International Journal of Biochemistry and Cell Biology 125 (2020) 105761

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Imaging in focus

Genetically encoded biosensors based on innovative scaffolds

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ARTICLE INFO

Keywords:
Genetically encoded biosensors
Fluorescent proteins
Selflabeling enzymes
Cell imaging
Protein engineering

ABSTRACT

Genetically encoded biosensors are indispensable tools for visualizing the spatiotemporal dynamics of analytes or processes in living cells in vitro and in vivo. Their widespread adaptation has gone hand in hand with the development of sensors for new analytes or processes and improved functionality and robustness. In this review, we highlight some of the recent advances in genetically encoded biosensor development, with a special focus on novel and innovative scaffolds that will lead to new possibilities in the future.

1. Introduction

One of the key goals of cell biology is knowing and understanding how a cell's constituent molecules behave and interact in their highly dynamic environment. Crucial in this regard is the ability to gauge this behavior with high spatiotemporal resolution (i.e. subcellular and subsecond resolution) and minimal invasiveness. Until the late nineties, this was possible almost exclusively through the use of organic dyes that stain specific molecules or structures, or report on specific reactions happening inside the cell (Johnson, 2010; Tsien, 1989). The introduction of the genetically encoded calcium biosensor Cameleon (Miyawaki et al., 1997) in 1997 revolutionized the field by allowing genetically encoded and targeted in situ monitoring of calcium dynamics with the minimal invasiveness and excellent spatiotemporal resolution afforded by fluorescence microscopy.

Over the last 23 years, enormous progress has been made in the field of genetically encoded biosensors, and a plethora of new and improved sensors has been developed. The field of genetically encoded biosensor design and engineering is continuously evolving and novel sensors, often with innovative architectures, are being developed regularly. In this review, we will touch upon some original and often unforeseen design strategies that will undoubtedly spark ideas for novel approaches and applications in the future.

2. General design principles of genetically encoded biosensors

In this first section, we will take a look at some of the general design principles of genetically encoded biosensors. This is a generalizing overview, and many sensors deviate from the overall principles described in this section. However, this overview will set the stage and introduce general principles.

Overall, genetically encoded biosensors consist of two main constituents: the sensing domain and the reporting domain. Their combined action translates a state or change in cell physiology into a signal that can be read out using for instance fluorescence microscopy.

2.1. The sensing domain

The sensing domain, also called a molecular recognition element, is the part of the genetic construct that is responsible for sensing the presence or absence of an interaction, analyte or (enzymatic) activity (Fig. 1A/D, B/E and C/F, respectively). The sensing domain can consist of a single polypeptide chain or of two (or more) separate proteins or protein fragments. Upon analyte binding or enzymatic modification, the sensing domain undergoes a conformational change or changes its inter- or intramolecular interaction.

One of the paradigmatic sensing domains is the calmodulin-M13 (or any other calmodulin-binding peptide) moiety (Bayley et al., 1996). Binding of Ca^{2+} ions to calmodulin leads to binding of the peptide to calmodulin, which is accompanied by a large conformational change. Similarly, periplasmic binding proteins are bacterial proteins that undergo large conformational changes (so-called Venus Flytrap changes (Felder et al., 1999)) upon analyte binding (Dwyer and Hellinga, 2004) and have been engineered to sense a wide range of analytes (Ribeiro et al., 2019).

A similarly widespread approach to sensing domain design is the use of intramolecular complexation after enzymatic modification. For instance, phosphoamino acid binding domains recognize their cognate peptide only after its phosphorylation by a kinase of interest. Fusing the phosphoamino acid binding domain with a specific kinase's target

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https://doi.org/10.1016/j.biocel.2020.105761
Received 20 March 2020; Received in revised form 27 April 2020; Accepted 29 April 2020
Available online 03 June 2020
1357-2725/

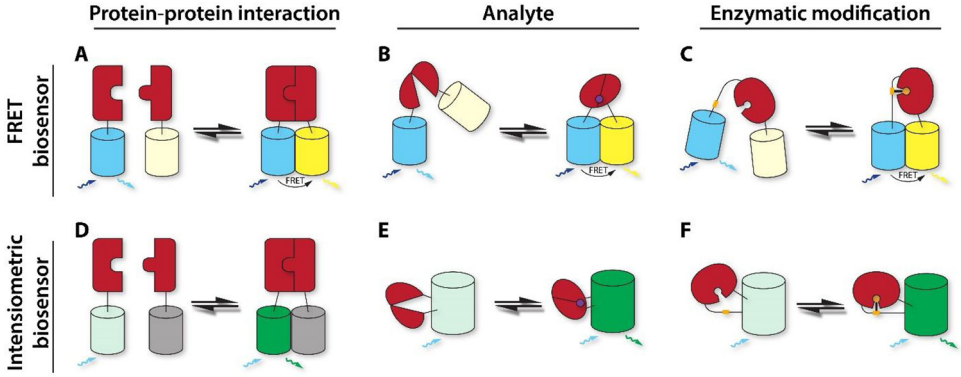

Fig. 1. Genetically encoded biosensors based on FRET or intensiometric read-out can report on protein-protein interactions, analyte binding or enzymatic modification.

peptide, constitutes a binding domain which, upon peptide phosphorylation, undergoes a conformational change (Fig. 1C, F). This design strategy is used in e.g. the AKAR (Zhang et al., 2001) and EKAR (Harvey et al., 2008) series of sensors.

