Fluorescence in Living Systems: Applications in Chemical Biology

Virginia W. Cornish and Sarah S. Gallagher, *Columbia University, New York, New York*

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Advanced Article

Article Contents

Increasingly, studies of biomolecules are progressing from understanding how isolated biomolecules work *in vitro* to asking how macromolecular machines, signaling pathways, and other biological networks function in the complex environment of the living cell. Just as chemical methods greatly impacted fundamental studies of the structure and function of biomolecules *in vitro* in the last century, chemical methods are now being developed to study biomolecule function *in vivo*. This review focuses on chemical biology tools being developed to provide fluorescent reporters for biomolecules in living cells. The wealth of *in vitro* fluorescent reporters is the underpinning of much of this research, but it is beyond the scope of this review. Emphasis is placed on protein tagging, beginning with the fluorescent proteins and more recently moving to chemical tagging methods. In addition, chemical tools for fluorescence imaging of other classes of biomolecules and dynamic modifications to biomolecules in living cells are also overviewed.

Chemical methods for labeling biomolecules site specifically with biophysical probes have significantly impacted fundamental studies of biomolecules *in vitro* and provided practical tools for drug discovery in the last century. Now, chemical biology is providing tools to report on biomolecules inside a living cell. The thiol-reactive probes that are so effective at labeling a purified biomolecule *in vitro* simply cannot be adapted to label an individual biomolecule of interest *in vivo* in the sea of biomolecules and other reactive species present in the cell. Thus, advances in organic chemistry and DNA technology are being combined to allow individual proteins, nucleic acids, ions, and even signaling molecules and metabolites to be labeled selectively and imaged in a living cell. Initial advances, such as the fluorescent proteins, have largely relied on direct tagging of the molecule of interest and have had a major impact on biomedical research. Chemical tags have the potential to provide smaller tags that minimally perturb biomolecule function and to allow incorporation of fluorophores with increased brightness and new functionalities. The early molecular reporters for ions are now mainstay tools in biomedical research. In addition, these ion reporters show the potential to offer a general motif for molecular reporters for other molecules that are too small to be tagged directly and for monitoring dynamic chemical changes to molecules *in vivo*. Advances in the design and directed evolution of molecular recognition are significantly impacting molecular reporter design. Fluoresecent reporters have already significantly impacted fundamental biomedical research and drug discovery even though at this point they primarily provide largely descriptive information, such as the location of protein expression or the timing of a change in intracellular calcium ion concentration. As the design of molecular reporters grows more sophisticated and is interfaced with advances in microscopic imaging, the goal is to allow temporal resolution of individual biomolecular interactions to understand the mechanisms of macromolecular machines, signaling pathways, and biological networks in the context of a living cell.

The Fluorescent Proteins

With the introduction of the fluorescent proteins (FPs) as a selective, genetic protein tag, live-cell imaging became a mainstay tool in cell biology. Naturally occurring and engineered FPs have been optimized for spectral variation and increased brightness and other properties. FP tags are used routinely to observe the timing and location of protein expression *in vivo*, which often provide significant mechanistic insight. For applications in the *omics* and drug discovery, the FPs allow high-throughput

imaging of large numbers of samples. The FPs are now being adapted to enable temporal resolution of biomolecular interactions to understand the mechanisms of macromolecular machines, signaling pathways, and biological networks in the context of the cell through single-molecule tracking, Förster resonance energy transfer, and other approaches. Finally, as detailed in the subsequent sections, the FPs are providing inspiration and being adapted themselves to report on other classes of biomolecules and dynamic events *in vivo*. Here, we provide an overview of the FP technology. Excellent, more comprehensive reviews of the FPs are given elsewhere (1–3).

Fluorescent proteins are powerful tools for molecular imaging in living cells

In 1994, Chalfie et al. (4) and Tsien et al. (5) independently reported that a naturally occurring fluorescent protein from the jellyfish *Aequorea victoria* could be used for molecular imaging in living cells. The green fluorescent protein (GFP) from *A. victoria* is a 238 amino acid protein, which on folding spontaneously forms a fluorescent chromophore by rearrangement and oxidation of a Ser, Tyr, and Gly residue in the core of the 11-stranded β-barrel (**Fig. 1a**) (6, 7). A protein can be selectively tagged with GFP *in vivo* simply by introducing a plasmid encoding the protein-GFP fusion by standard transfection techniques and then imaged in the living cell using confocal microscopy.

The significance of the FPs is that they allow protein distribution to be monitored in a minimally perturbed, living cell. Thus, the protein's location is directly observed in real time, as opposed to inferred from reconstituted *in vitro* systems, fixed cells, or genetic knockouts. Even the straightforward dynamic localization experiments doable with today's mainstream technology can be very informative mechanistically. For example, conflicting hypotheses about the role of Gal3p in activation of the Gal genes in yeast were resolved by imaging studies showing that Gal3p resided in the cytoplasm and so must act by sequestering the transcriptional repressor Gal80p, not by serving as a transcription factor itself in the nucleus (8). Furthermore, the FPs facilitate genome-wide studies (the *omics*). For example, global analysis of *Saccharomyces cerevisiae* strains encoding most of the ∼6600 yeast proteins tagged with GFP provided the subcellular localization of every yeast protein—a labor-intensive analysis just for individual proteins with traditional biochemical methods (notably ∼30% of the proteins could not be detected, presumably because the signal to noise obtained with EGFP is not sufficient to detect proteins expressed at low concentrations or dispersed among several cellular compartments) (9). Finally, FPs are used routinely in high-throughput assays for drug discovery (10).

