Fluorogenic protein-based strategies for detection, actuation and sensing

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Abstract

Fluorescence imaging has become an indispensable tool in cell and molecular biology. GFP-like fluorescent proteins have revolutionized fluorescence microscopy, giving experimenters exquisite control over the localization and specificity of tagged constructs. However, these systems present certain drawbacks and as such, alternative systems based on a fluorogenic interaction between a chromophore and a protein have been developed. While these systems were initially designed as fluorescent labels, they also present new opportunities for the development of novel labeling and detection strategies. This review focuses on new labeling protocols, actuation methods, and biosensors based on fluorogenic protein systems.

1. Introduction

A. Fluorescent protein based reporters have revolutionized biological imaging

The discovery and subsequent development of GFP has fundamentally changed the way that biological questions can be asked and answered.[1] The ability to genetically tag a protein of interest or sense a cellular process in real-time allows us to interrogate the subcellular localization, timing, and magnitude of a particular phenomenon. The subsequent expansion in the color palette of fluorescent proteins increased the number of targets that can be imaged at once.[2] The development of photoactivatable fluorescent proteins and the advent of superresolution microscopy further expanded the detail with which intracellular structures and processes can be examined.[3] Indeed, fluorescent proteins have become ubiquitous to the point that they are used as a practical element in many standard protocols, e.g. as a reporter for the successful generation of a stable cell line.

However, there are certain drawbacks associated with classic autofluorescent proteins such as GFP or mCherry.[4,5] Their chromophores form via the cyclization, dehydration, and finally, oxidation of a triplet of amino acids (Ser-Tyr-Gly in GFP), which limits both their maturation time and imposes an absolute requirement for oxygen. The production of hydrogen peroxide during the maturation of GFP-like proteins can complicate *in vivo* experiments, as hydrogen peroxide is a known mediator of cell survival, growth, differentiation, and is implicated in diseases.[6-9] Furthermore, they often exhibit complicated photophysics that impedes quantitative analysis.[10,11] Despite efforts to engineer monomeric variants, many fluorescent proteins retain their tendency to oligomerize, which renders imaging in crowded environments particularly difficult. Finally, fluorescent proteins are comprised of a rigid and rather large (~26 kDa) β barrel that can impede the proper secretion,[12] folding[13] and/or function of many proteins.

To address these concerns and to further expand the possibilities of biological imaging, a suite of alternatives have been generated, retaining a protein tag, which affords the

classic advantages of protein tags – absolute labelling specificity and localization – but incorporating an external chromophore. These new systems are either based on natural fluorophores or synthetic fluorophores that must be added exogenously to the culture media. Furthermore, one can make the distinction between covalently and noncovalently linked chromophores, which changes the types of biological imaging that can be envisaged. While these new systems were originally conceived as a way to address the drawbacks of classical fluorescent proteins, they often present unique characteristics that can be used creatively to push the limits of biological imaging. Many of these systems are fluorogenic, meaning that the chromophore (often called fluorogen) exhibits little to no fluorescence when it is not bound to its receptor (**Figure 1**). This change in spectroscopic properties is typically due to a change in fluorescence quantum yield, chromophore absorption coefficient, or a spectral shift. Fluorogenicity imparts an inherent benefit in that background fluorescence is necessarily reduced, which is particularly interesting in the case of complex tissue samples and whole organisms. The different types of fluorogenic systems and their engineering has been recently reviewed elsewhere.[14,15] As such, this review will focus primarily on their implementation, with particular emphasis on labeling protocols and detection that have not been possible with classic autofluorescent protein systems.

Systems that use naturally occurring chromophores hijack them directly from cells, which thus solves the problem of chromophore delivery. Unfortunately, overexpression of these constructs can burden the cells and deprive them of important metabolic intermediates. To circumvent this issue, researchers can co-express more of the enzymes that generate the endogenous compound or add extra chromophore to the culture media, which partially negates the interest of using proteins that rely on endogenous chromophores. Since these chromophores are already present in cells, there are native proteins that already form interactions with them that can serve as scaffolds for the development or improvement of their natural chromophore-binding properties. However, this approach has inherent limitations due to the relatively small number of naturally-occurring, fluorogenic chromophores, meaning that only a small

chemical space can be explored to generate new complexes with novel spectroscopic properties.

