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Fluorescent amino acids: Modular building blocks for the assembly new tools for chemical biology

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ABSTRACT

Fluorescence spectroscopy is a powerful tool for probing complex biological processes. The ubiquity of peptide-protein and protein-protein interactions in these processes has made them important targets for fluorescence labeling to allow a sensitive readout of information concerning location, interactions with other biomolecules, and macromolecular dynamics. This chapter describes recent advances in design, properties and applications in the area of fluorescent amino acids (FIAAs). The ability to site-selectively incorporate fluorescent amino acid building blocks into a protein or peptide of interest provides the advantage of closely maintaining native function and appearance. The development of an array of fluorescent amino acids with a variety of properties such as environment sensitivity, chelation-enhanced fluorescence, and pro-fluorescence has allowed researchers to gain insight into biological processes, including protein conformational changes, binding events, enzyme activities, and protein trafficking and localization.

1. INTRODUCTION

The use of fluorescence as an analytical and diagnostic tool has become increasingly important in research ranging from fundamental studies in molecular and cellular biology, biological chemistry and biophysics to the applied fields of biotechnology and medicine. The utility of fluorescence-based approaches has been accelerated by the development of advanced techniques and instruments for monitoring and imaging fluorescence responses in diverse and complex systems. Fluorescence is highly sensitive, and most importantly, there are tremendous opportunities for manipulating the structural variety and fine-tuning the photophysical properties of molecular frameworks that exhibit fluorescence. Additionally, methods for the integration of fluorophores into macromolecular targets are rapidly evolving.

In the context of peptide and protein architectures, fluorescence has proven useful in probing protein structure and dynamics, including ligand-induced conformational changes, protein-protein and protein-nucleic acid interactions, protein trafficking, and enzyme activities.¹⁻³ The quest for insight into these ubiquitous biological processes, coupled with the limited range of naturally occurring fluorescent amino acids has led to development of versatile approaches for integrating fluorescent (or pro-fluorescent) motifs into peptides and proteins.^{4,5}

Currently, one of the most broadly applied methods for covalently labeling a protein with a fluorophore is to co-express the target with a naturally occurring fluorescent protein. Indeed, the discovery of the green fluorescent protein (GFP)⁶ and elucidation of the chemistry of the GFP chromophore by Tsien and coworkers has led to substantial improvements, providing an array of multicolored and analyte-responsive fluorescent proteins (FPs) with increased photostability.⁷⁻⁹ The success and applicability of fluorescent proteins is underscored by their widespread adoption throughout the research communities as a standard tool in protein imaging.

An alternative approach for covalently labeling target proteins with fluorophores is to exploit protein co-expression fusions that, while not intrinsically fluorescent, may undergo an enzymatic reaction to covalently self-label the encoded fusion tag with a

fluorophore, thereby imparting fluorescence to the protein target. For example, Johnsson and coworkers have pioneered methods which exploit fusing a protein of interest with the DNA O⁶-alkylguanine alkyltransferase. This enzyme efficiently reacts with benzylguanine derivatives, allowing covalent attachment of synthesized fluorophore-benzylguanine analogues to the protein of interest.¹⁰⁻¹² Such tags have become commercialized (SNAP, CLIP tags) and are now used extensively in live cell protein imaging. An alternative tag, based on similar strategy, is the haloalkane dehalogenase (HALO tag), which reacts covalently with halogenated alkanes.¹³ In principle, using this approach, any synthesized fluorophore-modified halogenated alkane can be ligated to the fused enzyme, thereby labeling the target protein.

Despite their widespread use, a drawback of fused FPs and self-modifying enzymes is their size (> 20 kDa), which may interfere with the function, localization and native interactions of the target protein. Smaller tags have been developed to address this limitation. For example, encoded amino acid sequences, which bind small molecules or metal ions and become fluorescent (FIAsH, ReASH, RhoBo, etc.),¹⁴⁻¹⁶ or are recognized by enzymes that can transfer a separate substrate moiety, which is fluorescent or can be orthogonally modified (biotin ligase, sortase, lipoic acid ligase, formylglycine-generating enzyme) have emerged as powerful strategies.¹⁷⁻²⁰

A complement to the aforementioned approaches is the application of fluorescent α -amino acid analogues, which have the advantage of being relatively non-perturbing replacements for the native encoded residues, thereby maintaining the overall native structure of a target peptide or protein. In addition, the modularity of α -amino acid building blocks makes them versatile components in the protein assembly toolbox and thus, once syntheses and methods for peptide and protein incorporation are developed new applications can be readily adopted. Finally, the increasing versatility of methods for selective incorporation of synthetic amino acids into peptides through solid phase peptide synthesis (SPPS) or into proteins via expressed protein ligation (EPL) or unnatural amino acid mutagenesis using modified translation systems enables the tailored assembly of innumerable targets with precisely-positioned fluorophores. Importantly, the relatively small molecular framework of typical α -amino acids makes them readily

amenable to modifications via organic synthesis, providing a range of fluorophore options with a variety of properties.

2. SCOPE OF THIS REVIEW

This review will present an overview and analysis of the current literature on fluorescent α -amino acids (FIAAs), which have been incorporated directly into peptides and proteins via chemical synthesis, protein semisynthesis and protein biosynthesis using the protein translation machinery. In particular, we focus on studies in which the integrated FIAA enables new applications in the study of peptide and protein interactions and activities. While we will not discuss the extensive applications of fluorescent labels, which are conjugated to reactive naturally-occurring amino acids (in particular cysteine), we will additionally present new opportunities for the selective incorporation of fluorophores using unnatural amino acids which can be labeled using bioorthogonal reaction chemistries. Also, the synthesis of described FIAAs will not be discussed in detail, but it is noteworthy to mention the wealth of advancements in the synthesis of α -amino acid including approaches which exploit asymmetric reaction methodologies (e.g. alkylation and hydrogenation), as well as methods which use starting materials from the chiral pool and enzyme-catalyzed resolution.²¹

3. SUMMARY OF APPROACHES

The advancement of solid phase peptide synthesis (SPPS) has enabled the incorporation of a wide variety of FIAAs into native peptide environments. In particular, the relatively recent and more widespread use of Fmoc-based SPPS, together with an ever-expanding range of commercially-available and orthogonally-protected amino acids, linker strategies and coupling reagents, which enable employment of milder reaction conditions, has further contributed to the robustness and versatility of SPPS with unnatural amino acids and FIAAs in particular. The motivation for many efforts in this area has been driven by the desire to develop residues with fluorescent side chain functionality that shows more advantageous photophysical properties and a broader range applications than the naturally-encoded fluorescent amino acid - tryptophan.²² With the need for more diverse and powerful reporter properties, research groups began to take the

approach of integrating known fluorophores into the side chains of α -amino acids and investigating the utility of these new FIAAs in peptide and protein structures. As substrates for larger proteins or as components of binding domains, modified peptide sequences can now be readily synthesized for interrogating the known processes that they participate in using fluorescence-based readouts. FIAAs have also become invaluable in probing protein structure, function, and interactions. For these studies, site-specific incorporation of fluorescent building blocks is crucial, where one may want to examine local dynamics of a particular region of a protein while avoiding perturbation of the protein's native function (see Figure 1 for a survey of approaches for the incorporation of FIAAs into proteins).

