Photoclick Chemistry

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Tetrazole Photoclick Chemistry: Reinvestigating Its Suitability as a Bioorthogonal Reaction and Potential Applications

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Abstract: *The bioorthogonality of tetrazole photoclick chemistry has been reassessed. Upon photolysis of a tetrazole, the highly reactive nitrile imine formed undergoes rapid nucleophilic reaction with a variety of nucleophiles present in a biological system, along with the expected cycloaddition with alkenes. The alternative use of the tetrazole photoclick reaction was thus explored: tetrazoles were incorporated into Bodipy and Acedan dyes, providing novel photo-crosslinkers with one- and two-photon fluorescence Turn-ON properties that may be developed into protein-detecting biosensors. Further introduction of these photo-activatable, fluorogenic moieties into staurosporine resulted in the corresponding probes capable of photoinduced, no-wash imaging of endogenous kinase activities in live mammalian cells.*

The discovery of new organic reactions has had a profound impact on the world. Only a few of the them, however, could be classified as bioorthogonal reactions.[1] A useful bioorthogonal reaction requires a highly efficient, chemoselective reaction to occur inside living systems with minimal interference from surrounding biological components. The light-induced tetrazole-alkene 1,3-dipolar cycloaddition (also known as tetrazole photoclick chemistry) was first reported as a bioorthogonal reaction in 2008,[2] and has been used in a number of chemical, biological, and materials applications.[3–5] Although the bioorthogonality of this reaction was studied in several reports,[2,3b–f] the conditions chosen (for example, use of excessive, highly reactive acrylamides/acrylates) rendered the conclusion biased. In many cases, only the fluorescent pyrazoline adducts were analyzed. We were intrigued by the biocompatibility of this reaction from a recent study in which tetrazoles were used as inhibitors of DNA methyltransferase 1;[6] we unexpectedly found that, upon UV irradiation, several tetrazoles readily reacted with biological nucleophiles. A subsequent literature survey revealed that, as early as 50 years ago, Huisgen et al. already reported the nucleophilic addition of thiophenol with the nitrile imine intermediate photolytically generated from 2,5-diphenyltetrazole.[7] More recent evidence also indicates the facile reaction between tetrazoles and various functional groups, including thiols, amines, acids, and heterocycles, under similar conditions.[3h,5a,8] Herein, we reassess the bioorthogonality of this reaction and propose its alternative applications in chemical biology.

Photoaffinity labeling (PAL) is a powerful method to covalently capture transient protein–ligand interactions.[9] The strategy has been applied in the form of affinity-based probes (A*f*BPs) for in situ proteome-wide profiling of drug–target interactions.[10,11] Recent advances in small-molecule bioimaging techniques[12] have made it possible to further couple such an "in situ profiling" approach with imaging of drug uptake and subcellular distribution.[11c,d,13] Dual-purpose A*f*BPs enable more accurate determination of genuine drug-target interactions by ensuring both imaging and profiling to be carried out inside native cellular environments, but few such probes are available due to a lack of suitable "minimalist" tags capable of both photo-crosslinking and visualization of labeled proteins in situ. By replacing a terminal alkyne with a cyclopropene, we recently succeeded in making alkyl diazirine-containing "minimalist" linkers compatible with live-cell imaging.[11c] Significant background labeling observed in the tetrazine reporter and the need of extensive post-ligation washing rendered this method impractical.[11c,14] Ideally, "no-wash" real-time imaging is more desirable, offering potential improvement in both temporal and spatial resolutions, but this would require the development of novel fluorescence Turn-ON reporters.[12,15] Another key component in A*f*BPs is the photo-crosslinking unit, which is typically made of an aryl azide, a benzophenone or a diazirine;[10,11] upon UV irradiation, a reactive intermediate is generated that could subsequently form a covalent bond with nearby protein targets. Given the extremely rapid and efficient photolysis of tetrazoles to nitrile imines, which, as earlier mentioned, react with different functional groups, and that some reported tetrazoles possess fluorescence Turn-ON properties,[2,3a] we wondered whether new classes of A*f*BPs could be developed, by using tetrazoles as novel photo-crosslinkers for both on-demand protein labeling and real-time Turn-ON imaging in situ. In the current study, by incorporating tetrazoles into Bodipy and Acedan dyes,[16] we have developed tetrazole-