Sensing domains do not need to be soluble protein domains. Voltage sensitive domains (VSDs), for instance, are membrane-bound moieties that change conformation as a result of changes in membrane potential and are frequently used to design voltage sensors (St-Pierre et al., 2015) (Fig. 5).

The diversity of sensing domains is enormous, but the general theme is that they relay a change in local molecular environment to a change in conformation or association status, which can then be exploited by a reporting domain to generate a detectable signal.

2.2. The reporting domain

The reporting domain is the part of the genetically encoded biosensor that produces a measurable signal, including but not limited to the modulation of a fluorescent read-out. The key challenge in the design of new and improved sensors is to identify a strategy by which to transduce the information captured in the sensing domain's present state to the reporting domain.

Reporting domains in genetically encoded biosensors have traditionally been comprised of one or two engineered fluorescent proteins (FPs) or parts thereof. In translocation-based sensors, a fluorescent protein is fused to a protein or protein domain whose spatial distribution is dependent on the presence of an analyte, and the localization of the fluorescence (e.g. membrane versus cytoplasm, cytoplasm versus nucleus) is the read-out signal. For instance, the strength of nuclear export signals (NESs) or nuclear localization signals (NLSs) is often influenced by the phosphorylation status of a nearby kinase target site. This system can be used to design a kinase biosensor by fusing the minimal NES or NLS to the kinase target site and a fluorescent protein. The nucleocytoplasmatic distribution of the FP is subsequently a measure of kinase activity. (Regot et al., 2014). Similarly, miniG proteins fused to a fluorescent protein translocate to the membrane upon activation of their cognate GPCR. (Wan et al., 2018)

In sensors based on Förster resonance energy transfer (FRET), the read-out is the FRET efficiency between two fluorophores (Terai et al., 2019). As the FRET efficiency is highly dependent on the distance and relative orientation between the two fluorophores, these sensors are built such that changes in the sensing domain's conformation alter the fluorophores' distance or relative orientation. Most straightforwardly, protein-protein interaction can be monitored using intermolecular FRET. In this setup, each of the interacting proteins is fused with a FRET donor or acceptor (Fig. 1A). The FRET efficiency is dependent on the distance between donor and acceptor fluorophore, as well as their relative orientation. As both the distance and relative orientation are influenced by the interaction between the fused proteins, the FRET efficiency is an excellent reporter of their interaction. One challenge of intermolecular FRET is that the donor and acceptor fluorophores may be present in different amounts, which complicates the readout of the FRET efficiency.

This challenge can be addressed by developing biosensors that make use of intramolecular FRET. These sensors are based on a single polypeptide chain with both donor and acceptor integrated in such a way that their FRET efficiency depends on the sensing domain's conformation (Fig. 1B-C). For instance, an improved version of the original Cameleon probe (called Yellow Cameleon 3.60 (Nagai et al., 2004)) consists of a circularly permuted yellow and a cyan fluorescent protein, with a calmodulin-M13 domain between them. Upon Ca^{2+}-binding, calmodulin binds the M13 peptide, bringing the cyan and yellow fluorophores closer together and/or changing their relative orientation, which results in a measureable FRET response. (Miyawaki et al., 2013)

In order to get accurate measurements from FRET-based biosensors, bleed-through between the donor and acceptor channel, slow or incomplete fluorescent protein maturation (Liu et al., 2018) or non-specific fluorescent protein oligomerization all need to be taken into account. Moreover, a considerable fraction of the biosensor contrast may not be generated by FRET, but rather by other processes that result in a reduced donor emission and/or increased acceptor emission, thought to arise via changes in the chromophore environment due to molecular interactions or local pH changes (Betolngar et al., 2015). Additionally, one sensor can already occupy a large fraction of the visible spectrum (Bajar et al., 2016; Watabe et al., 2020) which makes multiplexing multiple sensors hard. One way to address this is to combine non-fluorescent acceptors with readout of the sensor state using fluorescence lifetime imaging (FLIM), though the contrast between the different sensor states may be low (Demeautis et al., 2017). Other approaches are based on measuring homo-FRET between two identical fluorophores (Ross et al., 2018). An expansion of the FRET-based sensors is the BRET-based sensors (Salahpour, 2012), whereby the donor light is provided by a luminescent rather than a fluorescent protein.