With the merging of chemical synthesis and biology, the ability to exploit nature's machinery to modify the protein biopolymer with non-natural amino acid surrogates has become viable. Several groups have incorporated FIAAs into full-length proteins to enable their detection and to interrogate biological activities and interactions. In this respect, incorporation of the FIAAs into a protein (of which the synthesis is beyond the practicality of SPPS) is generally achieved by either 1) protein semisynthesis involving expressed protein ligation (EPL) – whereby a peptide fragment, which is synthesized via SPPS and contains the unnatural amino acid, is ligated with a recombinantly-expressed region of the desired protein (commonly via reaction of a C-terminal thioester with and N-terminal cysteine); or 2) Ribosomal machinery - using the chemical acylation of suppressor tRNAs or by exploiting orthogonal tRNA/AARS systems.

Techniques based on exploiting an organism's ribosomal machinery can be applied in theory to any protein, and can be particularly important when the target is a large protein that is not amenable to semisynthesis or difficult to site-selectively label with an exogenous reagent. Three main translation-based methods have become widespread: 1) exploiting promiscuity of wild type synthetases and mutants,²³ 2) expression using a chemically acylated tRNA which is then introduced into the expression system containing mRNA encoding a nonsense 3-base or 4-base codon.²⁴⁻²⁶ or 3) nonsense expression using an evolved orthogonal tRNA/aminoacyl-tRNA synthetase (AARS) pair recognizing a nonsense (commonly UAG or “amber”) codon.^{27,28} The final

method has resulted in the generation of several evolved tRNA/aaRS pairs, and incorporation of a variety of unnatural amino acids into proteins in *E. coli*, yeast, and mammalian cells.

4. IMPLEMENTATION

4.1 Probing interactions with solvatochromic FIAAs

One of the earliest extrinsic fluorophores that was applied for monitoring biological processes was the highly environment-sensitive (or solvatochromic) 6-propionyl-2-(dimethylamino)naphthalene (PRODAN, Figure 2).²⁹ This fluorophore shows a modest increase in quantum yield together with a significant blue shift of the emission λ_{max} of up to ~130 nm when changing exposure from aqueous protic to organic aprotic solvents.²⁹ An α -amino acid building block containing this fluorophore, 6-(2-dimethylaminonaphthoyl) alanine (DANA) was synthesized in 2002,³⁰ and has found use in peptides for monitoring peptide-protein and protein-protein interactions, where binding events often bring substrates in contact with more hydrophobic surfaces. An early study utilizing DANA illustrated its potential by reporting the binding of the phosphoserine-containing 14-3-3 peptide to the target 14-3-3 ζ -binding protein.³¹ By basing the peptide design on the available structural information, DANA was incorporated into the peptide in place of the Tyr residue which was localized at (-)2 relative to the phosphoSer. The residue at the (-)2 position was shown to be buried inside a hydrophobic pocket on binding the target protein by X-ray crystallography.³² A fluorescence increase of ~4-fold, as well as a blue shift of the emission maximum from 522 nm to 501 nm was observed upon binding of nonphosphorylated and phosphorylated peptide substrates to the 14-3-3 ζ -binding protein, respectively.

Contemporarily, Cohen *et al.* employed the amber suppression technique with a chemically-charged tRNA bearing the same amino acid fluorophore, which was named Aladan, to probe protein electrostatics.³³ By first incorporating Aladan into select sites of Kir2.1 and *Shaker* potassium channels it was concluded that Aladan was not only compatible with cellular biomachinery, but that substitutions in buried, aqueous, or lipid environments would still allow proper folding and functioning of the protein. As a proof

of utility of this FIAA to probe protein electrostatics, these researchers incorporated an Fmoc-variant of Aladan into the thermally-stable IgG binding domain GB1 protein (~6 kDa) via SPPS. By observing steady-state and time-resolved fluorescence data of GB1 mutants with exposed or buried Aladan, it was concluded that the interior of GB1 is polar and electrostatically heterogeneous. The ability to selectively position Aladan, an amino acid of similar proportions to tryptophan, within specified regions of a protein makes it a valuable amino acid for gaining insight into protein electrostatics and how the variation in this property may affect protein function, interactions, and stability.

Improving on the concept of environment-sensitive FIAA building blocks, residues based on the dimethylaminophthalimide and dimethylaminonaphthalimide systems were later developed with the goal of increasing the fluorescence changes that might accompany binding in target systems (Figure 2).^{34,35} The initial FIAA in this family was based on 4-(*N,N*-dimethylamino)-phthalimide (4-DMAP), a fluorophore which was known to exhibit robust changes in quantum yield (~70 fold) and λ_{maxEm} spectral shifts (~100 nm) on changes in the surrounding media.³⁶ Furthermore, the corresponding FIAA, 4-(*N,N*-dimethylamino)-phthalimide propionic acid (4-DAPA), had the advantage of being a closer size mimic to tyrosine, making it less likely to disrupt native peptide structure/interactions. On comparison with DANA, 4-DAPA gave a ~6-fold enhancement in fluorescence emission on binding the 14-3-3 ζ protein when placed at the phosphoSer(-2) Tyr position in the 14-3-3 binding peptide, with a blue shift in the emission maximum from 570 nm to 531 nm.³⁷ In an elegant study comparing the 4-dimethylaminonaphthalimide FIAAs, 4-DAPA and the synthesized 6-*N,N*-dimethylamino-2,3-naphthalimide amino acid (6-DMNA), antigenic peptides capable of binding to major histocompatibility complex (MHC) were made incorporating each of these FIAAs and used to track regulated cell-surface peptide binding activity in primary human monocyte derived dendritic cells. On binding class II MHCs, peptides containing these FIAAs gave substantial changes in fluorescence emission (>1000-fold for 6-DMNA, >35-fold for 4-DAPA) and fluorescence lifetimes (11.8 ns vs. 4.8 ns; 7.3 ns vs. 2.2 ns, complex vs. free peptide for 6-DMNA, 4-DAPA, respectively). In addition, docking of DANA, 6-DMNA, and 4-DAPA within a crystal structure of HLA-DR MHC protein and

the HA peptide show the importance of shape, size for hydrophobic binding of the amino acid, and potentially linker length (Figure 3). A crystal structure of the 4-DAPA-HA peptide-bound HLA-DR protein was also determined, confirming the occupation of the 4-DAPA side chain in the expected hydrophobic pocket.³⁸ These phthalimide and naphthalimide-based FIAAs have also been incorporated into peptides recognizing PDZ and SH2 domains revealing their versatility in diverse applications.³⁹⁻⁴²

The success of the dimethylaminonaphthalimide family led to their further exploration as environment-sensitive FIAAs. For example, the 4-(*N,N*-dimethylamino)naphthalimide amino acid (4-DMNA) was developed to complement 4-DAPA and 6-DMNA, and also yielded excellent improvements in stability towards hydrolysis and ease of synthesis.⁴³ When integrated at position 8 in the M13 peptide, which binds avidly to calcium-activated calmodulin, 4-DMNA exhibited a remarkable fluorescence enhancement. On binding, ~106-fold enhancement in emission intensity over background, as well as a blue shift from 550 nm to 505 nm is observed. This change compares favorably with other fluorophores in the dimethylaminophthalimide class, which exhibited ~26-fold (4-DAPA) and ~19-fold (6-DMNA) enhancements in the same context. Additionally, relative to the established solvatochromic PRODAN (4-fold), dansyl (10-fold) and NBD (3-fold) FIAA derivatives, the dimethylaminonaphthalimide family exhibited superior signal-to-background in these studies (Figure 2). Further synthetic efforts have yielded several thiol-reactive 4-DMN bioconjugation reagents with varying linker lengths, allowing for further analysis and development of solvatochromic peptides using general protein labeling experiments.⁴⁴