B. Fluorogenic protein systems

All systems that rely on endogenous chromophores are based on one of three compounds: flavin mononucleotide (FMN), biliverdin, or bilirubin (**Figure 2 and 3, Table 1**). Systems that rely on complexation of FMN are typically non-covalent complexes that fluoresce in the cyan-green wavelength range.[16,17] These systems are based on bacterial photoreceptors comprised of light, oxygen, and voltage (LOV) sensing domains, which form a reversible covalent bond with FMN via a conserved cysteine residue over the course of their photocycle. Cysteine replacement with alanine combined with additional directed evolution allowed the generation of complexes that interact non-covalently in which FMN fluorescence is unquenched.[18,19] There is only one protein thus far that uses bilirubin as a chromophore.[20] UnaG was originally isolated from eel and forms a non-covalent complex that emits in the green wavelength region. It binds endogenous bilirubin in mammalian cells, but exogenous bilirubin must be added to organisms that do not synthesize bilirubin, such as bacteria. Finally, a suite of biliverdin-interacting sytems have been developed that emit in the red and infrared.[21-27] The IFP and iRFP families are based on bacteriophytochromes (**Figure 3**), which naturally bind biliverdin covalently. They were generated by protein engineering to remove their natural photoactivity and improve their fluorescence efficiency. Recently, phycobiliproteins (**Figure 3**) have also been used to generate near infrared fluorogenic systems. A recent addition, smURFP, was developed from a phycobiliprotein, which began as a phycocyanobilin-binding hexamer and was engineered to finally be a dimer that self-catalyzes the covalent addition of biliverdin.[27] More recently, a different phycobiliprotein from a thermophilic organism was used to develop a series of extremely stable and pH-tolerant, monomeric, infrared systems.[28] Due to their emission wavelengths, which are particularly interesting for *in vivo* imaging as very few biological molecules absorb in this region, significant effort has been directed towards the

development of various sensing systems, resulting in some of the most advanced fluorogenic sensors for bioimaging.

Systems that rely on synthetic, fluorogenic chromophores present their own advantages and challenges. On one hand, synthetic fluorogens can be tailored to generate complexes with the desired spectral and photophysical properties. To some extent, the possibilities for the development of new fluorogen systems are limited only by the chemist's imagination. On the other hand, as these fluorogenic chromophores are synthetic, the development of a protein tag that interacts specifically and with high signal-to-noise can be challenging and often has required several rounds of optimization. Furthermore, fluorogen delivery can impede fluorescent complex generation, which places effective limits on the size and types of fluorogenic chromophores that can be developed. However, the physico-chemical properties of the fluorogens can be used creatively to provide labelling strategies that are not possible with traditional methods, such as the specific tracking of membrane-bound pools of protein.

Hybrid systems comprised of a small protein tag and a synthetic fluorogenic chromophore can be generally divided into two classes (**Figures 2 and 3, Table 1**): self-labeling tags, which interact with the fluorogenic chromophore covalently, or fluorogen-binding proteins that form non-covalent complexes with their cognate chromophores. Site-specific self-labeling systems react with particular substrates to label an amino acid residue on the protein to generate irreversibly, covalently-labelled fusion proteins. The most-extensively developed systems are the SNAP-tag/CLIP-tag and Halo-tag (**Figures 2 and 3**). SNAP-tag is 20 kDa protein generated *via* directed evolution from O^6-alkylguanine-DNA alkyltransferase to transfer the functionalized benzyl group of O^6-benzylguanine derivatives to its active site cysteine.[29,30] CLIP-tag was engineered from SNAP-tag to react selectively with O^2-benzylcytosine substrates.[31] Halo-tag is derived from a bacterial haloalkane dehydrogenase to react with chloroalkane ligands.[32] Derivatives of silicon-rhodamine have been developed for use with these systems to generate fluorogenic labeling protocols.[33,34] A more recent

addition to the self-labeling systems is the PYP-tag, which is based on the bacterial photoreceptor, photoactive yellow protein.[35] Fluorogenicity in this system either arises from environmentally sensitive fluorogens, that fluoresece in the non-polar environment of the tag,[36,37] or from intramolecular quenchers that are eliminated during the labeling reaction.[38-40] The first non-covalent system, termed fluorogen-activating proteins (FAPs), were developed by directed evolution from single-chain antibodies to bind variants of Malachite Green and Thiazole Orange with sub-nanomolar affinity.[41] The original FAPs required the formation of a disulfide bond, limiting their use to relatively oxidizing environments such as the secretory pathway and the cell surface. More recently, various FAP systems have been developed that do not rely on disulfide bond formation.[42-44] FAP systems based on single-chain antibodies are relatively large, resulting in fluorescent complexes on the order of GFP or larger. A smaller alternative non-covalent system is the Fluoresence-activating and Absorption-Shifiting Tag (FAST), which was recently developed by directed evolution of the bacterial photoreceptor photoactive yellow protein (PYP).[45] FAST reversibly and quickly binds a family of hydroxybenzylidene rhodanine derivatives to generate complexes with emission wavelengths from green-yellow to orange-red.[45,46] Furthermore, it has been shown to be an effective fluorescence marker in bacteria, yeast, mammalian cell culture, and zebrafish. The development of fluorogen-binding proteins has been expanded to the use of other scaffolds such as fibronectin,[47] CRABPII,[48] as well as designed ankyrin repeat proteins (DARPs)[49].

