doi:10.2533/chimia.2025.749 Chimia 79 (2025) 749–758 © E. Dressler, M. S. Frei

Chemigenetic Approaches for the Development of Fluorescent Biosensors for Biological Imaging

Elias Dressler and Michelle S. Frei*

Abstract: Fluorescent biosensors are essential for probing analyte dynamics and enzyme activities with high spatial and temporal resolution in living cells by functional microscopy. Recently hybrid, or so called chemigenetic, biosensors have emerged, that integrate the strengths of synthetic fluorophores – such as spectral diversity, high brightness and photostability – with the specificity and sensitivity of genetically encoded sensing units. Beyond enhancing optical performance, synthetic chemistry can also expand the repertoire of sensing units themselves, creating opportunities for novel biosensor designs sensing previously inaccessible analytes. In this review, we summarize the protein-labeling strategies used in chemigenetic biosensor design with particular emphasis on self-labeling protein tags. We further discuss biosensor design principles, representative applications, and emerging advances that highlight the growing impact of chemigenetic biosensors in functional microscopy.

Keywords: Functional microscopy · Protein engineering · Self-labeling protein tags · Synthetic fluorophores

Elias Dressler is a PhD student in the Frei Research Group at ETH Zurich. He received his BSc and MSc in Biotechnical Chemistry from the Technical University of Ilmenau in Germany. He completed his Master's Thesis at EPFL in the Laboratory of Biomedical Microfluidics, where he continued to work until starting his PhD in October 2024. His current research focuses on the development of chemigenetic biosensors for the intracellular measurement of enzyme activity.

Michelle S. Frei is a Tenure Track Assistant Professor in the Laboratory for Organic Chemistry at ETH Zurich. She obtained her PhD in Chemistry from EPFL in 2020, where she worked on synthetic fluorophores and labeling systems for advanced fluorescence microscopy. After a follow-up Postdoc at the MPI for Medical Research in Heidelberg she completed a Postdoc at the University of California San Diego, working on protein engineering for biosensor development. The Frei Research Group opened its doors in 2024 and focuses on the development of chemical tools for molecular imaging.

1. Bridging Two Worlds: Chemigenetic Strategies for Biological Imaging

To unravel the complexity of living systems, it is essential to study dynamic cellular processes in real time. Traditional biochemical methods, however, fail to capture the spatiotemporal dynamics of cell signaling and metabolic processes as they only provide snap shots and averaged information. Fluorescent biosensors in combination with fluorescence microscopy address this challenge by enabling real-time visualization of analyte concentrations and enzyme activities.[1,2] Biosensors generally consist of two main units: a sensing unit that interacts with the target analyte and a reporting unit that generates a fluorescent signal. Upon interaction of the analyte with the sensing unit, such as a binding event (Fig. 1A) or an enzymatic modification (Fig. 1B), the signal is transmitted, often through a conformational change, to the reporting unit, leading to a detectable change in fluorescence. This can take the form of a change in fluorescence intensity, spectral properties, Förster resonance energy transfer (FRET), fluorescence lifetime, or signal location. While the sensing unit is crucial to the sensitivity and selectivity of the biosensor, the photophysical properties of the reporting unit also impacts the overall performance.[3]

The first biosensors used to visualize real-time biological processes using fluorescence microscopy were of pure synthetic nature. These so-called chemosensors remain highly impactful to this day with some of the most commonly used Ca^{2+} biosensors being derived from fluorescent analogous of 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), developed by Roger Tsien in the 1980s.[4,5] As chemosensors utilize synthetic fluorophores they offer excellent photophysical properties. However, they are often limited in their cell membrane permeability and can be difficult to target to specific tissues or subcellular locations.[6,7] Hence, when fluorescent proteins were introduced in the 1990s, the field of biosensors started to shift towards fully genetically encoded biosensors, which combine fluorescent proteins with protein-based sensing units.[8,9] These biosensors can be expressed directly in living cells or organisms, enabling cell-type and organelle-specific imaging. They have no bioavailability issues and can utilize the large existing repertoire of highly selective binding domains from nature.[10,11] However, fluorescent proteins are limited in their brightness and photostability and are not readily available in the far-red spectral region.[10]

Since both chemosensors and genetically encoded biosensors have limitations, efforts to combine their strengths have long been pursued. Combination of synthetic and protein-based components could expand the range and specificity of available biosensors, improve their sensitivity, dynamic range, and spectral properties, as well as enhance their compatibility for multiplexing.[12–14] For instance, reporting units could be freely chosen from fluorescent

*Correspondence: Prof. Dr. M. S. Frei, E-mail: michelle.frei@org.chem.ethz.ch
Department of Chemistry and Applied Biosciences, ETH Zürich, CH-8093, Zürich

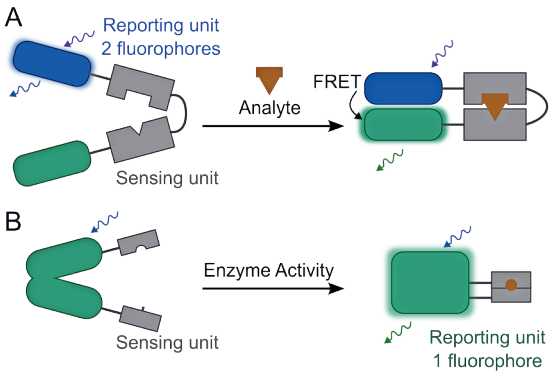

Fig. 1. Common biosensor designs. A) Biosensor using two fluorophores as a reporting unit. Analyte binding to the sensing unit results in a conformational change leading to an increase in FRET efficiency. B) Biosensor relying on a single fluorophore as the reporting unit. After the sensing unit is modified by an enzyme, the fluorophore changes its fluorescence intensity. Sensing units (binding *vs.* enzymatic) and reporting units (one *vs*. two fluorophores) can be mixed and matched.

proteins and synthetic fluorophores. The latter often offer superior photophysical properties, such as higher brightness, greater photostability, and extended spectral range that covers the far-red or even infrared region. Using synthetic fluorophores instead of fluorescent proteins also avoids issues such as slow and oxygen dependent maturation of fluorescent proteins.[15] Similarly sensing units can be selected from protein-based switches or synthetic recognition motifs covering many more analytes. While for many years such biosensors could only be constructed *in vitro*, the advent of efficient bioorthogonal protein labeling strategies has made it possible to generate hybrid or so called chemigenetic biosensors *in situ*. In this review, we discuss the protein labeling strategies that have been successfully used to make biosensors and highlight those that are promising to advance the field in the future. We then discuss the most common biosensor designs and their applications in functional microscopy. Lastly, we emphasize current challenges and potential future avenues of the field. Approaches where fully synthetic biosensors (reporting and sensing unit) are subcellularly targeted *via* a genetically encoded component are beyond the scope of this article and are described elsewhere.[16]

2. Synthetic Fluorophores used for Biosensor Generation

The main strengths of chemigenetic approaches lie in taking advantage of the photophysical properties of synthetic fluorophores. We therefore include a brief note about synthetic fluorophores but refer interested readers to the following reviews by ourselves and others.[17–19] While most protein labeling systems are compatible with a wide range of fluorophore classes, including cyanines, oxazines, and boron dipyrromethene (BODIPY) dyess, rhodamines remain the predominant choice.[20,21] Rhodamines are a family of xanthene-based fluorophores that offer high brightness and photostability, as well as cell membrane permeability, making them the preferred choice for intracellular applications. They are available in various spectral regions, also far-red, which is beneficial for biological imaging.[22] Another key advantage is their fluorogenicity, based on the open-close equilibrium between the non-fluorescent lactone **1** and the fluorescent zwitterion **2**, which minimizes background signal from the unreacted fluorophore (Fig. 2A). Fluorogenicity can additionally be harnessed as a sensing mechanism to report on changes in the environment. This is also why other environmentally sensitive fluorophores such as the amino acid derivative 3-(6-acetylnaphthalen-2-ylamino)-2-aminopropionic acid (ANAP (**3**), Fig. 2B) or the 4-hydroxybenzylidene rhodanine derivative HBR-3,5DOM ((**4**), Fig. 2C) have often been implemented in chemigenetic biosensors. Various types of environmentally sensitive fluorophores exist, changing their fluorescence properties according to different parameters *e.g.* polarity, pH, or viscosity.[23,24] With optimized and tailor-made fluorophores being developed at high speed, the chemigenetic toolbox is constantly being expanded in diverse directions allowing us to investigate more complex biological phenomena.[25]

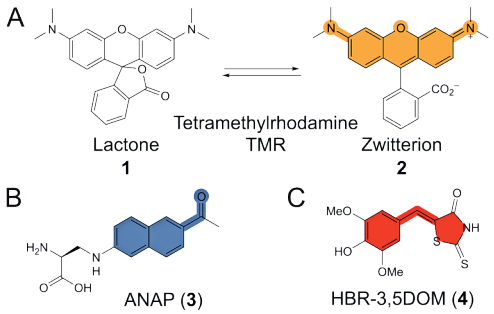

Fig. 2. Structures of common fluorophores. A) Tetramethylrhodamine (TMR) in the non-fluorescent lactone **1** and fluorescent zwitterionic form **2**. B) Environmentally sensitive fluorescent amino acid ANAP (**3**). C) Rhodanine derivative HBR-3,5-DOM (**4**).

3. Protein Labeling Methods used for Chemigenetic Biosensor Design

Proteins can be labeled using various methods. They differ in their specificity and labeling kinetics, rely on tags of different size, and use reagents that have different cell membrane permeability.[26] In the following we discuss the strategies used to generate chemigenetic biosensors starting with those of minimal 'tag' size.