Properties that determine imaging capabilities of the fluorescent proteins

The critical performance criteria for fluorescent proteins are brightness, photostability, expression (both robustness and oligomerization), chromophore maturation half-life, and spectral variation (3). Through characterization of naturally occurring FPs from different organisms and molecular evolution, FPs spanning the visible region have been obtained. Although a few very bright FPs are available, the best variants tend to have brightnesses on the order of $50\,mM^{-1}cm^{-1}$ (1). Brightness, which is the product of the extinction coefficient and the quantum yield, is critical for good resolution. Photostabilities for the best FPs are reported to approach $t_{1/2} = 100$ seconds under optimal conditions (3). [A caveat, measurements of photostabilities are highly condition dependent, which complicates comparisons of photostabilites from the published literature (3)]. Photostability of the chromophore is important for two reasons. First, it allows use of higher intensity excitation light, which yields higher intensity fluorescence signal. Second, the molecule can be tracked for longer time periods because the half-life to bleaching is longer. FPs continue to suffer from formation of higher order oligomers, which can interfere with biological function of fusion proteins (11). Several FPs, however, have been engineered to favor the monomeric form (12–16). Through molecular evolution, fast-folding variants have been obtained (17), but for most FPs, the chromophore requires ∼1 hour to form inside the protein β-barrel (1, 3, 12, 14, 16, 18–21). For multicolor tagging, the difficulty is that when the FPs are

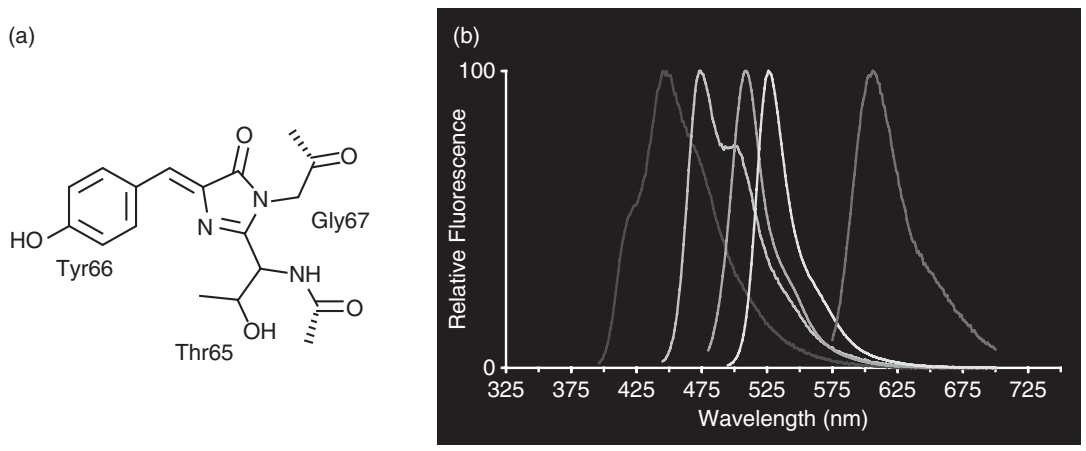

Figure 1 The fluorescent proteins. a) Cartoon of the chromophore that forms in the core of the GFP. b) Emission spectra of FP spectral variants that illustrate the broad-emission spectra of the FPs.

Table 1 Photophysical properties of multicolor and photoactivatable FPs versus Cy dyes

Dye	Excitation (nm)	Emission (nm)	Brightness ($mM^{-1}cm^{-1}$)	Photostability[d] (s)
Cerulean[a]	433	475	27	36
mOrange[a]	548	562	49	9
mPlum[a]	590	649	4	53
PA-GFP[b]	504	517	19	~174
Cy3B[c]	550	570	87[d]	ND[e]
Cy5[c]	646	662	>70[d]	ND[e]
Cy5.5[c]	683	707	>70[d]	ND[e]

[a]Three best FP spectral variants for multicolor tagging as reported by Shaner et al. (3).
[b]Photoactivatable GFP variant reported by Patterson and Lippincott-Schwartz (22).
[c]Three optimal Cy dyes for multicolor tagging from Molecular Probes.
[d]The photostabilities of the Cy dyes have not been determined under experimental conditions that would allow for comparison to the FP values reported here. However, the Cy dyes are used routinely for single molecule experiments and have good photostabilities (Reference).
[e]Sanborn, ME, Connolly BK, Gurunathan K, Levitus M. Fluorescence Properties an Photophysics of the Sulfoindocyanine Cy3 Linked Covalently to DNA. J. Phy. Chem. B 2007;111:11064–11074.

evolved for spectral variation, they often experience losses in their other photophysical properties (**Table 1**) (22)—although continual improvements of individual FPs are being made using directed evolution and fluorescence-activated cell sorting. Also, because the FPs have broad absorption and emission spectra and are difficult to engineer for longer-wavelength absorption and emission, three or (at best) four FP wavelength variants can be imaged simultaneously with conventional methods (**Fig. 1b**). With spectral deconvolution imaging, however, it is increasingly possible for experts in the field to image even greater numbers of spectral variants simultaneously. By way of example, the photophysical properties of the three best FPs for multicolor imaging are shown in **Table 1**. Thus, no single FP is optimal for all performance criteria, and it is important to choose the right FP carefully for a given application.