2. Fluorogenic proteins provide new strategies for labeling and detection

Alternative protein-based fluorescent labeling strategies provide new possibilities for labeling and detection. Moving towards the infrared is desirable so as to take advantage of the near-infrared (NIR) transparency window, where there is relatively little absorbance from endogenous biomolecules and background autofluorescence is reduced. Moreover, imaging in the near-infrared can be done in small animals. As fluorescent proteins from jellyfish or coral are limited to excitation maxima <610 nm,

there are no classic autofluorescent systems available for imaging in the NIR. However, there are several fluorogenic systems that bind biliverdin covalently to yield complexes that can be excited between 640 and 700 nm and emit between 670 and 720 nm (**Figure 2**).[21-27,50] The first developed systems were dimers, but the various wavelengths available still allowed for *in vivo* multiplexing in whole animals.[25] More recently, monomeric systems have been developed, which provide interesting opportunities for sensor development in the NIR (*vide infra*).[24,26,50] All of these systems have been tested in larger model organisms including mice,[21-27,51] *Drosophila*,[23,24] and zebrafish[24] using fluorescence and/or tomographic techniques. However, the availability of biliverdin remains an issue with most of these systems, which often require the co-overexpression of heme oxygenase-1 to compensate for the overexpression of the protein.[23,24]

Alternative systems to autofluorescent proteins can be used to specifically label proteins for live-cell microscopy. An important distinction lies in the lack of requirement for chromophore maturation as the covalent or non-covalent interaction with the chromophore occurs with a pre-formed chromophore. Thus, the formation of a fluorescent complex can be faster and oxygen-independent. While it was demonstrated that UnaG was capable of labeling mammalian cells in hypoxic conditions,[20] flavin-binding fluorescent proteins (FbFPs), in particular, have been examined for their utility under oxygen-poor conditions. Various studies have been published that clearly demonstrate the superiority of these systems for monitoring various intracellular processes in hypoxic conditions. The initial report of the development of the first FbFP evaluated its utility in the facultative anaerobe *Rhodobacter capsulatus* and demonstrated that the FbFP was produced and fluorescent in conditions where YFP was produced, but the chromophore was unable to mature.[18] A more detailed study comfirmed its ability to outperform traditional autofluorescent proteins and its utility as a quantitative reporter under anaerobic conditions.[52] Since their development, FbFPs have been validated as reporters in hypoxic conditions in fungi,[53] mammalian cells,[54] and several strains of bacteria.[55-57]

On the other hand, hybrid systems comprised of a protein tag and small molecule fluorogen expand the types of labeling protocols possible. Self-labeling systems such as SNAP-, CLIP-, or Halo-tag have taken advantage of the versatility of fluorophores available via chemical synthesis to generate a series of fluorogenic, far- and infra-red fluorophores for super-resolution microscopy techniques and single molecule studies. Most fluorogenic chromophores for these systems rely on the ground-state isomerization of silicon-rhodamine derivatives, whereby the spirolactone ring opens upon binding to the protein tag.[33] These fluorogenic chromophores can be used for STED as well as superresolution techniques such as GSDIM (ground state depletion followed by individual molecule return) and dSTORM (direct stochastic optical reconstruction microscopy). Tuning of the silicon-rhodamine fluorphores for better brightness and photostability by the addition of an azetidine group yielded the dye, JF_{645}, which is one of the highest performing dyes for the self-labeling systems.[58] Furthermore the silicon-rhodamine derivatives have been shifted into the near-infrared[34] and other fluorogens have been developed to provide both orange,[59] and green[60] complexes, allowing for multicolor fluorogenic labeling (**Figure 2**). As the sole requirement is the introduction of the recognition group of the respective system, small molecule probes and sensors can be converted into targeted systems.

Systems based on a non-covalent interaction between the fluorogen and the protein tag provide more opportunities for creative labeling protocols. In particular, control over the amount of fluorogen used has proven useful for generating sub-stoichiometrically labelled proteins for super-resolution imaging and single particle tracking. While FAPs were initially demonstrated as competent probes for STED nanoscopy,[61] localization microscopy of actin-FAP fusions was also possible by controlling the amount of fluorogen used, allowing for live-cell superresolution microscopy in the far-red.[62] Single molecule studies using subsaturating concentrations of fluorogen on the multichain immune recognition receptor, FcεRI, revealed the intersubunit dynamics of the receptor and as well as its internalization in response to IgE detection.[63] Furthermore, control over the permeability of the fluorogen has been used to generate novel methods for the quantification and detection of surface-localized proteins. By using a cell-permeant and