3.1 *Small Molecule-based Protein Labeling*

Early chemigenetic biosensors relied on protein labeling of surface exposed primary amines (lysine **5**, *N*-terminus) *via* the reaction with isothiocyanates **6** or *N*-hydroxysuccinimide (NHS) esters **7** (Fig. 3A) or the reaction of thiols (cysteine **8**) using iodoacetamides **9** or maleimides **10** (Fig. 3B).[27] Indeed, isothiocyanate labeling of the regulatory and catalytic subunit of protein kinase A was used as early as the 1990s to generate a FRET biosensor for cyclic adenosine monophosphate (cAMP).[28] Similarly, environmentally sensitive fluorophores were incorporated in proteins exhibiting suitable conformational changes giving access to early biosensors.[29] However, the reactivity of the chemical reagents used in these approaches are non-specific. The conjugation must therefore be performed on purified protein *in vitro* and if live-cell measurements are desired the modified proteins should then be delivered into cells, typically through microinjection[30,31] or using cell-penetrating peptides.[32]

One approach to improve labeling specificity is traceless affinity-based protein labeling *via* ligand directed chemistry, where a fluorophore carrying an unspecific reactive linker is guided to the desired site using a specific ligand.[33,34] Once bound to the protein of interest a nearby nucleophile reacts with the linker, releasing the ligand (Fig. 3C), allowing endogenous proteins to be labeled and turned into biosensors.[35–37] Aside from isolated reports of intracellular biosensors[38] this work has mostly been employed to label cell surface proteins. Moreover, selecting the right fluorophore, ligand, and labeling site to produce a measurable signal remains challenging and often needs extensive optimization.[36] More modern approaches make use of truly bioorthogonal label-

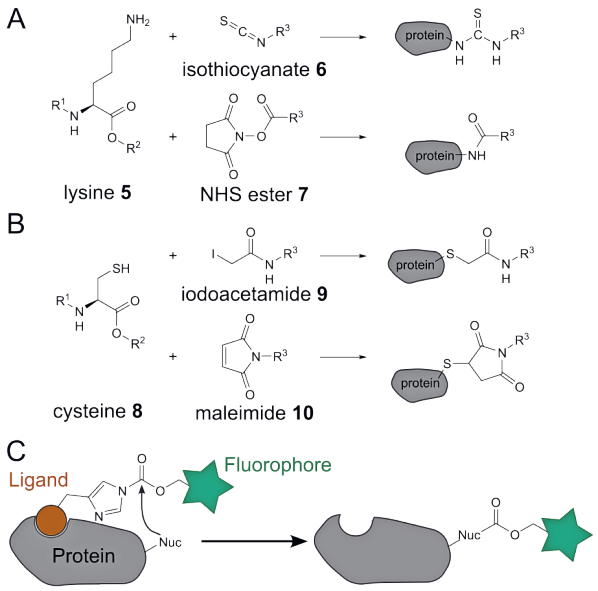

Fig. 3. Common small molecule-based protein labeling strategies. A) Labeling of lysine **5** residues or *N*-terminus *via* isothiocyanate **6** or NHS esters **7**. B) Cysteine **8** labeling using iodoacetamide **9** or maleimide **10** chemistry. C) Ligand directed chemistry to install a fluorophore on an endogenous protein using a cleavable acyl imidazole linker.

ing reactions, but these require the installation of the specific reactive groups at the desired position first.

3.2 *Genetic Code Expansion*

An alternative approach for site-specific protein labeling is genetic code expansion. In this approach, non-canonical amino acids (ncAAs) are incorporated into the protein of interest at defined positions during translation by introducing an orthogonal aminoacyl-tRNA/tRNA synthetase pair that recognizes the ncAA and reads a reassigned codon, usually the amber stop codon (Fig. 4A).[39] Using this approach fluorescent ncAAs can be incorporated directly into proteins.[40,41]

The environmentally sensitive fluorophore ANAP (**3**) (Fig. 2B) has been used to probe the conformational change of ion channels[42,43] in *Xenopus laevis* and mammalian cells. Similarly, ANAP (**3**) has served as a FRET donor together with fluorescent proteins.[44,45] More recently, genetic code expansion was used to incorporate a fluorescent molecular rotor into a protein scaffold generating a fluorescent protein, which was then turned into a biosensor for Ca^{2+}.[46] However, the range of fluorescent amino acids that can be incorporated *via* a suitable aminoacyl-tRNA/tRNA synthetase pair is currently limited. Most suitable fluorophores emit in the short-wavelength region and/or contain hydrophobic fluorophores that can interfere with the folding of proteins.[40]

To circumvent this challenge, genetic code expansion can be used in a two-step process, incorporating a ncAA carrying a small bioorthogonal click handle that is later reacted with a complementary handle bearing an appropriate fluorophore.[44,47] Among the available click-reactions, the inverse electron demand Diels-Alder (IEDDA) reaction between a dienophile such as cyclooctyne **11** (Fig. 4A) and tetrazine **12** is often the preferred method as it has faster labeling kinetics than strain promoted azide-alkyne cycloaddition and does not use cytotoxic Cu(I) as in copper catalyzed azide-alkyne cycloaddition. This two-step labeling procedure allows the incorporation of larger fluorophores with better photophysical properties or other functional handles and was used to generate a FRET biosensor.[48] However, the application of genetic code expansion to biosensor design is limited due to its technical complexity such as inefficient incorporation of the ncAA and disturbances of native signaling and metabolism. However, the minimal tag size offered makes it a promising approach for future biosensor generations.[49–51]

3.3 *Peptide Tags*

Peptide-based recognition tags enable the site-specific labeling of proteins *via* sequence-dependent reactivity, facilitating the selective conjugation of synthetic moieties. Peptide tags can easily be fused to proteins of interest and due to their small size the risk of disrupting protein function is minimal.[52] Several peptide tags can be directly labeled with a fluorophore. A prominent example is the tetracysteine motif (CCXXCC), which reacts covalently with organo-bisarsenic compounds, such as fluorescein-arsenical-helix binder (FlAsH (**13**), Fig. 4B).[53] FlAsH can be used as a FRET acceptor and was incorporated into a G protein-coupled receptor (GPCR) biosensor in combination with a cyan fluorescent protein for investigation in living cells.[54] Other similar strat-

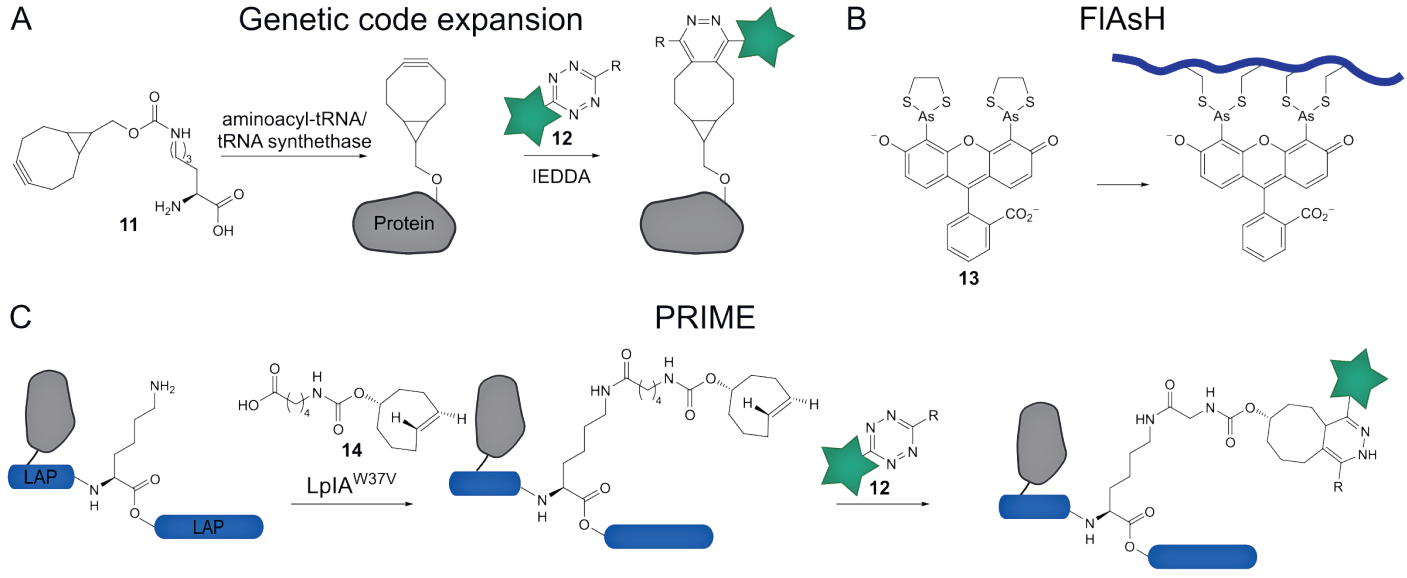

Fig. 4. A) Genetic code expansion reassigns a codon to an orthogonal tRNA–synthetase pair, enabling incorporation of ncAAs carrying fluorophores or click handles. Here cyclooctyne **11** reacts with tetrazine **12** *via* IEDDA. B) Structure of CCXXCC reactive fluorophore FlAsH (**13**). C) PRIME using $LplA^{W37V}$ to introduce a clickable lipoic acid derivative bearing a *trans*-cyclooctene **14**, which is then reacted with tetrazine **12**.

egies often do not show good cell membrane permeability and only find applications on the cell surface.[26]

Enzyme-mediated labeling strategies or probe incorporation mediated by enzymes (PRIME) leverage the sequence specificity of enzymes such as ligases or transferases to selectively install reporting units (one-step) or reactive handles (two-step) on a protein of interest. The most frequently used peptide tag to generate biosensors is based on an engineered lipoic acid ligase (LplA) that recognizes a 13 amino-acid LplA acceptor peptide (LAP) to which it attaches a substrate carrying a fatty acid handle. Instead of incorporating a fluorophore directly, the system can also be used to incorporate click handles such as trans-cyclooctene **14** for IEDDA reaction with tetrazine **12** (Fig. 4C).[55] This two-step approach has successfully been used to generate electrochromic voltage biosensors on the cell surface.[56,57] Although the small size of peptide tags is attractive, several of these approaches require additional enzymatic partners, or heavy metals that are toxic to the cells.[58] This restricts their use to the cell surface and limits their application in chemigenetic biosensor development.

3.4 *Protein Tags*

To enable specific protein labeling inside of living cells, tags based on entire proteins are commonly used. While they undergo highly specific and fast labeling reactions, they are much bigger than the labeling methods discussed so far. When integrating them with protein-based sensing units, the underlying protein scaffold needs to be manipulated to allow for efficient coupling between the sensing and reporting units. One common approach is to fuse sensing units to the C- or N-terminus of proteins. However, if these termini are spatially distant from the fluorophore-binding site it is often beneficial to generate circularly permuted (cp) protein versions, repositioning the termini closer to the ligand binding site.[59] Alternatively, sensing units can be inserted into a permissive position proximal to the fluorophore-binding site for which suitably flexible sites need to be identified. In the following the most commonly used protein tags and their cp and split variants are discussed.

3.4.1 *Noncovalent Protein Tags*

The antibiotic trimethoprim (TMP (**15**)) selectively and strongly binds to *E. coli* dihydrofolate reductase (eDHFR, Fig. 5A). This strong interaction can be used as a tag in which eDHFR is fused to the protein of interest and TMP (**15**) conjugated to a fluorescent probe.[60] This method can be used for labeling in living cells.[61] Moreover covalent versions of the TMP-tag have been introduced where a strategically placed cysteine reacts with a Michael acceptor installed on TMP (**15**).[62–64] A fluorogenic variant was also generated.[63] So far the TMP-tag has rarely been used in biosensor design.

