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Review: Engineering *in situ* biosensors for tracking cellular events

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This review will focus on advances in the development of such biosensor in tracking cellular events with a focus on their technical novelty.

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Introduction

Cellular events, such as dynamic changes in chromatin during cell differentiation, neurotransmitter uptakes, and protein assembly at transcription starting sites, occur at vastly different time and length scales inside a cell and are crucial for understanding fundamental biological mechanisms and providing useful insights for engineering novel cell types. Enabled by advances in bio-engineering and fluorescence microscopy, a broad array of bio-sensors has been developed in recent years to track cellular events with unprecedented time- and spatial-resolution. This review will focus on advances in the development of such biosensor in tracking cellular events with a focus on their technical novelty.

Biosensor in general

In situ biosensors are typically composed of two major units, namely a Recognition and a Transducer Domain as illustrated in Figure 1a. The Recognition domain is made of a defined amino acid (protein) or nucleic acid (DNA/RNA) sequence that can bind to the target of interest with high affinity and selectivity. These domains are typically selected based on naturally existing sequences and/or evolved using a directed approach. The Transducer Domains transduce and/or amplify the signal, that is, a binding event to the target of interest and provide a readout that can be recorded in various ways, for example, electrochemically and optically. These two domains are connected or integrated together to enable fast and accurate measurements of the signal of interest. Compared to biosensors that are primarily designed for *in vitro* applications (e.g. glucometer), additional factors must be accounted for in designing *in situ* biosensors, including compatibility with host strains and minimal perturbations of host cell functionality. Furthermore, sustainable expression of *in situ* biosensors is necessary for long-term monitoring of cellular activities and thus limits the type of biosensors that can be successfully adopted in such applications.

State-of-the art *in situ* biosensors

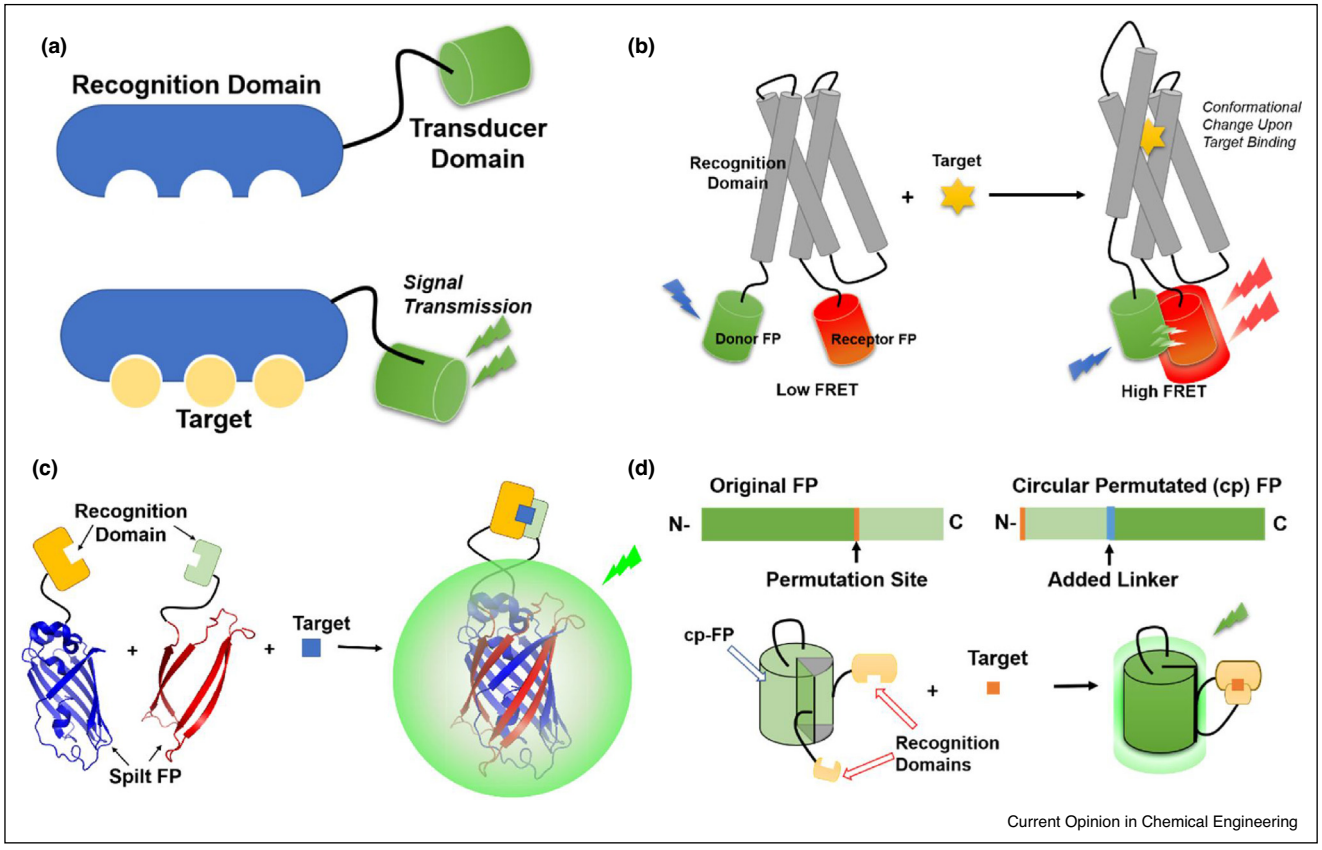
In situ biosensors can be coarsely categorized based on their chemical and biological compositions and will thus be discussed in that context, some representative biosensor are summarized in Table 1 based on their respective recognition and transducer domains.

