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Next-generation Genetically Encoded Fluorescent Biosensors Illuminate Cell Signaling and Metabolism

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Abstract

Genetically encoded fluorescent biosensors have revolutionized the study of cell signaling and metabolism, as they allow for live cell measurements with high spatiotemporal resolution. This success has spurred the development of tailormade biosensors that enable the study of dynamic phenomena on different time and length scales. In this review, we discuss different approaches to enhance and develop new biosensors. We summarize the technologies used to gain structural insights into biosensor design and comment on useful screening technologies. Furthermore, we give an overview of different fields of applications where biosensors have led to key advances over recent years. Finally, we give our perspective on where future work is bound to make a large impact.

Keywords

biosensor design; fluorescence microscopy; hybrid biosensors; super-resolution microscopy; in vivo imaging; biosensor multiplexing

1. Introduction

Cells constantly adapt their metabolism and cellular functions to extracellular and intracellular stimuli via an intricate signaling network. Proper regulation of this signaling network is key to cell survival, as malfunction can lead to disease. It is hence essential to understand the dynamic processes underlying these molecular networks, both in time and space. Genetically encoded fluorescent biosensors, paired with fluorescence microscopy, allow us to monitor the abundance and activity of different signaling molecules and metabolites in living cells. This helps untangle the precise spatiotemporal regulation of cell signaling and metabolism(37, 85).

Genetically encoded fluorescent biosensors generally consist of a sensing and a reporting unit. The sensing unit responds to the presence of an analyte or the activity of an enzyme by

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undergoing a change, often in its conformation. This change is transduced to the reporting unit, which typically comprises one or two fluorophores, eliciting a change in spectroscopic properties (e.g., intensity, excitation or emission spectra, etc.). Common designs involve modulating the distance and orientation of two fluorophores, leading to a change in Förster resonance energy transfer (FRET) (FRET-based biosensors, Figure 1a), or the fluorescence intensity of a single fluorophore (intensiometric biosensors, Figure 1b). Alternatively, the sensing unit can trigger the degradation or translocation of an attached fluorophore(37). Classically, fluorescent proteins (FPs) or their circularly permutated analogues (cpFPs) are used in the reporting unit. However, in recent years, strategies relying on exogenous synthetic fluorophores, combined with bioconjugation chemistry, have seen increasing application in biosensor design(136).

Over the last 20 years, biosensors have enabled investigations of diverse phenomena in cell signaling and metabolism. The insights gained have increased our thirst to probe molecular mechanisms in greater detail and in more physiological contexts. Meanwhile, advanced fluorescence microscopy techniques have emerged that facilitate studying biological systems at varying length- and timescales, from super-resolution microscopy to whole-organism imaging. These techniques impose specific photophysical requirements that demand bespoke biosensors. In this review, we first focus on reporting and sensing unit design and improvement. Then, we discuss efforts and strategies to streamline biosensor engineering. Finally, we highlight three areas where biosensors have seen increasing application in recent years.

2. Engineering of individual biosensor components

2.1. Sensing units

To access new targets and measure already accessible ones with greater sensitivity or better response kinetics, new sensing units are constantly being developed. Below, we focus on sensing units that undergo a conformational change and that are either adapted from naturally occurring protein switches or engineered from individual protein domains.

2.1.1. Natural sensing units—A wide variety of natural protein switches exhibit suitable conformational changes for biosensor design. Many can be classified according to their protein fold, and biosensors generated from representatives of the same class often follow similar design patterns. Popular protein classes include periplasmic binding proteins (PBPs)/solute binding proteins (SBPs) and G-protein-coupled-receptors (GPCRs) (29, 111, 117). Members of these protein families show large conformational changes upon binding to diverse analytes, such as metabolites, neurotransmitters/neuromodulators, etc., and have been used to generate both FRET-based and intensiometric biosensors (Figure 2a–b). PBPs give access to soluble biosensors that can be targeted to diverse subcellular compartments, whereas GPCR-based biosensors are membrane resident. More specialized sensing units have been derived from membrane-integral voltage sensing domains (VSDs) (126) or cyclic nucleotide binding domains (CNBDs)(83), yielding voltage and cyclic nucleotide biosensors, respectively (Figure 2c–d). For instance, a series of CNBDs from different species have been used to develop cAMP biosensors, including the most recent

FRET-based cAMP biosensor called cAMPFIRE(82). In contrast to full-length protein domains, short peptides that exhibit sensitivities to force(12) or temperature(56, 138) can be used to generate FRET-based biosensors by linking two fluorophores. Short peptides additionally serve as sensing units for the design of irreversible protease activity biosensors, which have found many applications in the study of caspase activity during different processes (81, 155).

When designing biosensors for new analytes, it is generally advisable to start from sensing units belonging to one of these classes if possible. Previous findings on related biosensors and structural information on conformational changes can help to inform biosensor engineering. However, this list of protein switches is not exhaustive, and other protein domains that undergo desirable conformational changes, e.g., eIF4E biding protein1 (mTORC1)(160), the ε subunit of F_0F_1-ATP synthase (ATP)(48), or the heterotrimeric AMP-activated protein kinase (AMPK) (AMP and ADP)(102) have successfully been used for biosensor design.

2.1.2. Synthetic sensing units—Multiple sensing units have been “synthesized” from different naturally occurring protein domains. Often, these domains work via a clamp-like motion or mutually exclusive binding(125).

Affinity clamps are engineered from two protein domains that reversibly bind each other upon stimulation (Figure 2e). The most prominent affinity clamp is based on calmodulin (CaM) and CaM-binding peptides derived from myosin light-chain kinase or CaM kinase II. Due to the importance of Ca^{2+} in neuronal signaling, this switch has found tremendous applications, and improvements are constantly being made. A recent milestone is the development of GCaMP8, in which a peptide from endothelial nitric oxide synthase was paired with the mutated CaM found in previous GCaMPs to generate biosensors with high sensitivity and improved response kinetics, enabling measurement of fast Ca^{2+} transients on the millisecond timescale(157).

Affinity clamps have also been engineered to measure enzyme activity such as kinase/phosphatase activities. The sensing unit consists of a kinase-specific peptide substrate and a phosphoaminoacid-binding domain (PAABD) that bind each other upon phosphorylation. While first applied to FRET-based biosensors(120), this design has more recently yielded single-FP-based kinase reporters(20, 86, 119), which exhibit excitation-ratiometric behavior and large dynamic ranges that enabled the measurement of protein kinase A (PKA) activity in awake mice during forced locomotion(152). Recent efforts are also focusing on optimizing substrate sequences to enhance biosensor specificity and dynamic range(74), and new work detailing the substrate specificities of ~300 Ser/Thr kinases is bound to be an invaluable resource(50).

