REVIEW

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Chemical Tools for Biomolecular Imaging

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ABSTRACT The visualization of biologically relevant molecules and activities inside living cells continues to transform cell biology into a truly quantitative science. However, despite the spectacular achievements in some areas of cell biology, the majority of cellular processes still operate invisibly, not illuminated by even our brightest laser beams. Further progress therefore will depend not only on improvements in instrumentation but also increasingly on the development of new fluorophores and fluorescent sensors to target these activities. In the following, we review some of the recent approaches to generating such sensors, the methods to attach them to selected biomolecules, and their applications to various biological problems.

The *in vivo* characterization of protein activities, key metabolites, and other important parameters has become one of the central quests in molecular cell biology. The development and application of tools that allow visualization of these parameters in living cells is therefore a highly active and interdisciplinary area of research. In the last decade, the majority of the new approaches to studying protein function or developing new sensors have been based on autofluorescent proteins (AFPs) (*1*). An impressive variety of AFP-based techniques emerged from these efforts, such as photoactivatable proteins for studying dynamic processes, indicators for membrane potentials, and sensors for certain ions and metabolites (*2*). However, despite all this protein engineering wizardry, AFPs as sensors for biological processes face limitations. First, AFPs are relatively bulky, in the best case monomers of ~240 residues, and size matters for all applications for which the distance between the AFP and the activity to be recorded is critical. Second, the spectral range of AFPs is limited (*2*). For example, no useful AFPs are available in the near-infrared region, and different pairs of AFPs for simultaneous FRET measurements in living cells have not yet been established. As a consequence, the AFP-based simultaneous measurements of different processes are still the exception (*3*). Third, for the construction of sensors for various crucial biomolecules and enzymatic activities, AFPs offer no obvious solution.

Chemistry has also always played an important role in the development of tools for biomolecular imaging. The >16,000 citations of the hallmark paper of Roger Tsien and his co-workers on improved versions of synthetic calcium sensors paint a clear picture (*4*). In this Review, we will summarize some of the recent contributions of chemistry to biomolecular imaging and how they can complement approaches based on AFPs to gain new insights into cellular processes.

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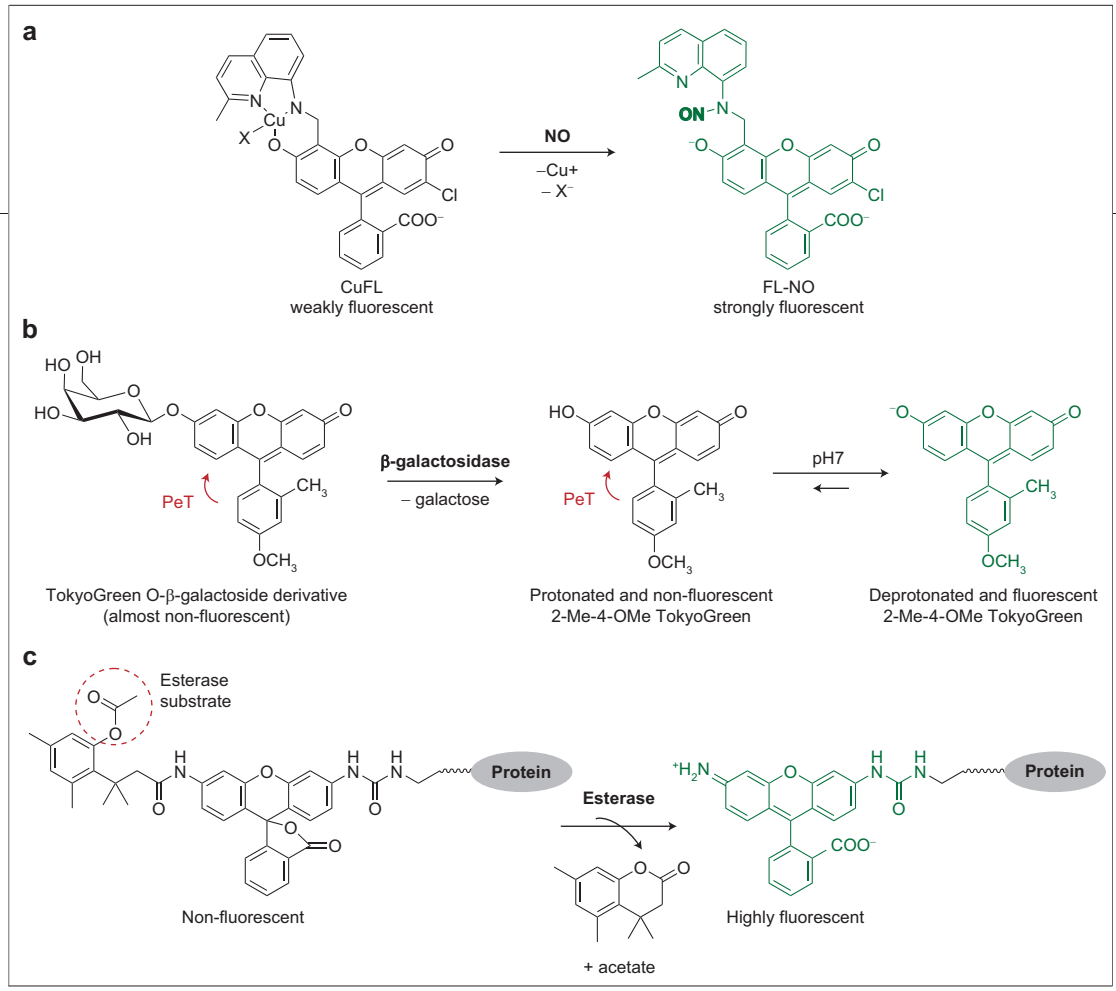