Protein-based sensors

Protein-based probes, for example, engineered recombinant proteins, nanobody, and Fab fragments, are commonly used for a wide range of *in situ* applications. Benefiting from the rich body of protein engineering literature, researchers have access to an array of engineering tools (experimental and computational), and protein database with functional and structural information readily available, enabling the design and development of protein probes with high specificity. Furthermore, proteins naturally exist in cells and are thus compatible with most *in situ* cell tracking applications.

In recent years, *in situ* probes have been developed to monitor an array of neuron activities, including membrane potential, neurotransmitter activity and ion transportation providing unprecedented opportunity to understand brain functions and thus considered as one of the most exciting developments in protein-based sensors. The Receptor Domain of this type of sensor is commonly engineered from native proteins for example, voltage-sensitive domains to monitor changes in membrane

Figure 1



Schematics of different *in situ* probe design. **(a)** Protein sensor with one target recognition domain and one single transducer domain (usually a single fluorescent protein). **(b)** An *in situ* probe design using Förster Resonance Energy Transfer (FRET). This type of probes contains two fluorescent proteins, one as a donor and the other as an acceptor. The two FPs are fused to the N and C terminus of a Recognition Domain which changes its conformation upon target binding thus changing the distance between the two FPs and therefore changing the FRET efficiency. **(c)** An illustration of Bimolecular fluorescence complementation (BiFC) sensors. FP is split into two fragments and fused to two halves of Recognition Domains that associate in the presence of targets of interest. Without a target the two halves of the FP do not form an active FP, thus do not emit any fluorescence. Upon target induced dimerization, the complementary fragments of the FP are brought into close proximity and reconstitute the active β-barrel structure therefore reactivating the FP. **(d)** cpFP is designed by changing the order of protein connectivity, an FP can be circular permutated at the permutation site but still maintain a similar 3D structure. The circular permutated FP can be fused with two recognition domains which associate upon target binding and restore the original connectivity of FP and transduce fluorescent signals.

