully exploit the evolvability of TF-biosensors.

7. **Stickiness as the source of evolvability.**

Based on their physical properties, biomolecules sometimes exhibit highly sophisticated sensor-like behaviors, such as the on/off switching of gene expression in response to chemical input, in the absence of molecular machines.

The physical chemistry of polyelectrolyte predicts that DNA can transition abruptly between aggregation and solubilization with a minimal change in the local chemical environment. Yoshikawa and his colleagues have demonstrated that double-stranded DNA (dsDNA) undergo soluble/insoluble transition as a first-order phase transition. This sharp transition can be induced by various chemical stimuli, including polycations, neutral polymers, divalent/trivalent cations, and even polyanions\textsuperscript{52}. Eukaryotic chromosomes are known to be organized in numerous micrometer-sized DNA “puffs” that are approximately 1 megabase in length\textsuperscript{53}; it is physically possible that small changes in the local chemical environment induce the phase separation of each individual puff independently. The puff-encoding genes could be excluded from the cytosolic aqueous system where all transcriptional machinery exists. Thus, the transcription of the puff-encoding genes should be set at the off-state. Upon a local decrease in the molecular crowding effect, the puff-encoding genes become readable, thus the on-state.

Subsequently, Maeda’s group tested whether they could confer this sensory properties directly to double-stranded DNA (dsDNA). First, they developed a method for the photochemical grafting of vinyl polymers onto dsDNA\textsuperscript{54}. They have thus
prepared polymer-jacketed dsDNA exhibiting various novel properties, such as temperature-induced aggregation, which reflect the property of the grafted polymers\textsuperscript{55–59}. Although the polymer-coated dsDNA has irreversibly lost its genetic information, it can be enzymatically ligated to other intact dsDNA\textsuperscript{60}. The grafting of thermosensitive polymer, poly(N-isopropylacrylamide), resulted in the temperature-control accessibility of the polymer-grafted DNA\textsuperscript{61,62}, as well as the dsDNA connected to it via enzymatic ligation\textsuperscript{63} (Fig. 4). This experiment demonstrates that information can be processed even without molecular machinery (such as TF) tailored for it. Besides the stability control of the TFs, as discussed previously, genetic information can be controlled by changing the local chemical composition/ solution properties surrounding the gene.

As of today, there is no report of research taking advantage of the solution properties of sensory components, TF (protein) and promoter (DNA), to improve signal-to-noise ratio or stringency of the laboratory evolved TF-based biosensors. But considering the highly condensed cellular environment, the demonstrations by the Yoshikawa group, and the growing evidence for the existence and biological function of membrane-less organelle\textsuperscript{64}, it is likely that solution properties of biomolecules should have the evolvability of TF-biosensors. By considering the designed biomolecules' physicochemical dynamics, including aggregative\textsuperscript{65}, kinetic, and phase separational\textsuperscript{52,55,64}, directed evolutionists can maximize the evolvability of the TF-biosensors.

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Figure captions

Figure 1 Transcription factor-based biosensors.
(a) The mechanism-of-action of the TetR-based tetracycline biosensor. In the absence of tetracycline, TetR binds to DNA tightly, blocking transcription of the gene under the control of Ptet. In the absence of tetracycline, transcription becomes unblocked, and the production of cellular fluorescence (green fluorescent protein (GFP)) increases in a dose-dependent manner.
(b) The natural divergence of the TetR family on ligand specificity. This phylogenetic tree was drawn using 220 TetR homologs using MEGA X (https://www.megasoftware.net).

Figure 2 Directed evolution of TF-biosensors.
(a) The entire reading frame of the gene encoding TetR was subjected to random mutagenesis. The resultant TF variant library was co-expressed with a selector (HSVtk-aph, an in-frame fusion of herpex simplex virus thymidine kinase and aminoglycoside 3’-phosphotransferase) under the control of Ptet. (b) After a single round of off (dP) and on (kanamycin) selection, (c) TF variant biosensors with the desirable profile were obtained. TMK: endogenous thymidine kinase, NDK: nucleoside diphosphate kinase.

Figure 3 Ligand addiction, a quick-and-dirty scenario for emerging sensory functions.
By accumulating destabilizing mutations (red dots), any proteins can be “tuned” into those that cannot fold by themselves. Only with ligand binding, these proteins can fold into the functional structure. As a result, TF variants can now activate GFP production.
on the condition that they remain bound to the ligand.

**Figure 4 TF functions by solubility switching of the genes.**

A fragment of dsDNA was grafted with temperature-responsive polymers and then ligated to the other intact dsDNA fragment encoding a reporter gene under the control of the constitutive promoter. The polymer-grafted fragment undergoes phase transition upon temperature change, and genes on the connected DNA are excluded from the aqueous system. By placing with the reporter gene encoding fluorescent or chemiluminescent proteins, DNA itself behaves as TF-based biosensors.
Fig. 1
Fig. 2
Fig. 3
Fig. 4
Graphical abstract