The solvatochromatic 4-DMNA has recently been incorporated into a sensor domain for the Rho GTPase Cdc42 via protein semisynthesis.⁴⁵ This domain, based on a Cdc42/Rac interactive binding (CRIB) domain of the Wiskott-Aldrich Syndrome Protein (WASP) was assembled via native chemical ligation between the C-terminal peptide fragment and the recombinant N-terminal WASP domain (residues 230-268) expressed as a GB1 fusion. Experiments with the sensor, Cdc42, and GTP- γ S (a non-hydrolyzable analog of GTP to activate the sensor) showed over a 17-fold increase in fluorescence emission as well as a hypsochromic shift from 534 nm to 514 nm relative to the GDP-bound inactive Cdc42. In addition to semisynthesis, native Cdc42 was mutated at residue

271 to a cysteine (F271A) based on a previous sensor design,⁴⁶ and the mutant was labeled with various 4-DMN thiol-reactive agents with different linker lengths. These sensors produced fluorescence changes ranging from 11-32 fold, underscoring the importance systematic screening to guide the placement of environment-sensitive fluorophores and their utility for reporting on protein interactions.

Another class of environment-sensitive amino acid building blocks based on the dansyl fluorophore, has been exploited for probing protein interactions and protein unfolding. Two amino acids, dansylalanine and dansyllysine, show a two-fold increase of quantum yield and ~50 nm hypsochromic shift upon changing from polar to nonpolar environments.⁴⁷ The sensitivity of these fluorophores makes them ideal tools for studying structural dynamics in proteins when they are site-specifically positioned in the protein without significant structural perturbation. The Schultz lab has shown, by incorporation into the human superoxide dismutase (hSOD) protein in yeast using an evolved tRNA/leucyl-tRNA synthetase pair, dansylalanine fluorescence intensity can be monitored in the presence of guanidinium hydrochloride to monitor protein unfolding.⁴⁸ More recently, a tRNA/tyrosyl-tRNA synthetase pair was used to incorporate a PRODAN amino acid into the same protein to study conformational changes.⁴⁹

In a recent study involving mammalian cells, dansylalanine has also been incorporated into neural stem cells using an orthogonal tyrosyl tRNA/TyrRS pair from *E. coli*. Using a lentiviral vector delivery system allowing for efficient expression of the orthogonal biomachinery throughout the differentiation process, Wang and coworkers site-selectively incorporated dansylalanine into the voltage sensitive domain (VSD) of *Ciona intestinalis* voltage-sensitive phosphatase in HCN-A94 cells. Dansylalanine was able to report, via environmentally-sensitive fluorescence, the conformational change of this domain in response to membrane polarization.⁵⁰ Dansyl amino acids have also been used in a scaffold for detecting concentrations of Hg²⁺ that approach toxic levels, as well as Zn²⁺.⁵¹⁻⁵³

Additionally, coumarins, known to exhibit sensitivity to solvent polarity and pH have also seen use in studying protein dynamics. One example of direct coumarin incorporation into proteins by the Schultz lab involved the fluorescent amino acid L-(7-hydroxycoumarin-4-yl) ethylglycine (7HC). For incorporation of the 7-hydroxycoumarin

amino acid, a mutant *Methanococcus jannaschii* tyrosyl amber suppressor tRNA/tyrosyl tRNA synthetase pair was evolved, incorporating the coumarin amino acid on recognition of the TAG stop codon. On incorporation into the protein holomyoglobin, 7HC fluorescence was monitored in correlation to helix unfolding in varied concentrations of urea, suggesting the utility of coumarin as a probe for local protein conformational changes.⁵⁴

4.2 Investigating phosphorylation by disrupting quenching of a pyrene FIAA

An application of FIAAs from the Lawrence laboratory focused on exploiting fluorescence enhancements of a pyrene-based amino acid, which occurred after tyrosine phosphorylation, to report on protein tyrosine kinase activity (Figure 4). In this study, pyrene fluorescence ($\lambda_{em} = 375$ nm) is quenched when involved in π - π stacking with a proximal tyrosine residue and when this stacking is disrupted due to tyrosine phosphorylation, quenching is abrogated. Amino acid derivatives of pyrene with different side-chain linker lengths (L-2,3-diaminopropionic acid (Dap), or L-2,4-diaminobutanoic acid (Dab)) were prepared and incorporated into tyrosine-containing peptide sequences (on either side of the phosphorylated tyrosine position). The selected peptides were designed to be substrates for the Src tyrosine kinase family, which phosphorylate tyrosines in this context. Incubation of the pyrene-FIAA-containing peptides with Src revealed increases in fluorescence intensity over time ranging from 1.8-5 fold.⁵⁵ The increase in fluorescence was attributed to a disruption of a π - π interaction between tyrosine and pyrene, corroborated by 2D nOe NMR data showing marked differences in the observed nOes between the pyrene and tyrosine protons in nonphosphorylated and phosphorylated states.⁵⁵

The pyrene amino acid approach was further applied in a protein kinase detection assay using a pyrene-containing peptide substrate for a serine kinase (PKA). In the assay, a quencher molecule forms non-covalent interactions with the peptide substrate and the incorporated pyrene moiety. On addition of ATP, PKA, and the phosphoserine binding domain 14-3-3 τ , pyrene fluorescence is restored due to displacement of the quencher by the phosphoserine-binding domain (Figure 4). After screening an array of quencher molecules against peptides with the pyrene FIAA at various positions, a very effective

probe was found using Rose Bengal or Aniline Blue as the quencher. With this design, termed “Deep Quench,” fluorescence increases up to 64-fold are observed.⁵⁶

Wang and coworkers have also exploited the difference in environment between phosphorylated and nonphosphorylated states by incorporating 7-hydroxycoumarin (7HC) into the signal transducer and activator of transcription 3 (STAT3) protein, which is phosphorylated at a Tyr705. In the context of stimulated cell lysates, up to 15-fold fluorescence enhancement can be observed relative to the nonphosphorylated Y705F mutant.⁵⁷

4.3 Interaction of FIAAs with metal ions: lanthanide binding tags (LBTs)

Peptides incorporating FIAAs capable of interacting with metal ions have found use in lanthanide-binding peptides. In this context, a specific peptide scaffold together with lanthanide metal-binding properties of encoded as well as synthetic amino acids can be exploited to great advantage for long wavelength imaging of proteins.

