Published in final edited form as:
Bioconjug Chem. 2012 September 19; 23(9): 1891–1901. doi:10.1021/bc300262h.

Specific Labeling of Zinc Finger Proteins using Non-canonical Amino Acids and Copper-free Click Chemistry

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Abstract

Zinc finger proteins (ZFPs) play a key role in transcriptional regulation and serve as invaluable tools for gene modification and genetic engineering. Development of efficient strategies for labeling metalloproteins such as ZFPs is essential for understanding and controlling biological processes. In this work, we engineered ZFPs containing cysteine-histidine (Cys2-His2) motifs by metabolic incorporation of the unnatural amino acid azidohomoalanine (AHA), followed by specific protein labeling via click chemistry. We show that cyclooctyne promoted [3 + 2] dipolar cycloaddition with azides, known as copper-free click chemistry, provides rapid and specific labeling of ZFPs at high yields as determined by mass spectrometry analysis. We observe that the DNA-binding activity of ZFPs labeled by conventional copper-mediated click chemistry was completely abolished, whereas ZFPs labeled by copper-free click chemistry retain their sequence-specific DNA-binding activity under native conditions, as determined by electrophoretic mobility shift assays, protein microarrays and kinetic binding assays based on Förster resonance energy transfer (FRET). Our work provides a general framework to label metalloproteins such as ZFPs by metabolic incorporation of unnatural amino acids followed by copper-free click chemistry.

Introduction

Zinc finger proteins (ZFPs) comprise a broad class of transcription factors in living organisms. ZFPs regulate gene expression by binding to target sites along chromosomal DNA with high specificity and moderate-to-high affinity.[1] Zinc fingers are classified based on amino acid residues coordinating zinc ions, with a major class containing a common structural element with two cysteine and two histidine residues complexed with a zinc ion (Cys2-His2 motif). Recent advances in ZFP engineering have enabled the design and construction of “programmable” transcription factors that target and bind to chromosomal DNA in a sequence-specific fashion, thereby enabling robust control of gene expression in live cells.[1–3] Synthetic ZFPs consist of tandem arrays of zinc finger modules assembled via molecular cloning. In addition, zinc finger nucleases (ZFNs) have been constructed by fusing zinc finger modules with the *Fok*I cleavage domain. ZFNs have been used for targeted manipulation of genomes in a variety of cell types, including human cell lines.[4–6]

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Supporting Information This information is available free of charge via the Internet at http://pubs.acs.org/

Using ZFNs, chromosomal DNA can be modified by producing double stranded DNA breaks to induce site-specific homologous recombination, thereby allowing for direct modification of any desired genomic locus. Zinc finger technology has capitalized on the success of rapid construction methods for ZFPs, including a modular assembly method, combinatorial selection of engineered multi-finger proteins (oligomerized pool engineering, OPEN) and context-dependent assembly methods (CoDA).[7–9]

Engineered ZFPs can be screened for sequence-specific DNA binding using powerful *in vivo* methods, including bacterial two-hybrid selection.[10] However, measurement of dynamic ZFP-DNA binding and binding kinetics is generally facilitated by *in vitro* methods or molecular-level biochemical tools.[11, 12] Biochemical analysis can yield valuable mechanistic information regarding DNA site recognition by zinc finger modules. Fluorescence-based techniques such as Förster resonance energy transfer (FRET) allow for measurement of dynamic, non-equilibrium processes that are difficult to access using traditional electrophoretic mobility shift assays or immunoprecipitation methods. Characterization of DNA-protein interactions using fluorescence-based methods generally requires site-specific conjugation of fluorescent probes to minimize perturbations to protein structure and to retain protein activity.[13] A common strategy for protein labeling is conjugation of fluorescent tags at existing or engineered cysteine moieties on the protein backbone. However, this labeling strategy is generally not feasible if cysteine moieties are essential for protein function. In the context of zinc fingers, an intact and unmodified Cys2-His2 motif is required for competency in ZFP-based DNA recognition, and this presents a major challenge in labeling ZFPs using cysteine-based methods.[14, 15] Targeted cysteine modification or non-specific conjugation of fluorescent molecules to ZFPs tends to diminish or completely abolish the DNA-binding ability of ZFPs.[16] From this perspective, there is a strong need for development of new methods for specific labeling of ZFPs with fluorescent probes or chemical functional moieties to facilitate the study of DNA recognition, protein function and dynamic binding of zinc finger modules.[12]

Development of new strategies for transcription factor labeling would enable fundamental research in gene network dynamics, transcriptional regulation and genetic engineering. Metabolic labeling of ZFPs by direct incorporation of bioorthogonal moieties (*e.g.,* azide) is a particularly attractive strategy to label proteins with fluorescent probes using click chemistry via copper-catalyzed azide–alkyne cycloaddition.[17] Click chemistry allows for rapid, robust and specific chemical reactions, thereby serving as a practical and widely-adopted labeling strategy.[18, 19] Reaction conditions for click chemistry are generally robust, being insensitive to variations in salt concentration or the presence of additional functional groups such as primary amines. Copper (Cu) catalysts are commonly used to activate click reactions between an azide and a terminal alkyne to generate triazole moieties, and a triazole ligand is typically used to boost reaction yields and to enhance reaction rates in aqueous media.[19, 20] Although a triazole ligand is not an absolute requirement for the azide–alkyne cycloaddition reaction, low reaction yields have been observed in the absence of the triazole ligand in typical aqueous buffers, and such low yields complicate quantitative studies of dynamic protein interactions.[21] In addition to copper-mediated azide-alkyne cycloaddition, copper-free strain-promoted cycloaddition can be used to avoid potentially toxic copper(I) (Cu^{+1}) ions for click reactions.[22] The conditions for copper-free click reactions are mild and biologically compatible, which facilitates bioorthogonal reactions and chemical conjugation in living cells.[23]

In this work, we report an efficient method for residue-specific labeling of ZFPs using direct metabolic incorporation of non-canonical amino acids followed by copper-free click chemistry. ZFPs consisting of tandem arrays of zinc finger modules are expressed by replacing natural methionine with azidohomoalanine (AHA) in cell cultures. In this way,

non-canonical AHA amino acids are directly incorporated at targeted locations along the protein backbone to introduce reactive sites for click chemistry. Using this strategy, we compare both copper-mediated and copper-free strain-promoted [3 + 2] cycloaddition as labeling methods, and we assay the DNA binding properties of modified ZFPs using a solution-based FRET method and a microarray-based DNA hybridization imaging technique. Finally, we validate the metabolic labeling strategy for ZFPs by demonstrating competent DNA recognition and binding of target sequences with high specificity and affinity. Overall, we show that metabolic incorporation of unnatural amino acids, coupled with Cu-free click chemistry, is a powerful approach to study transcription factor binding using a wide variety of fluorescence-based techniques.