potential in neuronal cells [1–3]. Natural glutamate, dopamine, and acetylcholine transporters were adapted to record changes in neurotransmitter activities [4••,5,6]. To obtain meaningful signal readouts, Transducer Domains are typically designed based on advanced fluorescent techniques, namely Förster Resonance Energy Transfer (FRET), Bimolecular Fluorescence Complementation (BiFC) and circular permutation of fluorescent proteins (cpFP) as illustrated in Figure 1b–d. Circular permutated fluorescent proteins are the primary Transducer Domains used to develop fluorescent reporters of neurotransmitter activity [4••,5,6] and membrane potentials [1,2] given its relative simplicity in signal monitoring. FRET sensors have also been designed to detect neurotransmitters, such as acetylcholine [7], dopamine, and norepinephrine [8]. These cell-based neurotransmitter fluorescent engineered reporters (CNiFERs) use GCPR based signaling to increase intracellular Ca^{2+} concentration, this concentration change is in turn measured by the genetically encoded Ca^{2+} sensor TN-XXL [9]. The Recognition Domain undergoes a conformational change upon binding to Ca^{2+} and uses FRET as a Transducer to quantify ion concentrations. The amount of fluorescent signal can then be directly correlated to the quantity of neurotransmitter activating the GPCR [7,8]. In another recent work, researchers have successfully constructed a BiFC probe monitoring proton-gated ion-channel activity in neurons by splitting acid-sensing ion channels into two parts, namely ASIC2a and ASIC2b (ASIC2a/2b) each fused to half of an FP [10].

Table 1

Highlights of biosensor types, their transduction domains, and major applications of these designs

Recognition domain	Transducer domain	Major applications
Engineered natural protein motifs	cpFP	Neurotransmitter [4••,5,6] and ion [1,2] transportation across membranes
	FRET pair	Neurotransmitter [7,8] and ion [3] transportation across membrane. Epigenetic sensors [20•,21]
	BiFC pair	Proton-gated ion-channel activity [10] and epigenetic sensors [11]
	Fluorescent protein	Epigenetic sensors [12,13,14••,15,23]
Nanobody/Fab fragment	FRET pair	Chromatin-chromatin interaction [16,17]
DNA Aptamer	FRET pair	ZIKA virus detection [33]
RNA Aptamer	Fluorescent RNA aptamer	Ribosomal RNA detection [38•]
dCas9	Fluorescent protein	Visualization of specific genomic location [43,44]
dCas13a	Fluorescent protein	Virus, for example, ZIKA and Dengue detection [46,48]

The recognition domains of such biosensors are typically engineered from naturally existing protein domains modified by protein engineering techniques, including site-directed mutagenesis, multimerization and directed evolution. For example, the creation of acetylcholine and dopamine sensors are based on acetylcholine-binding and dopamine-binding proteins found in humans, respectively. cpFPs were selectively inserted into specific sites maximizing the detection of structural change upon ligand binding [4••,6]. The length and sequence composition of the linker domain connecting the recognition and transducer domain also need to be optimized to enable sensitive detection. For example, to create the acetylcholine sensor (GACh) 723 random mutations were introduced to the linkers to screen for optimal fluorescent response [6].

Chromatin dynamics account for structural changes of DNA inside the cell nucleus and serves as the molecular basis for gene regulation and reprogramming. Monitoring dynamic chromatin changes during cell division and differentiation can thus offer unique insights into cell reprogramming machinery. Live cell probes have been developed in recent years with a focus on advancing our current understanding of the underlying epigenetic make-up of chromatin. These sensors utilize Receptor Domains that are engineered from natural epigenetic reader' domains or Fab fragments/nanobodies raised against the modification of interest. For example, engineered chromodomains and DNA methyl-binding domains have been used to detect histone and DNA methylation with high specificity, [11,12,13,14••,15]. Additionally, Nanobody and Fab fragments have also been raised for similar purposes [16,17]. Nanobody (~15 kDa) and Fab fragments (~50 kDa) are essentially 'miniaturized' monoclonal antibodies (mAb) with equivalent binding affinities but are of much smaller sizes [18,19]. FP can be directly applied as a Transducer Domain since probes bound to the modification of interest will have significantly slower diffusion and thus provide stronger fluorescent signal during imaging. Advanced fluorescence techniques, particularly FRET and BiFC have also been adopted to monitor chromatin dynamics using the developed probes [20•,21].