Recently, these lanthanide binding tags (LBTs) have been introduced as genetically encodable peptide tags that bind and sensitize lanthanide cations, thus imparting luminescence to a target protein on lanthanide binding. As previously mentioned, the size of encodable fluorescent protein tags can be problematic, as the added bulk may disrupt the native protein structure or function. LBTs are relatively small (17-20 amino acids), and bind the lanthanide series trivalent cations with high affinity (nM K_{Ds}) and selectivities relative to all of the physiologically relevant divalent cations. The version of these short tags which includes only the naturally encoded amino acids includes a strategically-positioned tryptophan residue which sensitizes bound Tb^{3+} , leading to a luminescence signal at 544 nm (Figure 5).^{58,59} Since lanthanide luminescence emission wavelengths are in the visible region and the luminescence lifetimes are long (msec), these tags are very well suited for applications in *in vitro* assays and in diagnostics. Additionally, LBTs have been genetically-encoded into or conjugated to a variety of proteins for use in monitoring protein-peptide interactions using luminescence resonance energy transfer (LRET).⁵⁹

Although the luminescence and low background of Tb^{3+} -bound LBTs is very

interesting, the excitation of the tryptophan sensitizer requires UV light, which is not ideal for cellular studies, and the emission wavelengths of the indole are only compatible with terbium sensitization, of which the emission wavelength may not be suitable for all potential applications. Addressing this shortcoming, two unnatural FIAAs were targeted for incorporation into LBTs based on the carbostyryl 124 (cs124) and the acridone (Acd) fluorophores (Figure 5), which exhibit emission properties compatible with sensitization of europium ions.⁶⁰ The lower energy excitation properties of these FIAAs (337 nm and 370 nm for cs124 and Acd, respectively), as well as known sensitization of europium (longer emission wavelength) made these amino acids desirable. In one study, cs124 and Acd were incorporated into LBTs via SPPS and shown to not only to sensitize at longer wavelengths but also sensitize Eu^{3+} , which luminesces at a longer wavelength (620 nm vs. 550 nm) than Tb^{3+} . After observing that the LBTs which included synthetic FIAAs also showed nM binding for lanthanides, it was demonstrated that a synthesized tag could be conjugated to the Crk(SH2) protein via native chemical ligation, potentially making it an effective tool for monitoring protein interactions and trafficking *in vivo*.⁶⁰

4.4 Sulfonamido oxime (Sox) amino acids and chelation enhanced fluorescence

Developing probes for kinase activity has been the target of much interest due to the crucial roles played by kinases in cellular signaling. The sensitivity of having a fluorescence-based output has afforded FIAAs an important role in the development of kinase probes and activity assays, as demonstrated with the application of pyrene-based FIAAs in Section 4.2. While several FIAAs have relied on changes in environment to generate a response to kinase activity, the Imperiali group has developed a fluorescent amino acid of which the emission is derived from metal chelation, termed chelation-enhanced fluorescence (CHEF, Figure 6). The chromophore, sulfonamido-oxime (termed Sox, earlier implemented to sense divalent zinc⁶¹) was synthesized initially as an alanine variant (Sox) and then later as a cysteine variant (C-Sox) to improve recognition and specificity. These FIAAs can be incorporated into kinase recognition peptide motifs and report on serine/threonine or tyrosine phosphorylation events via enhanced fluorescence emission in the presence of Mg^{2+} .⁶² The emission signal (~485 nm) relies on the formation of a chelate between the 8-hydroxyquinoline and the installed phosphoryl

group, which shows a 15-20-fold greater affinity for Mg^{2+} relative to the nonphosphorylated hydroxyl group. Peptide probes containing the Sox/C-Sox amino acid for a variety of kinases have been synthesized and implemented for kinase measurements with recombinant enzymes and in unfractionated cell lysates, yielding fluorescence enhancements of ~2-12-fold.⁶²⁻⁶⁴ It was also found that substitution of the sulfonamide moiety on C-Sox by various substituted triazole groups (installed via click chemistry) gave rise to a multitude of novel Mg^{2+} -chelating hydroxyquinoline FIAAs with fluorescence emission maxima that are shifted up to 40 nm.⁶⁵ These new click derivatives were incorporated into peptide substrates for study of the MK2 kinase and shown to be as efficiently turned over as C-Sox-containing peptides.⁶⁵

The ability to selectively target some kinases including ERK1/2 and p38 α , that are members of the MAK kinase family, which are known to be dysregulated in disease and represent important therapeutic targets relies on recognition elements that require extensive protein-protein interactions more complicated than those exhibited by the short peptide sequences on which many kinase probes have been based. In this context, a semisynthetic approach involving conjugation (via native chemical ligation) of a Sox-containing peptide containing an ERK1/2 consensus sequence to a recombinant N-terminal pointed domain (PNT) of transcription factor protein Ets-1. The added domain (~11 kDa) allows for specific docking of ERK1/2 to the C-Sox-based sensing module with enhanced affinity over the shorter Thr-Pro consensus peptide motif. This Sox-PNT sensor was capable of specifically detecting ERK1/2 activity in cell lysates relative to the more promiscuous C-Sox-peptide, showing a 4-10-fold increase in fluorescence readout.⁶⁶ Based on this scaffold approach and the benefits of the C-Sox amino acid, further sensors for specific kinases (p38, JNK, MAPK family) have been developed, providing insight into the modulation of kinase activities in diseased tissue samples.⁶⁷

Chelating FIAAs have found use in other applications as well. For example, Wang and coworkers have reported the synthesis and incorporation of the metal-chelating 2-amino-3-hydroxyquinoline-3-yl)-propanoic acid (HQ-Ala) into proteins not only as a CHEF fluorescent reporter, but also a handle for introduction of a heavy metal ion for X-ray crystallography phasing.⁶⁸ Using an orthogonal tyrosyl amber suppressor tRNA/tRNA synthetase, incorporation of HQ-Ala into a Z-domain protein showed a

significant increase in fluorescence at ~540 nm on excitation at 400 nm when titrated with as little as 10 μM Zn^{2+} . HQ-Ala was also introduced into O-acetylserine sulfhydrylase protein, incubated with Zn^{2+} and crystallized. Solving of the crystal structure showed no differences between the wild type structure and the Zn-coordinated HQ-Ala mutant, suggesting the potential for an alternative to other techniques such as selenomethionine incorporation for providing phasing information.

4.5 FIAAs employed in FRET experiments

Fluorophores and FIAAs have experienced much success as diagnostic and basic science tools due to ease in readout and sensitivity. Further information can be extracted by using more than one FIAA, for example in the application of Förster Resonance Energy Transfer (FRET). The use of FIAAs in FRET was first demonstrated by Stryer and Haugland in 1967 in the context of poly-L-proline oligomeric peptides.⁶⁹ Using an α -naphthyl group as a fluorescence donor and a dansyl fluorophore as the acceptor, energy transfer as a function of distance could be observed, demonstrating the potential of monitoring local and specific protein conformational changes via fluorescence.

Since 1967, methods to incorporate unnatural amino acids have advanced considerably, allowing for use of different unnatural entities within peptides and proteins. For example, acridone-based FIAAs, including the acrydonylalanine that was used as an LBT Eu^{3+} sensitizer, and benzoacridone (badAla) have been incorporated into peptides for use as a FRET pair to report on protease activity of caspase-3.⁷⁰ In an impressive recent example by Hohsaka and coworkers, amino acid derivatives of BODIPY fluorophores (BODIPYFL) were incorporated into calmodulin using the tRNA chemical-acylation approach pioneered by Sisido with a four-base anti-codon tRNA.^{24,26,71} After a screen of several BODIPYFL amino acid-acylated tRNAs in a cell-free translation system with streptavidin mRNA containing a four-base codon insert, it was determined that aminophenylalanine (AF) derivatives were most efficiently incorporated. Extending this to the calmodulin protein, two different BODIPYFL-AF amino acids (BODIPYFL-AF, BODIPY558-AF, Figure 7) were simultaneously incorporated using two different four-base codons at the N- and C-termini. Fluorescence spectra of the double-labeled mutant with excitation at 490 nm showed a decrease in donor (BODIPYFL-AF)

fluorescence at 515 nm and an increase in acceptor fluorescence at 575 nm, indicating FRET. Further calmodulin mutants with varied donor positions indicated FRET could be used to monitor conformational changes of calmodulin on Ca^{2+} -dependent peptide binding.⁷¹