Intensiometric sensors usually contain only a single fluorescent protein whose emission intensity is modulated by changes in the sensing domain's state. The resulting modulation can be detected as a local and reversible change in fluorescence intensity or excited-state lifetime. For instance, one of the more common design principles relies on having the sensing domain split into two constituent domains that are fused to the N- and C-terminus of a circularly permuted fluorescent protein. In another very common general design strategy, the sensing domain is fused inside the (circularly permuted) fluorescent protein's primary sequence (Fig. 1E/F). For instance, structural (Wang et al., 2008; Akerboom et al., 2009) and spectroscopic (Barnett et al., 2017) data indicates that the interaction between calmodulin and M13 in GCaMPs leads to a decrease of pK_a and concomitant increased

population of the anionic state. As the anionic chromophore is the major contributor to fluorescence, this change in chromophore environment explains the change in fluorescence intensity upon Ca^{2+} binding. Fluorescent protein-based pH sensors such as ecliptic pHluorin (Miesenböck et al., 1998) and pHTomato (Li and Tsien, 2012) rely on a change in molecular brightness that similarly depends on the protonation state of the chromophore, and thus the sensing domain is identical to the reporting domain.

The most common biosensors detect the presence of ions (such as Ca^{2+} (Pérez Koldenkova and Nagai, 2013) or H^{+} (Martynov et al., 2018)), small molecules (such as cAMP (Paramonov et al., 2015), glucose (Takanaga et al., 2008) or glutamate (Marvin et al., 2013; Helassa et al., 2018)) and enzymatic activities (such as kinases/phosphatases (Lin et al., 2019), and caspases (Zlobovskaya et al., 2016; Zhang et al., 2019)) thanks to the existence of sensing domains for which a breadth of structural and biophysical data is available. There are many great reviews available that discuss these sensors in terms of design and applications (Frommer et al., 2009; Ibraheem and Campbell, 2010; Piatkevich and Verkhusha, 2011; Palmer et al., 2011; Bizzarri et al., 2009; Tantama et al., 2012; Walia et al., 2018). Of outstanding quality because of its depth and completeness, is the 2018 review by Greenwald et al. (2018) that is accompanied by a searchable and community-driven database (https://biosensordb.ucsd.edu/index.php).

Regardless of the particular sensing or reporting domain, most sensors are based on the idea that analyte binding to or an enzymatic modification of the sensing domain results in a conformational change and a concomitant change in the reporting domain's emissive properties. As more and more sensors based upon these general design principles are being developed for broad-purpose or niche applications, new sensing domains and creative novel reporting strategies continue to emerge.

3. Novel sensing domains

3.1. GPCR-based biosensors

An interesting recent addition to the genetically encoded biosensor repertoire had been the design of intensiometric indicators of neurotransmitter-specific synaptic activity with GPCRs as sensing unit. (Patriarchi et al., 2018; Sun et al., 2018; Jing et al., 2019, 2018; Feng et al., 2019) GPCRs undergo a conformational change upon agonist binding, which is most prominent in the change in relative position of the fifth and sixth transmembrane helix, connected by the third intracellular loop (ICL3). This small conformational change can be exploited by replacing this ICL3 by a circularly permuted fluorescent protein, which results in a change of emissive properties of the fluorescent protein upon agonist binding (Fig. 2A).

The GPCR-based sensor design is modular in nature, as evidenced by the rapid development of GPCR-based sensors for different neurotransmitters (dopamine (Patriarchi et al., 2018; Sun et al., 2018), acetylcholine (Jing et al., 2018) and norepinephrine (Feng et al., 2019)). It is thus expected that sensors for other GPCR agonists, be it neurotransmitters, pheromones or chemokines or mechanical changes, will emerge in the near future. The likely development of color variants will, in combination with sensors for intracellular responses and/or secondary messengers, provide an increasingly more complete picture of the spatiotemporal regulation of cell physiology.

These sensors highlight a promising future direction for the field of genetically encoded biosensor design. Most intensiometric biosensor designs for analyte sensing are based upon sensing domains that undergo relatively large conformational changes, such as calcium binding-induced peptide occlusion in GCaMPs (Akerboom et al., 2009) or the Venus-flytrap architecture of periplasmic binding proteins (Felder et al., 1999; Sack et al., 1989). The conformational changes that are conveyed by agonist binding in GPCRs are much more subtle, in the order of a few angstroms (Rasmussen et al., 2011). This is small relative to the size of a protein but large compared to the length of hydrogen bonds, which influence the chromophore's pK_a and thereby act as the main driver of fluorescence intensity modulation.

GPCR-based biosensors provide a very convincing demonstration that small conformational changes are sufficient to yield a highly sensitive and bright read-out. Most biological interactions (between a protein and a small molecule, or between two proteins) directly result in a conformational change of the binding protein, called the induced-fit model of enzyme activity (Koshland, 1994). Taken together, these notions suggest that, at least in theory, genetically encoded biosensors could be developed for any binding or interaction event involving a proteinaceous binding partner. Of course, the fusion of a fluorescent protein or other sensing domain can result in modified binding properties in terms of affinity or specificity. Moreover, such fusions might lead to problems in terms of folding or trafficking or lead to aberrant phenotypes. Nonetheless, we envision that there a many more unexplored sensing domains available that display small but usable conformational changes upon binding or interaction.