FAST (Fluorescence-Activating and absorption-Shifting Tag)[65] was developed from photoactive yellow protein[66] and noncovalently binds small-molecule fluorogens such as 4-hydroxy-bezylidene rhodanine (HBR (**16**)), which are non-fluorescent under physiological conditions and become fluorescent only when deprotonated upon binding to FAST (Fig. 5B). Various color variants of HBR (**16**) and corresponding FAST variants have been developed,[67,68] some of which allow for orthogonal labeling and spectral multiplexing.[69] Moreover, many rhodanine-FAST combinations show different fluorescence lifetimes enabling multiplexing in the lifetime dimension.[70,71] Topological variants such as splitFAST were engineered by dividing the protein into two fragments that reassemble into a functional FAST when brought into close proximity, enabling fluorescent readout of transient protein–protein interactions.[72] Similarly, a cpFAST was engineered that allows the attachment of sensing units at new termini, leading to conditional binding of the rhodanine.[73] While FAST (14 kDa) is significantly smaller than other protein tags, it also relies on a specific fluorophore and is hence not as versatile as covalent tags that can be used with various fluorophore classes.

3.4.2 *Covalent Protein Tags – Self-labeling Proteins*

Self-labeling proteins (SLPs) overcome many limitations of peptide tags as they directly react with small-molecule ligands in a bioorthogonal and covalent manner, eliminating the need for separate enzymes or toxic cofactors.[74,75]

SNAP-tag and the faster-labeling derivative SNAPf (E30R) use a reaction between an active site cysteine and an O^6-benzylguanine (BG (**17**)) derivative to form a covalent bond between the protein and the chemical component (Fig. 5C).[75–77] In combination with synthetic fluorophores this has been extensively used for protein labeling and fluorescence microscopy in living cells. However, in recent years it has become clear that SNAP-tag is often outperformed by HaloTag due to its slower labeling kinetics, reduced fluorogenic response, and the limited cell membrane permeability of SNAP-tag ligands.[78] An improved variant, SNAP-tag2, that uses a modified ligand, shows increased stability and a 100-fold higher labeling rate than SNAP-tag, making it one of the most robust SLPs available for chemigenetic biosensor design.[79] To obtain an orthogonal SLP allowing simultaneous labeling of multiple proteins, the Johnsson lab engineered CLIP-tag from SNAP-tag by altering its ligand recognition to accept O^2-benzylcytosine derivatives instead of BG (**17**).[80] cp variants for biosensor design were generated[81] and both SNAP-tag and CLIP-tag have been split at various sites and used for applications such as chemical dimerization or in bimolecular fluorescence complementation (BiFC) assays.[82–84]

HaloTag7 was derived from a bacterial dehalogenase and is the most widely used SLP for applications in live-cell imaging and biosensor design.[85] HaloTag covalently reacts with chloroalkane (CA (**18**)) enabling the irreversible attachment of various fluorophores or chemical handles to proteins (Fig. 5D).[86] HaloTag is currently the fastest SLP, showing labeling speeds of up to $10^8\ M^{-1}s^{-1}$ with rhodamines but reacting slower with other fluorophores.[87,88] Protein variants with different fluorophore specificities such as styrylpyridinium derivatives,[89] benzothiadiazole-based fluorophores,[90] or negatively charged fluorophores[91] have been developed. In addition, the access tunnel of HaloTag has been investigated to better understand ligand specificity and labeling efficiency.[92] Incorporation of an aromatic ring into the classical HaloTag ligand accelerated protein binding, especially with non-rhodamine ligands.[93]

Further HaloTag variants were engineered to modulate the fluorescence properties of the bound fluorophore. Frei *et al.* pioneered this approach providing HaloTag variants with altered fluorescence lifetimes when labeled with one and the same fluorophore. This enabled multiplexing *via* fluorescence lifetime imaging microscopy[94] and inspired the design of chemigenetic lifetime biosensors.[95,96] These findings further open up new and interesting avenues for tool engineering, some of which are currently pursued in the Frei Research Group.

HaloTag tolerates circular permutations at various sites, particularly in the lid region where the fluorophore is located.[97] One site (143/144) has been identified as particularly valuable for the generation of biosensors.[98] Insertion of protein domains is also possible at the same site or in the loop between residues 155–156 or at R179.[96,99,100] Multiple flexible loops were also identified as suitable split sites of HaloTag and the resulting constructs used for BiFC assays.[101,102]

More recently asymmetrical split HaloTags were introduced where cpHalo(154/156) was split into a short peptide (145–154) and a large fragment cpHaloΔ(141/156).[103] The large fragment was later reengineered to exhibit improved thermostability and activity (cpHaloΔ2).[104] Similarly, a high affin-

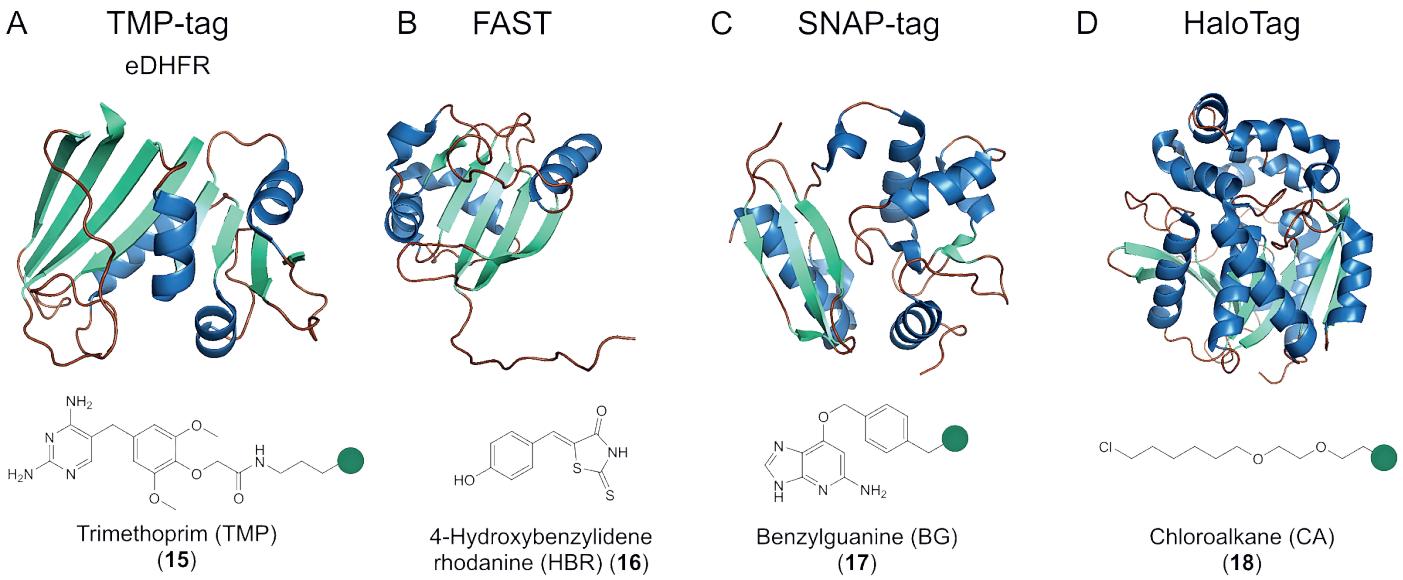

Fig. 5. Structures of the four most common protein tags along with the molecular structure of their ligands. A) TMP-tag based on the 18 kDa eDHFR which binds TMP (**15**) noncovalently (PDB ID: 5DFR). B) 14 kDa FAST and its binder HBR (**16**) which only becomes fluorescent upon binding to the protein tag (PDB ID: 7AVA). C) SNAP-tag (19 kDa) where the active site cysteine attacks the benzylic position of BG (**17**) forming a covalent bond (PDB ID: 6Y8P). D) HaloTag (33 kDa) in which D106 displaces the chlorine atom in the CA (**18**) in a nucleophilic attack forming a covalent ester bond (PDB ID: 6Y7A). α-helices in blue, β-sheets in cyan and loops in brown.

ity system cpHaloΔ3 in combination with several peptides was introduced.[105]

Although covalent labeling of HaloTag has multiple advantages, reversible binding to circumvent photobleaching or allow blinking has attracted attention in recent years. To address these needs, non-covalent versions of the HaloTag system have been introduced by either modifying the HaloTag protein or the CA ligand (**18**).[106,107] These systems could also help to tackle photobleaching in biosensor applications. Taken together, the HaloTag system has evolved into a versatile imaging platform capable of supporting a variety of biosensing applications.

4. Most Common Designs of Chemigenetic Biosensors

Protein tags can be selectively and efficiently labeled, even directly inside living cells. They are therefore frequently used to bridge the chemical and genetic components in chemigenetic biosensors. Here, we discuss the most common biosensor design strategies and their applications in functional microscopy.

4.1 *Chemigenetic Approaches to Introduce Chemical Sensing Units*

Synthetic organic chemistry can be exploited to build sensing units that do not exist in nature. Chemigenetic approaches can be used to attach them close to a fluorophore to modulate its fluorescence. The Campbell lab has recently introduced biosensors based on cp green fluorescent protein (cpGFP) inserted into HaloTag labeled with Ca^{2+} or Na^{+} chelating moieties such as BAPTA-CA (**19**)[100] or crown ethers (Fig. 6A).[108] The chelator is in close proximity of the GFP chromophore and metal binding induces a change in fluorescence intensity.

Furthermore, most fully genetically encoded biosensors are based on conformational changes in their protein-based sensing unit, however, not all sensing units show large conformational changes. To expand sensing modalities to other ligands, the competitive binding of a tethered ligand can be used. SNAP-tag-based indicators with a fluorescent intramolecular tether (SNIFIT), introduced by the Johnsson lab,[109] combine two SLPs (*e.g.* SNAP and CLIP-tag) within a biosensor. One SLP is labeled with a FRET donor, an intramolecular tether as well as a small-molecule ligand that can bind to a protein-based binding domain also present in the biosensor (Fig. 6B). For instance, in a NADPH/$NADP^{+}$ SNIFIT, the SNAP-tag was labeled with CP-TMR-SMX **20**, which consists of O^4-benzyl-2-chloro-6-aminopyrimidine (CP), TMR as the FRET donor and sulfamethoxazole (SMX) as a protein binder. The other SLP is labeled with the FRET acceptor siliconrhodamine-CA (SiR-CA (**21**)). The presence of the analyte either displaces the intramolecular ligand or enables its binding to the binding domain. This results in a large conformational change, inducing a change in FRET efficiency. SNIFIT biosensors have been used on the cell surface[110] and were later diversified to intracellular analytes such as glutamate[111] and GABA.[112] More recently biosensors based on different SLP combinations (*e.g.* SNAP-tag and HaloTag) as well as cross-overs with GFP have been used to generate biosensors for nicotinamide adenine dinucleotides[113] and coenzyme A.[114] The use of SLPs to introduce an intramolecular tether to the sensing unit allows the artificial generation of protein switches with large conformational changes, accessing analytes for which no suitable protein-based sensing units are available. However, these biosensors require dual labeling which can be challenging for the rather large component consistent of a fluorophore-tether and small-molecule binder as the increased size often leads to reduced cell-membrane permeability and therefore inefficient labeling.