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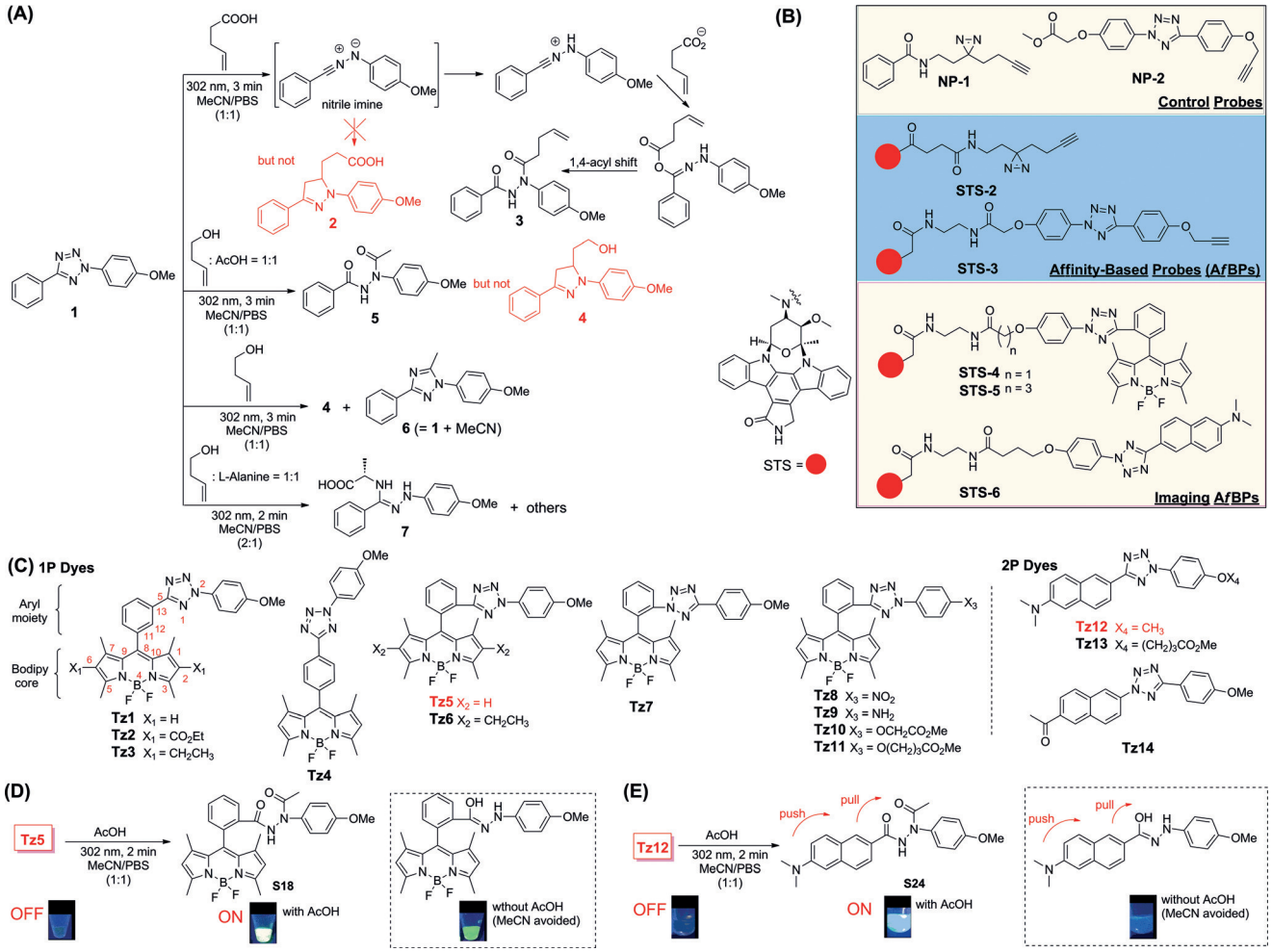