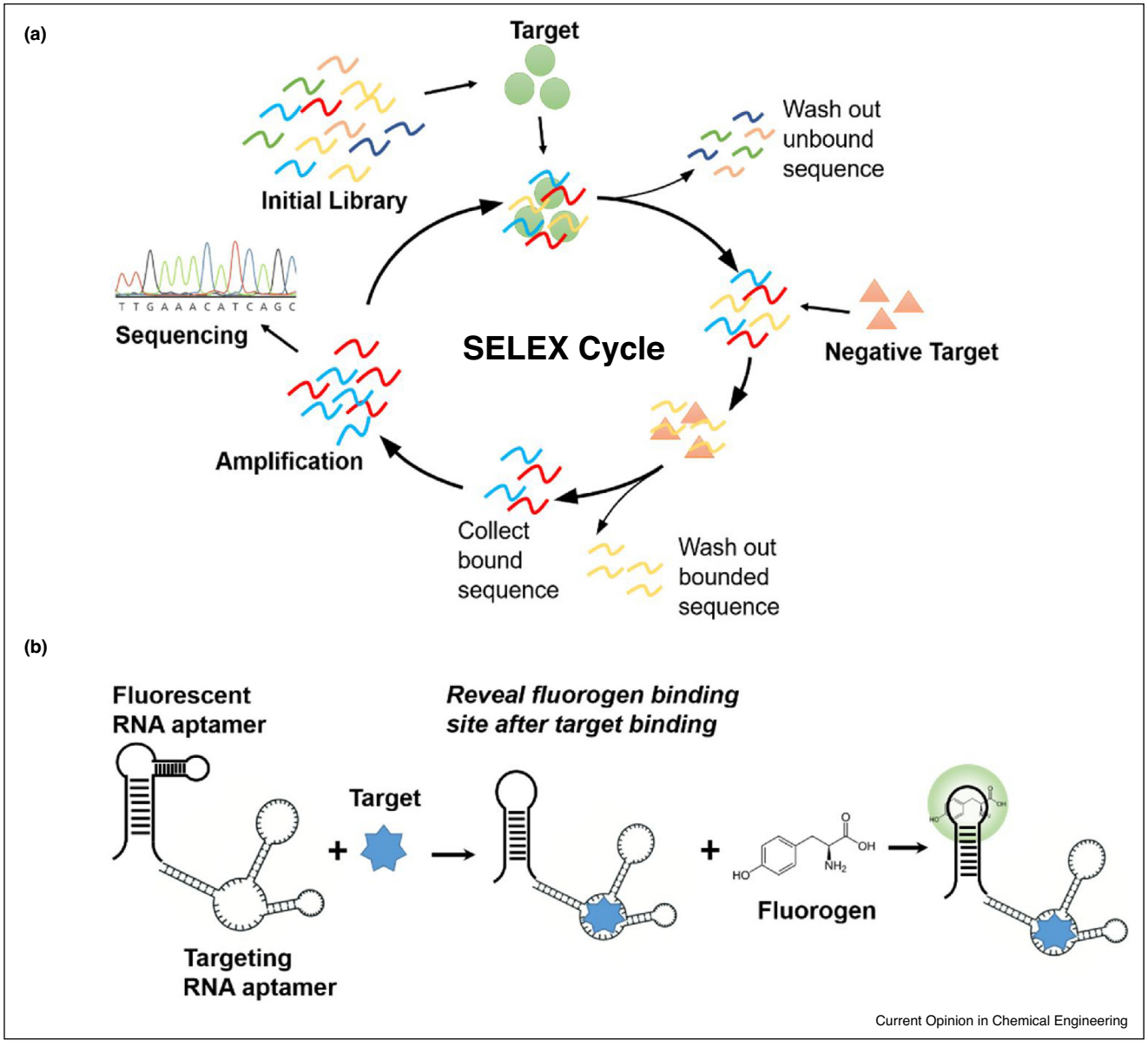
Sensitivity, selectivity and dynamic range are three important metrics for evaluating biosensors. Sensitivity of biosensors can be modulated by both the recognition and transducer domains. The most common way to modify the sensitivity of protein sensors is through the mutation of amino acids in or near the binding pocket of the recognition domain to alter binding affinity and kinetics [4••]. Furthermore, transducer domains with low signal-to-noise ratio, for example, BiFC versus full-length FP, can also improve the overall performance of biosensors. Selectivity of biosensors are determined primarily by recognition domains. Site-directed mutagenesis at the binding pocket can be used to improve the specificity of biosensors [8]. Computational tools that predict protein structure and stability, such as FoldX, can facilitate the engineering efforts but cannot offer a complete replacement of laboratory work due to limited accuracy [22]. Additionally, inclusion of tandem repeats of recognition domains can improve the sensitivity and selectivity of biosensors as demonstrated by our group and others [12,14••,23]. Dynamic range of *in situ* biosensors are typically dependent on their expression level in cells and can thus be modulated using engineered promoter sequences and other transcription regulators [24,25].