Figure 1. Synthetic fluorescent sensors for detecting small molecules or enzymatic activities. a) Fluorescein-based sensor (CuFL) for detecting NO. b) Fluorescein derivatives (dubbed TokyoGreens) (*12*) as sensors for β-galactosidase activity. c) Rhodamine-based probes as sensors for esterase activity. Esterases release the phenol of the so-called trimethyl lock group (*14*), and this leads to rapid lactonization and liberation of the fluorophore.

The key characteristics for each new fluorescent sensor are its selectivity and sensitivity. It is instructive to discuss how these problems have been addressed in recent examples and to point out some general lessons that may prove important for the design of the next generation of fluorescent sensors.

Tracking Messengers. Small molecules as second messengers play a central role in signal transduction. One such ubiquitous messenger is nitric oxide (NO), which is involved in various physiological and pathophysiological processes (*5*). Key to a detailed characterization of its function is a sensitive and robust measurement of its concentration in living cells. Here, approaches based on synthetic molecules as well as AFPs are available and can therefore be directly compared.

Diaminofluoresceins and diaminocyanines have been described as small-molecule-based sensors for the detection of NO (*6, 7*). However, these molecules do not sense NO directly but react with oxidized NO products to yield a highly fluorescent product. A fluorescent sensor that allows for the first time a direct detection of NO was recently introduced by Lippard and colleagues (*8*). This sensor is based on a Cu(II) complex of a fluorescein derivative (CuFL), which by itself possesses relatively low fluorescence (Figure 1, panel a). Through the irreversible reaction of CuFL with NO, which leads to a reduction of Cu(II) to Cu(I), a nitrosated fluorescein derivative is formed that possesses an ~11-fold increased fluorescence. Importantly, the sensor shows high specificity for NO over other reactive nitrogen and oxygen species and is cell-permeable; neither CuFL itself nor the reaction products with NO display any significant cytotoxicity. These features allowed the authors to visualize NO formation by constitutive and inducible NO synthases in living macrophages and neuroblastoma cells. Comparison of CuFL with the previously mentioned diaminofluorescein derivatives showed that CuFL detects NO with faster response rates and higher sensitivity. Thus, CuFL represents a significant step toward the design of sensitive and selective synthetic NO sensors. However, some aspects of this sensor still need improvement. First, CuFL, like all other synthetic fluorescent sensors of NO, undergoes an irreversible reaction

with NO. This significantly limits its application for measuring dynamic changes in NO concentrations. Clearly, the high reactivity of NO makes the construction of a reversible sensor a challenging task. Furthermore, in the experiments described by Stephen Lippard's group, cells were imaged immediately after incubation with CuFL, and it remains to be shown whether the sensor will be suited for live imaging. Last, for the precise determination of NO concentrations, particularly in tissues, a ratiometric fluorescent sensor would be desirable, because it adjusts for variations arising from differences in senor concentration, excitation efficiency, and sample thickness (*9*).