Materials and Methods

Molecular Cloning

Standard protocols in molecular biology were used for molecular cloning, as described in Supporting Information (SI).[24] Plasmid DNA was isolated from cell cultures using miniprep kits (Qiagen). Plasmid DNA was digested using restriction endonucleases (New England Biolabs), and gene inserts and linearized vectors were purified by agarose gel electrophoresis and gel extraction kits (Qiagen). All molecular sub-cloning was performed using *E. coli* strain DH5-alpha, pUC19 plasmid DNA, and a modified version of pcDNA 3.0 plasmid DNA (Invitrogen).

Design and Modular Assembly of ZFPs

The design and assembly of engineered zinc finger proteins was carried out as previously described.[25] Detailed procedures describing ZFP assembly are provided in Supporting Information (SI).

Protein Expression and Purification

Engineered ZFPs were produced using *E. coli* protein expression host platforms (M15MA and B834) and purified using fast protein liquid chromatography (FPLC) by affinity-based and size exclusion methods, as described in Supporting Information (SI).

Homology Modeling

Zif268 (pdb 1A1L_A) was used as a model protein for generating protein structures. Homology models were constructed using the Swiss Model server, and protein structures were rendered using VMD (Visual Molecular Dynamics, University of Illinois, Urbana-Champaign).[26]

Click Chemistry Reactions for Azide-modified Proteins

For copper-mediated click chemistry reactions, purified protein (~μM concentration) was mixed with copper sulfate (final concentration of 100 μM), tris[(1-benzyl-*1H*-1,2,3-triazol-4yl)methyl] amine (TBTA) (200 μM) and ascorbic acid (0.5 mM), followed by the addition of Cy5-alkyne (~5–30x molar excess relative to protein). Ascorbic acid was freshly prepared for each batch reaction. For copper-free click chemistry reactions, fluorescein-dibenzylcyclooctyne (DBCO, Click Chemistry Tools, Scottsdale, AZ) or Cy5-DBCO was directly mixed with protein solutions. Cu-free click chemistry reactions were performed in protein elution buffer immediately following gel filtration (Superdex) or metal-ion affinity exchange chromatography (Ni-NTA), with the latter reaction containing 1 M imidazole. Reaction yields were independent of the buffer composition for reactions proceeding at room temperature (25 °C) in the presence of up to ~100x molar excess dye reactant. To monitor the reaction yield after the reaction, mixtures were snap frozen in ethanol/dry ice

solution to stop the reaction. For MALDI-TOF mass spectrometry analysis, unreacted dye was removed by methanol/chloroform extraction. Labeled protein samples were analyzed by MALDI-TOF mass spectrometry, SDS-PAGE and fluorescence imaging using a Typhoon scanner (GE healthcare) for cyanine dye-labeled proteins or a STORM scanner (GE healthcare) for fluorescein-labeled proteins.

Fluorescence Anisotropy

A 26-base oligonucleotide ("target1") encoding for a 9-base ZFP binding site and containing a fluorescein dye (6-FAM) at the 5′ terminus was obtained from Integrated DNA Technologies (Coralville, IA). The oligonucleotide sequence for "target1" was: 5′-6-FAM – TATGGAACTCATCAGCCTCATTTCTA-3′, where the ZFP binding site is shown as the underlined sequence. The "target1" oligo was converted to duplex DNA by hybridization with a complementary oligonucleotide, wherein the target and complementary strands were annealed by elevating the temperature to 95 °C for 5 minutes, followed by gradual cooling to room temperature. Fluorescence measurements were performed using a Cary Eclipse Fluorescence Spectrophotometer (Varian) equipped with excitation and emission polarizers, automated temperature control and magnetic solution stirrers. ZFP-DNA binding was performed at 20 °C in the following reaction buffer: 20 mM Tris/Tris HCl (pH 7.9), 100 mM NaCl, 0.1 mM $ZnCl_2$, 1 mM TCEP, 20% glycerol, 0.05% Nonidet-40, 2 ng/mL dI-dC and 50 μg/ml BSA. The optimal buffer composition for DNA binding was determined over the course of several control experiments. Fluorescence anisotropy was measured in solutions containing 20 nM 6-FAM labeled target1-dsDNA (duplex DNA) using bandwidths of 20 nm. Fluorescence anisotropy (r) was calculated by:

$$r=\frac{I_{VV}-G\times I_{VH}}{I_{VV}+2G\times I_{VH}},\ \text{with}\ G=\frac{I_{HV}}{I_{HH}} \quad \text{(Eq. 1)}$$

where the instrument G factor is defined as the ratio of detection sensitivities for vertically and horizontally polarized light in the system, and H and V refer to the horizontal and vertical positions, respectively, of the excitation (first subscript) and emission (second subscript) polarizers.[27] For these measurements, the instrument G factor was carefully measured, and we observed that glycerol did not affect fluorescence measurements. For data analysis, a nonlinear regression method was used to fit the experimental data. In addition, anisotropy data was analyzed using a Hill equation to characterize the extent of cooperative protein binding.[28] Normalized anisotropy values were fit to a Hill equation:

$$\theta=\frac{[\text{protein}]^n}{K_D+[\text{protein}]^n} \quad \text{(Eq. 2)}$$

where θ is the fraction of occupied DNA bound to protein, K_D is the dissociation constant and n is the Hill coefficient describing the extent of cooperative binding.

FRET measurements

A 25-base oligonucleotide ("target2") encoding for a 9-base ZFP target DNA binding site and containing a Cy5 dye at the 5′ terminus was obtained from Integrated DNA Technologies. The oligo sequence for "target2" was: 5′-Cy5-TATGGAACTCATCAGCCTCATTTCT-3′, where the ZFP binding site is shown as the underlined sequence. The "target2" oligo was converted to duplex DNA by hybridization with a complementary oligonucleotide. Fluorescence measurements were performed at 20 °C using a Cary Eclipse Fluorescence Spectrophotometer. For FRET measurements, the ZFP-DNA binding buffer was: 20 mM Tris/Tris HCl (pH 7.9), 100 mM NaCl, 0.1 mM $ZnCl_2$, 1 mM TCEP, 20% glycerol, 0.05% Nonidet-40, 2 ng/mL dI-dC and 50 μg/ml BSA.

In addition, Cy3-DBCO-ZF2 protein was prepared in binding buffer at 600 nM, and "target2" Cy5-DNA was titrated to the desired final concentration. After incubating the reaction for 10 minutes at room temperature (25 °C), fluorescence spectra were measured. All samples were measured in triplicate, and disposable cuvettes were used to minimize residual fluorescence from previous measurements.