Through advances in imaging technology, progress has been made to adapt the FPs for single-molecule imaging in the last few years (11). It has been shown that engineered photo-activatable and photo-switchable FPs can be used to increase the resolution of molecular imaging using methods like photoactivation localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) (1, 2, 22–26). The most recent publications show resolution on the order of 10 nm with PALM (2), and the first extensions of this technique from fixed to live cells (27). FP donor–acceptor pairs are being engineered for Förster resonance energy transfer (FRET) to report on biomolecule interactions (28). FRET pairs that can read out biomolecule interactions *in vivo* have the potential to impact fundamental mechanistic studies and drug discovery significantly. However, interpretation of FRET measurements in the complex cellular environment remains challenging given the photophysical limitations of existing reporters and orientation effects of energy transfer. Finally, with state of the art microscopy, leaders in the field have been able to image immobilized proteins such as α-tubulin in microtubule filaments (29, 30) and most recently a DNA-binding protein bound to DNA (31) at the single molecule level in live cells.

Chemical Tagging Methods

Motivated by the broad impact of the FPs, chemical tags have begun to be developed for labeling proteins *in vivo*. These chemical tags retain the specificity of protein labeling through genetic encoding, but they have the potential to provide smaller, more robust tags and modular use of small molecule probes with increased brightness and new functionalities. Three main strategies are taken in the design of chemical tags. First, an intact protein is used as the tag and labeled noncovalently or covalently with its small-molecule ligand or substrate. Second, a short peptide tag is labeled noncovalently or covalently with a small molecule. Third, "unnatural amino acid mutagenesis" is used for the direct incorporation of an amino acid fluorophore or "chemical handle" that can be subsequently modified. As discussed below, generally a trade-off occurs between the size of the tag and its selectivity and hence utility for labeling intracellular proteins. Other than the FlAsH tag, the chemical tags are just beginning to be employed as research tools for cell biology—thus, the utility of the chemical tags will only be tested in the coming years. Here, we overview the different chemical tagging methods. Several very good, more detailed reviews of the chemical tagging technologies are available (32–35).

Protein tags

Rather than tagging the protein of interest (POI) with a FP, the POI can be tagged with a protein receptor or enzyme. The protein tag can then subsequently be labeled with a cell-permeable small molecule ligand- or substrate-probe heterodimer. Several critical design issues exist for these protein receptor-ligand or enzyme-substrate tags. First, the small-molecule ligand or substrate must be readily cell permeable. Second, the synthesis of the ligand or substrate derivatives should be straightforward and minimally disruptive to the receptor binding or enzyme reaction. Third, the protein receptor or enzyme should be small, monomeric, and well behaved for minimal perturbation of the biological pathway being studied. Last, the protein label must not bind non-specifically to endogenous proteins or other macromolecules or otherwise partition to particular organelles. To