Umezawa and colleagues recently introduced a genetically encoded AFP-based sensor that yields a ratiometric signal for the determination of NO levels in living cells (*10*). This sensor does not detect NO directly but instead detects the product of the NO-activated soluble guanylate cyclase (sGC) cyclic guanosine monophosphate (cGMP). Binding of cGMP leads to a change in FRET intensity of a cyan fluorescent protein/yellow fluorescent protein-based cGMP sensor. Because a single molecule of NO leads to the production of many cGMP molecules through activation of sGC, the system possesses a very high sensitivity (detection limit ~0.1 nM of NO) and is therefore suited to visualizing changes of NO concentrations in living cells. However, this sensitivity comes at the cost of the specificity of the signal. cGMP is a second messenger in its own right; it also can be induced by other biological stimuli, or it can induce other biological stimuli itself. Although the ratiometric measurement of NO by the change in FRET efficiency is feasible in cell culture, a ratio change of only 1.5–1.7 makes it a challenge for the average cell biology lab to study those effects in tissues or even more complex environments.

Creating Fluorescent Sensors for Enzymatic Activities by Design. The sensing of NO by CuFL is based on an irreversible modification of the sensor that results in a change of its spectroscopic properties. Similarly, the sensing of calcium by the various synthetic ion sensors is based on a change of the spectroscopic properties of the fluorophores through the reversible binding of the ion. In the past, the design of such fluorescent sensors was often based on empirical knowledge, luck, and a trial-and-error strategy. Recent work by Nagano and colleagues showed how a detailed understanding of the spectroscopic properties of fluorophores can be exploited to design superior fluorophores and sensors (*11–13*). Systematic studies revealed that the quantum efficiency of fluorescence of fluorescein and dipyrromethane boron difluoride derivatives is controlled through intramolecular photoinduced electron transfer (PeT) from either an acceptor to the donor fluorophore or from a donor to the acceptor fluorophore. In the case of fluorescein, the xanthene moiety is the fluorophore, and the benzene can play a role as donor or acceptor for PeT, depending on the respective redox potentials (*11, 12*). This knowledge can be exploited for the rational design of various fluorescent sensors. For example, when benzene moieties were selected that were capable of donor PeT to the neutral xanthene moiety but not to its electron-rich deprotonated form, a highly sensitive fluorescent sensor for the ubiquitous reporter enzyme β-galactosidase was generated (Figure 1, panel b) (*12*). Compared with the previously used fluorescein-based sensors for β-galactosidase, this sensor displayed a faster and more linear response to β-galactosidase activity and should become a useful tool in various assays. More importantly, the same design principles should lead to the generation of fluorescent sensors for a wide variety of other biologically relevant molecules and activities.

Another noteworthy example of how clever design and synthesis of fluorophores can yield sensitive and reliable probes for enzymatic activities was recently reported by Raines and colleagues (Figure 1, panel c) (*14*). A fluorogenic esterase substrate was chemically coupled to ribonuclease (RNase) A, and the endocytosis of the labeled protein was followed by fluorescence microscopy. Esterases present in endocytic vesicles liberated the fluorophore and allowed the study of endocytosis of RNase A in human cells with temporal and spatial resolution. Clearly, the design of new synthetic fluorescent sensors for the numerous biological processes for which visualization is not yet possible is urgently needed and remains an exciting and rich field for chemical biologists.