Similarly, to measure the activity of small GTPases, affinity clamps were designed based on the binding of a small GTPase (e.g., Ras) to a cognate binding domain upon GTP loading(93). Using this approach, biosensors were generated for small GTPases including Ras, Rap1, Rac1, RhoA, or Cdc42. Unfortunately, this approach requires the incorporation

of the full-length GTPase within the biosensor and does not allow the measurement of endogenous GTPase activity.

Alternatively, mutually exclusive binding, where an analyte is used to outcompete another binding partner, can be used (Figure 2f). A unimolecular pseudoligand strategy enabled the generation of FRET-based biosensors for phosphoinositides(8, 44) or the GTPase Ras. For the latter, a low-affinity pseudoligand of the Ras binding domain (RBD) of Raf1 was derived from Ras, fused to the RBD and sandwiched between a FRET-pair. In the absence of active Ras, pseudoligand-RBD binding induces a closed, high-FRET conformation of the RasAR biosensor. Binding of GTP-bound Ras outcompetes the pseudoligand, producing an open, low-FRET biosensor conformation. As Ras is not part of the biosensor, RasAR was used to measure the activity of cellular Ras isoforms(143). Similarly, semisynthetic fluorescent biosensor proteins (Snifits) use an intramolecular ligand that competes for the same binding site as the target analyte. In contrast to the pseudoligand strategy, which is fully genetically encoded, Snifits use a small-molecule ligand that is localized via a self-labeling protein (SLP) tag(147). Even though these biosensors require a labeling step and use ligands that are not commercially available, they enable biosensors with higher dynamic ranges than those based on fully genetically encoded sensing units. Furthermore, Snifits expand the current repertoire of sensing units to new analytes, as demonstrated by a recent biosensor for coenzyme A (CoA). CoA-Snifit enabled measurements of CoA concentrations in different subcellular locations and gave insight into CoA biosynthesis and transport(148). Mutually exclusive binding was also employed for a de novo-designed bimolecular system (64). The LOCKR system consists of a cage and latch fused via a small linker and a key that can bind the cage once the latch is released. Latch release is induced via binding of the target to a functional motif on the latch. Within this modular design, the functional motif can easily be exchanged to develop switches for diverse targets(110). Combined with a FRET-pair and using an RBD as the motif, LOCKR was recently rendered into a fluorescent Ras biosensor, albeit so far with rather limited dynamic range(153). Thus, synthetic switches enable versatile biosensor designs that complement the diversity of natural sensing unit classes.

2.2. Reporting unit

Engineering of the reporting unit focuses on optimizing the spectral properties of fluorophores, such as excitation/emission maxima, brightness, and photostability, to improve signal-to-background ratios, while keeping the fluorophore insensitive to other cellular changes. Red-shifting spectral properties has received special attention, as it enhances signal-to-background by reducing autofluorescence and light scattering while simultaneously allowing for more physiological measurements due to lower phototoxicity than blue-green light. Moreover, the far-red, near-infrared (NIR) window is uniquely privileged for deep tissue imaging due to low tissue absorbance and scattering.

2.2.1. FP-based reporting units—FP chromophores arise via either intrinsic cyclization, oxidation, and dehydration of three amino acids (Figure 3a)(115) or incorporation of an endogenous cellular cofactor such as biliverdin(113). While the former

strictly depends on oxygen, preventing use under anaerobic conditions, the latter might require supplementation of the cofactor depending on the organism used(23).

Matured chromophore FPs: Currently, FP brightness is generally highest in the green-yellow region, and accordingly, the brightest biosensors can be found in this region. For instance, the current brightest Ca^{2+} biosensor, NEMO, is based on mNeonGreen(68). However, even the brightest biosensors perform suboptimally over time if they have low photostability. Hence, more photostable green-yellow FPs have been developed such as mGold(66) and the dimeric stayGold(46), which still await to be explored for biosensor engineering. Furthermore, slow maturation can complicate biosensor performance, especially in degradation-based biosensors, and hence fast-maturing FPs such as Achilles were developed(151). Similarly, many efforts have been undertaken to generate red-shifted FPs optimizing multiple properties such as brightness, photostability and maturation kinetics simultaneously. However, red-shifting is often concomitant with decreased brightness, necessitating various compromises. For instance, the emission maximum of the brightest red FPs (RFPs) with applications in biosensors – superTagRFP (stagRFP)(91) and Azalea-B5(9) – is still below 600 nm. To access spectral properties closer to 700 nm, biliverdin-based FPs or hybrid approaches are used.

Endogenous cofactor FPs: FPs based on endogenous cofactors can overcome oxygen dependence and maturation issues common to other FPs. Moreover, some are smaller in size than traditional FPs, which can be beneficial(16, 23). Nevertheless, the most commonly exploited property is the far-red/NIR emission of biliverdin-based iRFPs (Figure 3b)(22). They were for instance employed as FRET-pairs (e.g. miRFP670 and miRFP720) in Ca^{2+}(123), Rac1 GTPase, or kinase biosensors(122). The latter were enhanced through the use of a smaller, cyanobacteriochrome-derived miRFP (miRFP670-nano), which boosted their dynamic range(101). iRFP was also used to generate single-NIR FP Ca^{2+} biosensors that can easily be multiplexed with red, green or cyan/yellow biosensors(108, 109). Great strides have already been made in the field of iRFPs and challenges such as low abundance of biliverdin in cell culture or drosophila were circumvented via supplementation of biliverdin or co-expression of heme-oxidase(22). Nevertheless, we expect that the field will be pushed further to generate even brighter iRFPs and biosensors.

2.2.2. Synthetic fluorophores—Instead of relying on endogenous fluorophores, hybrid biosensors make use of synthetic fluorophores and biorthogonal conjugation methods(136). Indeed, some of the very first biosensors were generated by in vitro conjugation of synthetic fluorophores to protein switches, followed by injection into mammalian cells(129). Hybrid biosensors profit from the beneficial properties of synthetic fluorophores, including high brightness and photostability, tunable spectral properties, and oxygen independence(38). Here, we summarize the covalent and non-covalent bioconjugation methods that were successfully used for biosensor design. Approaches that necessitate in vitro reconstitution, or hybrid approaches where the genetically encoded component is solely used to target a purely chemical biosensor, are not discussed.

Covalent systems: The most common approach to generate hybrid biosensors is via SLP tags and fluorescent ligands. SLP tags are small protein domains that specifically and covalently react with a chemical ligand such as benzylguanine for SNAP-tag(53) and a chloroalkane in the case of HaloTag (Figure 3c)(77). SLP-fluorophore systems can be used analogously to FPs, and both FRET-based and intensiometric hybrid biosensors are currently available. The former were pioneered by the Johnsson laboratory via the generation of Snifits, in which SLP tags are part of the sensing and reporting unit(147). Using SLPs as the reporting unit enables the selection of red-shifted fluorophores to generate green/far-red FRET-based biosensors with high dynamic ranges. More recently, classical SNAP-tag and HaloTag fluorophore-ligands were used as FRET-pairs in combination with protein-based sensing units (PKA, ERK, and Ca^{2+}). The biosensors are spectrally versatile, as they can be flexibly labeled with diverse fluorophores. However, all of these biosensors had rather limited dynamic ranges(137). In contrast, engineering a favorable protein interface between *Aequorea victoria*-derived FPs and HaloTag labeled with rhodamine-based fluorophores significantly increased FRET-efficiency, resulting in FRET-based Ca^{2+}, ATP, and NAD^{+} biosensors with unprecedented dynamic ranges and easily tunable spectral properties(41). This design should be applicable to improve biosensors for other analytes and to shift their spectral properties. HaloTag was further implemented as a FRET-donor in electrochromic voltage biosensors called Voltron(2, 145). The use of a synthetic fluorophore enhances the brightness and photostability of these retinal/rhodopsin-based biosensors, thereby enabling prolonged measurements in mice, fruit flies and zebrafish(2, 3).