Figure 1. A) The photoinduced reaction between tetrazole **1** and pent-4-enoic acid or but-3-en-1-ol, in the presence of competing nucleophiles. B) Structures of the STS-derived A*f*BPs, including **STS-2/3** and imaging-based **STS-4/5/6**. Control probes (**NP-1/2**) are also shown. C) Structures of tetrazole-containing one- and two-photon probes, **Tz1–11** (left) and **Tz12–14**, based on Bodipy and Acedan dyes, respectively. D), E) Proposed fluorescence Turn-ON mechanisms of D) **Tz5** and E) **Tz12** upon reaction with AcOH in aqueous solution. For comparison, proposed products formed in reactions (by avoiding MeCN in solvents) without AcOH were shown (boxed).

based photo-crosslinkers possessing one- and two-photon fluorescence Turn-ON properties, some of which were developed into protein-detecting biosensors (Figure 1 C). Further introduction of these photo-activatable, fluorogenic moieties into staurosporine (STS, a potent reversible pan-kinase inhibitor) resulted in the corresponding kinase-detecting A*f*BPs, **STS-4/5/6**, which were capable of photoinduced, no-wash imaging of endogenous kinase activities in live mammalian cells (Figure 1 B).

We initially performed a photoclick reaction between **1** and pent-4-enoic acid (100 x) under previously reported conditions (Figures 1 A; Supporting Information, Figure S1);[2] to our surprise, only product **3** was isolated (71 % yield), which was confirmed as the product of nucleophilic attack by the CO_2H group in pent-4-enoic acid to the nitrile imine intermediate of **1**, rather than the expected cycloaddition adduct **2**. This indicates CO_2H was a faster reactant than a simple alkene toward **1**. Next, competition experiments were carried out with **1** and a 1:1 mixture of but-3-en−1-ol and AcOH (Supporting Information, Figure S2); again the nucleophilic addition product **5** (from **1** + AcOH), rather than pyrazoline **4**, was obtained (75 % yield). The same reaction without AcOH produced a mixture of both **4** and **6**, a product subsequently confirmed to have originated from cycloaddition between the nitrile imine and acetonitrile (a reaction solvent; Supporting Information, Figure S3). We next carried out a competition reaction between **1** and a 1:1 mixture of but-3-en-1-ol and L-alanine (Supporting Information, Figure S4); along with the nucleophilic addition product **7** (**1** + L-alanine), as previously reported,[8] several other minor products were detected, presumably the cycloaddition product **6** and others. Another competition reaction between **1** and 1:1 acrylamide/AcOH showed both the cycloaddition product **S19** (**1** + acrylamide) and the nucleophilic addition product **5** (**1** + AcOH) were produced in nearly equal amounts (Supporting Information, Figure S7). Finally, kinetic studies of **1** with AcOH alone indicated the photoinduced nucleophilic addition between the tetrazole and AcOH could be completed in 2 min (Supporting Information, Figure S6). These new lines of evidence, together with previous reports,[3h,5a,7,8] clearly show the tetrazole photoclick chemistry is not bioorthogonal, and could potentially react with various

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biological nucleophiles. Interestingly, of all functional groups tested, CO_2H appeared most reactive at physiological *p*H, better than other common nucleophiles including thiols, amines, and alcohols. Its reactivity toward **1** rivaled that of highly reactive acrylamides/acrylates.