4.6 Fluorescent coumarins, alkyne analogs, and bioorthogonal labeling

Tirrell and coworkers have taken advantage of the ability of some wild type aminoacyl tRNA synthetases to charge tRNAs with unnatural amino acid substrates similar in shape to their natural counterparts. In this relatively simple approach, they have incorporated several amino acid analogs of methionine with reasonable efficiency using the less discriminating methionyl-tRNA synthetase (MetRS) from *Methanococcus jannaschii* into *E. Coli*.⁷² One of these methionine analogs, homopropargylglycine has received attention due to its efficient incorporation and ability to undergo click chemistry, essentially providing a handle for site-selective modification of a protein. In an elegant example of selective protein labeling in bacterial cells, Beatty *et. al.* showed that in methionine auxotrophic *E. coli* cells containing a plasmid expressing the Barstar protein (two Met sites), click chemistry with the pro-fluorescent dye 3-azido-7-hydroxycoumarin produced fluorescent enhancement only in the alkynyl-labeled cells.⁷³ This specific, *in vivo* labeling approach was also employed with an alkynyl analog of phenylalanine (ethynylphenylalanine) using an auxotrophic strain that overexpresses a phenylalanyl-tRNA synthetase mutant. A later study employed the same method with azidohomoalanine (Aha) and a BODIPY-conjugated cyclooctyne for imaging of mammalian cells.⁷⁴ Additional studies by the Tirrell lab have involved methionyl-tRNA synthetase (MetRS) mutants which accept unnatural amino acids such as azidonorleucine (NLL).⁷⁵ Using cells bearing a plasmid copy of the NLL-MetRS, it was demonstrated that only proteins made in these cells could be tagged with affinity reagents (such as biotin-FLAG-alkyne) or fluorescent dyes (dimethylaminocoumarin alkyne, TAMRA-alkyne) and imaged or enriched in complex cellular mixtures (Figure 8).⁷⁵ Recently, additional MetRS mutant screening strategies have been employed to further improve efficiency and selectivity for NLL.⁷⁶ Overall, the simplicity of the expression protocols for incorporating these analogs, as well as practical modified protein yields, make this a

suitable method for fluorescent protein labeling either *in vitro* or *in vivo*.

Lemke *et al.* demonstrated that 3-azido-7-hydroxycoumarin can also be incorporated into proteins site-specifically via metal-free click chemistry using an unnatural ring-strained-conjugated lysine amino acid based on cyclooctyne.^{77,78} To accomplish this, they employed a pyrrolysine *Methanosarcina bakeri/mazei* tRNA/pylRS pair evolved by Chin and coworkers.⁷⁹ Lemke evolved the pair to accommodate the extra bulk of the cyclooctyne amino acid and used it to incorporate the cyclooctyne moiety into the mCherry protein (Figure 9). Labeling with the fluorogenic coumarin azide confirmed incorporation of the cyclooctyne amino acid, suggesting the potential to tag any protein with a variety of cargo *in vivo*. Most recently, Chin has evolved this tRNA/pylRS pair to incorporate a norbornene-linked amino acid into sfGFP, myoglobin, and T7 lysozyme.⁸⁰ This study showed that norbornene is able to undergo a highly efficient metal-free click reaction with a variety of tetrazine-fluorophore conjugates (TAMRA, BODIPY-TMR, and BODIPY-FL), all of which show quenched fluorescence until being clicked to norbornene (Figure 9). By encoding the norbornene amino acid into an EGFR-GFP fusion, metal-free labeling with a TMR-conjugated tetrazine on a mammalian cell surface could be visualized.

5. CONCLUSIONS/OUTLOOK

The presented literature review on FIAAs illustrates the importance of having a vast FIAA toolkit encompassing a variety of fluorescent scaffolds with diverse spectroscopic properties, together with practical methods of incorporation for probing protein function and interactions. The utility and benefit of fluorescent amino acids in research owes much success to continuing advancements in chemical synthesis, synthetic biology methods, and sensitivity of imaging tools. Future employment of FIAAs will see not only improved fluorophore scaffold designs and advanced methods for multiple-FIAA incorporation, but also greater integration of imaging techniques to observe dynamic yet delicate cellular functions in more complex systems. For example, Chapman *et al.* has incorporated the 7-hydroxycoumarin amino acid into the bacterial tubulin homologue FtsZ cytoskeleton protein to visualize subcellular localization.⁸¹ FtsZ assembles into a contractile ring termed “Z-rings” during cytokinesis, and attempts to

fuse a fluorescent protein on FtsZ have yielded non-functional proteins. Not only did site-selective incorporation of coumarin amino acid produce functional FtsZ protein, visualization of Z-rings could be imaged 40 min after induction in *E. coli*. This underscores the power of fluorescent amino acids in real-time imaging and the importance of imparting fluorescence to a protein without perturbing its native function. Even more recently, Schuman and coworkers were able to show that larval zebrafish could metabolically incorporate azidohomoalanine into newly synthesized proteins, which then could be imaged by clicking on an Alexafluor-488-alkyne.⁸² As chemistry and biology become further intertwined, FIAAs will continue to be at the forefront of aiding researchers in gaining insight into fundamental yet potent questions regarding life's essential biological functions involving protein interactions, recognition, and synthesis.

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Figure Captions

Figure 1. Summary of approaches for incorporating unnatural amino acids into proteins and peptides.