Furthermore, HaloTag was used as the reporting unit in single-fluorophore intensiometric biosensors in combination with environmentally sensitive fluorophores, whose brightness is influenced by the protein environment. (cp)HaloTag used in combination with different protein switches gave access to the far-red Ca^{2+} biosensor HaloCaMP and the voltage biosensors HASAP and HArcLight(27). HASAP was more sensitive than the electrochromic Voltron when labeled with the same far-red fluorophore. A related strategy inserted a Ca^{2+}-sensing unit into a HaloTag loop region and paired the resulting biosensor with a color-shifting fluorophore to generate a ratiometric RHCaMP(140).

Bioconjugation chemistry approaches such as ligand-directed chemistry(118) or probe incorporation mediated by enzyme paired with click-chemistry(75, 146) have also been used to install fluorophores directly on protein switches. The former can even produce endogenous hybrid biosensors without the need for overexpression. However, labeling of intracellular proteins and the generalization to diverse protein switches remains challenging(118).

Non-covalent systems: Compared with covalent systems, non-covalent approaches allow further enhancement of photostability, as the fluorophores exchange over time. The most commonly used non-covalent system is the fluorogen activating protein (FAP) FAST (fluorescence activating and absorption shifting tag, Figure 3d)(106). This 14-kDa protein binds hydroxybenzylidene rhodanines, which only become fluorescent once they are conformationally locked by FAST. The fluorophore can thus be used in excess. cpFAST was successfully used to engineer a Ca^{2+} biosensor, whose fluorophore binding affinity is influenced by the conformation of M13-CaM(132). Later, a split FAST system was used

to report on Ca^{2+}, protein-protein interactions and caspase activity(130). Importantly, split-FAST complementation is fully reversible, in contrast to split FPs, and can therefore report on transient interactions without locking them in place. More recently, orthogonal color variants of FAST (green and redFAST) were developed which found applications in Fucci biosensors and helped visualize cell-cycle stages almost instantaneously after fertilization in zebrafish embryos, a time regime inaccessible to maturation-limited FPs(131). Further color variants of FAST, ranging in emission from 450 to 650 nm, were used as FRET-acceptors in FRET-based biosensors to measure aurora kinase A activity(15).

Other FAP-based biosensors include a biosensor for measuring O-glycosylation via the conditional binding of malachite green to its FAP(13) and biosensors based on the binding of de novo-designed miniFAPs to 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) (30). The intrinsic pH sensitivity of the fluorophore was used to generate pH biosensors, and Ca^{2+}-responsive elements were inserted to generate Ca^{2+}-sensitive miniFAPs(57). Last but not least, split miniFAPs were generated for measurement of dynamic protein-protein interactions(57).

Hybrid systems can enable brighter, more photostable and far-red biosensors that are otherwise challenging to access via classical FPs. Furthermore, oxygen independence and virtually absent maturation help produce biosensors for anaerobic systems and fast phenomena. However, hybrid systems come at the price of an additional labeling step that can lead to increased background. Factors such as fluorophore permeability, bioavailability, and fluorogenicity, as well as labeling specificity, should therefore be carefully evaluated.

3. Approaches to enhance biosensors and design new ones: From rational engineering to high-throughput screening

While the engineering of individual biosensor components can enhance their performance, it is often necessary to optimize the biosensor as a whole. The coupling between sensing and reporting units often does not follow a generalizable concept and is only poorly understood. Researchers therefore try to increase the molecular understanding of biosensors via structural and computational approaches while at the same time developing methods to screen large biosensor libraries (Figure 4).

3.1. Rational design based on structural and computational information

Structural information on individual sensing and reporting units, as well as existing biosensors, often provide the starting point for new biosensor design or inform targeted screening campaigns. Depending on the size of the biosensor or its domains, different methods have been used. For instance, crystal structures of FPs, SLPs, or FAST have guided efforts to reposition protein termini for circular permutation(27, 97, 132), find positions tolerant of sensing unit insertion, and investigate FRET-pairs and their interaction interfaces(41, 60). Moreover, the conformational changes undergone by molecular switches such as PBPs have been investigated(31, 34), letting researchers infer the place of largest movement or flexible insertion sites. However, crystal structures of full-length biosensors are rare; to date, GCaMP2 and a more recent aspartate biosensor are the only biosensors

for which both bound and unbound structures have been solved(5, 42, 141). Nonetheless, the available structures have helped us better understand different biosensor classes. For example, the Ca^{2+}-bound form of the Twitch2B biosensor, the only FRET-based biosensor for which a crystal structure is available, revealed that the linkers connecting the minimal sensing unit to the two FPs formed helical structures held in place by polar interactions. This information was used to reengineer and rigidify the linkers, thereby increasing FRET-efficiency(135).

X-ray crystallography gives access to limited dynamic information, and hence solution-based methods such as small-angle X-ray scattering (SAXS) or nuclear magnetic resonance (NMR) are being used. The former has mostly been employed to investigate the movement and organization of FRET-based biosensors(32, 87, 112, 133). For example, SAXS combined with coarse-grained molecular dynamics (MD) simulations revealed that at least one FP in a FRET-pair requires tight allosteric coupling to the sensing unit, while the second FP is allowed to be more flexible(112). In contrast to SAXS, NMR is more restricted by the size of the biosensor. Thus, NMR is typically used on individual sensing units, as performed for the TN-XXL and Twitch Ca^{2+} biosensors(36, 133), or to answer specific questions about biosensor dynamics via the use of specialized NMR methods. For instance, paramagnetic NMR of Twitch-2B loaded with dysprosium (Dy^{2+}) allowed investigators to probe the dynamics of the FPs with regard to the sensing unit and thereby explain the lower measured FRET-efficiency as compared to the value calculated from the crystal structure(135).