Given the unique photoinduced reactivity of tetrazoles toward biological nucleophiles, especially CO_2H groups which are abundant in proteins, we reasoned that they might serve as general photoreactive protein-labeling reagents, and be used to develop novel A*f*BPs (Figure 1B and Figure 2), which is currently dominated by aforementioned photoreactive groups with C–H and N–H insertion chemistry.[9–11] We therefore designed **NP-2** to test the general photoreactive and protein-labeling properties of tetrazoles when compared to common photoreactive groups (that is, the alkyl diazirine-containing **NP-1**[11d]). To assess whether tetrazoles could be used in A*f*BPs for in situ drug profiling, we also synthesized **STS-3** by covalently attaching **NP-2** to STS (Figure 1B). Similar to previously reported **STS-2** (an alkyl diazirine-containing A*f*BP[11b]), **STS-3** was expected to serve as a general kinase-profiling probe by first non-covalently binding to target kinases by its STS warhead, followed by UV-initiated photo-crosslinking by its tetrazole moiety, thus converting the transient drug-target interaction into stable covalent linkage. As shown in Figure 2B, as general photoreactive reagents, both **NP-1** and **NP-2** (100 μM) were able to effectively label bovine serum albumin (BSA; 0.5 μg/lane) under UV-irradiated conditions. At a lower probe concentration (20 μM), only **NP-2** produced a prominent labeled band. These results indicate tetrazoles such as **NP-2** are excellent photo-crosslinkers with apparently improved protein crosslinking ability (when compared to **NP-1**). Similar to **STS-2**, the tetrazole-containing **STS-3** also showed potent inhibitory activities against recombinant PKA (protein kinase A), with an IC_{50} value approximately twofold higher than that of STS (Figure 2A). As A*f*BPs, **STS-2/3** effectively labeled recombinant PKA at sub-micromolar probe concentration in an UV-dependent manner in as little as 1–10 min, whereas under similar conditions, no PKA labeling was observed for **NP-1/2** (Figures 2C; Supporting Information, Figures S9, S10). This indicates PKA labeling by **STS-2/3** depended on both their specific protein-binding affinity and photo-crosslinking. To map potential labeling sites of **STS-3** in PKA, probe-labeled samples were analyzed by LC-MS/MS (Supporting Information, Figure S11); a labeled peptide with modification at its Glu (E) position was positively identified. This agrees well with our earlier finding that CO_2H groups were amongst the most reactive biological nucleophiles toward nitrile imines under physiological conditions. In a more complex scenario, both **STS-2** and **STS-3** were equally effective as in situ A*f*BPs (Figure 2D–F). In HepG2 mammalian cell lysates spiked with recombinant PKA (10% of total lysates), a single 42-kDa fluorescent band was detected, which was competed away by 10× STS (Figure 2D). In live HepG2 cells, similar to **STS-2** as previously reported,[11b] successful labeling of endogenous PKA was detected in **STS-3**-treated cells upon click chemistry (with Biotin-N_3) followed by pull-down (PD; with avidin beads) and western blotting (WB) analysis. Multiple fluorescently labeled bands were also visible in the same labeled samples (clicked with TER-N_3 followed by SDS-PAGE/in-gel fluorescence scanning; Figure 2E). They were competed away by 10× STS, indicative of additional STS-binding endogenous targets besides PKA. Interestingly, the amount of endogenous PKA labeled by **STS-3** appeared less in PD/WB samples than that by **STS-2** (bottom gel), which might be due to the improved photo-crosslinking efficiency of tetrazole in **STS-3**, resulting in more effective labeling of other STS-binding targets (top gel). As terminal alkyne-containing A*f*BPs, **STS-2/3** were not suitable for live-cell imaging owing to the need of a Cu^I catalyst.[11a,b] Both of them nevertheless were effective in visualizing subcellular probe localization in fixed mammalian cells (Figure 2F); strong fluorescence signals were observed throughout the entire cell excluding the nucleus in **STS-2/3**-treated cells. Consistent observations that tetrazole-based probes produced stronger protein-labeling signals than alkyl diazirine-based probes under various settings (Figure 2B–F) clearly validate the potential of tetrazoles as general