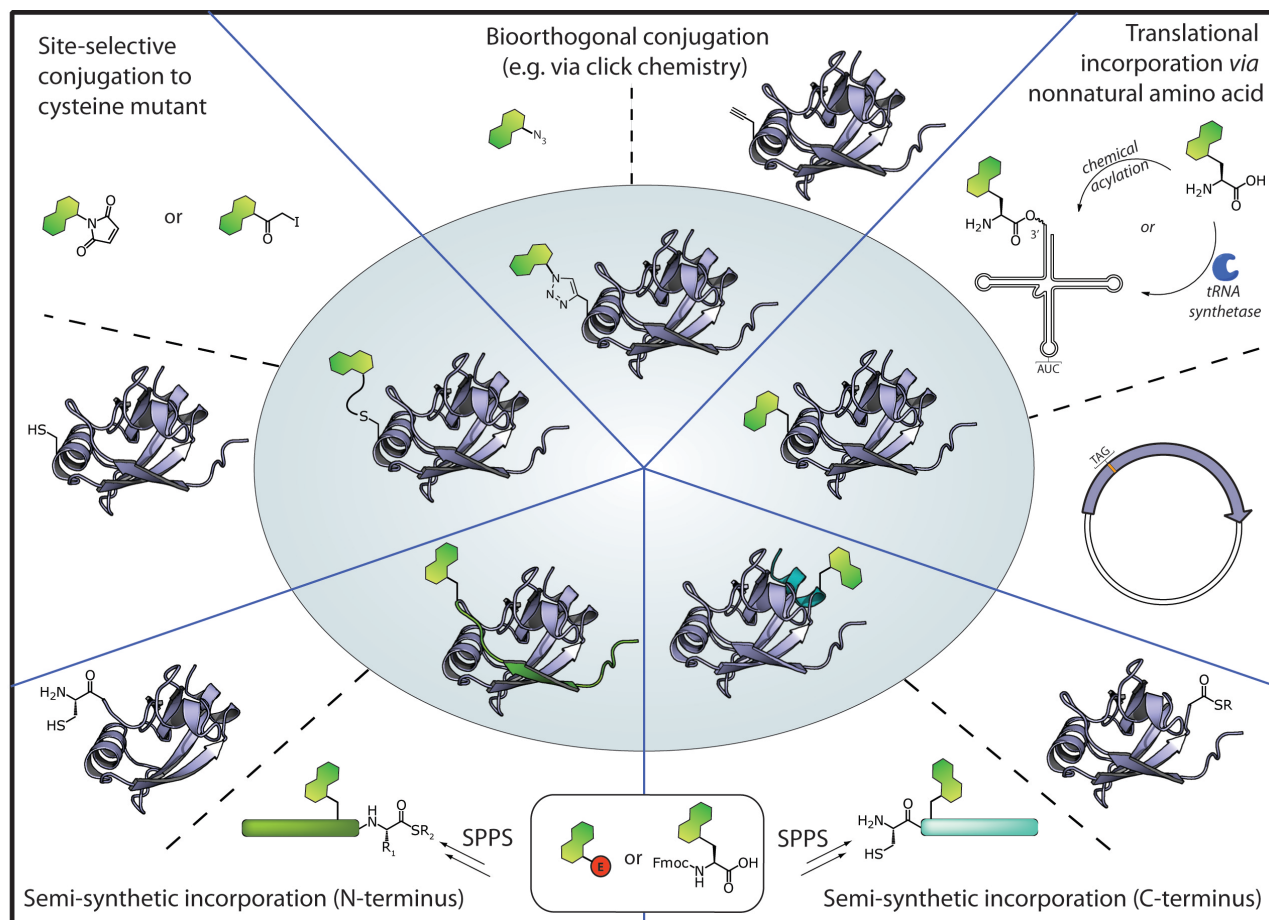


Figure 2. Comparison of some environment-sensitive fluorophores and their solvachromatic properties. Fluorescence data reported in context of a KR rich peptide.⁴²

^adata reported for PRODAN-labeled cysteine.

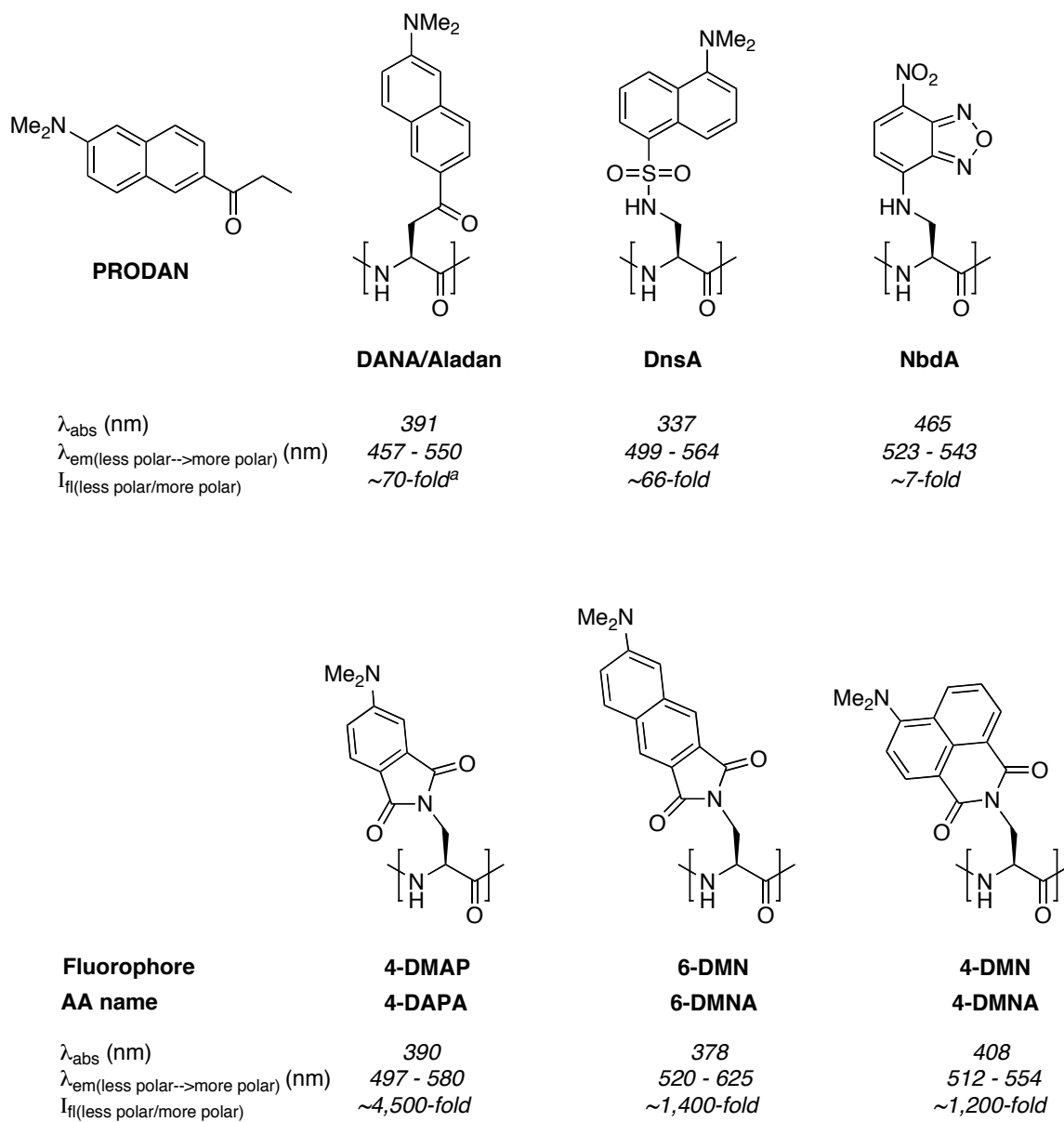


Figure 3. DANA, 4-DAPA and 6-DMNA modeled *in silico* into the hydrophobic P1 pocket in place of tyrosine in the crystal structure of the DR1–HA peptide complex. Reprinted with permission from ref. 38. Copyright 2007 Nature Publishing Group.

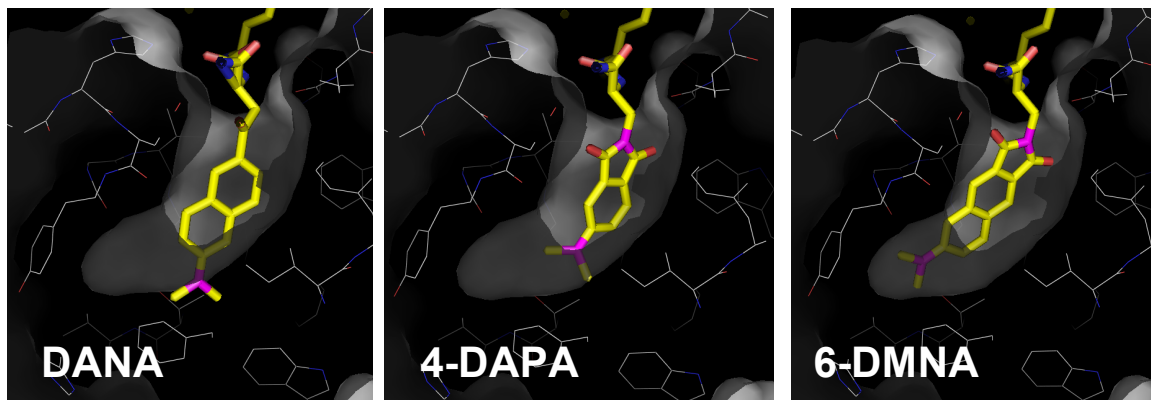


Figure 4. Pyrene FIAAs (left) and the employed kinase sensor design. Reprinted with permission from ref. 56. Copyright 2006 American Chemical Society.