Although protein sensors are frequently used for *in situ* applications, several drawbacks exist, specifically, excessive expression of protein probes may disturb the normal functionality of cells; in addition fluorescent proteins are typically used as Transducer Domains which requires the use of advanced fluorescent microscopy. Given the high modularity, versatility, and selectivity of protein probes, however, it appears that the development of protein-based biosensors will continue at a rapid pace for broad applications in various research and industrial settings.

Aptamer-based sensors

Nucleic acids (DNA and RNA) can also be used as biosensors *in situ*. These oligonucleotides, which are also known as aptamers, recognize specific targets of interest via non-covalent interactions [26]. Compared to protein-based sensors, the design of the Recognition Domain in an aptamer sensor follows a standard protocol, namely Systematic Evolution of Ligands by Exponential enrichment (abbreviated as SELEX [27], see also Figure 2a).

Figure 2



(a) A schematic outline of SELEX procedure. Briefly, we start with an aptamer library containing random DNA/RNA sequences, introduce the target of interest, separate aptamers bound to the target and enrich them via PCR. A negative selection round is also included to improve target selectivity of enriched aptamers. The procedure is repeated successively 10–15 times or until the DNA/RNA aptamer sequence converges. The converged sequence will then be tested for its binding affinity and selectivity. **(b)** Schematics of a light-up (fluorescent) RNA aptamer probe. A light-up RNA aptamer is fused with a targeting RNA aptamer. When the probe is introduced with a fluorogen and a target molecule, the complex will become fluorescent.

Aptamers for various targets, ranging from metabolites (e.g. cortisol and xanthine) [28,29] to specific proteins (e.g. BoHV-1) [30] have been identified with K_d values ranging from nM to mM.

For *in situ* applications, these aptamers are typically conjugated with organic fluorescent dyes as their Transducer Domain. For example, an aptamer can be coupled with a fluorescent donor and an acceptor dye with overlapping emission and excitation spectra. Upon binding to the analyte, the aptamer changes its conformation, altering the distance between the donor-acceptor pair and thus changing the FRET efficiency similar as described in Figure 1b. *In situ* FRET-based aptamers have been developed for imaging potassium ions and adenosine triphosphate in living cells [31,32]. Metal nanoparticles (NP), that is, Au-NP can also be used as a Transducer Domain to provide colorimetric readouts upon target-

binding. For example, aptamers specific for ZIKV-E (envelopment protein of ZIKA virus) can be immobilized on Au-NP and used to detect ZIKA virus in the range of 0.4–100 nM [33].