Measuring Ions. Monitoring the concentrations of molecules or ions in the presence of high concentrations of other molecules with similar properties requires fluorescent sensors of exquisite selectivity. Zinc, although considered a trace element, is an abundant metal ion whose concentration within eukaryotic cells is ~100 μM (*15, 16*). Zinc is bound to various proteins, including transcription factors, and acts as a cofactor in

several enzymes. The total concentration of zinc in cells is relatively high, whereas the concentration of free or rapidly exchangeable zinc is very low; estimates of the concentration of free zinc in prokaryotic cells are in the femtomolar range (*17*). Measuring free or rapidly exchangeable zinc at picomolar or lower concentrations in the presence of high concentrations of calcium and magnesium thus requires a highly specific and sensitive fluorescent sensor. Although a variety of synthetic fluorescent sensors for zinc have been developed and applied in recent years, their performance did not allow the reliable measurement of free zinc in resting eukaryotic cells (*18*). To address this problem, the groups of Thompson and Fierke have taken advantage of the strong and highly specific binding of zinc by the enzyme carbonic anhydrase (CA) to create a semisynthetic ratiometric fluorescent sensor (Figure 2) (*19, 20*). CA binds zinc in its active site with picomolar affinity and very high specificity over other metal ions. Fluorescent aryl sulfonamides bind to the zinc in the active site of holo-CA. When CA is covalently labeled on a specific cysteine residue with another fluorophore, a zinc-dependent FRET between the two fluorophores can be observed (Figure 2). To measure the zinc concentration in the cytosol of cultured mammalian cells, the scientists first expressed the enzyme as a fusion protein with a so-called transduction domain and then modified it at the free cysteine with Alexa Fluor 594 (AF594). The transduction domain is necessary for uptake of the protein sensor into mammalian cells (*21*); the aryl sulfonamide itself is membrane-permeable. With this sensor, the concentration of exchangeable zinc in a PC-12 cell culture line was measured to be ~5–10 pM. One advantage of this protein-based sensor is the ease by which the affinity for zinc as well as its exchange rate can be controlled through site-directed mutagenesis of the enzyme. A disadvantage is that the semisynthetic sensor needs to be introduced into the cells with the help of transducing peptides or some other invasive approach. This makes applications of the sensor for measurements in tissues and animals probably unsuitable. A possible solution to this problem would be to directly express CA in the cells of interest as a fusion protein with an AFP or with a tag that can be specifically labeled inside living cells with synthetic fluorophores (*22*).

KEYWORDS

AGT-based protein labeling: A protein is expressed as a fusion protein with a mutant of O^6-alkylguanine-DNA alkyltransferase (AGT is also abbreviated as SNAP-tag) and covalently labeled with synthetic probes through the specific reaction of AGT with benzylguanine derivatives carrying the synthetic probe.

Autofluorescent proteins (AFPs): Proteins that naturally fluorescence, such as green fluorescent protein. These AFPs are often fused to other proteins and used to follow the movement of the protein in the cell.

Carrier-protein-based protein labeling: A protein is expressed as a fusion protein with a carrier protein and covalently labeled with synthetic probes by phosphopantetheine transferases and CoA derivatives carrying the synthetic probe.

Fluorescence microscopy: A microscopy technique used to study samples that can be made to fluoresce. The sample can fluoresce naturally or can be treated with fluorescing chemicals.

FlAsH: A biarsenical fluorophore based on fluorscein that specifically binds to peptides containing a tetracysteine motif.

Fluorophore: A component of a molecule, often a functional group in a molecule, which will absorb energy of a specific wavelength and re-emit energy at a different but specific wavelength.

Tracking Proteins in Time and Space. As for ions and metabolites, the development of technologies that allow us to visualize proteins transformed our understanding of their behavior in living cells (*1, 22*). AFPs as reporters for protein localization and movement played an important role in this transition (*1*). The successes and limitations of AFPs have motivated scientists to pursue various alternative approaches (*22–24*). Similar to the use of AFPs, the protein of interest is expressed as a fusion protein with an additional polypeptide, a so-called tag. In contrast to AFPs, the role of the peptide is not to spontaneously form a fluorophore but rather to react with a synthetic fluorophore (or any other spectroscopic probe) in a highly specific manner. Several tags have been introduced over the years, but so far relatively few of these technologies have found applications outside the lab of the inventors. Among these are the tetracysteine tag (*25*), mutants of O^6-alkylguanine-DNA alkyltransferase (AGT; also known as SNAP-tag *26, 27*), carrier proteins (CPs) (*28, 29*), as well as the HALO-tag (*30*). A more complete overview of the different labeling approaches can be found in several recently published reviews (*23, 31, 32*). What are the strengths of these new tags compared with AFPs? First, the properties of the self-labeling tag itself might be advantageous compared with those of AFPs. These include applications for which the size of the tag is crucial (*33*) or conditions under which AFPs do not form their fluorophore, such as anaerobic environments (*34*). Second, the new tags allow labeling with a variety of fluorophores that possess spectroscopic properties, such as photostability or red-shifted excitation and emission maxima, which cannot be found in any of the currently existing AFPs (*35*). Third, the possibility of controlling the localization and time point of the labeling gives the researcher an additional degree of freedom to study protein function in