an cell-impermeant fluorogen the dynamics of BK channel plasma membrane residency was examined.[64,65] This system was further extended by conjugation of a coumarin to a malachite green-based fluorogen and co-labeling with a cell-impermeant malachite green fluorogen yielded a three-color, two-FRET read-out of the extracellular, intracellular, and total protein pools.[66] This concept has also been demonstrated with FAST, where labelling with a cell-permeant and impermeant fluorogen allowed for the assessment of small molecule effects on GPCR trafficking using flow cytometry.[67] While non-covalent systems such as the FAPs tightly bind their fluorogens to generate stable complexes with slow off rates and function similarly to classic fluorescent proteins and self-labeling systems, systems that exhibit dynamic exchange with their cognate fluorogens can be used for multiplexed imaging with classic fluorescent proteins or with multiple fluorogens with different exchange dynamics. Multiplexed imaging with FAST by fluorogen exchange has been demonstrated both with reversibly photoswitchable fluorescent proteins[45] as well as with two fluorogens with different colors and exchange dynamics[46]. In the case of photoswitchable proteins, washing and photoswitching steps can be alternated to monitor either FAST fluorescence or Dronpa fluorescence.[45] When using two fluorogens with different colors and exchange dynamics, displacement of one fluorogen by another yields signals that are anti-correlated in time, allowing one to distinguish FAST fluorescence even in the presence of other markers that fluoresce in the green and/or red.[46]

3. Fluorogenic proteins can be ROS delivery systems

Alternative protein-based fluorescent labeling strategies also offer new opportunities for actuation using the targeted delivery of singlet oxygen and other reactive oxygen species. In particular, the selective ablation of cells is of particular interest for the detection of cell function in complex tissues as well as for photodynamic therapy. Chemical photosensitizers often suffer from off-target effects and non-desirable accumulation in tissues, however genetically-targetable photosensitizers would help to mitigate these issues. Fluorogenic protein systems have emerged as excellent tools for the specific subcellular generation of singlet oxygen. Specifically, miniSOG is a singlet

oxygen generator engineered from the flavin-binding LOV2 domain that retains a modest fluorescence quantum yield.[68] It was initially developed for the localized polymerization of diaminobenzidine for correlated electron microscopy (CLEM), and further studies have evaluated the efficacy of various mutants of miniSOG and their ability to produce various reactive oxygen species (ROS).[69] However, miniSOG and its variants suffer from photodegradation, which limits its use as a fluorescent tag for CLEM. To circumvent this issue, tandem heterodimers of miniSOG and phiLOV2.1 were generated to combine the efficient ROS generation of miniSOG and the photostability of phiLOV2.1.[70] MiniSOG was also demonstrated to be an effective photoablation reagent for the target killing of cells in *C. elegans*.[71] Since then, miniSOG has been used extensively as a label and photosensitizer for chromophore assisted light inactivation of proteins (CALI). For example, fusions of miniSOG to the synaptic proteins VAMP2 and synaptophysin was used to selectively inactivate presynaptic vesicular release in cultured neurons and *C. elegans*.[72] It was also used to overcome the distance limitations of Förster resonance energy transfer (FRET) to map protein-protein interactions over a longer distance using singlet oxygen triplet energy transfer (STET).[73] For these studies, the phytochrome-based IFP1.4 was used as a singlet oxygen sensor and miniSOG was used as the single oxygen generator. The fluorescence intensity of IFP1.4 is selectively decreased by reaction with singlet oxygen in a distance-dependent manner, which was used to confirm the topology of a large, multi-protein complex that is otherwise inaccessible by other standard techniques.

Blue light has a reduced penetration depth with respect to longer wavelengths. Using thin tissue slices or transparent organisms can circumvent the limited penetration depth of blue light. However, longer wavelength photoablation probes allow for greater tissue penetration for use in other complex organisms or tissue samples. To overcome the limitation of blue light excitation, FAP-based systems utilizing malachite green derivatives substituted with heavy atoms have been developed.[74] The resulting system is excited at 666 nm, which is more adapted for deep tissue imaging. Furthermore, the dye is selectively photoactive only in the protein cavity, which provides an extra layer of experimental control and reduces off-target effects. The system was successively used to selectively ablate cells into both larval zebrafish as well as adult zebrafish.

4. Using fluorogenic proteins to develop biosensors to detect intracellular events

One of the most interesting applications of fluorescence microscopy is the tracking and detection of biochemical events in space and time using optical biosensors. A large suite of biosensors based on autofluorescent proteins currently exists and certain members have become essential components in the molecular biology toolbox to understand dynamic cellular events. However, these sensors suffer from the same limitations as autofluorescent proteins, and furthermore, the reliance on a single type of fluorescence moiety has limited the types and topologies of sensors that have been developed. An expansion in the sensor toolbox necessitates the development of alternative types of sensors based on other types of fluorescent systems. Fluorogenic protein systems, while still relatively new, have already provided interesting sensor platforms with which to develop entirely new detection systems that take advantage of the fluorescence modality as well as the protein structure.