Electrophoretic Mobility Shift Assay (EMSA)

Protein-DNA binding was assayed by EMSA using a protocol as previously described.[29] Briefly, an oligonucleotide ("target3") encoding for the ZFP binding sequence and containing a Cy3 dye at the 5′ terminus was used. The sequence for "target3" was: 5′-Cy3-AATATGGAACTCATCAGCCTCATTTCTAAATATGAAGAGTT-AAGACGTAATG′. The "target3" oligonucleotide was converted to duplex DNA by hybridization with a complementary oligonucleotide. Alternatively, an amplicon (100 bp or 200 bp in length) containing the target binding sequence was obtained by PCR amplification using phage lambda DNA as a template in a standard PCR reaction (Supporting Information). Following PCR, amplicons were purified by PCR cleanup (Qiagen). For EMSA, protein and DNA were mixed in desired stoichiometric proportions and incubated in binding buffer at room temperature (25 °C) for 20 minutes. Following incubation, the mixture was supplemented with DNA loading dye for gel electrophoresis and run on an 8% polyacrylamide gel. Gels were imaged using ethidium bromide (EtBr) staining and UV illumination for PCR amplified DNA or a Typhoon scanner for cyanine dye-labeled DNA.

Microarray Measurements

PEGylated glass slides were prepared using PEG-biotin-NHS ester (MW 3500 g/mol) and mPEG-NHS ester (MW 5000 g/mol) as previously described.[30] PEGylated glass slides were treated with protein-DNA binding buffer, followed by incubation with neutravidin (0.5 mg/mL). Next, Cy3-biotin-DNA (1 μM in water), which has the same sequence as "target3" DNA, was printed as 1 nL spots on neutravidin treated PEGylated slides using a TeleChem Stealth SMP3 pin (Perkin Elmer) as previously reported.[31] Briefly, the Cy3-biotin-DNA was diluted to a final concentration of 1 μM in deionized water, and the DNA solution was printed as spots on the functionalized glass slides in duplicate. After printing, slides were incubated with the DNA solution spots for 5 minutes. Glass slides were rinsed 4x with binding buffer to remove excess, unbound Cy3-DNA-biotin from the glass surface. Next, Cy5-ZFP (20 nM) in binding buffer solution was applied to Cy3-DNA surface array, followed by incubation for 5 minutes at a relative humidity of 55–60% at ambient temperature 25 °C. Glass slide arrays were rinsed with DNA-protein binding buffer to remove unbound Cy5-ZFP labeled proteins. DNA-protein complexes were imaged using a GenePix Pro 6.5 double laser scanner at wavelengths of 535 nm and 635 nm (Axon Instruments). For imaging, the photomultiplier tube voltage was set to 550, and the gain was set to 30%. For display, pseudo-colors were applied to Cy3 and Cy5 (green and red, respectively), and pseudo-colored images were merged using Image J (NIH) to show DNA-protein co-localization as overlapping colors in composite images.

Results and Discussion

Engineering Zinc Finger Proteins for Specific DNA Binding

Recent advances in ZFP technology have enabled the design of chimeric ZFPs with the ability to bind to targeted DNA sequences. Individual zinc finger modules can be linked in tandem arrays, thereby enabling assembled ZFPs to bind to recognize DNA sequences of variable length. Using this approach, we cloned several multi-finger ZFPs using a modular assembly method (Table 1).[7] A homology model structure for one of the engineered three-finger ZFPs (ZFP1) studied in this work is illustrated in Figure 1a, which shows that zinc

fingers are stabilized by coordination of a zinc ion in a cysteine-histidine structural binding motif.

In an initial set of experiments, we expressed and purified several of the engineered ZFPs using natural amino acids. The DNA binding capacity for a three-finger ZFP (ZFP6) was characterized using an electrophoretic mobility shift assay (EMSA), as shown in Figure 1b. In this experiment, purified ZFP was mixed with equimolar amounts of DNA oligonucleotides encoding for the 9-base pair recognition sequence and non-specific DNA without the target sequence. Electrophoretic shift assays clearly show that the target DNA band is up-shifted due to the formation of a specific protein-DNA complex (binding constant K_d = 180 nM), whereas non-specific DNA did not yield a significant shift. As a control experiment, we determined that a single base pair mismatch in the target DNA recognition sequence prevents specific binding of ZFPs to target sequences (Figure S1). Based on these results, we concluded that chimeric ZFPs were capable of binding to target DNA with high affinity and specificity.

Residue Specific Non-canonical Amino Acid Incorporation

Specific labeling of ZFPs with fluorescent tags for quantitative binding assays presents several challenges. To specifically label ZFPs with fluorescent tags, we pursued three distinct approaches: 1) introduction of mutant cysteine residues as reactive sites, 2) labeling via fluorescent protein fusion domains, and 3) residue-specific substitution of amino acids. In the first approach, we engineered cysteine residues at the N- or C- terminus of a three-finger ZFP, followed by reaction with a maleimide-conjugated fluorescent dye. We observed that the DNA binding capacity for ZFPs labeled using this strategy was completely abolished, and we conjecture that the natural Cys2-His2 structural motif was perturbed due to cross-reactivity between the labeling probe and natural cysteine moieties using this strategy.[32] In a second approach, we generated N- and C-terminal fusions between ZFPs and mCherry or Cerulean fluorescent proteins. We found that the binding equilibrium of ZFP-fusion proteins is comparable to the parent ZFPs (Figure S2), and ZFPs with N- and C-terminal fusions exhibited similar specificity to target DNA compared to unlabeled ZFPs. In a series of control experiments, we verified that fluorescent proteins alone did not confer DNA-binding properties to ZFP-fusion proteins. However, in contrast to small molecule organic dyes, fluorescent proteins generally exhibit lower levels of brightness and photostability, which could potentially complicate quantitative fluorescence binding experiments or single molecule detection assays.