In the absence of experimental structural information, modeling and structure prediction, such as alpha-fold(51), can be used as starting points for biosensor design. MD simulations were, for instance, used to model the linker connecting the CNBD with CFP in a cAMP biosensor. The findings were then used to rigidify the linker, leading to improved FRET-efficiency(73). Computational approaches were also used to reengineer sensing units such as a promiscuous SBP whose glycine binding preference was significantly increased over L-serine and GABA binding. The resulting FRET-based glycine biosensor was used to measure the influence of this inhibitory neurotransmitter in the extracellular space of hippocampal tissue by both 1P and 2P microscopy(156). Furthermore, de novo protein design can be used to generate reporting and sensing units as described above (Section 2.1.2 and 2.2.2.). However, to date, there is no report of a full biosensor (e.g., sensing and reporting unit) generated via de novo design. Future endeavors will show if such multicomponent designs will be successful.

3.2. Screening-based techniques

While structural and computational information can help generate biosensor prototypes, these generally require further fine-tuning via screening and directed evolution. Often, this entails simultaneous optimization of the two biosensor states for multiple (e.g., sensitivity, selectivity, signal-to-background-ratio) and dynamic (e.g., response kinetics, photostability) parameters. To tackle this challenge, various factors such as biosensor stimulation, library size, model system, or throughput have to be considered and chosen according to the biosensor's final application.

3.2.1. Screens using imaging-based systems or plate readers—Due to the ease of handling *E. coli*, biosensors can be directly screened in up to 100,000 bacterial colonies using a fluorescence imaging system before and after stimulation. Depending on the biosensor type, stimulation is either performed via the conditional expression of an enzyme (e.g., inducible promotor)(47) or by supplementation of the analyte. If the analyte shows good permeability and the biosensor is efficiently trafficked to the periplasm, the analyte can be directly applied on intact colonies. Alternatively, bacterial colonies can be blotted onto filter paper, permeabilized and then stimulated(133). This system was successfully applied to generate different color variants of Ca^{2+} biosensors(158), as well as biosensors for enzyme activities including histone trimethylation(47) and kinase activity(14, 152). Due to its high throughput, colony screening is often used to pre-screen candidates for further testing in more complex systems.

Bacterial lysate screens are popular for soluble biosensors that show good expression and can be robustly stimulated in a cell-free environment. Lysate screening can be paired with plate reader measurements, thereby allowing screens of several hundreds to thousands of biosensor variants. Both GCaMP3(134) and GCaMP5(4) were screened in lysates. Unfortunately, the correlation between biosensor performance in lysates and more intact preparations was found to be weak. Later studies hence focused on screening directly in the system of final application, e.g., mammalian cells, but were initially plagued by low throughput(105). Advances in microscope automation, automated stimulation, and the rapid generation of stable cell lines made it possible to increase throughput. Pairing an automated imaging system with robotic cell picking allowed optimization of multiple parameters of voltage indicators through simultaneous screening of 300,000 HEK293T cells. The enhanced indicator showed applicability in mouse brain slices, zebrafish and *C. elegans*(104). Furthermore, screening in neurons instead of non-excitable cells has become attractive for the development of Ca^{2+} biosensors. Pioneered during the development of the GCaMP6 series(21), electric field stimulation in a 96-well plate format(43) and the efficient transfection of neurons via lentivirus helped to increase throughput. GCaMP7/8(25, 157) and RCaMP(24) were later optimized in a similar fashion. However, the increasing complexity of the screening process and instrumentation necessary to screen in neurons requires considerable time investment, and hence, rapid screens in bacterial colonies or bacterial lysate remain more accessible.

3.2.2. Increasing throughput using microfluidics and fluorescence activated cell sorting (FACS)—To further increase throughput, microfluidic or FACS approaches are being pursued. Custom-made microfluidic devices paired with fluorescence microscopy allow flexible measurement of different biosensor parameters via multi-point detection(35, 80). This has been used to screen a Ca^{2+} biosensor library in *E. coli* in both the absence and presence of Ca^{2+}(159). A more recent approach used droplet microfluidics and in vitro transcription/translation to generate semipermeable gel shell beads containing a biosensor library. The beads are permeable to small-molecules and can therefore be used as ideal vessels to screen different biosensor parameters. However, fluorescence measurements were performed upon bead immobilization and not under flow conditions(61).

Instead of using single-cell sorting, pooled sorts via FACS and high-throughput sequencing can be used to optimize biosensors(103). Either iterative rounds of screening in the presence and absence of analyte are used to enrich desirable biosensors(95), or identical libraries are sorted in parallel under different stimulation conditions, selecting the best candidates based on sequencing data(58, 74). Here, sorting cells into multiple bins allows multiple parameters to be investigated at the same time(58). These methodologies have been used to rapidly optimize the insertion site of an FP into maltose or trehalose binding domains(58, 95), optimize linker regions of a pyruvate biosensor(58) and optimize the substrate sequence of a FRET-based kinase activity biosensor(74).

4. New and exciting applications of biosensors

The previously described strategies to improve sensing and reporting units and engineer tailormade biosensors have opened up new areas of research and expanded existing ones. Below, we summarize three fields of applications where we believe advances in biosensor engineering are bound to make game-changing contributions.

4.1. Multiplexing

To understand the complex networks of cell signaling and metabolism, the spatiotemporal dependence and interplay between different molecules needs to be investigated. While this information can be inferred by monitoring biosensors one-by-one, multiplexing several biosensors simultaneously allows us to investigate such spatiotemporal relationships on a single-cell level, accounting for cell-to-cell heterogeneity. These unique data sets can moreover inform computational models, moving us towards a systems-level understanding(37, 54).

4.1.1. Spectrum-based multiplexing—Traditional multiplexing relies on spectral properties to distinguish biosensors by imaging in orthogonal spectral channels. FRET-based biosensors have large spectral footprints that complicate multiplexing efforts, and strategies to reduce the spectral occupancy of FRET-based biosensors are thus highly sought after. For instance, the FRET pair can be replaced by dimerization-dependent FPs (ddFPs), which increase their fluorescence intensity when brought into proximity(6, 7). Several biosensors for analytes such as caspase activity, Ca^{2+}, PIP2, cAMP(28, 44), kinase activity(86) and small GTPases(55) were generated using this concept. Alternatively, two spectrally identical FPs can be used as a homo-FRET pair, with responses monitored by changes in fluorescence polarization (anisotropy). Starting from available FRET-based biosensors for different kinase activities or small-molecule analytes, analogous homo-FRET biosensors called FLAREs were developed, enabling multiplexed imaging of three FRET biosensors, a red PKA, yellow ERK, and cyan Ca^{2+} FLARE, using anisotropy imaging(116). More recently, blue, yellow and red homo-FRET caspase biosensors were generated and used to investigate the interdependence of caspases 3, 8 and 9(40). While ddFP- and homo-FRET-based biosensors only occupy one spectral channel and are thus better suited for multiplexing, both show small dynamic ranges compared with traditional FRET-based biosensors. Further engineering and optimization are thus needed before their widespread use can be achieved.