3.2. Intracellular antibodies for biosensor design

The most straightforward way to use fluorescent proteins in cell biology is to genetically fuse them to a protein of interest involved in the process under study, either in an overexpression construct or as a genomic insertion. That way, the spatiotemporal distribution of the protein of interest can be followed using fluorescence microscopy. However, such a genetic fusion is only seldom innocuous, especially in the case of overexpression (Hanson and Ziegler, 2004; Gibson et al., 2013). Moreover, genetic fusions are not always possible, for instance when monitoring the concentration and/or distribution of non-proteinaceous molecules such as lipids (Wills et al., 2018).

An alternative strategy is to fuse the fluorescent protein to a protein that binds to the target molecule. While this approach could reduce the risk of observing aberrant phenotypes due to overexpression of the target protein (Sliogeryte et al., 2016), it depends on the availability of such binding partners, which need to be chosen or designed to not have an influence on the molecule of interest or cell physiology in general.

Intracellularly expressed antibodies, also called intrabodies, are

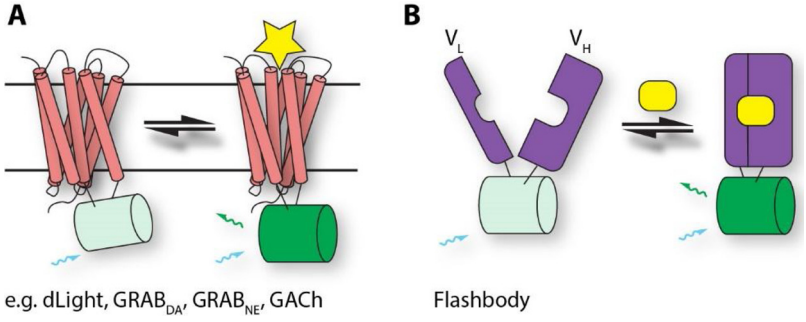

Fig. 2. *Novel sensing domains*. **A**. GPCR-based sensors consist of a GPCR (pink) with a circularly permuted fluorescent protein (pale green) fused in the third intracellular loop. Agonist (yellow) binding results in GPCR activation and a conformational change which leads to an increase in fluorescent protein brightness (dark green). **B**. The variable light and variable heavy domains (purple) of an antibody are fused to a circularly permuted fluorescent protein (pale green) that, upon analyte (yellow) binding, regains fluorescence (dark green) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

such binding partners and are attractive sensors for intracellular events when fused to a genetically encoded reporter such as a fluorescent protein (Griep et al., 1999). In theory, they can be designed in vitro to target any protein of interest, including variants with specific conformation (Irannejad et al., 2013; Cetin et al., 2017) or post-translational modification (Olson et al., 2008).

One of the biggest drawbacks of such sensors is the background signal provided by free, unbound sensors constructs. As a result, the sensor's signal and contrast is dependent on expression level of sensor and protein of interest, and the binding affinity between these; this also precludes quantitative measurements. To resolve some of these problems, a clever feedback mechanism on the transcriptional level has been designed to control sensor expression and, as a result, reduce background signal (though at the cost of very high sensor signal in the nucleus) (Gross et al., 2013). An even more attractive solution is an approach whereby a circularly permuted fluorescent protein is fused between the heavy and light chain variable region of an antibody single-chain variable region fragment. An example of such a design is the so-called Flashbody (Fig. 2B), which displays a 4-fold increase in fluorescence upon antigen binding (Wongso et al., 2017), resulting in a significant reduction in background fluorescence originating from unbound sensor. A recently developed biosensor design scaffold based on intrabodies proves to be modular and can be multiplexed across different analytes (Siciliano et al., 2018). It depends on the expression of a reporter gene, which allows the detection of multiple analytes, proteins or post-translational modifications in larger cell populations, but at the same time limits time resolution to the rate of transcription/translation and spatially to cellular resolution.

These genetically encoded biosensor designs are crucially dependent on the availability, performance and "engineerability" of one or more intrabodies for a given analyte. However, there is an enormous diversity of antibodies available as starting template, and a large body of research is going into the design and optimization of such intrabodies. They can be designed to recognize proteins for which no practically useful binding or sensing domains are available, and are highly conformationally selective (Rasmussen et al., 2011). Moreover, their overall structural similarities suggest that using anti- and intrabodies as genetically encoded biosensor scaffolds, might lead to modular sensor design (Siciliano et al., 2018). This means that intrabody-based biosensors will likely become more flexible, performant and useful in the near future.

4. Novel reporting domains

As we touched upon in the first part of this review, most genetically encoded biosensors, or at least the most widely used sensors, make use of fluorescent proteins to report changes in analyte binding or enzyme activity. However, novel reporting mechanisms are continuously being developed; we will highlight some of the ongoing developments.

4.1. Beyond GFP

One of the defining factors for biosensor performance is the molecular brightness, which depends on the one- or two-photon absorption cross-section and the fluorescence quantum yield. Not only does a higher molecular brightness lead to higher imaging sensitivity, it also allows faster imaging speeds. The obvious approach to design brighter probes is of course to use brighter FPs as reporting domain.