Some of the earliest types of autofluorescent protein-based biosensors involved sensitizing the fluorescence emission intensity to environmental changes such as chloride, pH, and oxygen. Similarly, pH and oxygen sensors have been developed based on fluorogenic protein systems. The site-specific incorporation of tyrosine analogues as photo-induced electron transfer probes in iLOV sensitized the fluorescence of iLOV to pH of the surroundings via the environmentally-sensitive tyrosine analogue, which has a shifted pK_A relative to that of tyrosine.[75] Depending on the tyrosine analogue employed, the pK_A of the side chain can vary from 5.3 to 9.2, effectively matching the expected pH range in various organelles. Biosensors based on FAPs for pH have also been developed by adding a pH sensitive unit to the exogenous fluorogen, which has allowed the tracking of the trafficking of the β2-adrenergic receptor[76] as well as studying synaptic GABA receptor internalization.[77] This technology has recently been expanded to generate far-red ratiometric pH probes that rely on intramolecular FRET to quench the pH-sensitive Cy3 moiety, which is unquenched upon FAP binding.[78] Recently, a single-protein, ratiometric pH sensor was developed based on the CRABPII scaffold, which binds retinal analogues. Exchange of the original

fluorogen used to develop the system, merocyanine aldehyde,[48] to a julolidine retinal analogue yielded a pH-sensitive system where the titratable group is an amino acid side chain, rather than the chromophore itself.[79] To sense oxygen, an oxygen-insensitive FbFP was coupled to an oxygen-sensitive YFP to yield a FRET-based molecular oxygen sensor.[80] This system was used to monitor real-time oxygen levels in batch-grown *E. coli* culture. In addition to sensing molecular oxygen, the redox state of cells can be assessed using protein-based biosensors sensitized to redox state by the introduction of cysteine residues. Recently, such a sensor was developed based on UnaG.[81] The introduction of a disulfide bond via rational design in the β barrel of UnaG yielded a redox sensor usable in live cell microscopy for real-time measurements with an 800% dynamic range and a reduction potential of –275 mV.

Perhaps the most important and developed class of sensors based on autofluorescent proteins is the cation sensors. There are several cation sensors that have been developed based on fluorogenic protein systems. Some of the first cation sensors were developed based on FbFPs. The site-specific incorporation of tyrosine analogues not only yielded a pH sensor, but also system that selectively detects Mn(III).[75] Additionally, it was found that Cu(II) binds to iLOV with a K_d of 4.7 μM and quenches its fluorescence selectively and reversibly.[82] Unfortunately, neither of these sensors has been tested in live cell microscopy. Ca(II) sensing by fluorescent proteins has been essential in neuroscience and understanding Ca(II)-based signalling. A variant of UnaG inspired by classic Ca(II) sensors was recently developed, which releases bilirubin in response to increases in Ca(II) levels. However a classic turn-on sensor was not able to be developed.[83] The only fluorogenic protein that functions as a dynamic and reversible Ca(II) sensor was recently developed using a circularly permuted variant of FAST to condition the noncovalent fluorogen binding to Ca(II) recognition.[84] Of interest, the color of this sensor can be changed at will simply by changing the identity of the fluorogen used.

One of the advantages of the self-labeling technique is the ability to adapt small molecule sensors to the self-labeling technology by adding the appropriate chemical handle. However, there are relatively few sensors based on self-labeling tags that are fluorogenic. One of the earliest fluorogenic sensors based on self-labeling systems is

the Mag-S-Tz fluorogen, a Mg(II) small molecule sensor coupled to a tetrazine quencher, which specifically labels Halotag via a click reaction to generate a fluorogenic, ratiometric Mg(II) sensor.[85] The fluorogenicity of the sensor allows the subcellular localization of the sensor with little background in off target organelles without requiring wash-out. Furthermore, the modularity of the approach should be extendable to other analytes. A turn-off probe for alkyl halides was also developed using a fluorogenic Halotag ligand.[60] Halotag was modified to accommodate the environmentally sensitive dansyl moiety and it was shown that binding of various alkyl halides could quench the fluorogenic response. One of the most developed systems is a fluorogenic proteome-stress sensor.[86] To develop this sensor, an unstable Halotag mutant that is prone to aggregation was evolved. Proteome stress leads to aggregation of this so-called AgHalo tag and the formation of soluble aggregates is detected via a fluorogenic response. The fluorogenicity is based on a solvatochromic fluorophore, which increases its fluorescence in soluble aggregates where the hydrophobic core of the protein is exposed. The system was further expanded with multiple colors and detection thresholds, with some fluorogens even capable of detecting misfolded monomers of AgHalo.[87]