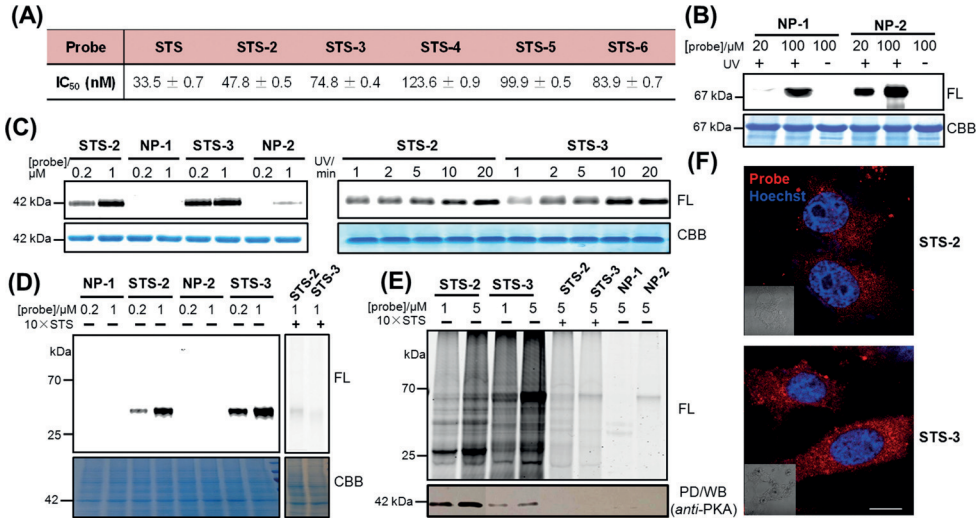

Figure 2. A) IC_{50} values of STS probes against recombinant PKA. B) Labeling profiles of BSA (0.5 μg) by **NP-1/2** (20 or 100 μM), with or without UV irradiation (10 min). Labeled proteins were clicked with TER-N_3 (Supporting Information, Table S1: SDS-PAGE and in-gel fluorescence scanning (FL)).[11b] CBB = Coomassie gel. C) Concentration-dependent and UV irradiation-time-dependent labeling of **STS-2/3** with recombinant PKA (0.5 μg/lane). (Left): 10-min UV irradiation; (right): 1 μM probe. D) Labeling of PKA-spiked HepG2 lysate (0.5 μg/lane) by **STS-2/3** (10 min UV irradiation), with or without 10× STS. E) Proteome reactivity profiles of live HepG2 cells treated with **STS-2/3** (30 min UV irradiation), with or without 10× STS. The corresponding pull-down (PD)/western blotting (WB) results are shown (bottom). F) Images of live HepG2 cells treated with **STS-2/3** (0.4 μM, 20 min UV irradiation), followed by cell fixation, permeabilization, click chemistry with TER-N_3, then image acquisition. Blue: Hoechst nuclear stain; red: TER channel. Scale bar: 10 μm.

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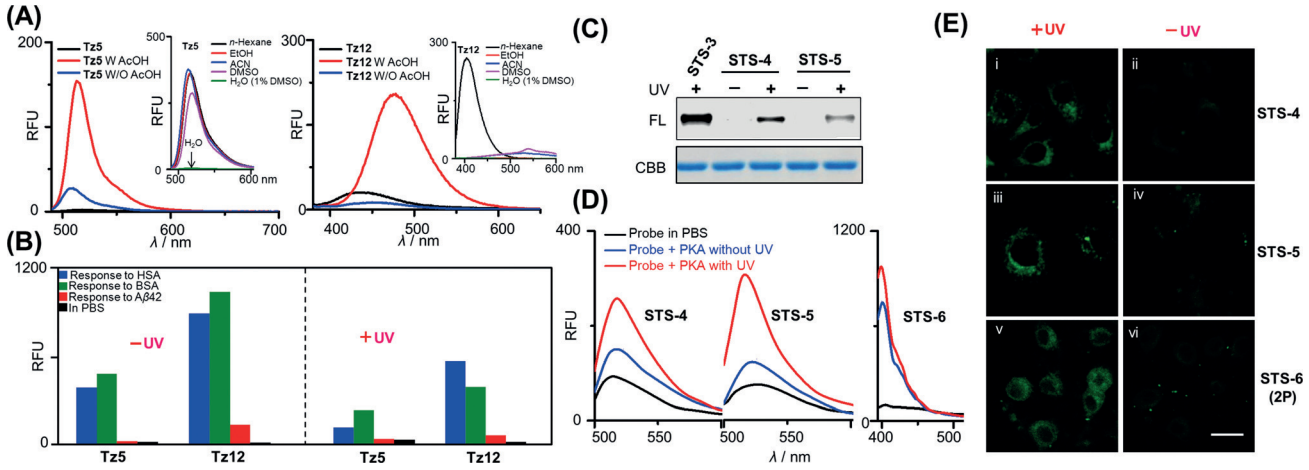