At the time of its publication in 2013, mNeonGreen (Shaner et al., 2013) was the brightest FPs available in the green-yellow wavelength spectrum, a position currently taken by the yet-unpublished AausFP1 (Lambert et al., 2019). Calcium sensors based on existing sensing domains, but using mNeonGreen as the reporter domain, have been coming out only recently. In general, the resulting sensors are brighter and similarly sensitive as their predecessors (Doronin et al., 2018; Zarowny et al., 2020; Subach et al., 2020). Similarly, red calcium indicators based on red FPs derived from DsRed (Zhao et al., 2011; Inoue et al., 2015; Dana et al., 2016), mRuby (Dana et al., 2016) and mKate (Shen et al., 2018) are being explored for their superior photophysical properties (Molina et al., 2019). This is done in neuron-based screens for testing their performance on reporting fast calcium transients resulting from action potential firing, additionally taking properties such as photostability, maturation and localization into account. A discrepancy between pro- and eukaryotic screens was observed during the development of the rsGreens (Duwé et al., 2015), which illustrates that though bacterial screens are a powerful way to increase brightness, they do not necessarily translate to eukaryotic expression systems. In general, biosensors' performance is best screened in an experimental setup mimicking their final use as closely as possible.

Very recently, an intensiometric calcium sensor based on the near-infrared fluorescent protein mIFP (Yu et al., 2015) called NIR-GECO1 was designed. (Qian et al., 2019) The practical use of this sensor is complicated by its low brightness (mainly because of the very low quantum yield), negative sensing mechanism (fluorescences decreases upon calcium binding) and dependence on the availability of biliverdin in the cell. However, NIR-GECO1 opens up exciting opportunities for expanded multicolor calcium imaging, for instance in combination with sensors for specific structures or other messenger molecules. Moreover, continued engineering efforts in terms of near-infrared fluorescent proteins (Oliinyk et al., 2019) and sensors using these long-wavelength emitting fluorophores will likely improve the brightness and potentially lead to (near-)infra-red sensors, which might be beneficial for imaging in deeper tissue layers, potentially in vivo.

4.2. Should it stay or should it go?

The majority of genetically encoded biosensors is designed to report on the spatiotemporal dynamics of analytes or enzymatic activity. However, experimental conditions sometimes preclude real-time monitoring, for instance during behavioral tasks in freely moving animals. CaMPARI (Fosque et al., 2015) and its improved variant CaMPARI2 (Moeyaert et al., 2018) are calcium- and light-gated optical sensors of neural activity, based on fluorescent proteins. Their red-to-green ratio is a permanent indicator of neural activity during 405-nm light irradiation, and can be read out *post-hoc*. Thanks to the development of a specific anti-red-CaMPARI antibody, the signal can be amplified using immunocytochemistry, for instance in fixed tissue. The green-to-red photoconversion is irreversible, meaning that repeated marking and read-out of the same region is barely possible. A recently reported alternative labeling approach called rsCaMPARI, however, allows marking, erasing and re-marking of the same sample using light pulses of different wavelengths (Sha et al., 2019).

Cal-Light (Lee et al., 2017) and FLARE (Wang et al., 2017) are two alternatives to the CaMPARI concept, in that they are both genetically encoded light- and calcium-gated sensors. However, their reporting domain is not necessarily a fluorescent output, but the expression of a reporter gene, which can code for any protein of choice, such as a fluorescent protein, a toxin or an optogenetic actuator. Both tools basically consist of a transcriptional activator that is, through a cleavable sequence, membrane anchored. Only after calcium-dependent recruitment of a protease (using the calmodulin-M13 system) and light-dependent unshielding of the proteolytic sequence, the transcription activator is released from the membrane. Although Cal-Light and FLARE are, just like CaMPARI, coincidence detectors of light and calcium, they serve very different purposes. First, the marking happens on a much slower time scale, which means that short behavioral spans cannot be selectively sampled, and reporter gene expression is evident only a few days after light exposure. However, the prospect of expressing any reporter gene, including optogenetic probes or toxins, creates exciting opportunities for deciphering causative relationships in neuronal networks.

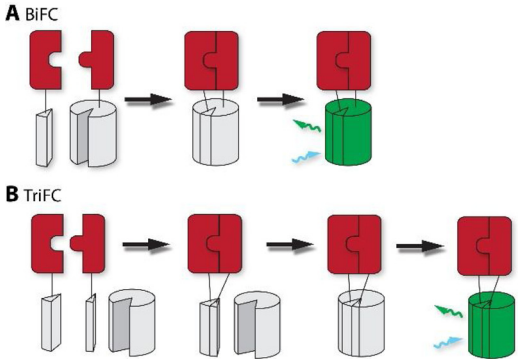

Fig. 3. *BiFC and TriFC*. **A**. In BiFC, a fluorescent protein is split in a large and a small fragment, both of them non-fluorescent on their own (grey) and fused to a bait and prey protein domain (dark red). Interaction of bait and prey protein domains results in reconstitution of the fluorescent protein, followed by maturation and the emergence of fluorescence (green). **B**. In TriFC, bait and prey protein domains are fused to two small parts of the fluorescent protein, with the large fragment unfused. Interaction of bait and prey results in reconstitution and maturation of the fluorescent protein (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