In a third approach, we pursued a bioorthogonal labeling strategy for ZFPs based on metabolic incorporation of unnatural amino acids (UAAa), followed by conjugation of organic dyes using azide-alkyne cycloaddition via click chemistry. For UAA incorporation into recombinant proteins, we adopted the method previously developed by the Tirrell group, wherein metabolic incorporation of UAAs is accomplished by universal replacement of one natural amino acid with a non-natural analog.[17, 33] In some cases, universal substitution of methionine can be used for protein labeling because the occurrence of methionine is low relative to other amino acids, yet most proteins have at least one methionine at the N-terminus. In *E. coli*, wild-type aminoacyl tRNA synthetases can charge cognate tRNAs with methionine analogues such as azidohomoalanine (AHA), and the ribosomal machinery universally incorporates the UAAs into growing protein chains during translation.[17, 34] To ensure universal replacement of natural amino acids such as methionine with UAAs, methionine auxotroph strains of *E. coli* are often employed as protein expression platforms. Alternative strategies based on suppression of nonsense mutations at amber codons have also been developed for site-specific incorporation of UAAs into recombinant proteins.[35, 36] In contrast to universal residue-specific replacement methods, site-specific incorporation methods can be used to introduce UAAs into specific target

locations within proteins, which minimizes the potential for perturbations to natural protein structure. From this perspective, site-specific incorporation of UAAs is a versatile approach for protein modification. However, the residue-specific methionine replacement strategy can be pursued when the substituted residue is confined to a well-defined target site.[37]

The vast library of zinc finger modules provides ample flexibility to engineer ZFPs containing only a single methionine residue at the protein N-terminus, distal from the DNA binding region of module. The modular assembly method allows for facile metabolic labeling of ZFPs with UAAs, because the library of zinc finger modules is degenerate for any given 3-base sequence of DNA.[6, 25] Therefore, we selected ZFP modules without methionine codons from the Zinc Finger Consortium library to exclude methionine residues from the DNA binding region, thereby preserving the DNA binding activity of the labeled protein. We used azidohomoalanine (AHA) as a methionine surrogate in *E. coli* methionine auxotroph strains (Scheme 1a), thereby replacing all methionine residues in recombinant proteins with AHA. Tandem arrays of zinc fingers were constructed consisting of two or three tandem modules to allow for specific binding to target DNA sequences (Table 1).

Metabolic incorporation of UAAs into ZFPs was carried out using the *E. coli* methionine auxotroph strain M15MA or B834 as a protein expression host. We observed that the methionine auxotroph strain M15MA efficiently incorporates AHA into expressed proteins in growth medium containing AHA, generally resulting in high levels of incorporation of AHA residues into recombinant proteins.[37] In the absence of methionine or AHA, *E. coli* cell cultures exhibited extremely poor cell growth over the course of several hours. Prior to inducing protein expression, basal expression was minimal due to the strong lacIq repressor module in the pQE80L expression plasmid. Following protein expression, ZFPs harboring metabolically incorporated UAAs were purified using metal-ion affinity chromatography (Ni-NTA resin), and proteins samples were carried forward to click chemistry reactions.

In the absence of expression tags, three-finger ZFPs generally exhibited low expression yields in *E. coli* methionine auxotroph strains used in this work (B834, M15MA or T7 Crystal). Without expression tags, the majority of three-finger ZFPs appeared in the insoluble fraction of cell cultures when expressed in methionine auxotroph strains. Moreover, we found that purification of ZFPs from the insoluble fraction of cell cultures was not feasible due to inefficient refolding of denatured ZFPs.[38] To enhance the expression yields of engineered ZFPs in methionine auxotroph strains, we used fluorescent proteins (FPs) as expression tags (Table 1), similar to previous studies using expression tags that do not interfere with DNA binding.[39, 40] We observed substantial improvements in expression yields for ZFPs with N- or C-terminal FP fusions, with a significant increase in the amount of expressed ZFP in the soluble fraction of cell cultures. In some cases, ZFP-FP fusion proteins were used for equilibrium DNA binding assays (Figure S2). However, for most three-finger ZFP constructs, we inserted a thrombin cleavage site between genes encoding for FPs and ZFP, thereby enabling the FP expression tags to be cleaved off of the ZFPs during purification after protein expression (Scheme S1). Using this approach, zinc finger modules can be cleaved from ZFP-FP constructs while the fusion protein is bound to the Ni-NTA affinity column, thereby generating high yields of three-finger ZFPs containing UAAs. We used biotinylated thrombin for the cleavage reaction, which enables facile removal of thrombin from ZFP samples using magnetic bead-based separation with streptavidin-coated microspheres. Purified ZFP was assayed using electrospray ionization mass spectrometry, which revealed highly purified protein at high yields (Figure S3).

The expression tag method described in this work provides several advantages for UAA incorporation using N-terminal methionine replacement. In prokaryotes, it is well known that the N-terminal methionine is typically excised in the post-expression modification by

methionine aminopeptidase (MetAP).[41] In a similar vein, MetAP can also remove N-terminal AHA, depending on the residue following the initial AHA.[37] Therefore, N-terminal expression tags intrinsically protect AHA residues from cleavage without the need for additional protein design or cloning steps.

Fluorescent Probe Conjugation using Click Chemistry with Linear Alkynes and Dibenzocyclooctyne

Following expression and purification, we labeled ZFPs with fluorescent dyes using two distinct strategies: Cu-mediated click chemistry using Cy5-conjugated with a propargyl group and Cu-free strain promoted [3 + 2] cycloaddition using Cy5-conjugated dibenzocycloocyne (DBCO), as denoted in Scheme 2. In the case of Cu-mediated click chemistry, we observed that addition of copper sulfate or the triazole ligand tris[(1-benzyl-1H-1,2,3-triazol-4yl)methyl] amine (TBTA), which is commonly used to accelerate Cu-mediated click cyclizations, resulted in protein precipitation and loss of protein function (Figure S7). Precipitated ZFPs showed a complete loss of DNA-binding activity (Figure 2), and precipitation of zinc finger proteins was irreversible. We attempted to resolubilize ZFPs using an excess amount of zinc ions in solution; however, the DNA binding activity of ZFP could not be recovered.

Zinc ion binding is a key requirement for efficient folding and activity of Cys2-His2 ZFPs because zinc finger modules possess $\beta\beta\alpha$ structures, which are stabilized by zinc binding. Dissociation constants for zinc binding are typically in the range of 10^{-9} to 10^{-11} M, whereas ligands such as EDTA bind extremely tightly with dissociation constants of approximately 10^{-16} M.[42, 43] Only a few minutes of exposure to strong chelating reagents such as EDTA will dissociate zinc ions from zinc finger proteins,[44] and loss of zinc ions induces structural perturbations. In zinc finger modules, consistent contacts are made between the DNA backbone and histidine residues. Specific contacts may be especially important in controlling docking arrangements because they couple the structural core of the zinc finger directly to the sugar phosphate backbone of DNA. Furthermore, metal ion substitution can induce significant structural changes in ZFPs.[43, 45] It is well known that zinc finger modules can associate with Cu^{2+}, Ni^{2+} or Co^{2+} ions, and the binding affinity is comparable to that of Zn^{2+}. However, the binding of divalent ions other than zinc can disrupt the $\beta\beta\alpha$ structure of zinc finger modules, and the DNA binding ability of ZFPs cannot be restored upon refolding.[46] Therefore, the addition of Cu^{2+} as a catalyst in Cu-mediated click reactions has the potential to induce structural perturbations in ZFPs.