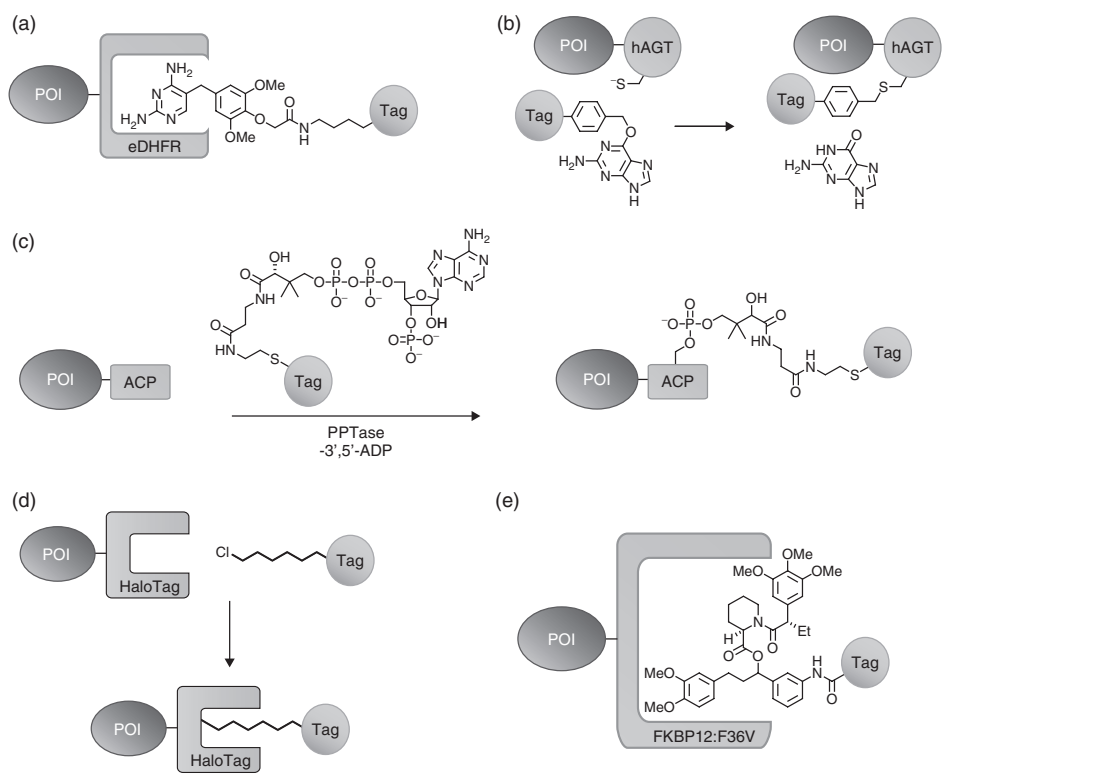

Figure 2 Chemical tagging methods. (a–e) Cartoons that depict different chemical-tagging methods. a) TMP-DHFR labeling strategy from Miller et al. (36), b) SNAP-Tag from Keppler et al. (37), c) CoA from Yin et al. (38) and George et al. (39), d) HaloTag from Promega Cell Notes (40), and e) SLF-FKBP12:F36V labeling strategy from Marks et al. (41).

date, the advantage of the protein tags over the other chemical tags is that they are sufficiently selective to enable intracellular proteins to be imaged with good resolution.

In collaboration with the Sheetz group, our laboratory has exploited the high-affinity interaction between dihydrofolate reductase (DHFR) and folate analogs to label proteins *in vivo* (**Fig. 2a**) (36–42). Briefly, rather than tagging the POI with a FP, we tag with the *Escherichia coli* DHFR. Because *E. coli* DHFR (eDHFR) binds trimpethoprim (TMP) with high affinity (1.9 nM K_D) and high selectivity (affinities for mammalian DHFRs are $K_D > 1\,\mu M$), the eDHFR tag can then be labeled selectively through a noncovalent interaction with cell-permeable TMP-probe heterodimers (43). The well-studied TMP-DHFR interaction provides a robust ligand–receptor pair to serve as a chemical tag. Consistent with the therapeutic use of TMP as an antibiotic, the TMP heterodimers have excellent cell permeability properties (44). As anticipated based on high-resolution structural data and SAR data, TMP can be modified at the para-methoxy position on the benzene ring with only minor perturbation of high-affinity binding to *E. coli* DHFR (45). The chemistry for derivatizing TMP is straightforward. Thus, different TMP heterodimers can be prepared readily. *E. coli* DHFR is a 159 amino acid, monomeric, well-behaved protein (46). Compared with the FPs, DHFR is advantageous because it is about two thirds the size, does not suffer from oligomerization and expression problems, folds rapidly, and does not bring the issue of chromophore maturation half-life. Presumably because of the high-affinity and high selectivity of TMP for DHFR, this tag can be used to image intracellular proteins with good resolution *in vivo*.

Through optimization of the TMP-probe heterodimers, as of 2007, we had TMP-green and TMP-red labels that could be used to label intracellular proteins with good resolution in a variety of mammalian cell types. First, we reported the use of methotrexate and DHFR for tagging proteins *in vivo* (47). The drawback to this system, however, was that it could only be used in DHFR(-/-) knockout cells because methotrexate also binds with high affinity to mammalian DHFRs. Then, we reported the use of TMP in place of methotrexate (36). Although TMP allowed labeling of wild-type (wt) mammalian cells, the initial TMP-fluorophore heterodimers reported could only label proteins highly concentrated and immobilized at the plasma membrane or in the nucleus. Finally, we developed optimized TMP-fluorophores and showed that these molecules were readily cell permeable and could label cytoplasmic proteins in a variety of cell types (42). In addition to providing improved TMP labels, this work establishes that standard linker and protecting group chemistry is sufficient to render ligand-probe heterodimers cell permeable. Important for use by the biology research community (i.e., laboratories not specializing in organic synthesis), the TMP tag is commercially available from Active Motif (Carlsbad, CA) as LigandLink. The current optimized TMP-green and TMP-red labels are both based on the fluorescein chromophore, which is not ideal because of its

poor photostability. Nonetheless, the TMP-red label provides an immediate alternative to the red FPs, which have suffered from oligomerization and other issues. The fluorescein chromophore in the optimized TMP-green tag is established in the published literature to allow chromophore-assisted laser inactivation (CALI) of proteins *in vivo* with spatial and temporal resolution. Recently, Cai et al. (48) have successfully used the TMP-green tag for CALI of intracellular myosin to test the hypothesis that the intracellular actomyosin network is actively involved in cell spreading at the periphery. The next steps are 1) to continue to develop TMP-fluorophores with improved photophysical properties that are cell permeable and 2) to develop orthogonal TMP-eDHFR pairs for multicolor labeling.

Based on the mechanism of the natural DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT), Keppler et al. (37) have developed the covalent "SNAP-tag" (**Fig. 2b**). AGT repairs O^6-alkylated guanine residues in damaged DNA by a single turnover alkylation of an active-site Cys residue. The researchers have shown that they can use guanine derivatives modified at the O^6 position with fluorophores to label proteins covalently fused to AGT in live cells. This system has many of the advantages of the TMP tagging system. AGT is a small (20kD), monomeric protein that is well behaved *in vivo*. Background labeling of endogenous mammalian AGT is minimal with use of the engineered, faster SNAP-tag variant (49). The guanine fluorophore heterodimers are cell permeable, and this technology can also be used to label cytoplasmic proteins *in vivo*.