Figure 3. A) Fluorescence emission spectra of **Tz5** (left) and **Tz12** (right) in H_2O, and their UV-irradiated products (with and without AcOH). Insets: emission spectra of **Tz5/12** in different solvents. See the Supporting Information, Figures S13–S15 for details. B) Fluorescence response of **Tz5/12** to HSA, BSA, Aβ42, and PBS, without (left) and with (right) UV irradiation. See the Supporting Information, Figure S16 for details. C) Fluorescent gel profiles of recombinant PKA (0.5 μg/lane) labeled by **STS-3/4/5** (5 μM probe) with or without UV irradiation (10 min). D) Fluorescence emission spectra of recombinant PKA (2 $\mu g\,\mu L^{-1}$) incubated with **STS-4/5/6** (10 μM) in PBS, with or without UV irradiation (10 min). E) Live-cell no-wash imaging of HepG2 cells with **STS-4/5/6** (10 μM for **STS-4/5**, 1 μM for **STS-6**), with (left) and without (right) UV irradiation (10–15 min on ice). Scale bar: 10 μm.

photo-crosslinkers and their future applications in the development of novel A*f*BPs, especially in cases where the size of the photo-crosslinkers is not a major issue.

Fluorescence Turn-ON effects were known in photoinduced tetrazole–alkene cycloaddition, which is due to the formation of structurally rigid pyrazolines.[2,3a] The nucleophilic addition products of **1** with biological nucleophiles (for example, **3**, **5**, **7**) were however non-fluorescent. Inspired by fluorogenic tetrazine-based probes in which the tetrazine serves as both a bioorthogonal reactant and a quencher,[17] we speculated tetrazoles, along with photoreactive protein-labeling properties, might be effective quenchers as well. Environment-sensitive Bodipy fluorophores are popular dyes in chemical biology.[16a] The fluorescence intensity of Bodipy increases substantially with increasing solvent viscosity owing to restricted rotation between the aryl moiety and the Bodipy core (Figure 1C). Recently, it was shown that tuning the electronic property of the aryl moiety could effectively modulate the fluorescence of Bodipy core.[18] With the aim to discover tetrazole-containing Turn-ON Bodipy dyes, the fluorescence increases of which may be caused by protein-binding and subsequent photoinduced protein-crosslinking events, 11 Bodipy analogues (**Tz1**–**11**) were synthesized (Figure 1C). Furthermore, **Tz12**–**14**, which are tetrazole-containing analogues of Acedan (an environment-sensitive two-photon dye[16b]), were made. Photophysical properties of all compounds were determined with and without UV irradiation (± AcOH). While most compounds exhibited both solvent- and UV-dependent fluorescence changes (Supporting Information, Table S3 and Figures S13–S15), we were particularly drawn to **Tz5** and **Tz12**, which showed the most ideal fluorogenic properties (Figure 3A); **Tz5** showed dramatic fluorescence increases in non-polar solvents, and more importantly an 80-fold increase in fluorescence upon UV-induced reaction with AcOH in H_2O, generating **S18** (70% yield; Figure 1D). Similarly, a UV-induced 12.5-fold increase in fluorescence was observed with **Tz12** upon reaction with AcOH (Figures 1E and Figure 3A). DFT calculations indicate the Turn-ON effect in **Tz5** might be due to intramolecular photoinduced electron transfer (PET) caused by rupture of the tetrazole ring on the aryl moiety (Figure 1D; Supporting Information, Table S2),[18] whereas in the case of **Tz12**, breaking its tetrazole ring followed by AcOH addition (giving **S24**) ended up restoring the push–pull intramolecular charge transfer (ICT) mechanism in the Acedan dye (Figure 1E).[16b] Recent reports indicate derivatives of thioflavin and Bodipy dyes are biosensors of proteins such as Aβ42 (a β-amyloid protein) and human serum albumin (HSA).[19] **Tz1**–**14**, with their environment-sensitive and fluorescence Turn-ON properties upon protein crosslinking, might be suitable sensors for such proteins, especially in cases where covalent fixation of protein–sensor complexes is needed for improved spatial resolution.[12] As shown in Figure 3B (see also the Supporting Information, Figure S16), along with several other probes, both **Tz5** and **Tz12** showed marked increases in fluorescence upon binding to HSA and BSA (an isoform of HSA), but not Aβ42, without UV irradiation. Upon UV irradiation, their fluorescence signals were attenuated, but still substantially higher than those of protein-free control samples. These data, though preliminary, show the tetrazole-based fluorophores could be developed into novel, on-demand protein-detecting covalent biosensors.