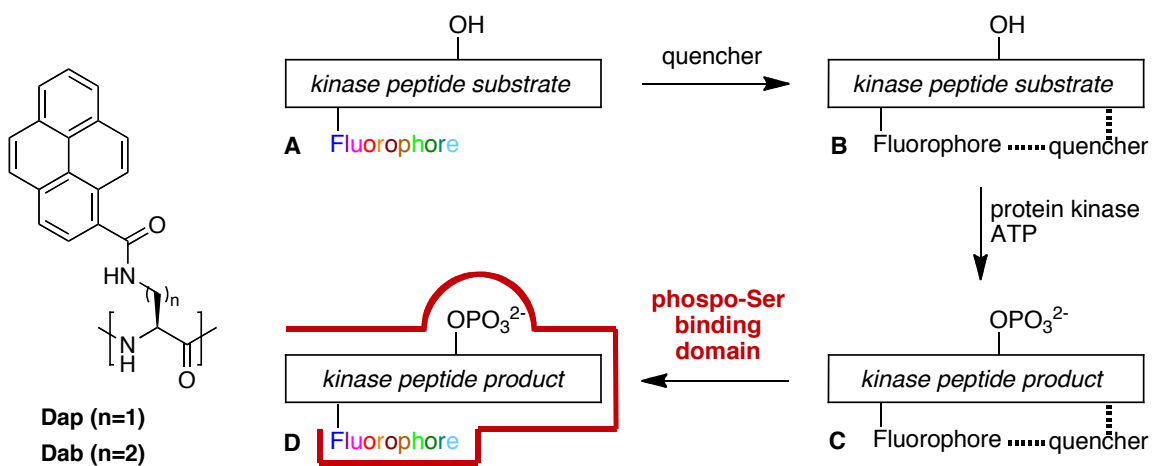


Figure 5. Exploiting acridonyl (Acd) and carbostyryl 124 (cs124) FIAAs in lanthanide sensitization for imparting luminescence to proteins via lanthanide binding tags (LBTs).

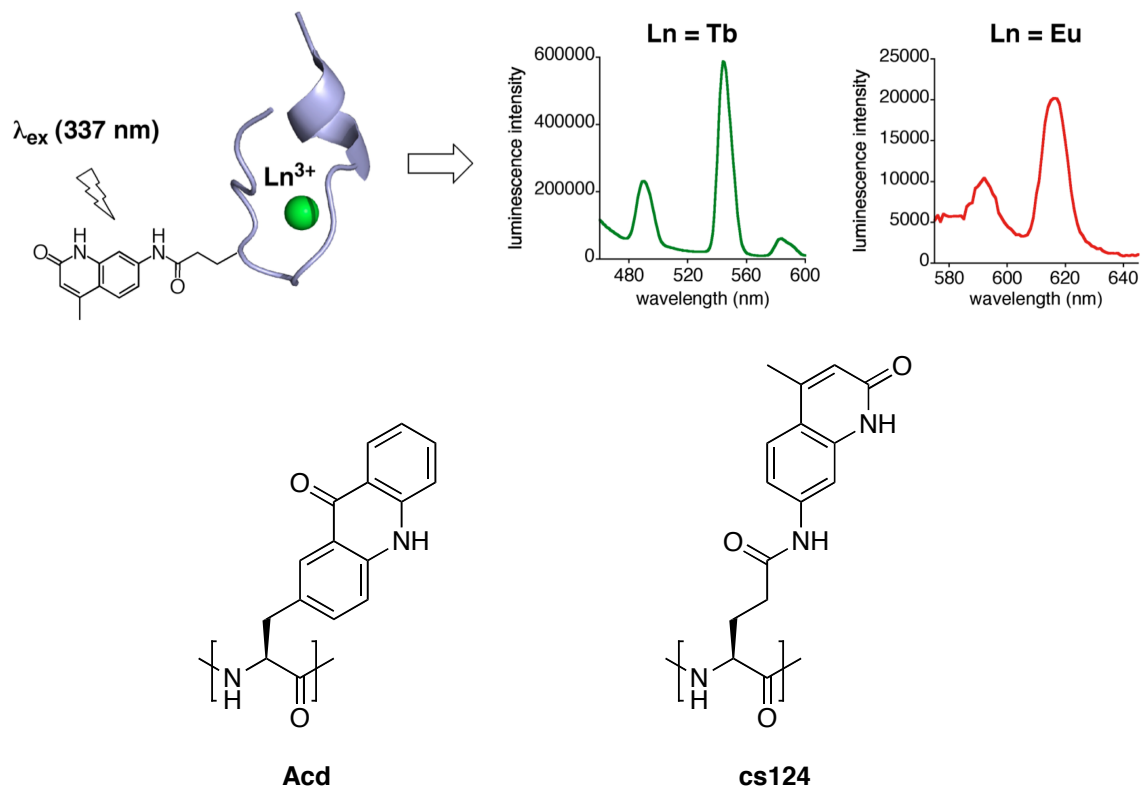


Figure 6. Sulfonamido oxine and 8-hydroxyquinoline (Oxn) FIAs (top); kinase activity sensing mechanism of these FIAs via chelation enhanced fluorescence (CHEF) (bottom).