The SNAP-tag has been extensively modified in the past few years and is now being used as a research tool for cell biology. The original AGT tag was reported in 2003 (37). Directed evolution was used to engineer AGT variants with resistance to an inhibitor of the wt AGT enzyme and an increased rate of reaction with the modified guanine probe substrates to overcome background labeling of endogenous wt AGT (49). Optimized guanine-probe heterodimers have been developed that span the visible spectrum and, importantly, can label cytoplasmic proteins (50). These small-molecule probes are commercially available from Covalys. In early 2008, Gautier et al. (51) reported the directed evolution of an orthogonal AGT variant, called CLIP, that selectively uses cytosine fluorophores as substrates. This publication is significant because it should allow two different proteins to be labeled simultaneously, and it shows the feasibility of evolving orthogonal variants. Additional publications will test the robustness of the SNAP-tag, CLIP-tag pair. Speaking to the utility of this method, the SNAP-tag was recently used to carry out a pulse-chase experiment to understand the timing of centromeric protein A expression in centromere determination and cell division (52).

Yin et al. (38) and George et al. (39) have shown that the acyl-carrier protein (ACP) or peptide carrier protein (PCP), respectively, can be labeled covalently with 4′-phosphopantetheine-probe heterodimers using the enzyme phosphopantetheine transferase (PPTase) (**Fig. 2c**). The 4′-phosphopantetheine-linked probes are transferred from coenzyme A (CoA) to a serine residue in ACP or PCP by PPTase. The advantage to this tag is that it is small (9 kD), enzyme catalyzed, and covalent. Through directed evolution, PCP has even been replaced with a short peptide tag, although the peptide tag, not surprisingly, is a less efficient substrate than the protein tag (53). The disadvantage is the use of the highly charged 4′-phosphopantetheine small molecule label, which likely will be challenging to render cell permeable. Thus, the ACP/PCP tag to date works well for labeling extracellular proteins, but use *in vivo* awaits additional development.

Promega Cell Notes have reported a covalent tagging system by similar modification of a natural dehalogenase enzyme—"HaloTag" (**Fig. 2d**) (40). The selective interaction of synthetic ligand of FK506 (SLF) with the FKBP12:F36V variant has been reported as a noncovalent chemical tag (**Fig. 2e**) (41). Although these systems seem promising, too few publications at this time use these systems to judge their practical performance.

Peptide tags

In theory, a small peptide tag is preferable to a protein tag because it is small and so minimizes perturbation of the biological pathway being studied. The challenge is to label a peptide selectively with a small-molecule probe in the complex environment of the cell. To achieve this selectivity, enzyme-catalysis is being used increasingly to label the peptide with a small-molecule probe substrate. The design issues for peptide tags are similar to those for protein tags. Again, the small-molecule probe must be readily cell permeable. If the peptide is labeled by binding of the small-molecule probe, the difficulty is designing a peptide–small-molecule interaction that is high affinity to ensure fast, selective peptide labeling. If the peptide is modified by an enzyme, then the enzyme must be engineered to work efficiently with a small-molecule analog substrate to allow for incorporation of the fluorescent probe. To date, the enzyme-modified peptide tags are useful for labeling extracelluar proteins but have not been reported to label intracellular proteins in living cells.

The first report of a chemical surrogate to the FPs for labeling proteins with small molecule fluorophores in living cells was FlAsH from Tsien et al. (54) in 1998 (**Fig. 3a**) (54–56). In design, FlAsH is the ideal chemical label. A short tetracysteine tag is attached to the protein of interest and then labeled with a bisarsenate chromophore. Compared with the ∼250–amino-acid FPs, a short peptide tag should be much less likely to impair natural protein function. A modular small-molecule probe is attractive because the same tag could be used to introduce probes with different functionalities. Although ReAsH provides some technical improvements (57), at this point the FlAsH technology still suffers practically from background labeling of Cys-rich proteins in the cell and toxicity of the bisarsenate compounds. Nonetheless, several publications use FlAsH as a tool for cell biology research (58), which show the utility of this general approach.

Ting and colleagues (59, 60) have adapted natural enzymes that modify short peptides with small molecules to provide a covalent tagging method. The advantages to this approach are the use of a short peptide, which should cause minimal perturbation to the natural biological system, and a covalent label to the small-molecule probe. To date, Ting and colleagues have reported peptide modification using transglutaminase, biotin ligase, and lipoic acid ligase enzymes (56, 59, 60). The biotin ligase system is the most developed of the three (**Fig. 3b**). So