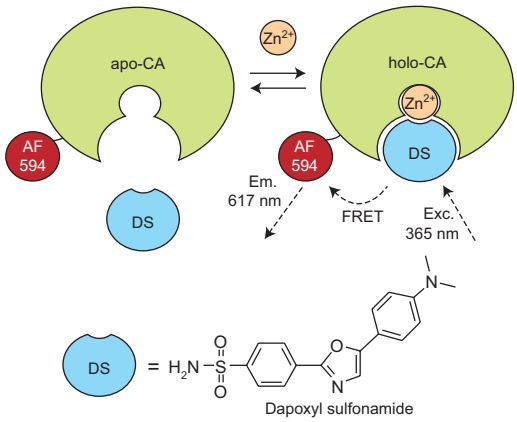

Figure 2. CA-based fluorescent sensor for measuring the concentration of zinc ions in living cells. CA is expressed as a fusion protein with a cell-penetrating peptide (not shown) and labeled with the synthetic fluorophore AF594 at Cys36. Binding of zinc by CA leads to subsequent binding of the fluorescent CA inhibitor dapoxyl sulphonamide (DS). Excitation of DS at 365 nm bound to CA results in efficient FRET to AF594 and emission at 617 nm. The measured emission at 617 nm upon excitation at 365 nm is standardized by dividing it by the emission at 617 nm measured upon direct excitation of AF594 at 540 nm.

time and space inside a living cell. In the following sections, we will focus on recent examples from the literature that exploit some of these benefits.

Time. The covalent labeling of proteins with synthetic fluorophores offers the choice of using different dyes at various times during the experiment. In living cells, where protein translation is not inhibited, this application will create distinct populations of otherwise identical molecules whose discriminating features are determined through the time point of the respective labeling of each population. The time interval between the additions of new label can be in the minute range, depending on the speed and efficiency of the reaction. The visualization of the formation of cellular substructures is one of the interesting applications that exploit this feature, provided that the subunits of this structure possess a low turnover to give sufficient time for subsequent rounds of labeling reactions. Two examples of this strategy were already reported. The first example is based on a labeling approach that leads to the formation of a stable complex between a biarsenical compound and proteins fused to a peptide containing a tetracysteine motif (*25*). Incubation of cells with FlAsH (green) or ReAsH (red), two biarsenical fluorophores, specifically label those proteins in the cell that carry the tetracysteine motif (*25*). Connexin43 was expressed as a fusion to a tetracysteine tag in order to use this technology to investigate the assembly of gap junction proteins in living cells. The cells were subsequently treated with pulses of FlAsH and ReAsH. Fluorescence microscopy of the labeled cells revealed that gap junction plaques are assembled from connexins from the outer edge of the plaques and are disassembled when their subunits are removed from the central core (*36*). The second example made use of the irreversible labeling of CP fusion proteins with fluorescent derivatives of coenzyme A (CoA). Specific phosphopantetheine transferases (PPTases) attach the fluorescent substitute of phosphopantetheine from the corresponding CoA substrates to the CP moiety of the fusion protein (*28*, *29*). CP was fused behind a signal sequence at the N-terminus of a protein that localizes on the surface of the cell to allow visualization of protein secretion and cell wall formation in yeast (*37*). Once secreted, this type of protein is immediately and covalently fixed to the rigid glucan layer of the cell wall. Because secretion in yeast is highly polarized and directed to different locations at the plasma membrane during the cell cycle, the subsequent addition of two and even three different CoA dyes allowed visualization of distinct areas where secretion has occurred during the experiment (Figure 3). One interesting outcome of this study was the visualization of a small fluorescent ring that completely surrounded the region where mother and daughter cells separate (*37*). The labeling of this structure from the outside of the cell indirectly demonstrates that secretion during cell separation occurs at the rim of the cells and that the new membrane is pulled from there to the center of the divi-