Copper-mediated click chemistry requires a Cu chelating reagent to increase reaction rates and yields. TBTA has suitable chelating capacity for copper(II) and is widely used for copper-mediated click reactions. Upon addition of TBTA to a solution of copper(II), the faint blue color immediately turns to a darker blue, which indicates copper chelation (Figure S7). Moreover, we observed that TBTA can strongly chelate zinc(II), as well as copper(II), as evidenced by observing a color change (green to blue-green) that occurs when zinc ions are added to solutions containing TBTA and Cu^{2+} (Figure S7c). Under typical copper-mediated click reaction conditions involving ZFPs, zinc ions are present at relatively high concentrations (~mM), in vast excess relative to the ZFP in solution. Therefore, it is likely that addition of TBTA as a catalytic ligand for copper-mediated click reactions will be bound by free zinc ions, rather than copper, which will reduce the efficiency of TBTA on the copper-mediated click reactions. In addition, we observed that addition of copper sulfate to solutions containing ZFPs resulted in immediate protein precipitation (Figure S7a), which indicates that Cu^{2+} ions can interact with ZFPs in undesired ways. We further found that TBTA or copper ions, alone or together, can induce the precipitation of ZFPs (Figure S7b). We found that the precipitation of ZFPs was irreversible, because it could not be reversed by extensive dialysis against a zinc-rich solution.

Although ZFP recovery after denaturation has rarely been successful,[47] a limited number of wild-type ZFPs have been demonstrated to refold after denaturation and extensive purification. However, protein refolding is time consuming, labor-intensive and generally a low-yield process.[48] For "synthetic" or engineered proteins such as ZFPs constructed by modular assembly methods, the overall protein fold and corresponding energy minimization may be suboptimal due to the "cut and paste" assembly method of the individual zinc finger modules, thereby making it very challenging to efficiently refold zinc finger proteins into the native $\beta\beta\alpha$ protein architecture stabilized by coordination of a zinc ion. To overcome the problems associated with ZFP denaturation due to loss of zinc ions, we used Cu-free strain promoted [3 + 2] cycloaddition, which does not require catalysts or ligands for protein labeling.[49, 50]

To demonstrate ZFP labeling using UAAs and Cu-free click chemistry, ZFPs were expressed and purified, and Cu-free click chemistry was used to label azide-modified ZFPs with fluorescein, Cy3 and Cy5-conjugated dibenzocyclooctyne (DBCO).[51] Click reactions were monitored using MALDI-TOF and ESI (Q-TOF) mass spectrometry analysis and fluorescence-based gel imaging (Figure 3). More than 70% of the mono-functionalized three-finger ZFP was labeled with Cy5 using Cu-free click chemistry, as revealed by MALDI-TOF analysis (Figure 3c). The labeling reaction appeared to saturate in excess of 20:1 molar ratio of DBCO to ZFP following overnight reaction at 4 °C, as shown in Figure 3d. In addition, we observed no cross reactivity between natural amino acids and fluorescein-DBCO, according to MALDI-TOF analysis, even in the presence of ~100x molar excess dye to protein in the reaction mixture, which is a clear advantage of bioorthogonal labeling chemistries. Bioorthogonal labeling strategies generally ensure highly-specific labeling with minimal perturbations to protein structure, provided that the target labeling site is carefully selected based on protein structure/function considerations. In addition, Cu-free click chemistry also provided flexibility in reaction conditions for protein labeling. ZFPs labeled with fluorescein-DBCO at 4 °C overnight compared favorably with protein samples labeled 25 °C for two hours in terms of reaction yields, though higher temperatures generally showed slightly improved reaction yields, as shown in Figure S5. Over the course of two hours, reactions carried out at 55 °C resulted in ~85% yield for ZFP5, whereas the reactions carried out at 25 °C resulted in ~63% yield. Moreover, we observed Cu-free click labeling of ZFPs to proceed with high yields at 4 °C with overnight incubation, giving yields comparable to labeling reactions at two hours at 25 °C. In order to preserve the stability of purified transcription factor proteins such as ZFPs, it may be preferable to perform labeling reactions at 4 °C.

Binding Assays for ZFPs Labeled by Cu-free Strain Promoted [3 + 2] Cycloaddition

We used fluorescence anisotropy to measure the binding isotherms of wild-type ZFP1 (Met-ZFP1), azide-labeled ZFP1 (AHA-ZFP1) and Cy5-DBCO-clicked ZFP1 (Cy5-ZFP1), as shown in Figure 4a. Metabolically-labeled ZFP1 (AHA-ZFP1) and Cy5-DBCO-clicked ZFP (Cy5-ZFP1) exhibit nearly identical DNA binding properties compared to wild-type Met-ZFP1, as revealed by fluorescence anisotropy. The dissociation constants K_d calculated from fluorescence anisotropy experiments were: 158 nM, 184 nM and 123 nM for Met-ZFP1, AHA-ZFP1 and Cy5-ZFP1, respectively. Therefore, the dissociation constants were nearly the same for these three ZFP1 protein species, within experimental error, based on a Hill model fitting equation (Materials and Methods). The measured values of dissociation constants for engineered ZFPs are in agreement with previously published data for ZFPs.[40]

We also investigated the DNA binding properties of labeled ZFPs using gel electrophoretic mobility shift assays (EMSA). In these experiments, Cy5-clicked zinc finger proteins (Cy5-ZFP2) were bound to Cy3-labeled duplex DNA containing the 9-base "target2" DNA site for the three-finger tandem array of assembled ZFPs (Materials and Methods). EMSA

revealed sequence-specific DNA binding of Cy5-ZFP2 to target Cy3-DNA in the presence of non-specific competitor DNA (Figure 4b), which agrees with fluorescence anisotropy data for the same proteins (Figure S4). In these experiments, we used a fixed concentration of DNA (1 nM) and varied the concentration of labeled ZFP, which revealed the specific binding of ZFPs protein to target DNA. Unlike fluorescence anisotropy, EMSA is a quasi-steady state method for assaying DNA binding; however, binding isotherms calculated from EMSA data were comparable to fluorescence anisotropy experiments, as shown in Figure S4. At a DNA:AHA-ZFP2 ratio of ~1:60, nearly all DNA is bound to the zinc finger protein, as shown in Figure 4b. In addition, EMSA shows a distinct single band corresponding to the bound ZFP-DNA complex, which is suggestive of a well-defined protein-DNA complex in the bound state. Overall, these experiments demonstrate the utility of specific labeling of three-finger ZFPs with conserved DNA binding capacity of transcription factor proteins to target DNA sites.