4.2 *Chemigenetic Approaches for FRET-based Biosensors*

Protein tags can be implemented in FRET based biosensors to replace either one or two fluorescent proteins. Protein tags allow various FRET pairs to be rapidly tested, including several fluorophores in the far-red region. For instance, the replacement of the yellow and cyan fluorescent proteins in kinase and Ca^{2+} sensors with SNAP-tag and HaloTag allowed Vecchia *et al.* to generate tunable biosensors with donor excitations ranging from 503–585 nm and acceptor excitation from 549–646 nm.[115] While the sensor performance depended on the specific sensor and the FRET pair used, replacement of fluorescent proteins in existing sensor backbones gave rapid access to red-shifted biosensors. However, often the resulting changes in the FRET ratio upon sensor activation were small and required further engineering, reflecting the different topology of SLPs compared to fluorescent proteins. This

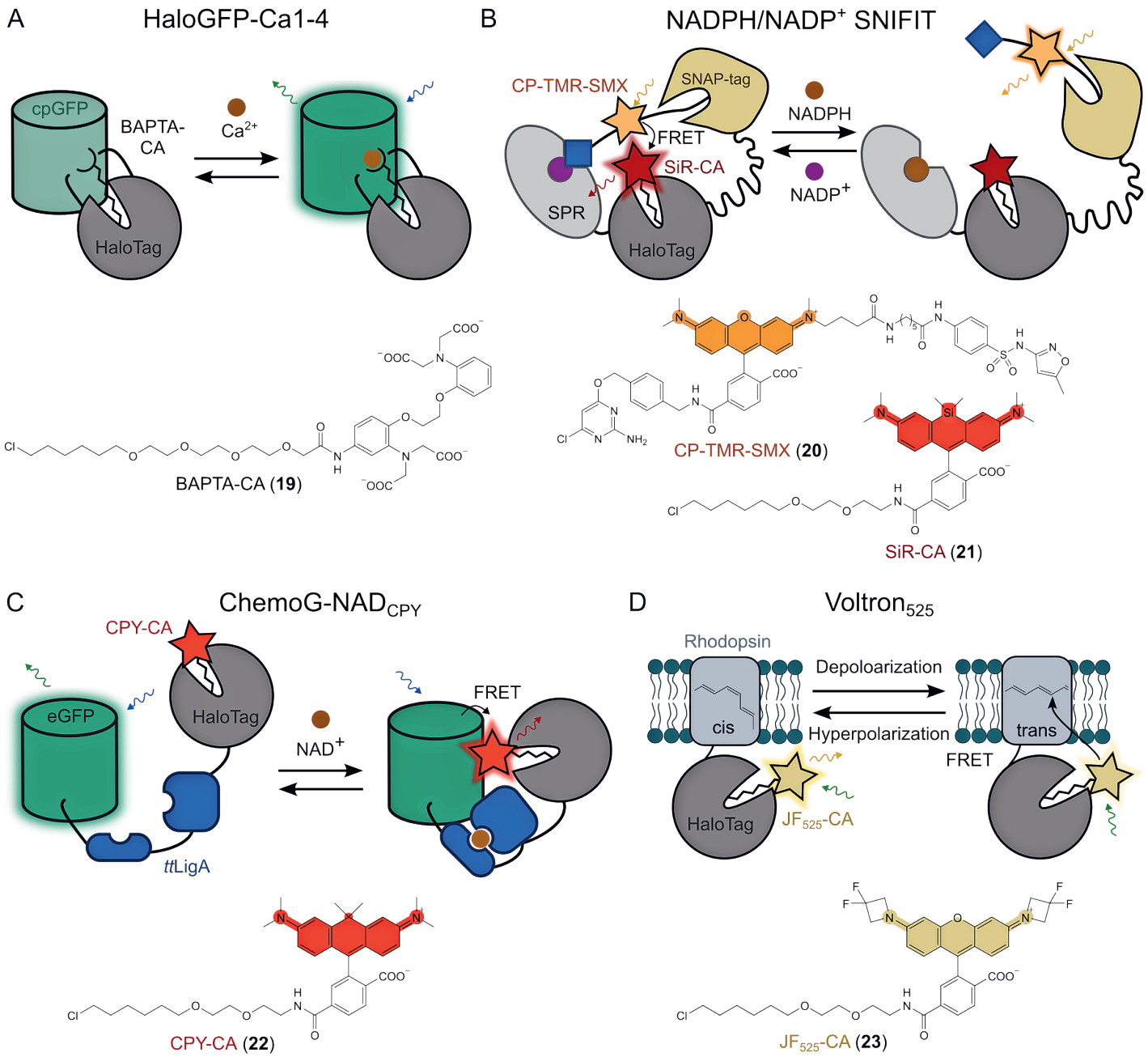

Fig. 6. Protein tags used in chemigenetic biosensors. A) HaloTag is used to bring the synthetic Ca^{2+} chelator BAPTA-CA (**19**) into close proximity of cpGFP. Binding of Ca^{2+} leads to an increase in fluorescence intensity. B) NADPH/$NADP^+$ SNIFIT based on SNAP-tag and HaloTag and the NADPH/$NADP^+$ binding protein SPR (sepiapterin reductase). SNAP-tag carries the intramolecular tether (CP-TMR-SMX **20**) consisting of the SNAP-tag ligand CP, TMR as the FERT donor and the SPR binder SMX. HaloTag is labeled with the FRET acceptor SiR-CA (**21**). C) ChemoG-NAD_{CPY} biosensor making use of high FRET efficiencies between eGFP and CPY-CA (**22**) labeled HaloTag. Binding and unbinding of NAD^+ to catalytically inactive Thermus thermophilus DNA ligase A (*tt*LigA) leads to a change in FRET efficiency. D) Voltage biosensor $Voltron_{525}$ which relies on FRET between JF_{525}-CA (**23**) labeled HaloTag and the retinal of rhodopsin.

challenge was circumvented by the development of a highly efficient FRET pair formed between *Aequorea Victoria* fluorescent protein homologs such as GFP as FRET donors and a rhodamine labeled HaloTag as a FRET acceptor. Insertion of sensing units for Ca^{2+}, ATP, and NAD^+ between the two FRET partners allowed the generation of chemigenetic biosensors with high dynamic ranges and minimal effort for optimization.[95] Moreover, the synthetic fluorophore could be switched easily giving access to different color variants such as the carbopyronine-CA (CPY-CA (**22**)) labeled NAD^+ biosensor with a dynamic range of almost 20-fold (Fig. 6C). As the high FRET efficiency is based on the formation of an interface between the labeled HaloTag and an *Aequorea Victoria* fluorescent protein homolog, red fluorescent proteins which are not derived from *Aequorea Victoria*, could not be implemented with the same success.[95,116]

FRET-based biosensors can also be generated using the FAST system, whose small size (14 kDa) allows for short distances between the FRET pair, enhancing FRET efficiencies. A promiscuous version of FAST (pFAST) enabled the rapid screening of different fluorogens as optimal FRET acceptors for the fluorescent proteins mTurquoise2 and eGFP. Both the red HBR-3,5DOM (**4**) (520/600 nm) and the dark acceptor HBIR-3M (514 nm) were identified and implemented in a biosensor for Aurora kinase A.[117]

Another type of biosensor that has benefited from replacing a fluorescent protein by HaloTag is the electrochromic voltage sensor based on microbial rhodopsin. Changes in membrane po-

tential modulate the extinction coefficient of the bound retinal chromophore *via* a *cis-trans* isomerization. The labeled HaloTag acts as a FRET donor and depending on the membrane potential varying FRET efficiencies can be measured. For instance, the green-orange fluorophore Janelia Fluor 525-CA (JF_{525}-CA (**23**)) can be used for the generation of a highly photostable biosensor called Voltron.[118] Later positive-going,[119] more sensitive,[120,121] and more photostable variants were presented.[122]

Protein tags serve as ideal platforms to introduce and rapidly test various fluorophores in FRET-based chemigenetic biosensors as no additional cloning steps are required. This versatility allows us to generate tailormade biosensors with ideal properties for multiplexing experiments (*e.g.* far-red) or photostable FRET donors for prolonged or high frequency measurements. Additionally, spectral fine-tuning allows the access of high-performance biosensors with optimized FRET efficiencies and high dynamic ranges.

4.3 *Chemigenetic Approaches for Single Color Biosensors*

Protein tags can also be used to replace fluorescent proteins in single fluorescent protein biosensors in which the attached sensing units modulate the photophysical and/or spectral properties of the fluorescent protein chromophore, relying on coupling between the reporting unit and the sensing unit. This is often specific for individual biosensors and only few generalizable concepts have been identified. For chemigenetic biosensors three main design principles have been pioneered: conditional binding of fluorogen, influencing fluorogenicity, and changes in quantum yield. The single color of these biosensors makes them especially suitable for multiplexing experiments to investigate multiple dynamic biological processes at the same time.

First, conditional binding of 4-hydroxy-3-methyl-benzylidiene-rhodanine (HMBR (**24**)) to cpFAST has been exploited to generate a biosensor for Ca^{2+} *via* the fusion of M13 and calmodulin a typical calcium sensing unit (Fig. 7A).[73] The same concept was later expanded to glutamate, K^+, and ATP but the generated sensors were not tested for live-cell microscopy.[123] Similarly, splitFAST has been used to generate biosensors as the two halves, NFAST and CFAST, as well as the fluorogen bind reversibly.[72] This can be exploited to study protein complex formation and Ca^{2+} signaling.