To convert **STS-3** into photoinduced, no-wash imaging A*f*BPs, we next made **STS-4/5/6** (Figure 1B), which were effectively probes modified with **Tz5** and **Tz12**, respectively. We anticipated the quenched fluorescence in **Tz5** and **Tz12** moieties of these A*f*BPs would be liberated upon binding to the hydrophobic protein targets and ensuing photo-crosslinking. The IC_{50} values of these probes against PKA were 2–4-fold higher than STS (Figure 2A), indicating the bulky fluorophores did not significantly impede their kinase-binding property. Next, fluorescent labeling of PKA was carried

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out under UV irradiation conditions (Figure 3C); compared to **STS-3** (whose labeled PKA was visualized after click chemistry with TER-N_3), **STS-4/5**, by virtue of their internal Bodipy moieties, enabled direct fluorescence detection of labeled PKA. **STS-6**-labeled PKA was not analyzed by SDS-PAGE/in-gel fluorescence scanning owing to the incompatibility of Acedan with our fluorescence scanner. Nevertheless, the protein-binding, UV-dependent, fluorescence Turn-ON effect of all three probes were confirmed spectrometrically (Figure 3D); in all cases, further increases in fluorescence were observed in UV-irradiated samples, indicating the successful formation of covalent protein–probe adducts. Finally, as in situ imaging-enabled A*f*BPs, **STS-4/5/6** were used to image endogenous kinase activities in live HepG2 cells under "no-wash" conditions (Figure 3E); while only weak fluorescence signals were detected in probe-treated cells prior to UV irradiation (indicating non-covalent probe-target binding), apparent fluorescence increases were observed when the samples were UV-irradiated and imaged. Similar to **STS-2/3** (Figure 2F), these fluorescence signals were distributed throughout the entire cell except nucleus, and resistant to repeated cell washes, indicating the probes were indeed covalently bound to endogenous STS-binding cellular targets.

In summary, we have confirmed the tetrazole photoclick chemistry is not a bioorthogonal reaction. Instead, we suggest tetrazoles could be used as general photo-crosslinking reagents given its photoinduced reactivity toward biological nucleophiles. With the ability of linking protein-crosslinking and fluorescence Turn-ON properties together, we have successfully developed novel tetrazole-containing Bodipy and Acedan dyes, which could act as potential protein-detecting biosensors. Further conversion of these photo-activatable, fluorogenic compounds into kinase-detecting probes enabled on-demand, no-wash imaging of endogenous kinase activities in live mammalian cells. The unique properties of these novel photo-crosslinkers should find wide applications in chemical biology, especially where their relatively large size is not a key issue.

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