Systems using fluorogenic probes have been designed to monitor protease activity in living cells, which can indicate changes in cell viability or disease state. In particular, imaging protease activity using a fluorogenic system rather than a FRET-based system is particularly attractive due to the increased signal-to-noise. The first caspase reporter to be used for *in vivo* imaging was developed from mIFP.[88] The original IFP and iRFP systems were dimeric, which hindered the development of effective sensor system. However, with the later publications of mIFP and miRFP, which are monomeric systems, infrared protein-based sensors development became possible. To design a caspase sensor based on mIFP, the protein was first circularly permuted and the previous N- and C-termini were linked with a sequence containing a protease recognition site.[88] The N-terminal sequence was also truncated to introduce a conformation strain in the system so as to impede the access of the catalytic cysteine to the active site, thus preventing the attachment of biliverdin and thus the formation of a fluorescent complex. Split GFP was also added to the sensor to force the association of

the two fragments of mIFP, even after the protease-sensitive linker is cut. This has the added benefit of rendering the sensor ratiometric. Changing the protease-sensitive linker changes the specificity for a given protease. This system was ultimately used to image caspase activity in embryo morphogenesis and tumorigenesis in *Drosophila*.[88]

Perhaps one of the most interesting applications of fluorogenic reporters is for the detection of protein-protein interactions. Protein-protein interactions are typically detected using fluorescence microscopy either using FRET pairs fused to two interacting proteins or by bimolecular fluorescence complementation. Bimolecular fluorescence complementation using split reporters based on the GFP family is hindered by the irreversibility of the system, which prevents the real-time detection of protein interaction dynamics. The suite of fluorogenic proteins provides an attractive alternative scaffold to develop reversible and dynamic systems, since the protein structure differs drastically from that of GFP. A split protein system based on UnaG was developed and was benchmarked with the rapamycin-inducible interaction of FRB and FKBP.[89] While the system was able to be dissociated by the addition of FK506, which disrupts the FRB-FKBP interaction, it has not been tested on other protein-protein interactions. The first developed split protein system based on the infrared fluorogenic proteins was iSplit, developed from iRFP.[90] However, the system was irreversible and dimeric, which hinders its utility. A second system was then developed based on the IFP1.4 scaffold, which was shown to be reversible and capable of detection of spatiotemporal dynamics of protein-protein interactions in yeast and mammalian cell culture.[91] However, the system suffered from low brightness and a requirement for exogenous biliverdin. Additionally, it was later reported that IFP1.4 can form dimers at high concentrations,[24] and it remains unclear as to whether this affects the IFP-based split reporter. Finally, a split system called miSplit based on the miRFP system has also been developed, which has the advantage of being an inherently brighter protein than IFP as well as being monomeric and available with multiple emission wavelengths.[26] After demonstration that the split reporter can detect rapamycin-inducible FRB-FKBP association, it was also demonstrated that two color split labelling was possible as each color of split shares a protein fragment. The system was then applied to the detection of mRNA in live cells using two high affinity RNA-protein interactions based on the MS2

bacteriophage coat protein, MCP, and the PP7 bacteriophage coat protein, PCP. Unfortunately, the association of the split fragments is irreversible, so the detection of dynamic interactions with this system remains impossible.

5. Conclusion

Fluorogenic protein systems present certain advantages that address the drawbacks of the widely used autofluorescent protein palette. Since they are based on different mechanisms of chromophore incorporation, they do not require oxygen and can exhibit much shorter times for fluorescent complex formation. The development of fluorogenic proteins that utilize either endogenous or exogenous chromophores gives the experimenter an extra layer of control and choice in the type of reporter system used depending on the type of imaging desired. While systems that use endogenous chromophores have fewer issues with chromophore permeability and delivery, the use of exogenous chromophores can engender creative and robust labeling protocols to access information that was previously inaccessible or to allow multiplexed detection. Furthermore, systems based on synthetic chromophores can take advantage of the photostability and brightness inherent in small molecule fluorophores, while still utilizing a protein tag, which allows for precise subcellular labelling.

One of the most exciting applications for fluorogenic systems is in the development of sensors and actuators to detect and trigger intracellular events. In particular, protein scaffolds that are different from the well-established GFP-based sensors allow for the development of new sensor types that rely on different mechanisms of coupling between fluorescence and detection. Sensors based on GFP-like proteins often rely on a specific interaction between the analyte detection module and the fluorescent protein. While these designs should still function with fluorogenic protein systems, the nature of the fluorogen interaction should also allow for the generation of scaffolds that rely on more general mechanisms of conformational coupling or energy transfer. Fluorogenic systems can furthermore provide scaffolds for

the development of new sensing moieties as well as allow their implementation in a diverse array of organisms and conditions.

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Notes

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Figure Legends

Figure 1. General concept of fluorogen-based reporters.

Figure 2. Natural and synthetic fluorogen structures used in protein-based fluorogenic systems.