Solution-based FRET Binding Assays & Microarray Assays for Labeled ZFPs

To demonstrate the utility of labeling ZFPs using UAAs and Cu-free click chemistry for *in vitro* DNA binding assays or single molecule fluorescence experiments, we studied the binding between Cy3-clicked ZFP2 (Cy3-ZFP2) and Cy5-labeled target DNA (Cy5-DNA, “target2” DNA) using Förster resonance energy transfer (FRET). We designed a FRET dye pair using a Cy3-donor/Cy5-acceptor located on the labeled ZFP and target DNA, respectively. The binding site for DNA was designed to be located at a distance of 5 nm from DNA terminus containing the Cy5 dye molecule, which is within the Förster radius of 56 Å for a Cy3/Cy5 dye pair.[27] In these experiments, we used a fixed concentration of Cy3-ZFP2 in solution, and we varied the amount of Cy5-DNA while monitoring the FRET signal (Figure 5a). Samples were illuminated using an excitation wavelength of 520 nm to excite the Cy3 donor dye. Upon addition of Cy5-acceptor DNA to solution, we observed a decrease in Cy3 emission and a concomitant increase in Cy5 emission arising from FRET between the Cy3/Cy5 dye pair within the ZFP-DNA complex. FRET signals reached a maximum for solutions containing a 7.5:1 ratio of ZFP:DNA (600 nM Cy3-ZFP2 with 80 nM Cy5-DNA). As a control experiment, 80 nM Cy5-DNA alone did not show any considerable emission at 670 nm in the absence of Cy3-ZFP2. Overall, the FRET experiments reported here serve as proof-of-principle demonstration for studying dye-labeled transcription factors using fluorescence-based methods, which will enable kinetic binding experiments or transcription factor localization studies via bulk-phase or single molecule fluorescence imaging.

Inspired by equilibrium FRET measurements, we monitored the dynamic dissociation of ZFPs to target DNA, as shown in Figure 5b. Initially, an excess amount of unlabeled target DNA was added to a solution of Cy3-ZFP2 and Cy5-DNA, and the decrease in FRET was monitored over time. The fluorescence signal was fit to a single exponential decay, and we determined a characteristic time for dissociation as $t_{1/2} = 57$ seconds. Kinetic binding/unbinding measurements typically require specialized equipment such as surface plasmon resonance (Biacore) or related techniques.[52] However, in our work, direct labeling of transcription factors allows for these measurements to be obtained using standard fluorescence spectrophotometers.

Several surface-based methods are available to study protein-DNA or protein-protein binding interactions, including label-free surface plasmon resonance, surface-based fluorescence detection using DNA microarrays and single molecule fluorescence methods. Fluorescence-based single molecule techniques typically require surface passivation with PEG or polyelectrolyte multilayers, followed by immobilization of the target biomolecule to a glass surface. Here, we studied ZFP-DNA binding interactions on a glass surface passivated with polyethylene glycol (PEG) using specific chemistry for biomolecule

attachment (Figure 5c). In this way, we adapted DNA binding studies involving labeled ZFPs to assays involving solid supports, such as general microarray-based screening tools for proteomics or genomics. We used a microarray system for detection of ZFP-DNA binding that utilizes similar surface chemistry for surface-immobilized single molecule fluorescence experiments.

In this experiment, glass slides were first pretreated with PEG to minimize non-specific protein binding. Next, Cy3-DNA (green) was printed on the PEGylated glass surface within a confined area, and Cy5-ZFP2 (red) was applied to the DNA-printed glass slide, incubated for 5 minutes and copiously rinsed to remove unbound protein. Overlaid fluorescence images of Cy3-DNA-biotin and Cy5-ZFP2 show specific binding interactions between DNA and ZFP, as revealed by a distinctive yellow color. In particular, the ZFP binding pattern (red) precisely co-localizes with the DNA printing area (green), with the low background being the result of the hydrophilic PEG-treated surface.

Conclusions

ZFPs comprise a broad class of transcription factor (TF) proteins. TFs are fundamental elements of genetic control and play a key role in transcriptional regulation in living organisms. In this work, we developed an efficient method for specific labeling of ZFPs using metabolic incorporation of UAAs, followed by copper-free click chemistry. Modular ZFPs containing tandem arrays of three zinc fingers were successfully expressed using a universal amino acid replacement strategy by substituting azide-containing AHA for natural methionine in auxotroph strains. We demonstrate residue-specific labeling with dye-conjugated DBCO using copper-free click chemistry, generally achieving reaction yields in excess of ~70%. Moreover, the specific DNA binding activity was retained for ZFPs labeled via copper-free click chemistry. To our knowledge, this work represents an important advance in residue-specific labeling of ZFPs, which can be useful conducting *in vitro* screens for assembled ZFPs via solution-based FRET assays or microarray-based hybridization assays. Furthermore, this work will open new avenues for the molecular-level exploration of dynamic processes involving ZFP binding in solution-based assays and in surface based fluorescence experiments using surface-immobilized protein strategies.

Bioorthogonal chemistries such as copper-catalyzed azide-alkyne cycloaddition broaden the toolset for investigation of protein-protein or protein-DNA interactions using solution-phase or surface-based assays. For microarrays or surface-based biosensing assays, biomolecules such as DNA or proteins are typically linked to surfaces using specific chemical attachment, and the binding partners are introduced in solution. Many surface-based assays rely on biotin-avidin linkage chemistry to immobilize biomolecules, which strongly motivates the need for additional orthogonal chemistries for fluorescent labeling of biomolecules. In the case of ZFPs, copper mediated click chemistry completely abolishes the binding activity of zinc fingers to target DNA by disruption of the Cys2-His2 zinc ion complex caused by additives in the reaction, such as copper and chelating reagent. On the other hand, copper-free click chemistry provides an efficient strategy for residue-specific labeling of ZFP by incorporation of unnatural amino acids and copper-free click chemistry, which circumvents cross-reactivity with natural amino acid residues and retains protein function.

Overall, we anticipate that this labeling strategy will be useful in quantifying transcription factor dynamics using fluorescence-based methods, which will motivate new studies in gene network dynamics or regulation.[11] Until now, the study of transcription factor localization and dynamics has mainly relied on using fluorescent proteins as labeling probes. Using UAAs and copper-free click chemistry, ZFPs can be labeled with small molecule organic dyes for improved fluorescence brightness, which will enable quantitative studies of

transcription factor binding kinetics.[53] Finally, the labeling method described in this work provides a general framework for labeling metalloproteins containing iron, zinc or copper cofactors for mechanistic studies of protein function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a Packard Fellowship from the David and Lucile Packard Foundation and an NIH Pathway to Independence Award (4R00HG004183-03 to C.M.S.) and an NIH MERIT Award (5R37DK-15556 to J.A.K.). We thank Professor A. J. Link (Princeton University) for generously providing *E. coli* auxotroph strain M15MA.