Compared to DNA aptamers, RNA aptamers are uniquely advantageous because of the existence of a family of 'fluorescent' light-up RNA aptamers and their pairing cognate fluorogenic ligands. These ligands only fluoresce upon binding to their pairing aptamers. These types of RNA aptamers such as Spinach [34], Broccoli [35], malachite green [36], and SRB-2 [37] can be fused to another RNA aptamer(s) using a modular approach and thus function as a Transducer Domain in RNA-aptamer sensors as illustrated in Figure 2b. In a recent study by Sunbul *et al.*, SRB-2 aptamers were fused to an RNA-binding aptamer to enable successive imaging of ribosomal and messenger RNA in both bacterial and mammalian cells revealing their distinctive localization patterns in real time [38•]. 'Fluorescent' RNA aptamers that blink at near-infrared wavelength have also recently been developed and can bind to silicon rhodamines to enable superresolution imaging in live cells [39]. A novel platform combing fluorescent RNA aptamer and protein sensor has been established to monitor RNA-protein interactions *in situ* via FRET [40].

Aptamer-based biosensors are emerging as one of the most favorable options for *in situ* tracking of biological events because of their modular design and potential adaptability to many targets of interest, particularly novel targets with few existing biological information. Although DNA aptamers are easier to screen for and more economical to produce, RNA aptamers offer greater flexibility for live-cell imaging and can be expressed *in situ*. Low selectivity and off-target effects remain as the major concerns for the application of RNA-aptamers since an aptamer normally only recognizes part of the chemical/biological structure of its target and thus making the differentiation of enantiomers and derivates extremely challenging.

CRISPR-based probes

CRISPR/Cas9 is a two-component protein-RNA system that allows for precise editing of selected DNA sequences [41]. Deactivated Cas9 (dCas9) contains a single nucleotide polymorphism that inhibits the DNA cleavage activity of Cas9 and can be used to target a specific DNA of interest [42] offering unique capability of tracking sequence-specific events *in situ*.

dCas9 has been used to track the dynamics of selected gene loci. Integration of fluorescence *in situ* hybridization (FISH) with dCas9 has led to the development of CAS-FISH complexes that can bind to a specific gene locus without the need of performing harsh DNA denaturation as is required in traditional FISH [43]. This system uses gRNA/dCas9 as the Recognition Domain for identifying the DNA sequence, and a fluorescent protein bound to the dCas9 as a Transducer Domain, allowing for the visualization of the genomic location. The addition of fluorophores of distinctive wavelengths enables the visualization of multiple genetic loci in one cell, providing unprecedented spatial information about different genetic locations and their ability to interact [44]. dCas9 has also been combined with other epigenetic reader domains to enable gene-specific quantification of epigenetic modifications. For example, the fusion of dCas9 to cat3a, an engineered version of the DNMT3A enzyme, has been used to track methyltransferase activity in cells. Fluorescence visualization of the cat3a shows the relative position of methylation as the cell develops [45•]. Similarly, dCas9 has been combined with other epigenetic reader domains, for example, methyl binding domain and chromodomain, to reveal gene-specific changes in DNA and histone methylation i*n situ* [11] via a BiFC strategy.

Among many types of naturally existing Cas9 variants, Cas13a is unique in its ability to target RNA [46]. dCas13a has thus been used to detect specific miRNA *in situ* [47•]. Since dCas13a has high sequence specificity, it has been used to detect specific viral sequences (e.g. Zika and Dengue) in a system known as SHERLOCK [48]. Like the other Cas proteins, SHERLOCK used the gRNA/Cas13 as a Recognition Domain while FPs act as transducers.

CRISPR-based biosensors are uniquely positioned to provide gene-specific readouts inside a cell nucleus bridging single-cell based *in vitro* tracking with conventional genomic and biochemical studies. CRISPR-based biosensors represent a fast-growing field of biosensors that have the potential to revolutionize how imaging analysis can contribute to addressing fundamental biological questions associated with defined gene targets. Significant challenges, however, exist primarily arising from target specificity of the existing CRISPR systems.