Another strategy to reduce FRET-based biosensors to one spectral channel is the use of so-called dark acceptors, which are mutant FPs that absorb light and accept energy but have no emission. Dark acceptors are paired with fluorescence lifetime imaging microscopy (FLIM)-based FRET measurements, in which FRET-efficiency is determined by monitoring changes in the excited-state lifetime of the FRET donor only. Several dark FPs have been developed, including ShadowG(94) and dark mCherry(96), which were implemented in FLIM-FRET biosensors and used for multiplexing with intensiometric biosensors.

Nevertheless, the options for multiplexing FRET-based biosensors remain limited, and biosensors that contain a single-fluorophore (e.g., cpFP-based sensors) continue to offer the greatest flexibility. Currently, four biosensors occupying blue, cyan, yellow and red spectral regions have been successfully multiplexed, providing information on PKC and PKA activities, as well as cAMP and Ca^{2+} concentrations(86). Incorporating red-shifted biosensors could further extend these efforts, with the NIR Ca^{2+} biosensor NIR-GECO already enabling co-imaging with the CFP-YFP based PKA biosensor AKAR4 and the red cAMP biosensor pinkFlamindo(109). However, multiplexing of five biosensors has yet to be reported. To go beyond five spectral channels, spectral detection can be used, although biosensors have so far not been multiplexed using this technology(18, 19). Ultimately, multiplexing via spectral properties will always be limited by the finite spectrum, and expansion to higher degrees will require the use of additional parameters.

4.1.2. Complementary approaches to infer biosensor identity—Biosensors with similar spectral properties can be distinguished along other dimensions to achieve multiplexed imaging (Figure 5a). For instance, the identity of a biosensor, which analyte it measures, can be determined via its spatial location. Recently, multiplexing of six single-FP-based biosensors was made possible through targeting some of them to different, visually distinct subcellular locations within the same cell(86). As an alternative to classical organelle targeting, biosensors have been fused to self-assembling peptides that form stable signaling reporter islands (SiRIs) containing distinct immune-epitopes, allowing post hoc determination of biosensor identity through fixation and immunostaining(72). This spatial multiplexing approach enabled successful monitoring of four GFP-based biosensors simultaneously in living cells. Ideally new clustering motifs can be identified to scale this method to more biosensors in the future.

Similarly, multiplexing approaches can leverage time as an additional parameter to infer biosensor identity. This is most often performed with the help of photoswitchable or photochromic FPs. A FRET-based biosensor carrying a photochromic donor FP can be distinguished from a spectrally identical non-switchable biosensor via the switching behavior of the donor and FRET signal(114). Similarly, the turn-off kinetics of several green and red switchable FPs were used to distinguish kinase translocation reporters (KTRs), allowing multiplexing of four biosensors in one channel(107).

Taken together, these additional parameters can dramatically expand the degree of multiplexing. Nevertheless, certain trade-offs should be acknowledged. For example, spatial signaling information is largely inaccessible using SiRIs when performing spatial multiplexing, whereas the kinetic nature of photochromic biosensor measurements typically

entails sacrificing some temporal resolution. Multiplexing strategies therefore need to be chosen carefully, taking the spatial and temporal organization of the processes in question into account.

4.2. Super-resolution microscopy compatible biosensors

In recent years, it has become clearer that signaling networks are spatially compartmentalized and that the sizes of many signaling compartments are below the diffraction limit. Many such signaling nanodomains are known to be associated with various second messengers, such as Ca^{2+} and cAMP(10, 11). Investigating these nanodomains and understanding their biological functions requires approaches with the necessary sub-diffraction resolution, driving efforts to pair biosensors with super-resolution microscopy(149). However, super-resolution microscopy techniques impose unique requirements that are not fulfilled by every biosensor, and hence specialized biosensors have been developed.

For instance, a rather straightforward approach is the use of bimolecular fragment complementation (BiFC) of photoconvertible FPs in single-molecule localization microscopy. However, due to the irreversibility of FP complementation, these biosensors cannot report on dynamic phenomena(79). Alternatively, an intensiometric H_2O_2 biosensor (HyPer2) based on a photostable YFP was combined with stimulated emission depletion (STED) microscopy. Local H_2O_2 production was measured upon growth factor stimulation of receptor tyrosine kinases. Neighboring microtubules showed differing increases in H_2O_2 concentration, confirming the existence of H_2O_2 domains as small as 100–200 nm. However, the biosensor bleached considerably, and only few consecutive STED frames could be measured(88). A more recent strategy paired a photoswitchable Ca^{2+} biosensor based on rsEGFP2 with reversible saturable optical linear fluorescence transitions (RESOLFT) microscopy (Figure 5b). The generated rsGCAMP1.4 biosensor, while too dim in the Ca^{2+} unbound state, nevertheless allowed measurement of high Ca^{2+} concentrations such as the Ca^{2+} distribution in the endoplasmic reticulum(90). Brighter variants are anticipated to enable measurements in both the bound and unbound states in the future.

In contrast to HyPer2 and rsGCaMP1.4, which use intensity as the readout, other fluorophore properties can be harnessed(149). For instance, changes in blinking behavior were used in kinase activity biosensors working via fluorescence fluctuation increase by contact (FLINC, Figure 5c). Here, the proximity of the FPs Dronpa and TagRFP-T influences the blinking behavior of the latter. Changes in FLINC can be measured via photochromic stochastic optical fluctuation imaging (pcSOFI), which was used to generate functional super-resolved maps of PKA and ERK activity, revealing the formation of activity clusters roughly 300 nm in diameter(92). Similarly to HyPer2, long-term measurements using FLINC biosensors were limited by photobleaching of TagRFP-T. Taken together, functional super-resolution microscopy should benefit from biosensors with good photostability and high brightness in the on and off states. Hybrid approaches based on synthetic fluorophores are therefore promising candidates for future applications.

4.3. Applications under more physiological conditions

A major goal of biosensor imaging efforts is to move beyond cultured cells and investigate cell signaling and metabolism in more physiological settings, such as in primary cells, tissue sections, animals or plants. Thus, biosensors are increasingly being paired with advanced fluorescence microscopy techniques such as intravital imaging, 2P imaging, or fiber optometry(1, 65).