4.3. Nice to meet you

A classical method for the visualization of protein-protein interactions is bimolecular fluorescence complementation (BiFC). A fluorescent protein is split in two non-fluorescent fragments which are fused to a “bait” and a “prey” protein. When the two split FP fragment are brought into close proximity, for instance upon interaction of bait and prey, the fluorescent protein reconstitutes and becomes fluorescent (Fig. 3A). Similar complementation systems using luciferase, TEV protease or, prototypically, beta-galactosidase as reporter proteins have been extensively used (Kerppola, 2008). However, after heterodimerization, the fluorescent protein’s chromophore needs to be formed, which can take over one hour (Reid and Flynn, 1997). This makes real-time monitoring of interactions impossible. Moreover, background reassembly has been reported and the reconstituted fluorescent protein is relatively stable, which means that background can accumulate and that dynamic interactions cannot be accurately studied using BiFC (Romei and Boxer, 2019).

An improvement on BiFC is trimolecular fluorescence complementation (TriFC) (Cabantous et al., 2013), whereby the fluorescent protein is split in an unfused large fragment and two peptide fragments, to which a “bait” and a “prey” protein are bound (Fig. 3B). Only upon interaction of bait and prey with the larger unfused fragment, the fluorescent protein is reconstituted, the chromophore is formed and the protein fluoresces. This trimolecular approach largely solves the problem of background fluorescence complementation, and only short peptides have to be fused to bait and prey, potentially reducing disturbances to the system. However, the reconstituted fluorescent protein is also very stable, and consequently dynamic processes cannot be imaged. An interesting and innovative modification of the TriFC system is a caspase sensor whereby the TriFC complementation peptides are locked in an unfavorable parallel conformation, which is released by the caspase’s proteolytic activity, leading to fluorescence complementation and signal detection (Zhang et al., 2019).

Several alternative or complementary methods for sensing protein-protein interactions have been developed that circumvent (some of) the drawbacks with complementation approaches. Dimerization dependent fluorescent proteins (ddFPs) are sets of fluorescent protein mutants that form low-affinity heterodimers. One of the ddFP partners is dimly fluorescent in its monomeric form and the other is non-fluorescent because it does not form a chromophore. Upon heterodimerization, the non-fluorescent partner induces a 10- to 60-fold increase in brightness of the dimly fluorescent partner (Fig. 1D) (Alford et al., 2012a, b). Because the affinity is tuned such that no heterodimerization takes place at typical overexpression concentrations, and because the chromophores are already formed, the ddFP approach allows for real-time biosensing of protein-protein association and dissociation.

While ddFPs yield better contrast than the conceptually similar nanobody-based eGFP Enhancer or Minimizer (Kirchhofer et al., 2010), they still suffer from a limited brightness and contrast, and the need for two relatively large fluorescent protein moieties. However, genetically encoded biosensors using ddFPs rather than a FRET might be more straightforward in the design and data analysis and occupy only a single color channel, allowing multiplexing. (Hertel et al., 2020; Mehta et al., 2018) Since the green and red ddFPs are derived from the same DsRed red fluorescent protein, the authors reasoned that, by having the dimly fluorescent ddFP partners compete for the same non-fluorescent protein, a ratiometric sensor reporting domain can be constructed, which they called fluorescent protein exchange (FPX) (Ding et al., 2015).

4.4. Chemigenetic hybrids

All sensors which we described so far are based on fluorescent proteins, which fluoresce in vivo without the addition of external cofactors or chromophores and can be genetically fused, resulting in the ultimate labeling specificity. Although small molecule dyes have significant advantages over fluorescent proteins (such as a high brightness and photostability) (Toseland, 2013), they can, by themselves, not bring the labeling specificity of genetic fusions.

Self-labeling enzymes (SLEs) are proteins that irreversibly bind molecules (e.g. fluorophores) in the cell bearing specific binding groups, for instance a reactive chloroalkane linker in the case of HaloTag (Los et al., 2008) or a benzylguanine substrate in the case of SNAP-tag (Mollwitz et al., 2012). By designing organic fluorescent dyes coupled to such reactive groups, in combination with genetic expression of the SLE, it is possible to combine the superior photophysics of small molecules dyes with the labeling specificity conveyed by genetic fusions (Fig. 4A). Moreover, continued advances in the design, synthesis and performance of fluorescent dyes for live cell and in vivo imaging (such as the Janelia Fluor dyes (Grimm et al., 2015, 2016; Grimm et al., 2017)) have resulted in dyes across the visible spectrum that are bright, photostable, cell-permeant and bind their target specifically. These features make them attractive alternatives for, or complements to, fluorescent proteins.

One of caveats of SLE-based labeling approaches is background fluorescence from unbound dye, which necessitates high dye concentrations and tedious washing after labeling. As an alternative, fluorogenic dyes exist in a non-fluorescent, cell-permeable form, and upon intracellular target binding convert into a fluorescent form (Fig. 4A). A classic example of such a fluorogenic probe is silicon rhodamine (Lukinavičius et al., 2013), and several improved rhodamine-based fluorogens have been reported recently (Grimm et al., 2017; Lukinavičius et al., 2016; Grimm et al., 2013; Wang et al., 2020).