Figure 3. Fluorogenic protein scaffolds. (Top) Fluorogenic proteins that use natural chromophores: IFP2.0 (4CQH), smURFP (4PO5 manually docked with biliverdin), UnaG (4I3C), and iLOV (4EES). (Bottom) fluorogenic protein systems that use synthetic chromophores: Halo-tag (5UXZ), SNAP-tag (3KZZ), FAP L5* (4KH), and FAST (1NWZ manually docked with HMBR)

Table 1. Fluorogen-based markers. Abbreviations are as follows: λ_{abs}, wavelength of maximal absorption; λ_{em}, wavelength of maximal emission; ε, molar absorption coefficient at λ_{abs}; ϕ, fluorescence quantum yield; K_D, thermodynamic dissociation constant. Structures of the fluorogens are given in Figure 1

Tag	Fluorogen	Binding mode	Oligomeric state	λ_{abs} (nm)	λ_{em} (nm)	ε ($M^{-1}cm^{-1}$)	ϕ (%)	K_D	Ref.
BsFbFP	FMN	Non-covalent	Dimer	449	495	13,900	39		[92]
EcFbFP	FMN	Non-covalent	Dimer	448	496	14,500	44		[92]
PpFbFP	FMN	Non-covalent	Dimer	450	496	13,900	27		[92]
iLOV	FMN	Non-covalent	Monomer	447	497		44		[19]
phiLOV2.1	FMN	Non-covalent	Monomer	450	497		20		[92]
miniSOG	FMN	Non-covalent	Monomer	447	497	14,200	41	170 pM	[92]
IFP1.4	Biliverdin	Covalent	Dimer	684	708	92,000	7.0		[21]
iRFP	Biliverdin	Covalent	Dimer	692	713	105,000	5.9		[22]
iRFP670	Biliverdin	Covalent	Dimer	643	670	114,000	11.1		[25]
iRFP682	Biliverdin	Covalent	Dimer	663	682	90,000	11.3		[25]
iRFP702	Biliverdin	Covalent	Dimer	673	702	93,000	8.2		[25]
iRFP720	Biliverdin	Covalent	Dimer	702	720	96,000	6.0		[25]
IFP2.0	Biliverdin	Covalent	Dimer	690	711	98,000	8.1		[23]
mIFP	Biliverdin	Covalent	Monomer	683	705	82,000	8.4		[24]
miRFP670	Biliverdin	Covalent	Monomer	642	670	87,400	14		[26]
miRFP703	Biliverdin	Covalent	Monomer	674	703	90,900	8.6		[26]
miRFP709	Biliverdin	Covalent	Monomer	683	709	78,400	5.4		[26]
smURFP	Biliverdin	Covalent	Dimer	642	670	180,000	18		[27]
BDFP1.1	Biliverdin	Covalent	Dimer	682	707	68,700	5.9		[28]
BDFP1.5	Biliverdin	Covalent	Monomer	688	711	74,000	5.0		[28]
UnaG	Bilirubin	Non-covalent	Monomer	498	527	77,300	51	98 pM	[20]
FAP HL1.01	Thiazole Orange	Non-covalent	Monomer	509	530	60,000	47	1.7 nM	[41]
FAP H6	Malachite Green	Non-covalent	Monomer	635	656	105,000	25	38 nM	[41]
FAP dL5**	Malachite Green	Non-covalent	Tandem Dimer	638	666	103,000	20	18 pM	[74]
Self-labeling proteins	SiR650	Covalent	Monomer	645	661	100,000	39		[33]
Self-labeling proteins	SiR700	Covalent	Monomer	687	716	100,000			[34]
Self-labeling proteins	JF646	Covalent	Monomer	646	664	152,000	54		[59]
Self-labeling proteins	JF585	Covalent	Monomer	585	609	156,000	78		[59]
Self-labeling proteins	JF635	Covalent	Monomer	635	652	167,000	56		[59]
FAST	HMBR	Non-covalent	Monomer	481	540	45,000	23	0.23 µM	[46]
FAST	HBR-3,5DM	Non-covalent	Monomer	499	562	48,000	49	0.08 µM	[46]
FAST	HBR-3,5DOM	Non-covalent	Monomer	518	600	39,000	31	0.97 µM	[46]