4.3.1. Animals—In vivo applications largely focus on model organisms such as drosophila, zebra fish, and mice, as well as the central nervous system. Biosensors for Ca^{2+}, voltage, and neurotransmitters/neuromodulators are hence among the most optimized biosensors, and excellent reviews are available(26, 67). However, biosensors have also been used for in vivo measurements in other fields of biology, including cancer and metabolism(17, 71, 84). Common challenges are connected to poor biosensor expression and insufficient penetration of excitation and emission light. The former is being tackled by advances in transduction technologies such as adeno-associated viruses (AAVs) with cell-type dependent promotors(39, 67) or the generation of transgenic animals(52). The low penetration depth and high scattering of visible light can be circumvented by working in the NIR window, as discussed previously (Section 2.2), or via 2P excitation (Figure 5d–e). For instance, the recently developed voltage reporter JEDI-2P showed ideal 2P properties and response kinetics to measure voltage changes in deep tissue, including layer 5 neurons of the visual cortex of awake mice(76). Furthermore, the stability of the method allowed measuring of subthreshold voltage correlations in paired neurons upon locomotion. Such measurements were so far only accessible via invasive electrophysiology. 2P-FLIM-FRET measurements are also attractive for FRET-based biosensors, as only one channel needs to be measured. This was showcased with the recently developed cAMP biosensor cAMPFIRE, with which the heterogeneous cAMP responses of layer 2/3 neurons were measured in running mice(82). Optical fiber photometry can be used to achieve even deeper imaging but involves sacrificing spatial resolution in addition to being rather invasive(142). Newer methods using much thinner multi-mode fibers allow for better resolution and less tissue damage, making them highly promising, as highlighted by initial measurements performed using Ca^{2+} indicators(100). Considering that in vivo measurements are often also hampered by suboptimal biosensor brightness and photostability, developments such as the hybrid biosensor series Voltron, which allowed measuring more neurons for longer periods, are bound to push in vivo biosensor measurements to the next level(2, 3).

4.3.2. Plants—Genetically encoded biosensors have found many applications in plant biology, especially because most chemical probes cannot penetrate plant cells(139, 150). Plant-specific problems such as post-transcriptional gene silencing have been overcome via the choice of promotor and a plant mutant deficient in RNA polymerase activity required for post-transcriptional gene silencing. However, it is still necessary to carefully screen transgenic plants for sufficient expression. Many biosensors originally developed for research in mammalian cells can directly be adapted and used in *Arabidopsis thaliana*. Others were specifically developed for the investigation of important plant metabolites such as sucrose or glucose(33, 62), phytohormones(49), or plant specific kinases(154). A special breakthrough was the development of a FRET-based biosensor for auxin, one of the most

important plant signaling molecules involved in diverse processes. The biosensor enabled the first measurements of the effects of gravitation on the auxin transport system, and auxin redistribution could be detected as fast as 1 min in root tips. Moreover, the biosensor allowed monitoring of subcellular auxin pools, which was not possible previously(45).

4.3.3. Further applications—The utility of genetically encoded biosensors can be further expanded by using alternative read-out modalities, including bioluminescence, optoacoustics, or ultrasound. This allows applications where fluorescence as a read-out is limiting, such as in vivo imaging. Bioluminescence relies on optical detection, but unlike fluorescence does not require excitation by an external light source. This drastically reduces background signal and phototoxicity and allows for deeper imaging in vivo(161). Luciferases can be used as donors in BRET-based biosensors that can be adapted directly from FRET-based designs(59). Alternatively, split luciferases or insertion into luciferases can be used(99, 128, 144). Current limitations are mainly associated with the low light output and insufficiently red-shifted emission spectra of luciferase-luciferin pairs. Moreover, substrate delivery, stability and toxicity are of concern, and hence new luciferases and luciferins are being developed(78, 127). Efforts to endogenously encode all components of bioluminescent biosensors are also ongoing, and the first biosensor system where the luciferin is produced endogenously was recently described(124).

In optoacoustic (OA) imaging, optical stimulation is used to generate an acoustic signal via absorbance and heat production. While spatial resolution is usually sacrificed, much higher tissue penetration can be achieved(98). Labels for optoacoustic imaging should show high extinction coefficients but low quantum yields(89, 90). In recent years, biosensors based on NIR FPs(69, 70) or switchable FPs, which can enhance contrast, were used in functional OA imaging(90).

Finally, the development of air-filled protein nanostructures, called gas vesicles (GVs), has made ultrasound imaging possible on a molecular scale(121). A first acoustic biosensor, reporting on protease activity of tobacco etch virus (TEV), was engineered by introducing a TEV cleavage site into the scaffolding gas vesicle protein C (GvpC). Upon TEV activity, the protein was cleaved, leading to a gas vesicle with a less stiff shell and hence more non-linear ultrasound contrast(63). All three of these imaging modalities enable enhanced tissue penetration, and more biosensors and applications are expected in the coming years.

5. Conclusion

Genetically encoded biosensors enable spatiotemporally resolved measurements and therefore significantly contribute to our understanding of the intricate networks of cell signaling and metabolism. Recent endeavors have focused on enhancing biosensors so that cellular processes can be dissected more accurately, for instance, via biosensor multiplexing and super-resolution measurements, or under more physiologically relevant conditions. Although these applications differ in terms of microscopy, they converge on a few key requirements for biosensors: high brightness, high photostability, far-red to NIR emission properties and accurate/fast response kinetics. Thus, many biosensor engineering efforts focus on these goals. We have discussed how some of these goals can be addressed

systematically, as with the use of NIR FPs or hybrid approaches, while others (e.g., response kinetics) still necessitate large screening campaigns. At the same time, the community is constantly generating biosensors for new analytes and exploring new biological areas.

However, to fully exploit biosensors in different contexts, several limitations still need to be overcome. For instance, NIR FP-based systems need to be rendered brighter so they can be used more reliably. Hybrid approaches often suffer from high background or issues delivering the synthetic fluorophore. More knowledge is needed about the in vivo behavior of different fluorophore classes, and biorthogonal labeling technologies must be expanded. Similarly, functional super-resolution microscopy pushes current biosensors to their limit; less photo-intensive microscopy methods, more photostable and brighter biosensors such as hybrid systems, as well as improved image analysis methods are most likely to bring this field forward. Furthermore, advances in de novo protein design and use of deep learning for data analysis are predicted to streamline screening endeavors and thereby impact biosensor engineering. Last but not least, clever biosensor designs that rely on non-classical fluorescent (e.g., blinking/switching kinetics) or other optical (bioluminescent) or acoustic (optoacoustic and ultrasound) detection strategies can open up new, unforeseen avenues, and we eagerly await the generation of more such biosensors in the near future, helping us to investigate outstanding questions in cell signaling and metabolism.

Terms and Definitions List:

Circular permutation
Process of repositioning a protein’s original N- and C termini, giving access to different topologies

Förster resonance energy transfer (FRET)
Non-radiative energy transfer between two spectrally compatible chromophores (donor and acceptor), with transfer efficiency dependent on chromophore orientation and distance

Chromophore
Chemical moiety within a (bio)molecule that is responsible for absorbing visible light

Photostability
Property of a fluorophore described by a characteristic time before a fluorophore succumbs to irreversible photodamage

Brightness
Fluorophore characteristic determined by extinction coefficient and fluorescence quantum yield

FP maturation
Time required for a FP to fully form its chromophore via cyclization, oxidation, and dehydration

Two photon (2P) microscopy

Imaging technique wherein the near-simultaneous absorption of two long-wavelength photons excite a fluorophore, increasing penetration depth and reducing background

Fluorescence lifetime imaging microscopy (FLIM)-FRET
Imaging modality in which FRET efficiency is calculated by measuring changes in the fluorescence lifetime of the donor fluorophore