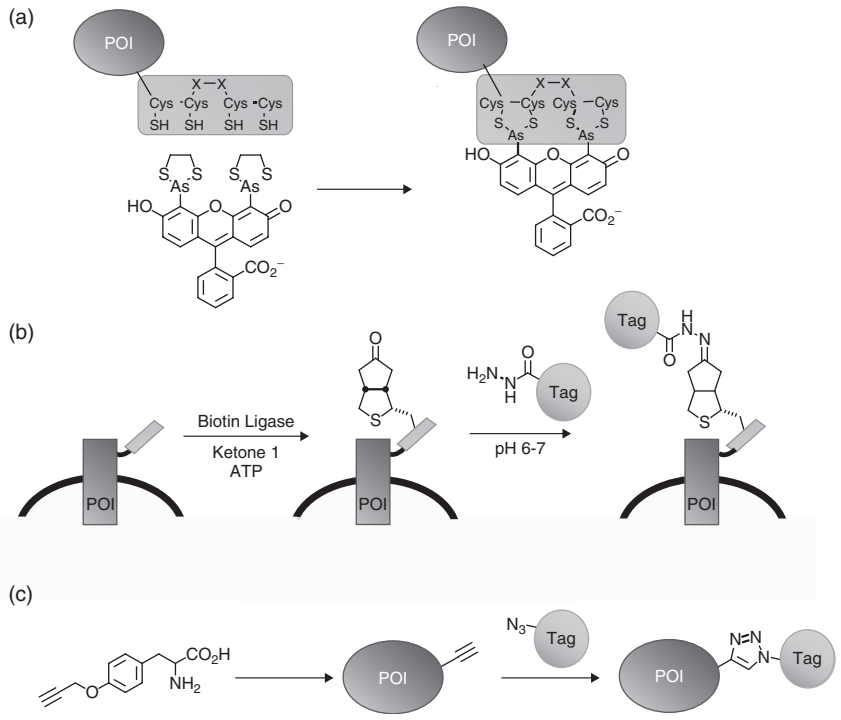

Figure 3 Chemical tagging methods. (a–c) Cartoons that depict different chemical tagging methods. a) FlAsH from Griffin et al. (54), b) Biotin ligase from Chen et al. (55), and c) Unnatural amino acid incorporation followed by click chemistry (56).

far, the one technical limitation to these methods is that the natural small-molecule substrate; for example, biotin can only be minimally perturbed (61). Whereas a biotin ketone derivative can be incorporated and then modified with orthogonal chemistry, a biotin–fluorophore heterodimer cannot be incorporated directly (55). With no driving force for a fast k_{on}, the second orthogonal chemistry labeling step is slow. In practice, what this has meant is that these methods are very attractive for labeling proteins at the cell surface, but they are not yet practical for labeling intracellular proteins. In theory, however, with extensive modification it should be possible to overcome these technical hurdles for direct incorporation of a small-molecule probe analog substrate.

The biotin ligase chemical labeling system was first reported in 2005 (55). Briefly, the *E. coli* biotin ligase enzyme, whose natural function is biotinylation of proteins that contain a peptide recognition motif, is used to modify a 15–amino-acid peptide tag with a biotin analog. A biotin ketone isostere (55), azide isostere (61), and alkyne isostere (61) have all been ligated to the peptide tag using biotin ligases from different organisms. Ting and colleagues have used this technology to label cell surface proteins with both small molecule fluorophores and quantum dots (55, 62).

Similar to the FlAsH technology, a poly-His peptide tag has been labeled with nickel-nitrilotriacetic acid-probe heterodimers (63). The difficulty, however, with this tag is that the Ni^{2+} quenches the chromophore fluorescence and the affinity of the poly-His tag for the ligand is moderate (low μM K_D). In an interesting approach, Marks et al. (64) evolved a 38–amino-acid peptide that binds the Texas red fluorophore with high affinity and used this peptide tag for live cell imaging. An N-terminal Cys residue generated *in vivo* with a sequence-specific protease has been labeled with thioesters, which is analogous to native chemical ligation (65). These different approaches illustrate the potential for chemical methods developed for *in vitro* protein labeling to be adapted for labeling biomolecules *in vivo*.

Direct incorporation of amino acid analogs

Finally "unnatural amino acid mutagenesis" is being used to incorporate small-molecule probes as the amino-acid side chain in living cells (**Fig. 3c**) (66). In this approach, the unnatural amino acid is either incorporated nonspecifically relying on the natural substrate promiscuity of the different components of the translation machinery or specifically using an evolved, orthogonal tRNA/aminoacyl-tRNA synthetase pair for incorporation of the analog in response to an engineered stop codon in the protein-coding sequence. In the long term, this technology should allow direct incorporation of a fluorescent amino acid into a protein as the protein is being synthesized in the living cell (67, 68). At this point, however, significant technical hurdles still must be overcome to adapt this technology for live cell imaging as opposed to protein production. For example, although the translation machinery does accept many amino-acid analogs, generally the large fluorophores cannot be incorporated (69). To date, the only fluorophores incorporated *in vivo* using an evolved tRNA/aminoacyl-tRNA synthase pair are coumarin and a dansyl fluorophore (68, 70)—neither fluorophore is sufficiently red shifted for good resolution from background cellular autofluorescence. Although a unique functional group like an

azide can be incorporated and then subsequently modified with a fluorophore-alkyne derivative (71), with no driving force for fluorophore binding, the chemical modification is slow. In addition, in its current embodiment, there is no specificity, as the fluorophore would be incorporated at every copy of the stop codon in the cell. Promiscuous incorporation of unique chemical handles such as an azide can be very useful in the right application (56); however, it is not a general solution.