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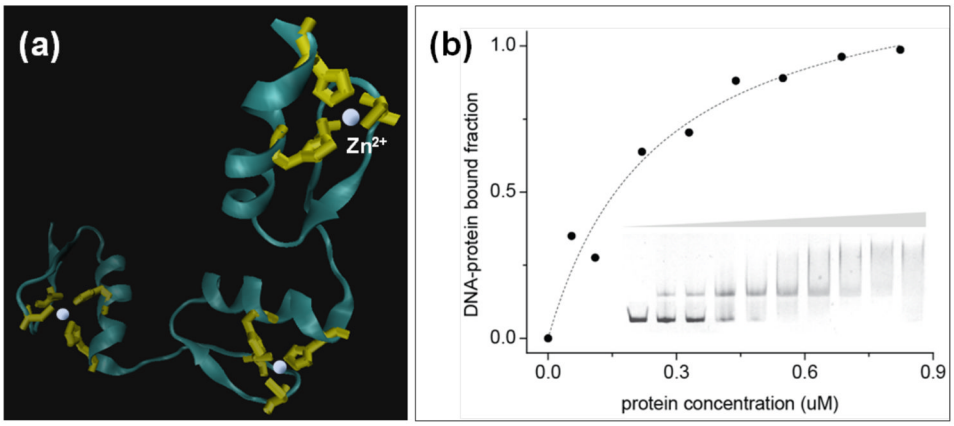


Figure 1.

Engineered ZFPs bind target DNA in a sequence-specific fashion. (a) Homology model structure of a modular ZFP consisting of a tandem array of three repeating zinc finger modules, each containing a zinc ion in a Cys2-His2 complex (yellow). (b) Electrophoretic gel mobility shift assay (EMSA) shows that a three-finger ZFP (ZFP6) binds specifically to target DNA. The bound fraction was quantified to determine the equilibrium binding constant K_d. (inset) EMSA image shows that ZFP6 forms a specific complex with target DNA in the presence of non-specific competitor DNA.

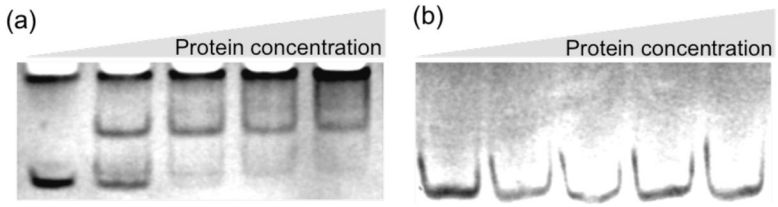


Figure 2.
Gel electrophoretic mobility shift assay (EMSA) for a three-finger ZFP. (a) Under native conditions, ZFPs clearly form a specific complex with target DNA. (b) Upon addition of the triazole ligand TBTA and copper ions, ZFPs no longer form a specific complex with DNA, and the DNA binding activity is ablated.

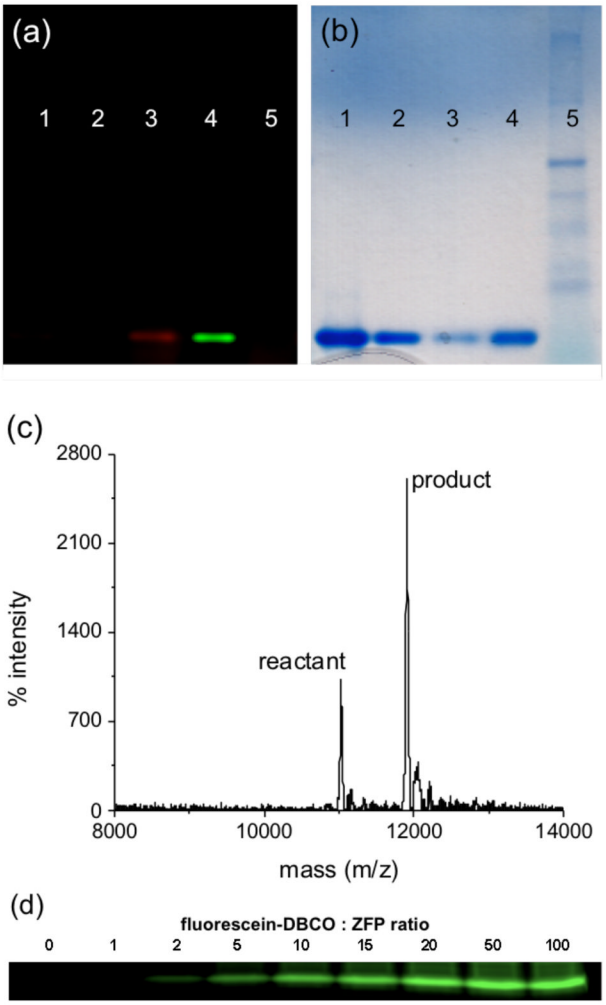


Figure 3.

Copper-free click chemistry for specific labeling of ZFPs with fluorescent tags. (a) Fluorescent gel image and (b) SDS-PAGE analysis for a gel showing dye conjugation to three-finger ZFPs following copper-free click reactions. *Lane 1*: Met-ZFP1, *Lane 2*: AHA-ZFP1, *Lane 3*: Cy5-clicked-ZFP1, *Lane 4*: Cy3-clicked-ZFP1, *Lane 5*: protein MW ladder. (c) Protein mass spectrophotometric analysis (MALDI-TOF) of labeled ZFPs. AHA-ZFP1 (MW 11035 Da) was reacted with Cy5-DBCO (MW 915 Da) using copper-free click chemistry, and the shift in mass confirms conjugation of a single Cy5 dye to ZFP1. Reaction yield calculated from the peak height is 72%. (d) Fluorescent gel showing that dye loading on ZFP5 increases upon increasing the stoichiometric ratio between fluorescein-DBCO and ZFP5 in the click reaction (1 hour at 25 °C). An equal mass of protein was loaded in each lane.