KEYWORDS

FRET: Fluorescence resonance energy transfer (or Förster resonance energy transfer) in which a fluorescent donor is excited at its specific fluorescence excitation wavelength. This excited state is then nonradiatively transferred to a second molecule *via* a long-range dipole–dipole coupling mechanism to a second molecule, the acceptor. This method is often used to measure the distance between the donor and acceptor groups.

Photoinduced electron transfer (PeT): An electron transfer resulting from an electronic state produced by the resonant interaction of electromagnetic radiation with matter. PeT is observed in certain dyes and has gained substantial interest for sensor development.

Protein transduction domains: Polypeptides or proteins that can be used to deliver cargo across the plasma membrane into cells.

Pulse-chase labeling: A method to follow the assembly of structures or the movements of proteins in the cell. A fluorescent dye is added to a cell that expresses a certain fusion protein that will be specifically labeled with this dye. The dye is then washed away and replaced with a second or even a third dye to consecutively label those fusion proteins that were made between the labeling intervals. Pictures are taken at defined times to follow the locations of the labeled proteins in the cell.

ReAsH: Biarsenical fluorophore based on resorufin that specifically binds to peptides containing a tetracysteine motif.

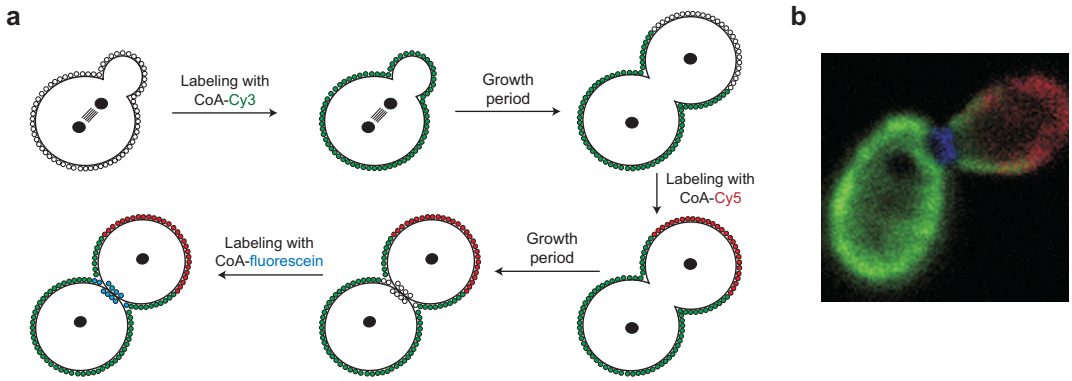

Figure 3. Pulse-chase labeling of CP fusion proteins for studying cell wall formation in yeast. a) Cartoon of a pulse-chase experiment with a budding yeast cell expressing the cell wall protein Sag1p as a CP fusion protein (white circles). b) Fluorescent micrograph of a yeast cell expressing CP-Sag1p on its surface after labeling the cell subsequently with CoA-Cy3 (green), CoA-Cy5 (red), and CoA-fluorescein (blue).

sion plane to separate the cytosols of the dividing cells (*38, 39*). A different mechanism is seen in plant cells where the vesicles fuse at the center to form two new membranes that grow toward the rim.

A similar application of the CP technology can be envisioned for the investigation of the assembly of the extracellular matrix that surrounds mammalian cells. The proteins of the matrix require efficient translocation across the membrane, extensive modification and processing, and a complicated assembly process and cross-linking reactions on the surface of the cell. Finding a place where a bulky polypeptide addition might not interfere with the maturation and function of these proteins is therefore not trivial. A recent development to reduce the size of the CP tag from originally 76 to 11 residues without compromising too much on the specificity and the kinetics of the labeling reaction might meet this demand (*40*).