Second, the fluorescence of fluorogenic rhodamines can be modulated by the attachment of suitable sensing units. Sensing leads to a shift in their open-close equilibrium, impacting the apparent extinction coefficient and hence the fluorescence intensity. Appending the calcium sensing unit M13 and calmodulin to cpHaloTag(143/144) allowed the generation of a powerful Ca^{2+} biosensor HaloCaMP with an intensiometric response.[98] Around the same time a ratiometric biosensor rHCaMP[99] was generated that required labeling with a specialized color shifting rhodamine derivative instead of the fluorogenic Janelia Fluor 635-CA (JF_{635}-CA (**25**)).[124] Similarly, the voltage sensitive biosensors HASAP and HArcLight were generated using different topologies of HaloTag in combination with JF_{635}-CA (**25**).[98] A similar approach to HArcLight was previously reported but using the non-fluorogenic TMR-CA.[125] More recently, the use of fluorogenic rhodamines and HaloTag was extended to other small molecule analytes, replacing the Ca^{2+} specific sensing unit by the two halves of OxyR to generate a H_2O_2 biosensor[126] or by a G-protein coupled receptor giving access to a far-red biosensor for dopamine.[127]

Frei *et al*. furthermore demonstrated that not only small molecules could be sensed but also enzyme activity by appending kinase specific substrates and phosphoamino acid binding domains to cpHaloTag generating powerful kinase activity reporters HaloKARs (Fig. 7B). The best performing variant HaloAKAR (protein kinase A activity reporter) was employed in four- and five-color multiplexing experiments to untangle biased GPCR signaling.[128] While this strategy allows the generation of powerful intensiometric biosensors for various analytes, using JF_{635}-CA (**25**) limits the use of these biosensors to cell-based systems as its bioavailability is limited.[96,129] This was overcome for the dopamine sensor by using less fluorogenic but more bioavailable fluorophores such as Janelia Fluor 646-CA or SiR-CA (**21**).[127] However, this led to an almost 2-fold reduction in dynamic range compared to JF_{635}-CA (**25**).

A third strategy focuses on modulating the quantum yield of bound fluorophores rather than the open-close equilibrium. The resulting sensors do not rely on fluorogenic fluorophores; therefore, the fluorophore can be chosen based on other beneficial properties *e.g.* bioavailability. Based on the finding that Trp residues in close proximity to the fluorophore binding site of HaloTag lead to photoinduced electron transfer quenching and therefore a reduction in the quantum yield,[94] Farrants *et al.* generated the first Ca^{2+} biosensor, termed WHaloCaMP (Fig. 7C), using the bioavailable but non-fluorogenic fluorophore Janelia Fluor 669-CA

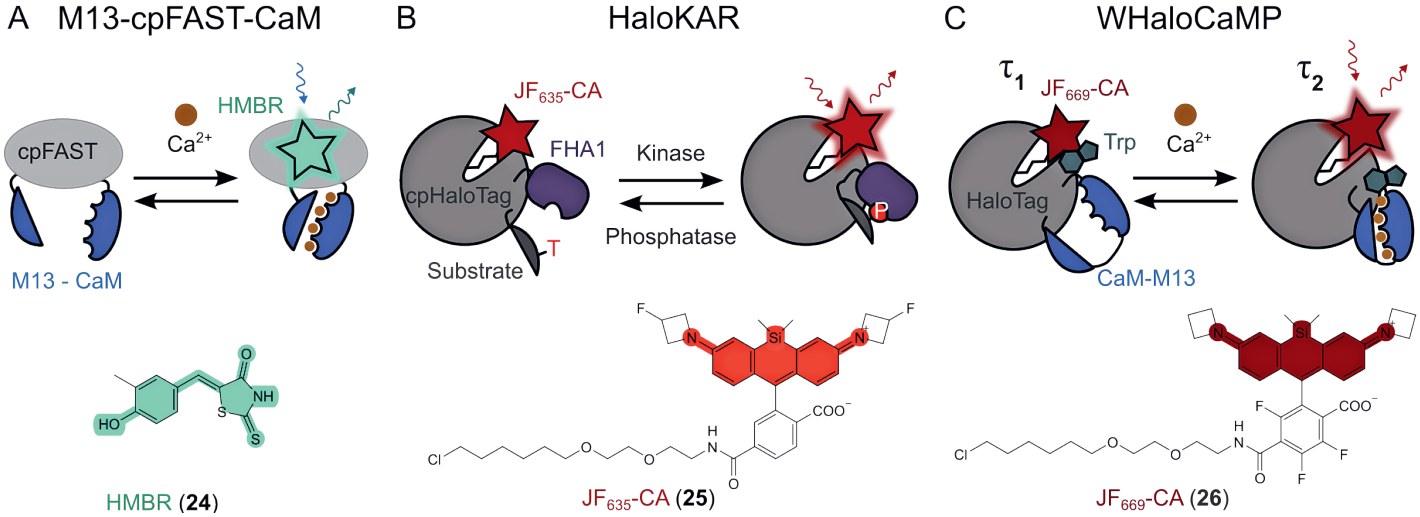

Fig. 7. Single fluorophore biosensors using protein tags. A) Ca^{2+} biosensor based on cpFAST fused to CaM and the peptide M13. Ca^{2+} binding triggers binding of HMBR (**24**). B) HaloTag-based kinase activity reporter HaloKAR using the fluorogenic JF_{635}-CA (**25**). Activity of a kinase leads to phosphorylation of the substrate followed by binding by the phosphoamino acid binding domain FHA1. C) The WHaloCaMP biosensor consists of the Ca^{2+} sensing unit CaM-M13 inserted into HaloTag-G171W and JF_{669}-CA (**26**). Changes in Ca^{2+} levels lead to changes in fluorescence intensity and fluorescence lifetime (τ) of the sensor.

(JF_{669}-CA (**26**)).[96] While this sensor used the previously identified G171W mutation found by Frei *et al*., T155W also led to promising results. This type of biosensor was well suited for two photon imaging of Ca^{2+} in a mouse's primary visual cortex and in addition to its intensiometric readout also showed a change in fluorescence lifetime.

5. Conclusion and Outlook

Chemigenetic approaches leverage synthetic chemistry as well as genetically encoded components to generate powerful tools for probing dynamic processes in living cells. In particular, the field of chemigenetic biosensors has accelerated rapidly through innovative approaches to couple organic fluorophores with protein domains. Major advances have been driven by the advent of SLPs and the FAST system. Over the last twenty years we have witnessed how these protein-based labeling systems have been used to replace fluorescent proteins in FRET-based biosensors, enhancing their performance and shifting their spectral properties into the far-red. Moreover, the SNIFIT concept has clearly shown how chemical components can not only be used to enhance the reporting unit but also how they can elevate the sensing unit, giving access to biosensors for new analytes. New topological variants and a concurrent development of synthetic fluorophores with properties such as high fluorogenicity then made it possible to generate powerful single fluorophore chemigenetic biosensors such as HaloCaMP and HaloKARs. Chemigenetic biosensors therefore allow us to investigate new analytes and do so in previously inaccessible contexts such as over longer time frames in deep tissue. While these chemigenetic systems increase the range of possible biosensor designs and offer performance advantages, they also present practical challenges. The synthetic component must be delivered and specifically conjugated to the genetically encoded component. For synthetic fluorophores unspecific reactions result in high background staining. Hence, future research will focus not only on the generation of new chemigenetic biosensors but also on the means to incorporate synthetic components specifically and efficiently, as well as the development of cell-membrane permeable synthetic components and fluorogenic fluorophores. Current endeavors have mostly focused on rhodamine-based fluorophores while other fluorophore classes remain underexplored. Taping into these reservoirs will further expand the scope of biosensors. Furthermore, having pushed the boundaries of what is possible with chemigenetic biosensors as compared to genetically encoded biosensors relying on fluorescent proteins, the underlying read-out principles (fluorescence intensity) remain the same and chemigenetic biosensors ultimately face the same challenges as fluorescent protein-based biosensors *e.g.* photobleaching, limited penetration depth, and multiplexing *etc*. Chemigenetic biosensors, however, have the added benefit that the chemical component can be switched out rapidly and therefore have the potential to allow for different read-out modalities as has been demonstrated with an optoacoustic chemigenetic biosensor[130] or the use of fluorescence lifetime.[96] The Frei Research Group is hence interested in moving beyond fluorescence intensity and is focusing its endeavors on fluorescence-lifetime based biosensors as well as strategies not relying on fluorescence.

Acknowledgements

We would like to thank H. Farrants (Janelia Research Campus) for input on the manuscript. The Frei Research Group is funded by ETH Zurich. This work was supported by the Swiss National Science Foundation (SNSF) under grant number CR00I5-235927.