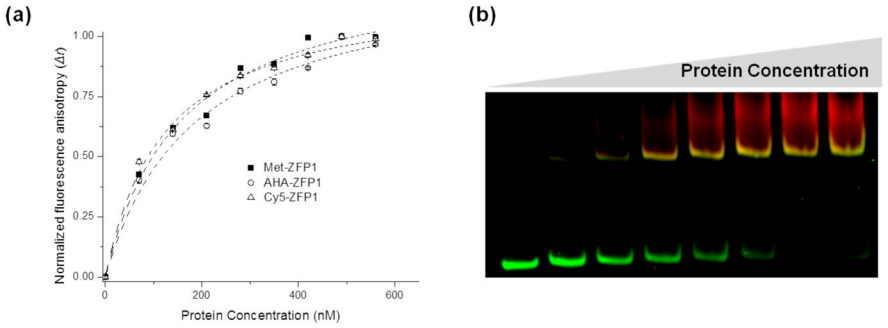


Figure 4.

DNA binding assays for labeled ZFPs. (a) Fluorescence anisotropy shows that fluorophore-conjugated ZFPs are competent for sequence-specific DNA binding. Three-finger ZFPs were studied before and after click reactions, including the wild-type protein (Met-ZFP1, square), metabolically-labeled azide containing protein (AHA-ZFP1, circle), and Cy5-clicked protein (Cy5-ZFP1, triangle). (b) Gel electrophoretic mobility shift assays (EMSA) show that labeled ZFPs effectively bind to target DNA in sequence-specific manner. Three-finger ZFPs (ZFP2) labeled with Cy5 (red) bind to Cy3-labeled DNA (green), and the protein-DNA complex (yellow) migrates through the gel with a clear mobility shift.

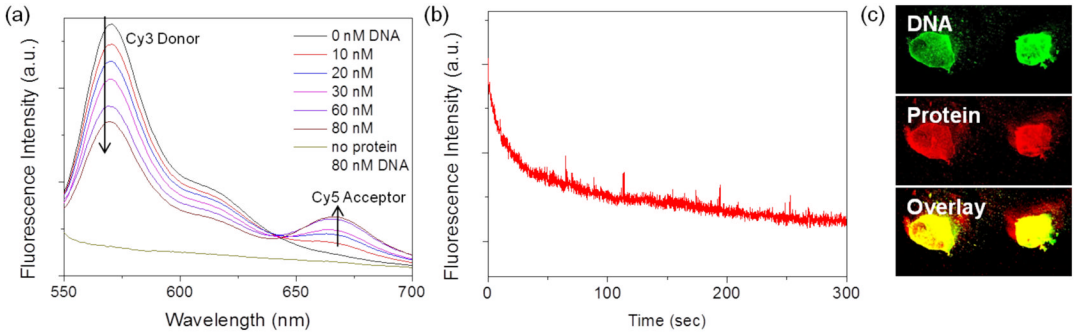
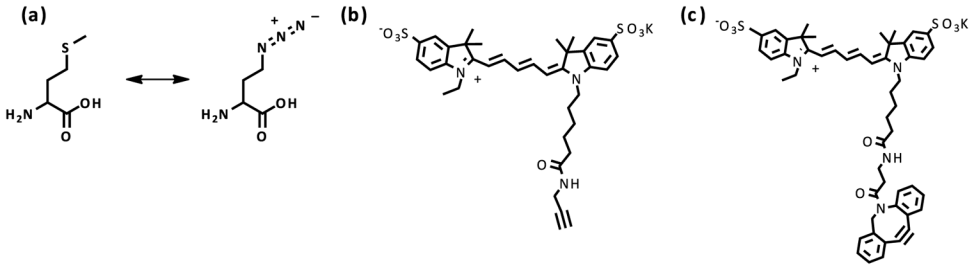
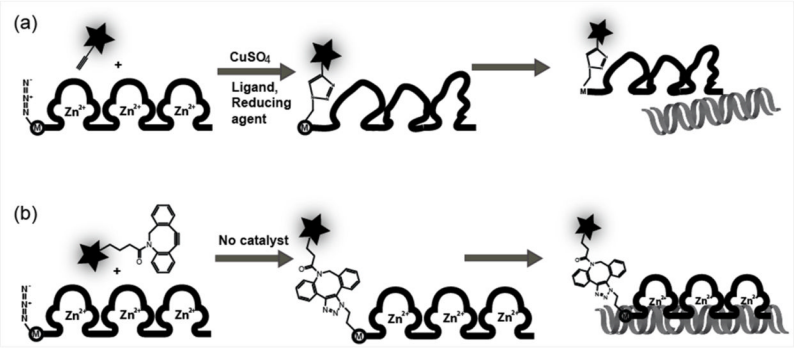


Figure 5.

Solution-based FRET and surface-based DNA binding assays for labeled ZFPs. (a) Solution-based FRET reveals specific binding interactions between Cy3 dye labeled ZFPs and target Cy5-DNA. The concentration of Cy3-ZFP2 was maintained at 600 nM, and the Cy5-DNA concentration was varied. (b) Unbinding kinetics for a bound complex of Cy5-ZFP2 and Cy3-DNA measured by FRET. (c) Microarrays demonstrate co-localization between Cy5-labeled ZFP2 and surface-immobilized Cy3-DNA encoding for the target DNA binding sequence.



Scheme 1.
Chemical structures of (a) methionine (Met) and the methionine analogue azidohomoalanine (AHA), (b) Cy5-alkyne, and (c) Cy5-DBCO.



Scheme 2.

Schematic of click chemistry-mediated covalent conjugation of fluorescent dyes to ZFPs containing metabolically-incorporated azide. (a) Copper-mediated click chemistry was used to conjugate dyes containing linear alkynes with ZFPs, however the ligand TBTA induced protein denaturation. (b) Copper-free click chemistry was used to conjugate dyes containing DBCO to ZFPs, thereby enabling ZFPs to retain sequence-specific DNA binding activity.

Table 1

Summary of engineered zinc finger proteins used in this work. Dissociation constants (K_d) were measured using fluorescence anisotropy (Materials and Methods). The 9-base DNA binding sequence for three-finger ZFPs (ZFP1–4 & ZFP6) was 5′-ATCAGCCTC-3′. Schematics of the protein constructs are shown in Scheme S1.

ZFP	Protein molecular weight (Da)	Dissociation constant, K_d (nM)	Number of zinc finger modules	Fusion protein (post purification)	Number of UAAs	Location of UAAs
ZFP1	11040	158	3	None	1	N-terminus
ZFP2	38944	191	3	Cerulean (N-terminal fusion)	4	Cerulean FP domain
ZFP3	39505	167	3	mCherry (N-terminal fusion)	11	mCherry FP domain
ZFP4	39505	175	3	mCherry (C-terminal fusion)	11	mCherry FP domain
ZFP5	8716	~2000	2	None	1	N-terminus
ZFP6	12040	180	3	None	0	N/A