Instead of the application of dyes in succession, adding a mixture of dyes will lead to the simultaneous labeling of the fusion protein with more than one label. For example, labeling a single protein with two different fluorophores forming an excellent FRET pair is attractive for studying the aggregation state of proteins in living cells through FRET. Fusing CP to the neurokinin-1 receptor (NK1R) and treating the CP-NK1R-expressing cells with mixtures containing different ratios of Cy3- and Cy5-CoA and phosphopantetheinyl allowed Vogel and colleagues to determine by quantitative FRET microscopy whether NK1R forms multimers or other forms of aggregates on the cell surface (*41*). In this study, it was shown that NK1R stays monomeric at its physiological concentration and clusters in microdomains whose diameters are ~10 nm.

For measuring similar processes in the cell, a mutant of AGT was recently created that can be specifically labeled with substrates that are not accepted by wild-type AGT (*42*). This permits the labeling of two different AGT fusion proteins with various fluorophores in the same cell or *in vitro*. Such doubly labeled AGT fusion proteins are attractive candidates for the construction of FRET-based sensors and FRET pairs orthogonal to those of AFPs.

Space. The photoactivation of specific AFPs creates visible proteins at the sites where the laser is pointed (*43*). This precision in space cannot be matched by chemical labeling strategies yet. One way to improve the local specificity of the labeling reactions might be to employ substances that preferentially accumulate at substructures or compartments of the cell. However, the extent to which synthetic molecules might be enriched in certain subcompartments of the cell without interacting with any of its components is probably limited.

An alternative approach is self-evident from the underlying mechanism of the CP technology. Here, the physical contact between PPTase and the CP fusion protein is required to transfer the dye to the protein of interest. Once the enzyme can be fixed, the reaction will only occur at the site where the enzyme is located. Labeling cell surface proteins from the extracellular site of the membrane by the exogenous addition of enzyme and substrate is already one example of this strategy (*29*). Membrane proteins that carry the tag on their extracellular site are only labeled once they reach the surface of the cell. Once the protein is labeled, its trafficking can be studied and its final destination in the cell precisely determined. In a recent example of the CoA-mediated

labeling of CP fusions, a peptidyl carrier protein-transferrin receptor fusion was modified with AF488 exclusively at the surface of the cell (*44*). A fluorescently labeled transferrin allowed to follow the comigration of the ligand–receptor pair by FRET microscopy to the intracellular compartment of the cell.

Immobilizing the enzyme onto beads that can then be moved on the surface of cells by laser tweezers will extend the same approach and restrict the generation of labeled molecules to a very small region of the cell. For intracellular purposes, a similar technique based on an enzyme–substrate pair has yet to be developed. Targeting the enzyme of such a pair to a certain compartment of the cell would restrict the origin of the labeled acceptor fusions to this compartment and would allow the movement of the acceptor fusion from there to other regions in the cell to be measured.

Although we discussed the approaches separately in this Review, we expect to see strategies that will combine the design and application of new fluorescent sensors with the possibility of specifically labeling proteins with synthetic molecules in living cells. The indicators can thus be precisely directed to selected sites and compartments within the cell to yield a better resolution of the spatial distribution of metabolites, ions, and enzymatic activities. The reported generation of a short peptide tag that specifically binds to synthetic calcium sensors in living cells is a step in this direction (*45*).

In this Review, we focused on chemical methods that complement features of the available AFPs for measuring cellular activities. These strategies are not simply alternatives to AFPs. Instead, it is our opinion that only a combination of different approaches will illuminate the multiple aspects of protein function and cellular pathways in living cells. The acceptance of chemical tools in cell biology depends foremost on their success in answering relevant questions that resisted other standard techniques in the lab. As we see increasing examples of this in the literature, a second critical but nonacademic issue emerges. The cell biologist cannot be expected to synthesize the substances that are developed by the chemist. For new chemical methods to have a broad applicability, universities must react quickly and be flexible in solving patent and licensing issues and companies must be more willing to take the risk of marketing and distributing substances that are not yet accepted.

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