Author Contributions

E. D. and M. F. cowrote this perspective.

Received: September 12, 2025

[1] A. M. M. Gest, A. Z. Sahan, Y. Zhong, W. Lin, S. Mehta, J. Zhang, *Chem. Rev*. **2024**, *124*, 12573, https://doi.org/10.1021/acs.chemrev.4c00293.
[2] E. C. Greenwald, S. Mehta, J. Zhang, *Chem. Rev*. **2018**, *118*, 11707, https://doi.org/10.1021/acs.chemrev.8b00333.
[3] M. S. Frei, S. Mehta, J. Zhang, *Annu. Rev. Biophys*. **2024**, *53*, 275, https://doi.org/10.1146/annurev-biophys-030722-021359.
[4] X. Zhou, K. J. Belavek, E. W. Miller, *Biochemistry* **2021**, *60*, 3547, https://doi.org/10.1021/acs.biochem.1c00350.
[5] R. Y. Tsien, *Biochemistry* **1980**, *19*, 2396, https://doi.org/10.1021/bi00552a018.
[6] D. Wu, A. C. Sedgwick, T. Gunnlaugsson, E. U. Akkaya, J. Yoon, T. D. James, *Chem. Soc. Rev*. **2017**, *46*, 7105, https://doi.org/10.1039/C7CS00240H.
[7] L. Wu, A. C. Sedgwick, X.-P. He, T. D. James, Eds. 'Fluorescent Chemosensors', The Royal Society Of Chemistry, **2023**, https://doi.org/10.1039/9781839167324.
[8] S. Swaminathan, *Nat. Cell Biol*. **2009**, *11*, S20, https://doi.org/10.1038/ncb1953.
[9] E. A. Rodriguez, R. E. Campbell, J. Y. Lin, M. Z. Lin, A. Miyawaki, A. E. Palmer, X. Shu, J. Zhang, R. Y. Tsien, *Trends Biochem. Sci.* **2017**, *42*, 111, https://doi.org/10.1016/j.tibs.2016.09.010.
[10] M. Wang, Y. Da, Y. Tian, *Chem. Soc. Rev*. **2023**, *52*, 1189, https://doi.org/10.1039/D2CS00419D.
[11] A. Ibraheem, R. E. Campbell, *Curr. Opin. Chem. Biol*. **2010**, *14*, 30, https://doi.org/10.1016/j.cbpa.2009.09.033.
[12] K. K. Tsao, S. Imai, M. Chang, S. Hario, T. Terai, R. E. Campbell, *Cell Chem. Biol*. **2024**, *31*, 1652, https://doi.org/10.1016/j.chembiol.2024.08.002.
[13] A. Gautier, A. G. Tebo, *Curr. Opin. Chem. Biol.* **2020**, *57*, 58, https://doi.org/10.1016/j.cbpa.2020.04.005.
[14] A. Wang, J. Feng, Y. Li, P. Zou, *ACS Chem. Neurosci*. **2018**, *9*, 639, https://doi.org/10.1021/acschemneuro.7b00455.
[15] F. Broch, A. Gautier, *ChemPlusChem* **2020**, *85*, 1487, https://doi.org/10.1002/cplu.202000413.
[16] D. Li, L. Liu, W.-H. Li, *ACS Chem. Biol.* **2015**, *10*, 1054, https://doi.org/10.1021/cb5007536.
[17] J. B. Grimm, L. D. Lavis, *Nat. Methods* **2022**, *19*, 149, https://doi.org/10.1038/s41592-021-01338-6.
[18] A. M. Nicoli, M. S. Frei, *Nat. Chem. Biol*. **2025**, https://doi.org/10.1038/s41589-025-02038-4.
[19] T. C. Lovell, B. P. Branchaud, R. Jasti, *Eur. J. Org. Chem*. **2024**, *27*, e202301196, https://doi.org/10.1002/ejoc.202301196.
[20] L. D. Lavis, R. T. Raines, *ACS Chem. Biol*. **2014**, *9*, 855, https://doi.org/10.1021/cb500078u.
[21] L. D. Lavis, *Annu. Rev. Biochem*. **2017**, *86*, 825, https://doi.org/10.1146/annurev-biochem-061516-044839.
[22] J. B. Grimm, A. N. Tkachuk, L. Xie, H. Choi, B. Mohar, N. Falco, K. Schaefer, R. Patel, Q. Zheng, Z. Liu, J. Lippincott-Schwartz, T. A. Brown, L. D. Lavis, *Nat. Methods* **2020**, *17*, 815, https://doi.org/10.1038/s41592-020-0909-6.
[23] A. S. Klymchenko, *Acc. Chem. Res*. **2017**, *50*, 366, https://doi.org/10.1021/acs.accounts.6b00517.
[24] M. Paez-Perez, M. K. Kuimova, *Angew. Chem. Int. Ed*. **2024**, *63*, e202311233, https://doi.org/10.1002/anie.202311233.
[25] S. Wang, B. Li, F. Zhang, *ACS Cent. Sci*. **2020**, *6*, 1302, https://doi.org/10.1021/acscentsci.0c00544.
[26] M. Minoshima, S. I. Reja, R. Hashimoto, K. Iijima, K. Kikuchi, *Chem. Rev*. **2024**, *124*, 6198, https://doi.org/10.1021/acs.chemrev.3c00549.
[27] O. Koniev, A. Wagner, *Chem. Soc. Rev*. **2015**, *44*, 5495, https://doi.org/10.1039/C5CS00048C.
[28] S. R. Adams, A. T. Harootunian, Y. J. Buechler, S. S. Taylor, R. Y. Tsien, *Nature* **1991**, *349*, 694, https://doi.org/10.1038/349694a0.
[29] T. Tamura, I. Hamachi, *ACS Chem. Biol*. **2014**, *9*, 2708, https://doi.org/10.1021/cb500661v.
[30] Y. Zhang, L. Yu, *BioEssays* **2008**, *30*, 606, https://doi.org/10.1002/bies.20759.
[31] D. S. Miller, Y. T. Lau, S. B. Horowitz, *Proc. Natl. Acad. Sci. U. S. A*. **1984**, *81*, 1426, https://doi.org/10.1073/pnas.81.5.1426.
[32] M. Lindgren, M. Hällbrink, A. Prochiantz, Ü. Langel, *Trends Pharmacol. Sci*. **2000**, *21*, 99, https://doi.org/10.1016/S0165-6147(00)01447-4.
[33] K. Shiraiwa, R. Cheng, H. Nonaka, T. Tamura, I. Hamachi, *Cell Chem. Biol*. **2020**, *27*, 970, https://doi.org/10.1016/j.chembiol.2020.06.016.
[34] M. L. W. J. Smeenk, J. Agramunt, K. M. Bonger, *Curr. Opin. Chem. Biol*. **2021**, *60*, 79, https://doi.org/10.1016/j.cbpa.2020.09.002.
[35] S. Tsukiji, I. Hamachi, *Curr. Opin. Chem. Biol*. **2014**, *21*, 136, https://doi.org/10.1016/j.cbpa.2014.07.012.
[36] T. Ueda, T. Tamura, I. Hamachi, *ACS Sens*. **2018**, *3*, 527, https://doi.org/10.1021/acssensors.7b00894.
[37] S. Sakamoto, S. Kiyonaka, I. Hamachi, *Curr. Opin. Chem. Biol*. **2019**, *50*, 10, https://doi.org/10.1016/j.cbpa.2019.02.011.