One of the earlier examples using these self-labeling enzymes for biosensor design is the SNAP-tag based indicator with a fluorescent intramolecular tether (or Snifit) sensor design (Brun et al., 2009; Masharina et al., 2012). In this innovative, though rather complicated setup, a binding event displaces a competing ligand from the active site, leading to a change in FRET efficiency. Similar designs based on a ligand-induced change in BRET efficiency have, due to their high signal, high sensitivity and straightforward read-out, been proposed as interesting probes for point-of-care diagnostics (Yu et al., 2018). The development of such sensors depends on the availability of competing ligand groups and the synthesis and cell permeability of a molecule consisting of the SLE ligand fused to a fluorophore and a competing ligand.

An alternative approach is based on fluorogen activating proteins (FAPs), which bring non-fluorescent chromophores into a fluorescent

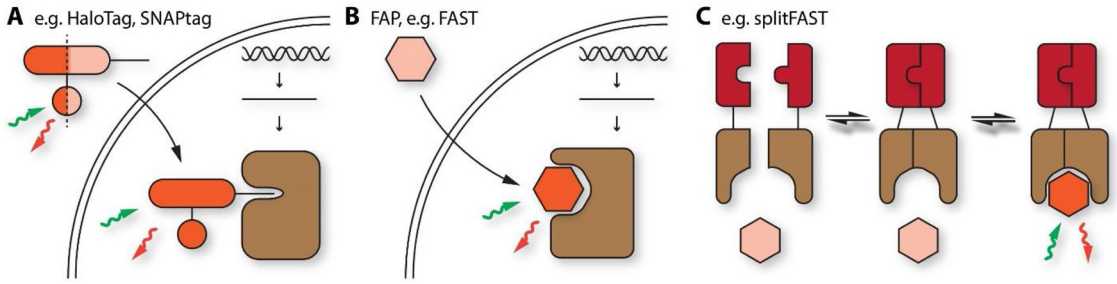

Fig. 4. *Chemigenetic hybrid reporting domains*. **A**. Self-labeling enzymes such as HaloTag and SNAPtag rely on cell-permeant dyes (orange) that specifically bind the Halo or SNAP protein (brown). **B**. Fluorogen-activating proteins such as FAST convert, through non-covalent binding, a cell-permeant molecule from a non-fluorescent (pale orange) into a fluorescent (bright orange) state. **C**. Upon interaction of bait and prey protein domains (dark red), the two splitFAST fragments (brown) heterodimerize and capture the fluorogen which leads to a fluorescent signal (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

state (Fig. 4B). An initial application of the concept relied on a single-chain antibody (scFv) with thiazole orange and malachite green as fluorogens (Szent-Gyorgyi et al., 2008). Since then, a wide range of FAPs and fluorogens has been developed (Xu and Hu, 2018; Perkins and Bruchez, 2020) and applied to biosensor design (Péresse and Gautier, 2019). Of particular interest for sensor design is Y-FAST (Plamont et al., 2016), a FAP engineered from photoactive yellow protein that, upon binding its ligand HMBR, displays high fluorogenicity and brightness. Different color variants of the original Y-FAST have recently been developed (Tebo et al., 2020). A split version of FAST, aptly called split-FAST (Fig. 4C) (Tebo and Gautier, 2019), shows great promise for further chemigenetic sensor design.

4.5. Voltage sensors take off

So far, we have mainly focused on genetically encoded biosensors that report on the presence or absence of an analyte or the detection of an enzymatic reaction product. One highly impactful type of genetically encoded sensors, however, is voltage sensors. When neurons get activated, their membrane potential flips from the resting potential of −70 mV to +40 mV, called a depolarization. Being able to visualize these depolarization events with high signal-to-noise ratio and fast kinetics, in combination with optogenetic stimulation of neurons is called all-optical electrophysiology. This set of techniques gives neuroscientists a way of studying firing behavior of large ensembles of neurons with a defined cell type simultaneously in vivo, which is nearly impossible to do using classical electrophysiology (Peron and Svoboda, 2011).

There are roughly two types of voltage sensors. One type is based on engineered microbial rhodopsins that are weakly fluorescent and change their emissive properties upon membrane depolarization (Kralj et al., 2012). However, because of their low brightness, they are hard to use in vivo. This problem has been largely circumvented by fusing a fluorescent protein donor protein to the rhodopsin, that in turn acts as an acceptor in a process called electrochromic FRET (Fig. 5A) (Zou et al., 2014). Although initially, the sensitivity of these probes was rather low, newer constructs such as Ace2N-mNeonGreen (Gong et al., 2015, 2014) and VARNAM (Kannan et al., 2018) enable in vivo imaging of neural activity. Despite these advances, voltage imaging of large populations of neurons in vivo over the timescale of behavioral events remains challenging using rhodopsin-based voltage sensors. This is due to a combination of modest brightness and rapid photobleaching of currently existing voltage sensors.