Photoswitchable or photochromic FPs
Fluorescent proteins that can be toggled between an on and off or a green and red state using light irradiation

Stimulated emission depletion microscopy (STED)
Scanning confocal microscopy-based technique wherein a depletion laser induces stimulated emission at the periphery of the diffraction limited excitation volume

Reversible saturable optical linear fluorescence transitions (RESOLFT)
Scanning confocal-based microscopy technique wherein fluorophores are reversibly switched to achieve super-resolution imaging

Stochastic optical fluctuation imaging (SOFI)
Widefield microscopy technique in which cross-correlation analysis of blinking fluorophores is used to generate super-resolution images

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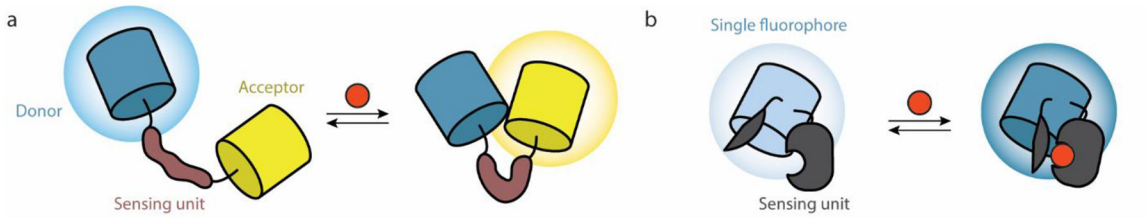

Figure 1:

Schematic of the two most common biosensor designs. (a) FRET-based biosensors consist of two fluorophores (i.e., FRET-donor and FRET-acceptor) fused to a sensing unit. (b) Intensiometric biosensor based on a single fluorophore inserted into a bipartite sensing unit. The two most common topologies are illustrated, though other configurations are possible.

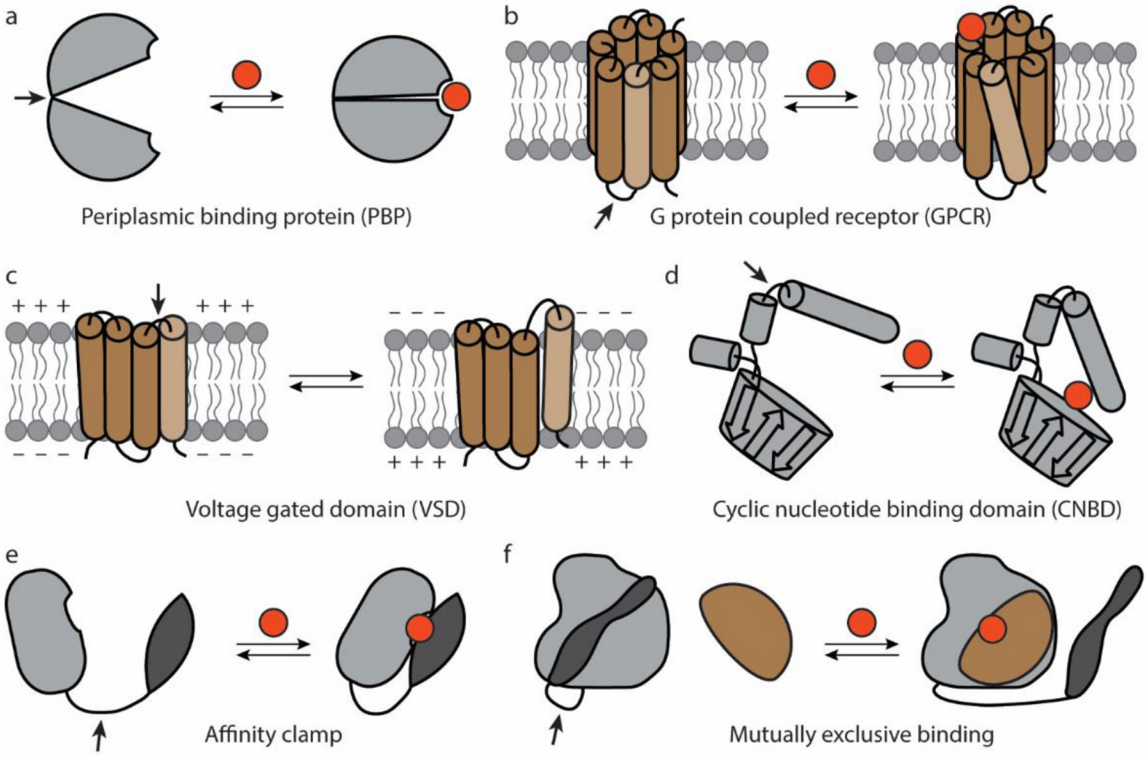

Figure 2:

Naturally occurring and synthetic sensing units. (a) PBPs show a large hinge-like motion upon analyte binding, which is well conserved among different family members. (b) GPCRs, a diverse class of structurally conserved transmembrane receptors, exhibit a conformational change in the 3rd intracellular loop upon analyte binding. (c) VSDs respond to changes in membrane polarization with a movement of the fourth transmembrane helix. (d) CNBDs are composed of a flexible α-helix and a β-sandwich with a conserved phosphate binding cassette. Binding/dissociation of the cyclic nucleotide triggers rearrangement of the α-helices. (e) Synthetic switch that works as an affinity clamp. (f) Analyte binding leads to dissociation of the two protein domains in mutually exclusive binding designs. Arrows indicated potential insertion sites for reporting units. Alternatively reporting units can also be attached to the termini.

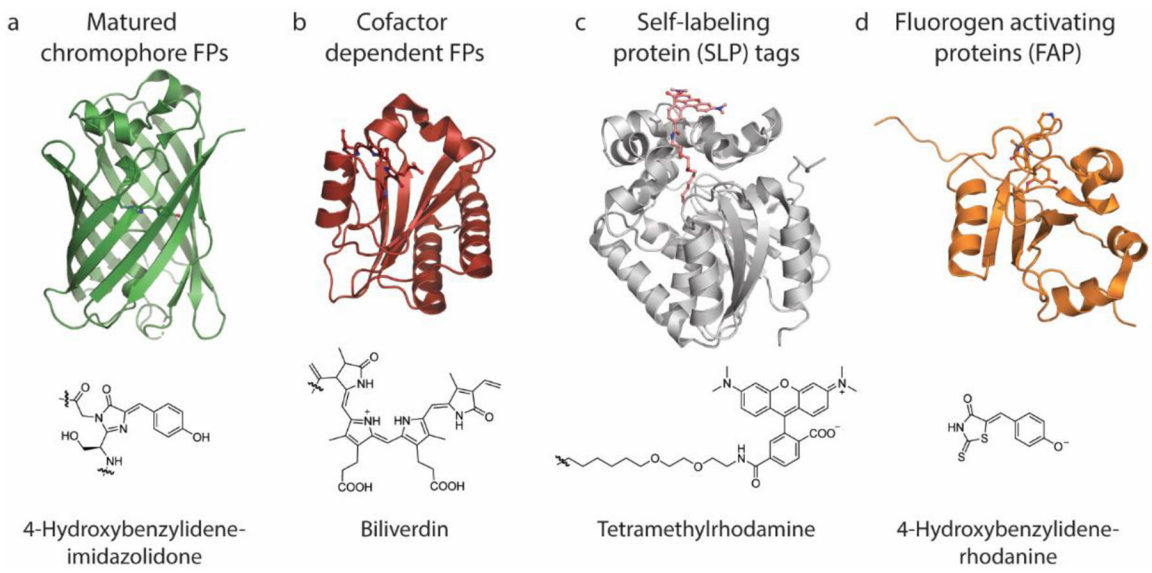

Figure 3:

Crystal structures of reporting units along with chemical structures of the corresponding chromophore/fluorophore. (a) FPs based on matured chromophores, illustrated by mNeonGreen (PDB ID: 5LTR). (b) NIR FPs such as miRFP670nano (PDB ID: 6MGH) are dependent on the cofactor biliverdin. miRFP670nano forms a covalent bond with biliverdin via Cys86. (c) SLP tags such as HaloTag7 (PDB ID: 6Y7A) can be labeled with a variety of different fluorophores. Tetramethylrhodamine is illustrated here. (d) The FAP FAST (PDB ID: 7AVA) non-covalently binds 4-hydroxybenzylidine rhodanines and its derivatives such as N871b, as indicated in the crystal structure.

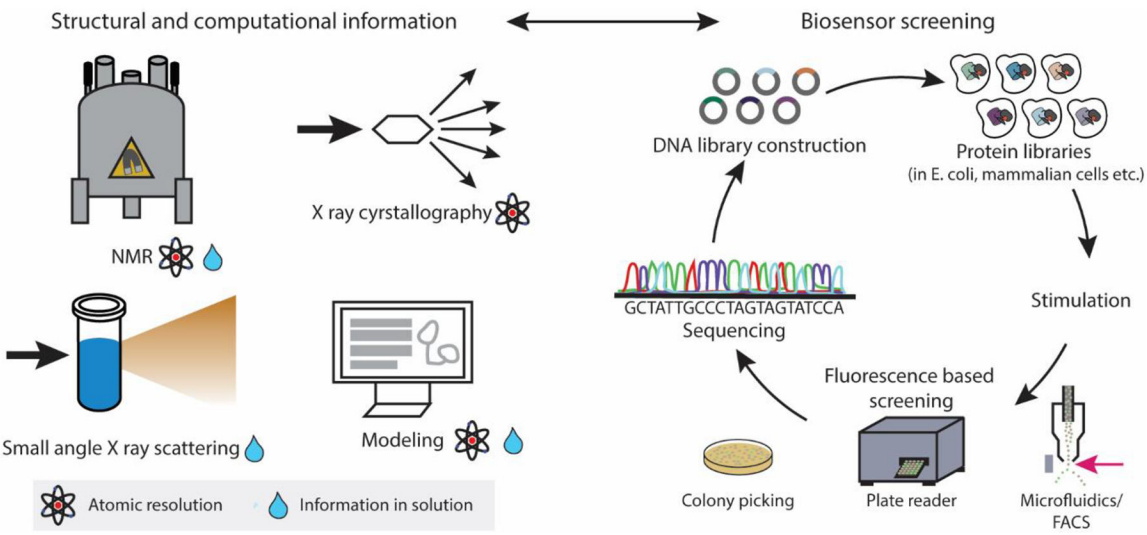

Figure 4:

Methods used to streamline biosensor engineering. Structural and computational information are used to help rational biosensor engineering and inform targeted screening campaigns. At the same time, high-throughput methods are adapted to help biosensor screening.

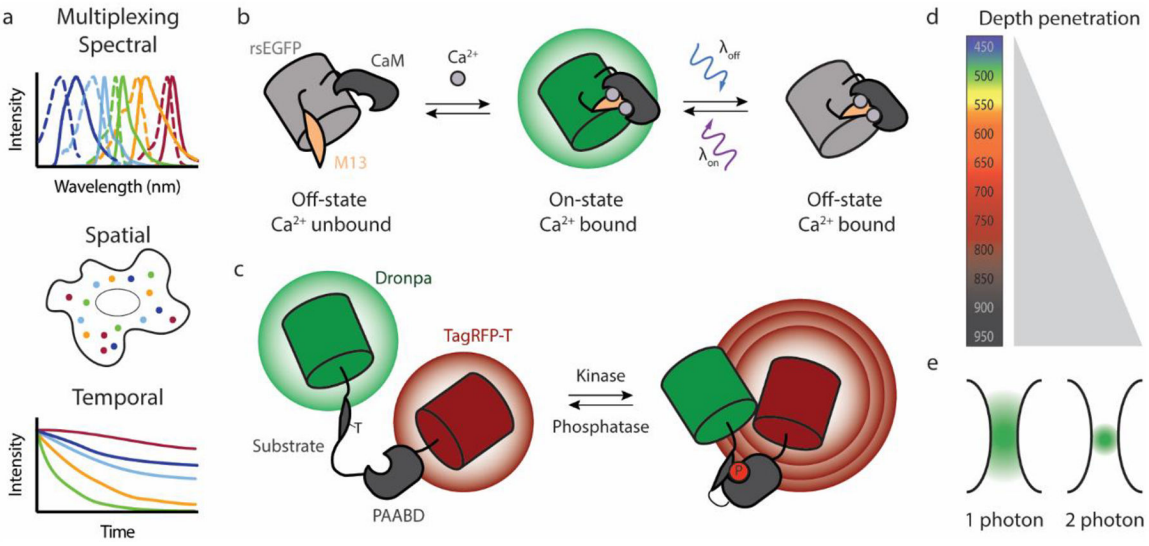

Figure 5:

Biosensors contribute to various application areas. (a) Biosensor multiplexing can help to investigate intertwined signaling pathways. Strategies generally rely on spectral, spatial, or temporal information to distinguish the signals from different biosensors. (b-c) Super-resolution compatible biosensors. Reversibly switchable Ca^{2+} biosensor generated from rsEGFP, CaM, and the M13 peptide(90). Only in the Ca^{2+} bound state can the biosensor be photo-switched using blue and UV light. The biosensor was paired with RESOLFT imaging to generate super-resolved maps of Ca^{2+} in the endoplasmic reticulum(90) (b). FLINC kinase activity biosensor for imaging via SOFI (92). The proximity of Dronpa to TagRFP-T induces fluorescence fluctuations in the emission of the latter (multiple circles) (c). (d) Biosensors compatible with far-red or NIR excitation help to perform in vivo imaging as the depth penetration of red-shifted light is greater. (e) In vivo imaging using 2P excitation offers increased penetration depth due to longer-wavelength illumination, while the smaller excitation area produced in 2P illumination helps reject out of focus light and obtain higher signal-to-background ratio.