[38] K. Matsuo, Y. Nishikawa, M. Masuda, I. Hamachi, *Angew. Chem. Int. Ed*. **2018**, *57*, 659, https://doi.org/10.1002/anie.201707972.
[39] A. Costello, A. A. Peterson, P.-H. Chen, R. Bagirzadeh, D. L. Lanster, A. H. Badran, *Chem. Rev*. **2024**, *124*, 11962, https://doi.org/10.1021/acs.chemrev.4c00275.
[40] S. Lee, J. Kim, M. Koh, *J. Mol. Biol*. **2022**, *434*, 167248, https://doi.org/10.1016/j.jmb.2021.167248.
[41] Z. Cheng, E. Kuru, A. Sachdeva, M. Vendrell, *Nat. Rev. Chem*. **2020**, *4*, 275, https://doi.org/10.1038/s41570-020-0186-z.
[42] T. Kalstrup, R. Blunck, *Proc. Natl. Acad. Sci. U. S. A*. **2018**, *115*, E6751, https://doi.org/10.1073/pnas.1719105115.
[43] M. A. Shandell, J. R. Quejada, M. Yazawa, V. W. Cornish, R. S. Kass, *Biophys. J.* **2019**, *117*, 1352, https://doi.org/10.1016/j.bpj.2019.08.028.
[44] H. B. Yi, S. Lee, K. Seo, H. Kim, M. Kim, H. S. Lee, *Chem. Rev*. **2024**, *124*, 7465, https://doi.org/10.1021/acs.chemrev.4c00112.
[45] J. H. Choi, S. Kim, O.-Y. Kang, S. Y. Choi, J. Y. Hyun, H. S. Lee, I. Shin, *Chem. Soc. Rev*. **2024**, *53*, 9446, https://doi.org/10.1039/D4CS00094C.
[46] L. Hu, W. Cao, Y. Jiang, W. Cai, X. Lou, T. Liu, *Nat. Chem*. **2024**, *16*, 1960, https://doi.org/10.1038/s41557-024-01675-x.
[47] C. Uttamapinant, J. D. Howe, K. Lang, V. Beránek, L. Davis, M. Mahesh, N. P. Barry, J. W. Chin, *J. Am. Chem. Soc*. **2015**, *137*, 4602, https://doi.org/10.1021/ja512838z.
[48] L. Xue, E. Prifti, K. Johnsson, *J. Am. Chem. Soc*. **2016**, *138*, 5258, https://doi.org/10.1021/jacs.6b03034.
[49] Y.-H. Tsai, S. J. Elsässer, Eds., 'Genetically Incorporated Non-Canonical Amino Acids: Methods and Protocols', Springer US, **2023**, https://doi.org/10.1007/978-1-0716-3251-2.
[50] M. Budiarta, M. Streit, G. Beliu, *Curr. Opin. Chem. Biol*. **2024**, *80*, 102445, https://doi.org/10.1016/j.cbpa.2024.102445.
[51] Y. Huang, P. Zhang, H. Wang, Y. Chen, T. Liu, X. Luo, *Chem. Rev*. **2025**, *125*, 523, https://doi.org/10.1021/acs.chemrev.4c00216.
[52] J. Lotze, U. Reinhardt, O. Seitz, A. G. Beck-Sickinger, *Mol. Biosyst*. **2016**, *12*, 1731, https://doi.org/10.1039/C6MB00023A.
[53] B. A. Griffin, S. R. Adams, R. Y. Tsien, *Science* **1998**, *281*, 269, https://doi.org/10.1126/science.281.5374.269.
[54] C. Hoffmann, G. Gaietta, M. Bünemann, S. R. Adams, S. Oberdorff-Maass, B. Behr, J.-P. Vilardaga, R. Y. Tsien, M. H. Ellisman, M. J. Lohse, *Nat. Methods* **2005**, *2*, 171, https://doi.org/10.1038/nmeth742.
[55] D. S. Liu, A. Tangpeerachaikul, R. Selvaraj, M. T. Taylor, J. M. Fox, A. Y. Ting, *J. Am. Chem. Soc*. **2012**, *134*, 792, https://doi.org/10.1021/ja209325n.
[56] Y. Xu, L. Peng, S. Wang, A. Wang, R. Ma, Y. Zhou, J. Yang, D. Sun, W. Lin, X. Chen, P. Zou, *Angew. Chem. Int. Ed.* **2018**, *57*, 3949, https://doi.org/10.1002/anie.201712614.
[57] S. Liu, C. Lin, Y. Xu, H. Luo, L. Peng, X. Zeng, H. Zheng, P. R. Chen, P. Zou, *Nat. Chem*. **2021**, *13*, 472, https://doi.org/10.1038/s41557-021-00641-1.
[58] M. F. Langhorst, S. Genisyuerek, C. A. O. Stuermer, *Histochem. Cell Biol*. **2006**, *125*, 743, https://doi.org/10.1007/s00418-005-0136-3.
[59] G. S. Baird, D. A. Zacharias, R. Y. Tsien, *Proc. Natl. Acad. Sci. U. S. A*. **1999**, *96*, 11241, https://doi.org/10.1073/pnas.96.20.11241.
[60] N. T. Calloway, M. Choob, A. Sanz, M. P. Sheetz, L. W. Miller, V. W. Cornish, *ChemBioChem* **2007**, *8*, 767, https://doi.org/10.1002/cbic.200600414.
[61] L. W. Miller, Y. Cai, M. P. Sheetz, V. W. Cornish, *Nat. Methods* **2005**, *2*, 255, https://doi.org/10.1038/nmeth749.
[62] S. S. Gallagher, J. E. Sable, M. P. Sheetz, V. W. Cornish, *ACS Chem. Biol*. **2009**, *4*, 547, https://doi.org/10.1021/cb900062k.
[63] J. Mo, J. Chen, Y. Shi, J. Sun, Y. Wu, T. Liu, J. Zhang, Y. Zheng, Y. Li, Z. Chen, *Angew. Chem. Int. Ed*. **2022**, *61*, e202207905, https://doi.org/10.1002/anie.202207905.
[64] Z. Chen, C. Jing, S. S. Gallagher, M. P. Sheetz, V. W. Cornish, *J. Am. Chem. Soc*. **2012**, *134*, 13692, https://doi.org/10.1021/ja303374p.
[65] M.-A. Plamont, E. Billon-Denis, S. Maurin, C. Gauron, F. M. Pimenta, C. G. Specht, J. Shi, J. Quérard, B. Pan, J. Rossignol, K. Moncoq, N. Morellet, M. Volovitch, E. Lescop, Y. Chen, A. Triller, S. Vriz, T. Le Saux, L. Jullien, A. Gautier, *Proc. Natl. Acad. Sci. U. S. A*. **2016**, *113*, 497, https://doi.org/10.1073/pnas.1513094113.
[66] Y. Hori, H. Ueno, S. Mizukami, K. Kikuchi, *J. Am. Chem. Soc*. **2009**, *131*, 16610, https://doi.org/10.1021/ja904800k.
[67] A. Gautier, *Acc. Chem. Res*. **2022**, *55*, 3125, https://doi.org/10.1021/acs.accounts.2c00098.
[68] L. El Hajji, B. Bunel, O. Joliot, C. Li, A. G. Tebo, C. Rampon, M. Volovitch, E. Fischer, N. Pietrancosta, F. Perez, X. Morin, S. Vriz, A. Gautier, *Nat. Commun*. **2025**, *16*, 2594, https://doi.org/10.1038/s41467-025-58017-9.
[69] A. G. Tebo, B. Moeyaert, M. Thauvin, I. Carlon-Andres, D. Böken, M. Volovitch, S. Padilla-Parra, P. Dedecker, S. Vriz, A. Gautier, *Nat. Chem. Biol*. **2021**, *17*, 30, https://doi.org/10.1038/s41589-020-0611-0.
[70] L. El Hajji, F. Lam, M. Avtodeeva, H. Benaissa, C. Rampon, M. Volovitch, S. Vriz, A. Gautier, *Adv. Sci*. **2024**, *11*, 2404354, https://doi.org/10.1002/advs.202404354.
[71] Y. A. Bogdanova, I. D. Solovyev, N. S. Baleeva, I. N. Myasnyanko, A. A. Gorshkova, D. A. Gorbachev, A. R. Gilvanov, S. A. Goncharuk, M. V. Goncharuk, K. S. Mineev, A. S. Arseniev, A. M. Bogdanov, A. P. Savitsky, M. S. Baranov, *Commun. Biol*. **2024**, *7*, 1, https://doi.org/10.1038/s42003-024-06501-1.
[72] A. G. Tebo, A. Gautier, *Nat. Commun*. **2019**, *10*, 2822, https://doi.org/10.1038/s41467-019-10855-0.
[73] A. G. Tebo, F. M. Pimenta, M. Zoumpoulaki, C. Kikuti, H. Sirkia, M.-A. Plamont, A. Houdusse, A. Gautier, *ACS Chem. Biol.* **2018**, *13*, 2392, https://doi.org/10.1021/acschembio.8b00417.
[74] C. A. Hoelzel, X. Zhang, *ChemBioChem* **2020**, *21*, 1935, https://doi.org/10.1002/cbic.202000037.
[75] N. Porzberg, K. Gries, K. Johnsson, *Annu. Rev. Biochem*. **2025**, *94*, 29, https://doi.org/10.1146/annurev-biochem-030222-121016.
[76] A. Keppler, S. Gendreizig, T. Gronemeyer, H. Pick, H. Vogel, K. Johnsson, *Nat. Biotechnol*. **2003**, *21*, 86, https://doi.org/10.1038/nbt765.
[77] X. Sun, A. Zhang, B. Baker, L. Sun, A. Howard, J. Buswell, D. Maurel, A. Masharina, K. Johnsson, C. J. Noren, M.-Q. Xu, I. R. Corrêa Jr., *ChemBioChem* **2011**, *12*, 2217, https://doi.org/10.1002/cbic.201100173.
[78] R. S. Erdmann, S. W. Baguley, J. H. Richens, R. F. Wissner, Z. Xi, E. S. Allgeyer, S. Zhong, A. D. Thompson, N. Lowe, R. Butler, J. Bewersdorf, J. E. Rothman, D. St Johnston, A. Schepartz, D. Toomre, *Cell Chem. Biol*. **2019**, *26*, 584, https://doi.org/10.1016/j.chembiol.2019.01.003.
[79] S. Kühn, V. Nasufovic, J. Wilhelm, J. Kompa, E. M. F. De Lange, Y.-H. Lin, C. Egoldt, J. Fischer, A. Lennoi, M. Tarnawski, J. Reinstein, R. Vlijm, J. Hiblot, K. Johnsson, *Nat. Chem. Biol*. **2025**, https://doi.org/10.1038/s41589-025-01942-z.
[80] A. Gautier, A. Juillerat, C. Heinis, I. R. Corrêa, M. Kindermann, F. Beaufils, K. Johnsson, *Chem. Biol*. **2008**, *15*, 128, https://doi.org/10.1016/j.chembiol.2008.01.007.
[81] D. Zhang, Z. Chen, Z. Du, B. Bao, N. Su, X. Chen, Y. Ge, Q. Lin, L. Yang, Y. Hua, S. Wang, X. Hua, F. Zuo, N. Li, R. Liu, L. Jiang, C. Bao, Y. Zhao, J. Loscalzo, Y. Yang, L. Zhu, *Cell Discov*ery **2023**, *9*, 56, https://doi.org/10.1038/s41421-023-00546-y.
[82] J. D. Cleveland, C. L. Tucker, *ACS Chem. Biol*. **2020**, *15*, 2212, https://doi.org/10.1021/acschembio.0c00412.
[83] M. Mie, T. Naoki, K. Uchida, E. Kobatake, *Analyst* **2012**, *137*, 4760, https://doi.org/10.1039/c2an35762c.
[84] M. Mie, T. Naoki, E. Kobatake, *Anal. Chem*. **2016**, *88*, 8166, https://doi.org/10.1021/acs.analchem.6b01906.
[85] G. V. Los, L. P. Encell, M. G. McDougall, D. D. Hartzell, N. Karassina, C. Zimprich, M. G. Wood, R. Learish, R. F. Ohana, M. Urh, D. Simpson, J. Mendez, K. Zimmerman, P. Otto, G. Vidugiris, J. Zhu, A. Darzins, D. H. Klaubert, R. F. Bulleit, K. V. Wood, *ACS Chem. Biol*. **2008**, *3*, 373, https://doi.org/10.1021/cb800025k.
[86] C. G. England, H. Luo, W. Cai, *Bioconjugate Chem*. **2015**, *26*, 975, https://doi.org/10.1021/acs.bioconjchem.5b00191.
[87] J. Wilhelm, S. Kühn, M. Tarnawski, G. Gotthard, J. Tünnermann, T. Tänzer, J. Karpenko, N. Mertes, L. Xue, U. Uhrig, J. Reinstein, J. Hiblot, K. Johnsson, *Biochemistry* **2021**, *60*, 2560, https://doi.org/10.1021/acs.biochem.1c00258.
[88] A. Cook, F. Walterspiel, C. Deo, *ChemBioChem* **2023**, *24*, e202300022, https://doi.org/10.1002/cbic.202300022.
[89] C. Miró-Vinyals, A. Stein, S. Fischer, T. R. Ward, A. Deliz Liang, *ChemBioChem* **2021**, *22*, 3398, https://doi.org/10.1002/cbic.202100424.
[90] B. J. Lampkin, B. J. Goldberg, J. A. Kritzer, *Chem. Sci*. **2024**, *15*, 17337, https://doi.org/10.1039/d4sc05090h.
[91] K. J. Koßmann, C. Ziegler, A. Angelin, R. Meyer, M. Skoupi, K. S. Rabe, C. M. Niemeyer, *ChemBioChem* **2016**, *17*, 1102, https://doi.org/10.1002/cbic.201600039.
[92] S. M. Marques, M. Slanska, K. Chmelova, R. Chaloupkova, M. Marek, S. Clark, J. Damborsky, E. T. Kool, D. Bednar, Z. Prokop, *JACS Au* **2022**, *2*, 1324, https://doi.org/10.1021/jacsau.2c00002.
[93] B. C. Shields, H. Yan, S. S. X. Lim, S. C. V. Burwell, C. M. Cammarata, E. A. Fleming, S. A. Yousefzadeh, V. Z. Goldenshtein, E. W. Kahuno, P. P. Vagadia, M. H. Loughran, L. Zhiquan, M. E. McDonnell, M. L. Scalabrino, M. Thapa, T. M. Hawley, G. D. Field, C. Hull, G. E. Schiltz, L. L. Glickfeld, A. B. Reitz, M. R. Tadross, *Nat. Methods* **2024**, *21*, 1288, https://doi.org/10.1038/s41592-024-02292-9.
[94] M. S. Frei, M. Tarnawski, M. J. Roberti, B. Koch, J. Hiblot, K. Johnsson, *Nat. Methods* **2022**, *19*, 65, https://doi.org/10.1038/s41592-021-01341-x.
[95] L. Hellweg, A. Edenhofer, L. Barck, M.-C. Huppertz, M. S. Frei, M. Tarnawski, A. Bergner, B. Koch, K. Johnsson, J. Hiblot, *Nat. Chem. Biol*. **2023**, *19*, 1147, https://doi.org/10.1038/s41589-023-01350-1.
[96] H. Farrants, Y. Shuai, W. C. Lemon, C. Monroy Hernandez, D. Zhang, S. Yang, R. Patel, G. Qiao, M. S. Frei, S. E. Plutkis, J. B. Grimm, T. L. Hanson, F. Tomaska, G. C. Turner, C. Stringer, P. J. Keller, A. G. Beyene, Y. Chen, Y. Liang, L. D.