A recent innovation in the field of voltage sensor development is the use of HaloTag as reporter domain. A highly promising new chemigenetic voltage sensor, called Voltron (Abdelfattah et al., 2019a), combines the rhodopsin Ace2N (Gong et al., 2015) with HaloTag bound to a fluorogenic rhodamine dye such as a Janelia Fluor dye (Fig. 5B) (Grimm et al., 2015). Voltron can report on neural activity for several

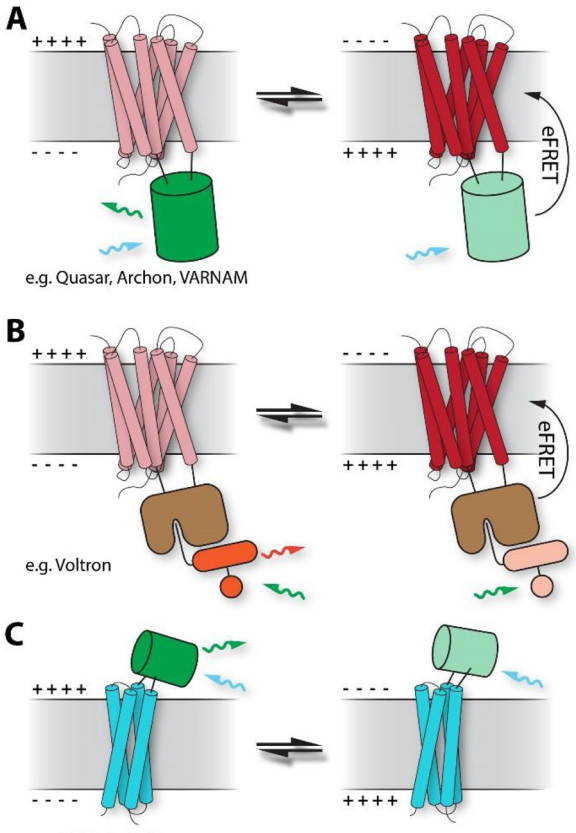

Fig. 5. *Voltage sensors*. **A**. Upon membrane depolarization, the rhodopsin changes from a less-absorbing (pale red) to a more-absorbing (dark red) state, causing the fluorescent protein (dark green) to engage in FRET, effectively reducing its brightness (pale green). **B**. In Voltron, a fluorogenic dye (bright orange) is bound to HaloTag (brown) fused to a rhodopsin such that membrane depolarization results in eFRET and a reduction in dye brightness (pale orange). **C**. A voltage-sensitive domain (blue) is fused to a circularly permuted fluorescent protein (green). Membrane depolarization leads to a conformational change in the voltage-sensitive domain, which leads to reduced fluorescence of the fluorescent protein (pale green) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

minutes in large neuron populations in vivo, with high brightness and low photobleaching. By simply selecting a different dye, the excitation and emission maxima of the probe can be selected across the visible range. The major drawback is the fact that Voltron is a negative sensor: action potentials elicit a reduction of fluorescence. However, the same group recently designed positive-going Voltron variants which they call

Positron. (Abdelfattah et al., 2019b) We expect that further engineering of rhodopsins will further increase the trafficking, sensitivity and contrast of genetically encoded voltage sensors in the years to come.

The second type of voltage sensors is based on voltage sensitive membrane protein domains which are terminally or internally fused to fluorescent proteins to result in ratiometric or intensiometric sensors of membrane potential (Fig. 5C) (St-Pierre et al., 2015; Bando et al., 2019). Replacing the fluorescent protein by (circularly permuted) HaloTag resulted, in a similar fashion as Voltron, in bright chemigenetic probes which can be color-tuned and have high photostability (Fig. 5D) (Deo et al., 2020). It is expected that also this type of voltage sensors will continue to evolve for higher contrast, sensitivity and brightness.

5. Outlook

Genetically encoded biosensors have revolutionized how processes and analytes inside the cell can be imaged with high sensitivity, specificity and/or spatiotemporal resolution. In this review, we have not tried to give an overview of what sensors are available, and how they can be used. Such an overview can be found elsewhere (e.g. Ref. Greenwald et al., 2018). Instead, we highlighted recent innovations that greatly expand what is possible with the genetically encoded biosensor toolbox.

Most sensors have focused on intracellular analytes and processes and how they influence cell physiology. Neurotransmitter biosensors and especially GPCR-based sensors, in combination with novel ways of multiplexing, will shift that focus to a more complete picture of how intra- and intercellular processes regulate cellular physiology in multicellular systems.

Continued advances in fluorescent protein technology and novel chemigenetic hybrid labeling schemes have resulted in novel and often unforeseen sensor designs. Some of these are specifically developed for niche applications, but some designs are, due to their general scope or modular buildup, bound to become new standards in the field of genetically encoded biosensors.

We are convinced that the developments in sensing and reporting mechanisms will continue to fill the genetically encoded biosensor toolbox, and will subsequently contribute to a more complete understanding of the dynamic organismal organization in healthy and diseased tissue.

Acknowledgements

B.M. holds a postdoctoral fellowship from the Research Foundation-Flanders (FWO Vlaanderen) and thanks FWO Vlaanderen for funding via grant 1514319N. P.D. thanks the FWO Vlaanderen for funding via grants G0B8817N and G090819N and the ERC for funding via ERC Starting Grant 714688 (NanoCellActivity).

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