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Figure 1

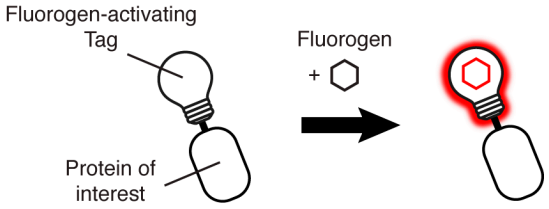


Figure 2

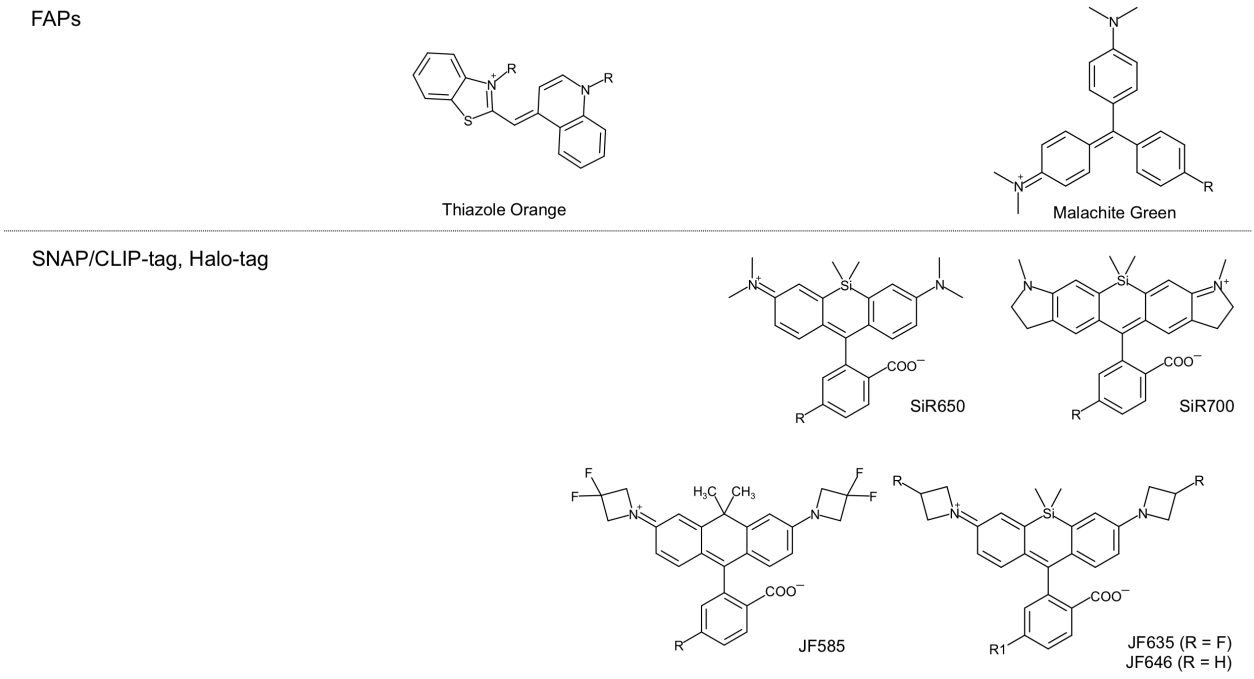
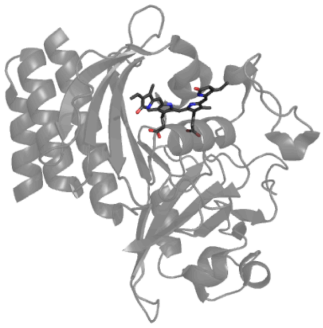

FAPs
Thiazole Orange
Malachite Green
SNAP/CLIP-tag, Halo-tag
SiR650
SiR700
JF585
JF635 (R = F)
JF646 (R = H)
FAST
HMBR
HBR-3,5DM
HBR-3,5DOM
400 nm
500 nm
600 nm
700 nm
FbFPs, UnaG, phytochromes, phycobiliproteins
FMN
Bilirubin
Biliverdin

Figure 3



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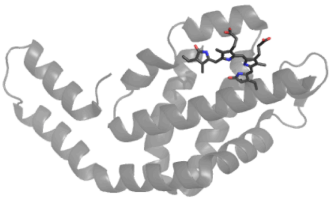

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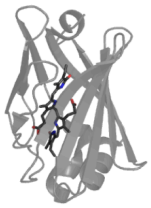

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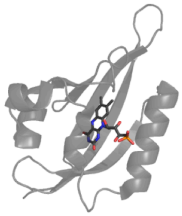

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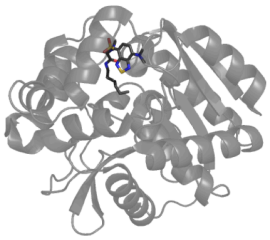

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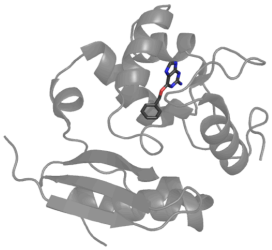

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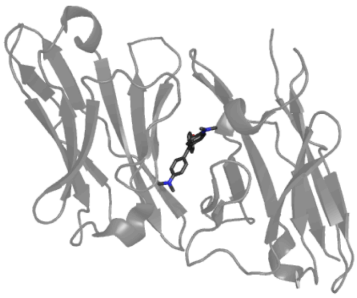
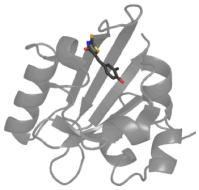


FAP



FAST