Summary

The chemical tagging methods have developed to the point that robust labels are now commercially available and can be employed by cell biologists. Although orthogonal tags are being engineered, in practice, the way to label multiple proteins at this point is simply to employ multiple, different chemical tags. For example, in theory the FlAsH, TMP-, SNAP-, and Halo-tags could be used simultaneously to label four different intracellular proteins. For extracellular proteins, the biotin ligase and PCP tags could be employed. The main technical hurdle for such multicolor tagging experiment is the development of cell-permeable, bright, photostable ligand-fluorophore heterodimers with spectral variation. For an *in vitro* experiment or labeling extracellular proteins in which there is no issue of cell permeability, this experiment can be performed today simply by plugging in the best small-molecule or quantum dot fluorophores. For *in vivo* experiments, the best small-molecule fluorophores still need to be optimized to improve their cell permeability with the chemical tags. Put more broadly, the development of small-molecule fluorophores that are cell permeable and have optimal properties for live-cell imaging is an important current challenge for organic chemistry. The advantage to the chemical tags is that they are more modular, and thus advances to the tag (e.g., DHFR and TMP) and to the label (e.g., fluorescein) can be mixed and matched—such that advances in this field can rapidly be generalized. Finally, the next step is to think creatively about new functionalities that could be built into the chemical tags to more fully exploit their modularity to incorporate probes that go beyond simple fluorescence.

Detecting Biomolecules Other Than Proteins

Beyond direct tagging of proteins to visualize their expression and localization, protein engineering and synthetic chemistry are being exploited to report on other classes of biomolecules in living cells. With the ability to then detect the substrate or product of a reaction or the reversible interactions of biomolecules, these tools have the ability to report on the dynamics of biological processes in their natural context. Myriad approaches are being taken in this developing field. Here, the dominant current method is highlighted, with references to current reviews that offer complete coverage of research in each subfield.

Nucleic acid

Analogous to protein tagging with FPs, FP nucleic-acid binding fusions have been engineered to tag RNA and DNA in living cells (72–74). Bertrand et al. (75) have shown that RNA appended with multiple MS2-binding protein RNA hairpins can be labeled with MS2-FP fusion proteins in live cells (**Fig. 4a**) (75–77). Likewise, DNA has been labeled with DNA-binding-protein-FP fusions (78). These approaches are successful and have been used to study biological mechanism. Alternatively, molecular beacons and complimentary nucleic-acid tags are used to image nucleic acids based on sequence (72). With growing appreciation for the biological importance of short, regulatory RNAs, new strategies will be required to report on short RNA molecules without perturbing their structure and function.

Ions

Well before the introduction of the FPs as a tool for live-cell imaging, Tsien adapted the small molecule chelator EGTA to provide a fluorescent indicator for Ca^{2+} (**Fig. 4b**), which is an important biological signaling molecule (79, 80). The optimized fura-2 Ca^{2+} indicator is cell permeable, selective for Ca^{2+} over other bivalent cations, provides a ratiometric fluorescent signal, and does not perturb the cellular Ca^{2+} signaling pathways (76). The ability to monitor changes in intracellular Ca^{2+} concentration with spatial and temporal resolution has significantly impacted fundamental studies of synaptic transmission, muscle contraction and cardiac function, and other biological-signaling pathways dependent on Ca^{2+} (81). The original 1985 publication by Grynkiewicz et al. (76) of the intracellular fura-2 Ca^{2+} fluorescent indicator has been cited over 16,000 times.

Following a similar strategy, small-molecule fluorescent reporters have been developed for other ions (82, 83). Moving beyond ions, an efficient nitric oxide sensor has been developed that is a copper complex of a fluorescein analog (84).

Alternatively, FPs have been engineered to report on changes in ion concentration (28). For example, in "camgaroos" the Ca^{2+}-binding protein calmodulin is inserted in place of Tyr145 in YFP (19, 85). Presumably because conformational changes in calmodulin are transmitted to YFP, a seven-fold increase occurs in fluorescence on Ca^{2+} binding. "Cameleons," however, use Ca^{2+} binding by calmodulin to induce a conformational change between a chimeric YFP-calmodulin-CFP reporter that is detected as a change in FRET signal (77). As discussed in the following section, both strategies are general and can be adapted for the detection of other classes of molecules. Notably, pH reporters have been engineered based on a *Aequorea* fluorescent protein scaffold (28).

Small molecules

Reporters for small molecules (molecules $\leq$1 kD) are challenging to design because typically small-molecule metabolites, signaling molecules, and other molecules cannot be modified directly without disrupting their function (11, 81). Unlike with ions, simple chelation strategies cannot be exploited for the selective molecular recognition of diverse small-molecule structures. The most successful general strategy reported to date for small-molecule imaging is the FRET sandwich reporter (28). As for Ca^{2+} above, a YFP-recognition-CFP reporter is engineered