Lavis, E. R. Schreiter, *Nat. Methods* **2024**, *21*, 1916, https://doi.org/10.1038/s41592-024-02411-6.
[97] N. R. Dall, C. A. T. F. Mendonça, H. L. Torres Vera, S. Marqusee, *Proc. Natl. Acad. Sci. U. S. A.* **2024**, *121*, e2321999121, https://doi.org/10.1073/pnas.2321999121.
[98] C. Deo, A. S. Abdelfattah, H. K. Bhargava, A. J. Berro, N. Falco, H. Farrants, B. Moeyaert, M. Chupanova, L. D. Lavis, E. R. Schreiter, *Nat. Chem. Biol*. **2021**, *17*, 718, https://doi.org/10.1038/s41589-021-00775-w.
[99] L. Wang, J. Hiblot, C. Popp, L. Xue, K. Johnsson, *Angew. Chem. Int. Ed*. **2020**, *132*, 22064, https://doi.org/10.1002/ange.202008357.
[100] W. Zhu, S. Takeuchi, S. Imai, T. Terada, T. Ueda, Y. Nasu, T. Terai, R. E. Campbell, *Nat. Chem. Biol*. **2023**, *19*, 38, https://doi.org/10.1038/s41589-022-01134-z.
[101] H. Ishikawa, F. Meng, N. Kondo, A. Iwamoto, Z. Matsuda, *Protein Eng. Des. Sel*. **2012**, *25*, 813, https://doi.org/10.1093/protein/gzs051.
[102] S. Shao, H. Zhang, Y. Zeng, Y. Li, C. Sun, Y. Sun, *Commun. Biol*. **2021**, *4*, 1, https://doi.org/10.1038/s42003-021-01896-7.
[103] M.-C. Huppertz, J. Wilhelm, V. Grenier, M. W. Schneider, T. Falt, N. Porzberg, D. Hausmann, D. C. Hoffmann, L. Hai, M. Tarnawski, G. Pino, K. Slanchev, I. Kolb, C. Acuna, L. M. Fenk, H. Baier, J. Hiblot, K. Johnsson, *Science* **2024**, *383*, 890, https://doi.org/10.1126/science.adg0812.
[104] J. Wilhelm, L. Nickel, Y.-H. Lin, J. Hiblot, K. Johnsson, *Protein Sci*. **2025**, *34*, e70123, https://doi.org/10.1002/pro.70123.
[105] Y.-H. Lin, J. Kompa, D. Sun, R. Mao, B. Koch, K. Hinnah, J. Wilhelm, N. Franz, S. Kühn, T. Menche, A. Adow, P. Breuer, J. Hiblot, K. Johnsson, *bioRxiv* **2025**, https://doi.org/10.1101/2025.06.15.659771.
[106] J. Kompa, J. Bruins, M. Glogger, J. Wilhelm, M. S. Frei, M. Tarnawski, E. D'Este, M. Heilemann, J. Hiblot, K. Johnsson, *J. Am. Chem. Soc*. **2023**, *145*, 3075, https://doi.org/10.1021/jacs.2c11969.
[107] M. Holtmannspötter, E. Wienbeuker, T. Dellmann, I. Watrinet, A. J. Garcia?Sáez, K. Johnsson, R. Kurre, J. Piehler, *Angew. Chem. Int. Ed*. **2023**, *62*, e202219050, https://doi.org/10.1002/anie.202219050.
[108] S. Takeuchi, S. Imai, T. Terai, R. E. Campbell, *RSC Chem. Biol*. **2025**, *6*, 170, https://doi.org/10.1039/D4CB00256C.
[109] M. A. Brun, K.-T. Tan, E. Nakata, M. J. Hinner, K. Johnsson, *J. Am. Chem. Soc.* **2009**, *131*, 5873, https://doi.org/10.1021/ja900149e.
[110] M. A. Brun, R. Griss, L. Reymond, K.-T. Tan, J. Piguet, R. J. R. W. Peters, H. Vogel, K. Johnsson, *J. Am. Chem. Soc*. **2011**, *133*, 16235, https://doi.org/10.1021/ja206915m.
[111] M. A. Brun, K. T. Tan, R. Griss, A. Kielkowska, L. Reymond, K. Johnsson, *J. Am. Chem. Soc*. **2012**, *134*, 7676, https://doi.org/10.1021/ja3002277.
[112] A. Masharina, L. Reymond, D. Maurel, K. Umezawa, K. Johnsson, *J. Am. Chem. Soc*. **2012**, *134*, 19026, https://doi.org/10.1021/ja306320s.
[113] O. Sallin, L. Reymond, C. Gondrand, F. Raith, B. Koch, K. Johnsson, *eLife* **2018**, *7*, e32638, https://doi.org/10.7554/eLife.32638.
[114] L. Xue, P. Schnacke, M. S. Frei, B. Koch, J. Hiblot, R. Wombacher, S. Fabritz, K. Johnsson, *Nat. Chem. Biol*. **2023**, *19*, 346, https://doi.org/10.1038/s41589-022-01172-7.
[115] M. D. Vecchia, A. Conte-Daban, B. Cappe, W. Vandenberg, P. Vandenabeele, F. B. Riquet, P. Dedecker, *ACS Sens*. **2022**, *7*, 2920, https://doi.org/10.1021/acssensors.2c00066.
[116] W. Wang, J. Yang, *ACS Sens*. **2024**, *9*, 5264, https://doi.org/10.1021/acssensors.4c01444.
[117] H. Benaissa, K. Ounoughi, I. Aujard, E. Fischer, R. Goïame, J. Nguyen, A. G. Tebo, C. Li, T. Le Saux, G. Bertolin, M. Tramier, L. Danglot, N. Pietrancosta, X. Morin, L. Jullien, A. Gautier, *Nat. Commun*. **2021**, *12*, 6989, https://doi.org/10.1038/s41467-021-27334-0.
[118] A. S. Abdelfattah, T. Kawashima, A. Singh, O. Novak, H. Liu, Y. Shuai, Y. C. Huang, L. Campagnola, S. C. Seeman, J. Yu, J. Zheng, J. B. Grimm, R. Patel, J. Friedrich, B. D. Mensh, L. Paninski, J. J. Macklin, G. J. Murphy, K. Podgorski, B. J. Lin, T. W. Chen, G. C. Turner, Z. Liu, M. Koyama, K. Svoboda, M. B. Ahrens, L. D. Lavis, E. R. Schreiter, *Science* **2019**, *364*, 699, https://doi.org/10.1126/science.aav6416.
[119] A. S. Abdelfattah, R. Valenti, J. Zheng, A. Wong, G. P. Team, K. Podgorski, M. Koyama, D. S. Kim, E. R. Schreiter, *Nat. Commun.* **2020**, *11*, 3444, https://doi.org/10.1038/s41467-020-17322-1.
[120] Y. Xu, M. Deng, S. Zhang, J. Yang, L. Peng, J. Chu, P. Zou, *ACS Chem. Neurosci*. **2019**, *10*, 4768, https://doi.org/10.1021/acschemneuro.9b00501.
[121] A. S. Abdelfattah, J. Zheng, A. Singh, Y.-C. Huang, D. Reep, G. Tsegaye, A. Tsang, B. J. Arthur, M. Rehorova, C. V. L. Olson, Y. Shuai, L. Zhang, T.-M. Fu, D. E. Milkie, M. V. Moya, T. D. Weber, A. L. Lemire, C. A. Baker, N. Falco, Q. Zheng, J. B. Grimm, M. C. Yip, D. Walpita, M. Chase, L. Campagnola, G. J. Murphy, A. M. Wong, C. R. Forest, J. Mertz, M. N. Economo, G. C. Turner, M. Koyama, B.-J. Lin, E. Betzig, O. Novak, L. D. Lavis, K. Svoboda, W. Korff, T.-W. Chen, E. R. Schreiter, J. P. Hasseman, I. Kolb, *Neuron* **2023**, *111*, 1547, https://doi.org/10.1016/j.neuron.2023.03.009.
[122] J. Ling, Y. Zhang, Y. Hei, J. Kompa, C. Yang, B. Wang, J. Zhang, J. Du, T. Rudi, K. Zhang, J. Sun, W. Wang, S. Fabritz, Y. Li, W. Deng, P. Zou, C. Chen, Z. Chen, *Proc. Natl. Acad. Sci. U. S. A*. **2025**, *122*, e2426354122, https://doi.org/10.1073/pnas.2426354122.
[123] F. Broch, L. El Hajji, N. Pietrancosta, A. Gautier, *ACS Sens*. **2023**, *8*, 3933, https://doi.org/10.1021/acssensors.3c01536.
[124] J. B. Grimm, A. K. Muthusamy, Y. Liang, T. A. Brown, W. C. Lemon, R. Patel, R. Lu, J. J. Macklin, P. J. Keller, N. Ji, L. D. Lavis, *Nat. Methods* **2017**, *14*, 987, https://doi.org/10.1038/nmeth.4403.
[125] H. Tsutsui, Y. Jinno, A. Tomita, Y. Okamura, *Biophys. J.* **2013**, *105*, 108, https://doi.org/10.1016/j.bpj.2013.05.051.
[126] J. D. Lee, A. Nguyen, C. E. Gibbs, Z. R. Jin, Y. Wang, A. Moghadasi, S. J. Wait, H. Choi, K. M. Evitts, A. Asencio, S. B. Bremner, S. Zuniga, V. Chavan, I. K. A. Pranoto, C. A. Williams, A. Smith, F. Moussavi-Harami, M. Regnier, D. Baker, J. E. Young, D. L. Mack, E. Nance, P. M. Boyle, A. Berndt, *Nat. Chem. Biol*. **2025**, https://doi.org/10.1038/s41589-025-01891-7.
[127] Y. Zheng, R. Cai, K. Wang, J. Zhang, Y. Zhuo, H. Dong, Y. Zhang, Y. Wang, F. Deng, E. Ji, Y. Cui, S. Fang, X. Zhang, H. Huang, K. Zhang, J. Wang, G. Li, X. Miao, Z. Wang, Y. Yang, S. Li, J. B. Grimm, K. Johnsson, E. R. Schreiter, L. D. Lavis, Z. Chen, Y. Mu, Y. Li, *Science* **2025**, *388*, eadt7705, https://doi.org/10.1126/science.adt7705.
[128] M. S. Frei, S. A. Sanchez, X. He, L. Liu, F. Schneider, Z. Wang, H. Hakozaki, Y. Li, A. C. Lyons, T. V. Rohm, J. M. Olefsky, L. Shi, J. Schöneberg, S. E. Fraser, S. Mehta, Y. Wang, J. Zhang, *Nat. Biotechnol.* **2025**, https://doi.org/10.1038/s41587-025-02642-8.
[129] J. B. Grimm, A. N. Tkachuk, R. Patel, S. T. Hennigan, A. Gutu, P. Dong, V. Gandin, A. M. Osowski, K. L. Holland, Z. J. Liu, T. A. Brown, L. D. Lavis, *J. Am. Chem. Soc*. **2023**, *145*, 23000, https://doi.org/10.1021/jacs.3c05273.
[130] A. Cook, N. Kaydanov, B. Ugarte-Uribe, J. C. Boffi, G. B. Kamm, R. Prevedel, C. Deo, *J. Am. Chem. Soc*. **2024**, *146*, 23963, https://doi.org/10.1021/jacs.4c07080.

License and Terms

This is an Open Access article under the terms of the Creative Commons Attribution License CC BY 4.0. The material may not be used for commercial purposes.

The license is subject to the CHIMIA terms and conditions: (https://chimia.ch/chimia/about).

The definitive version of this article is the electronic one that can be found at https://doi.org/10.2533/chimia.2025.749