Applications of *in situ* biosensors

Developmental toxicology

Developmental toxicology primarily accounts for damage in cells, tissues or organs induced by exposure to environmental pollutants and toxins during their developmental stage. For example, developmental exposure to heavy metals, such as methyl-mercury are known to be associated with increased risks of various types of cancers (e.g. liver cancer [49]) and/or neurodevelopmental diseases (e.g. Alzheimer's Disease [50]) in later-life. However, the underlying molecular mechanism remains elusive. To understand changes that are induced in cells due to the exposure, it is imperative to perform dynamic tracking of the potential underlying molecular features and verify its persistence. Live cell probes have been developed for this purpose. Specifically, FRET-based probes have been developed to track the concentration

of environmental chemicals in live cells over a long period of time (e.g. Arsenic [51], Mercury [52], Cadmium [53], and Nickle [54]). To reveal environmental impact on the epigenome, probes have been developed to track changes in H3K9me3, a potentially persistent epigenetic trait, after exposure to atrazine [14••]. To understand exposure effects on cell reprogramming, RNA-based fluorescent probes specific for Runx2 and Sox9 mRNA, two pluripotent genes crucial for mesenchymal stem cell differentiation, have been developed to track their mRNA levels during differentiation [55].

Neuron activity

Neuron activities (e.g. action potential and neurotransmitter activity) are conventionally monitored via electrophysiological approaches, that is, electro-encephalography (EEG) and patch-clamp requiring highly laboratory personnel. Different from other cell types, neurons function as a network with activities occurring at ultrafast time scales (∼ ms) generating another challenge for understanding brain functions. Biosensors compatible with fluorescent microscopy have been developed to address these challenges (see also Protein-Based Sensors Section above). Voltage-sensing domains have been engineered to report membrane potential changes occurring at ∼1 ms [3]. Neurotransmitter fluorescent reporter have been specifically designed using engineered receptors with optimized affinity to track neurotransmitter uptakes *in situ*. For example, recent works have demonstrated engineered glutamate-sensing fluorescent reporter (iGluSnFR) and dopamine-sensing fluorescent reporter (dLight) to monitor glutamate and dopamine release and intake in live cells [4••,5]. These biosensors show response times of 15 and 9.5 ms respectively when their target analyte is present. Both probes use engineered derivatives of natural receptors as their Recognition Domain and utilize cpFP as the transducer domain. Collectively, these tools have led brain research to a new era that multiple cellular events can be visualized simultaneously to understand neural circuits.

Outlooks

In situ biosensors offer an exciting opportunity to discover and redefine biological rules given their unique ability to track molecular changes at various time and length scales. The collective time-series information coupled with spatial details will enable us to build an interaction network of various molecular events and understand how they independently or synergistically contribute to the establishment of network/population behavior (for example brain activity) or a novel phenotype (for example response to microenvironment changes). Several major challenges remain. First, a large volume of time-series image information is expected to be collected from such endeavors. Identifying and extracting relevant features from image analysis is somewhat subjective. Data science tools that can facilitate the analysis and curation of time-series images are thus highly desirable. Second, compared to biosensors utilized in various *in vitro* applications, it is important to note that *in situ* sensors will require more rigorous validation inside a living cell to ensure minimal perturbations to cellular functionality and target specificity. Searching for probe designs with the highest affinity to the target of interest is not necessarily the best strategy to design *in situ* sensors since many biological features being explored by these sensors are also needed for maintaining biological activity and thus require access by other cellular protein factors. Striking a balance between target specificity and minimal cellular perturbation will require careful engineering of probes. Integration of biosensors for multiplexing applications is another area that calls for more attention since tracking multiple biological events at the same time is crucial for understanding synergy and interactions in a complex biological system.

Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

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