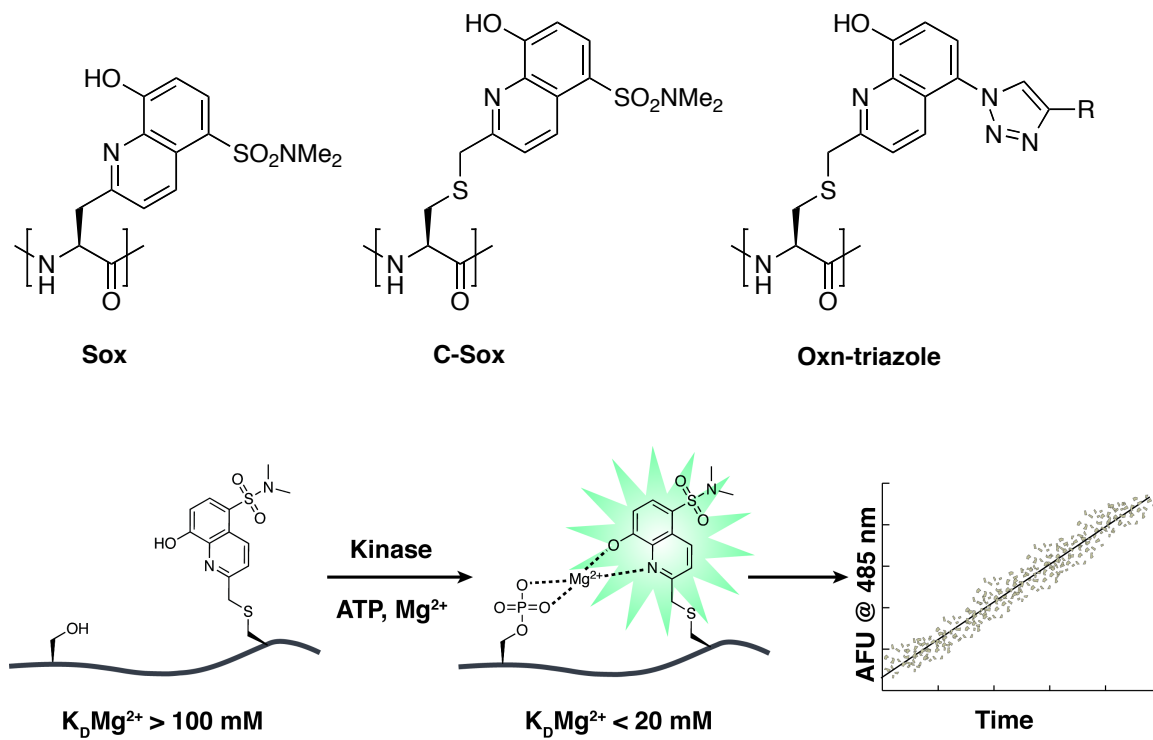


Figure 7. FIAAs with BODIPY fluorophores used for monitoring conformational changes in calmodulin via FRET.

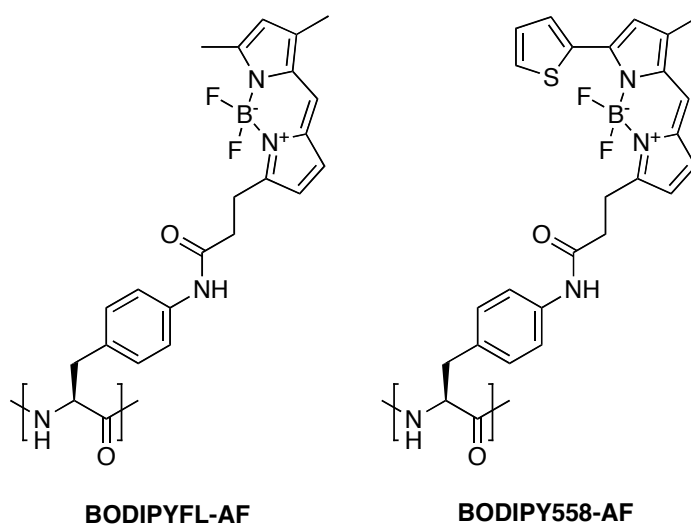


Figure 8. Cell-selective proteome labeling with incorporated azidonorleucine (NLL) using NLL-MetRS and bioorthogonal chemistry with biotin-FLAG-alkyne, profluorescent dimethylaminocoumarin, or TAMRA-alkyne. Reprinted with permission from ref. 75. Copyright 2009 Nature Publishing Group.

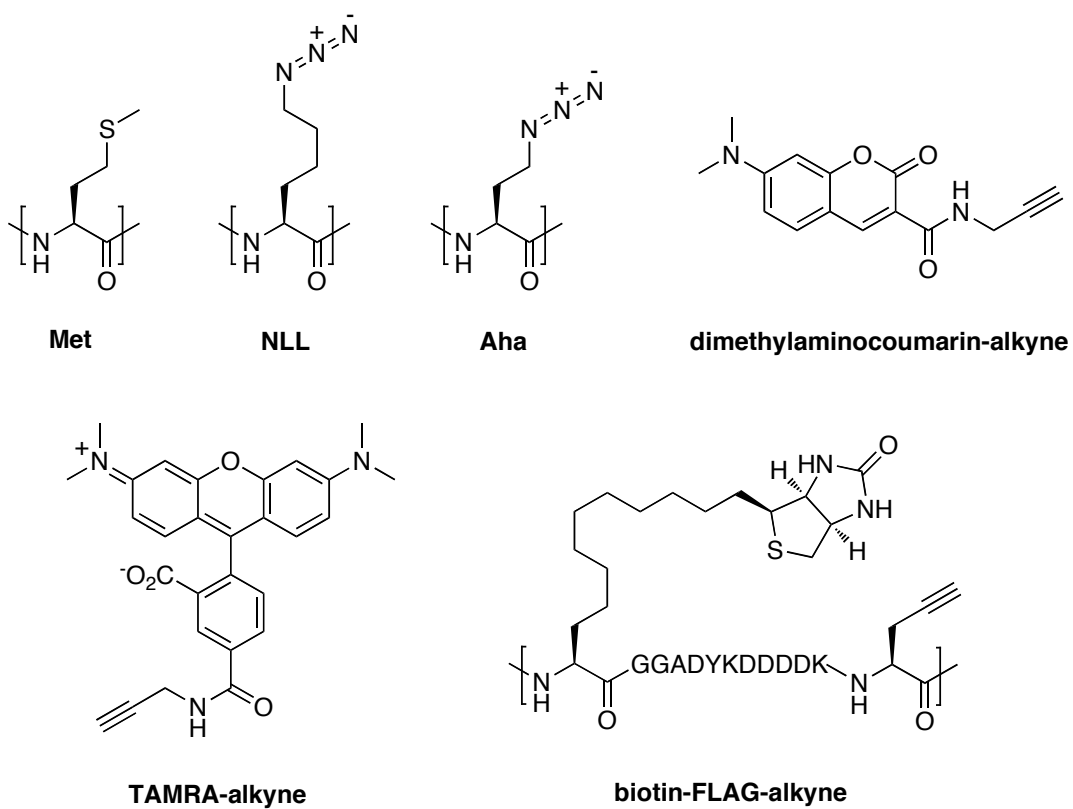
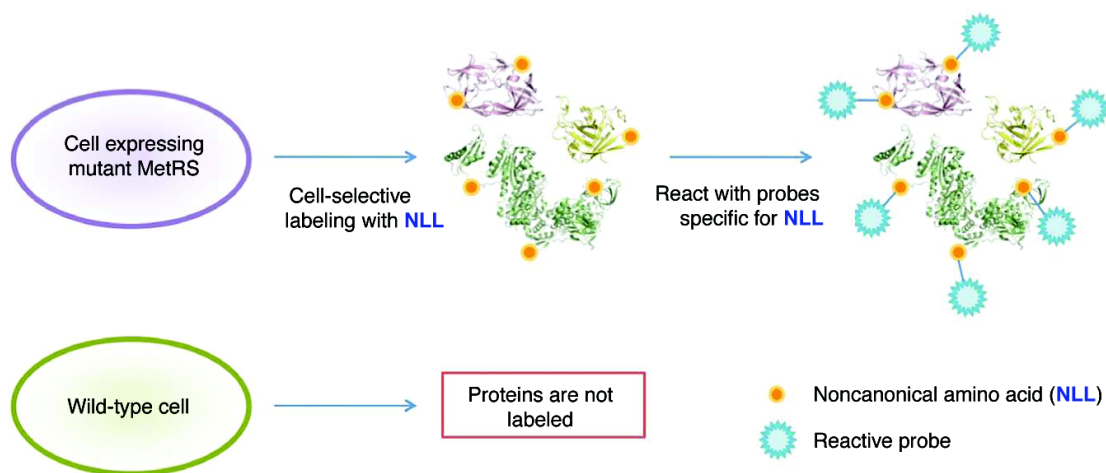


Figure 9. Ring-strained unnatural amino acids (cyclooctyne, norbornene) for bioorthogonal metal-free click chemistry with profluorescent substrates (coumarin azide, tetrazine-TAMRA-X).