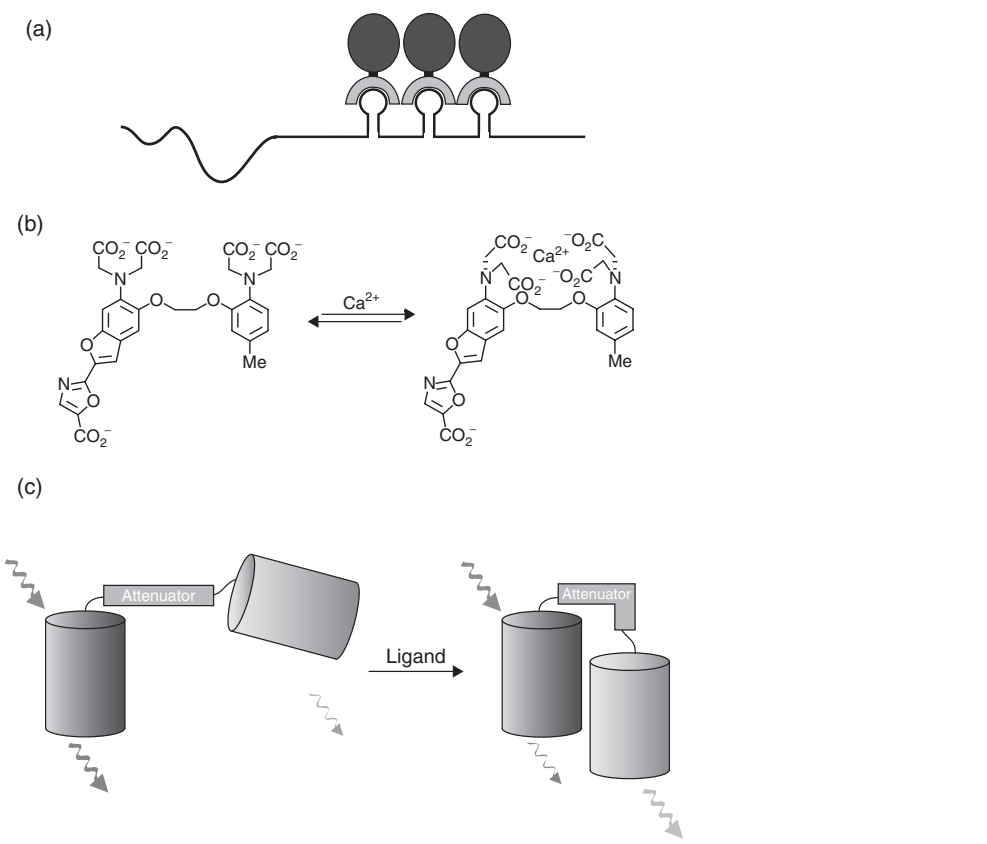

Figure 4 Detection of biomolecules. (a–c) Cartoons that depict different methods used for the detection of biomolecules *in vivo*. a) mRNA imaging using a MS2-FP fusion from Bertrand et al. (75), b) the fura-2 Ca^{2+} indicator developed by Grynkiewicz et al. (79), and c) a small-molecule imaging technique using a YFP-attenuator-CFP fusion that on ligand binding produces a change in the FRET signal (85).

such that binding of the small molecule by the recognition peptide induces a conformational change that alters the orientation between YFP and CFP and hence perturbs the FRET signal. For example, a FRET sandwich reporter has been developed for cAMP (**Fig. 4c**) (86).

In the case of localized signaling molecules, FP tagged binding proteins have been exploited to detect changes in local molecule concentration (28). This approach is widely used to report on the different phosphorylation states of phosphoinositides (PIPs) (87). Natural PIP binding proteins, which are specific for a given phosphorylation state, are fused to an FP. The change in fluorescence intensity at the membrane then serves as a read-out of the change in concentration of PIP. This approach has been rendered more quantitative by using two FP fusion proteins, in which the binding protein is constant, but a FRET donor and acceptor FP are used (88, 89). The change in PIP concentration at the membrane can then be read out as a change in FRET signal given that energy transfer will only occur efficiently when both the donor and acceptor are at the membrane.

Dynamic modifications

FP sandwich reporters have also been designed to report on posttranslational modifications, such as phosphorylation (90). For example, kinase activity has been read out as a change in FRET signal between YFP and CFP using a kinase substrate peptide-phosphopeptide binding protein fusion that undergoes a conformational change on peptide phosphorylation (91). Fluorophore-derivatized peptide substrates show promise as general biosensors for kinase and phosphatase activity (92), but they will need to be rendered cell permeable.

Longstanding *in vitro* fluorescent sensors for enzyme activity are beginning to be adapted for live-cell imaging. A small molecule FRET substrate for β-lactamase was reported by Zlokarnik (93), although to date it is used as a fluorescent gene-transcription reporter. A peptide-based reporter for capsase protease activity has been published for use *in vivo* (94). Wichmann et al. have developed small molecule FRET substrates for phospholipase A2 (95). Redox-sensitive optical switches have been adapted to provide fluorescent reporters for redox enzymes in living cells (96). Most recently FRET between a labeled enzyme and its substrate was used to reveal local regulation of phosphatase activity in COS-7 cells (97).

Finally reporters are being developed for other cellular events. FPs have been engineered to report on the redox state of a cellular compartment (98). Reporters that directly sense changes in voltage are being sought—a direct sensor of voltage would significantly impact the field of neurobiology (28). Very recently, a fluorescent protein complementation assay was used to provide a comprehensive map of protein–protein interactions in yeast (99).

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