Double Click Chemistry

Bioorthogonal Reaction Pairs Enable Simultaneous, Selective, Multi-Target Imaging**

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One of the most difficult challenges in synthetic chemistry is the ability to have precise control over chemical reactivity and selectivity. These demands are amplified when it is necessary to perform selective reactions in chemically complex environments, such as those found in biology. Thus, extremely selective and high-yielding bioorthogonal click chemistry reactions continue to gain popularity. Many advances in this area from visualizing glycans and lipids to activity-based peptide profiling have recently been reviewed.[1] A noteworthy progression in this field was the introduction of strain promoted copper-free azide–alkyne [3+2] cycloaddition chemistry by Bertozzi and co-workers, which allowed the use of this reaction in living systems.[2] This led to several new applications of this chemistry, as well as improvements upon its shortcomings, such as cycloaddition rate and aqueous solubility of the cyclooctyne.[3–7] Another more recently emerging reaction, the tetrazine-strained alkene [4+2] inverse electron demand Diels–Alder cycloaddition, was introduced for bioorthogonal applications in 2008.[8,9] Extremely fast reaction rates of *trans*-cyclooctene (TCO) with tetrazines (210–30 000 $L mol^{-1} s^{-1}$)[10] have made this pair an attractive choice for bioorthogonal labeling. Recent examples have included pre-targeted labeling of cancer cell surface receptors[9,11] and intracellular targets[12] with live cells, as well as in vivo tumor imaging with ^{18}F[13,14] or ^{111}In[15] radiolabeling and sensitive cancer cell detection applications.[16,17] Despite these substantial advances, the demands of chemical biology and modern biochemical labeling studies often require simultaneous tracking of multiple elements within a single system. For example, there is a need for new methods that would enable the simultaneous monitoring of multiple small biomolecules or drugs without impacting significantly their bioactivities. In the past few years, progress has been made toward this end in the use of sequential click reactions.[18–21] One recent example demonstrates elegantly the ability to perform sequential cycloaddition reactions of an azide and then a tetrazine on a reactive (*E,E*)-1,5-cyclooctadiene.[21] Although there are several excellent illustrations of using multiple click reactions in series, not all are biologically friendly and they have not been shown to proceed concurrently in biological systems without the need for additional reagents. Herein, we present the development and proof-of-principle validation of two bioorthogonal and mutually orthogonal reaction pairs using tetrazine–TCO and azide–cyclooctyne cycloaddition reactions in tandem to afford a platform for simultaneous labeling and imaging of multiple targets in biological environments. The results show that with the proper selection of reactants, these two reactions can be used at the same time in cells and still provide precise control of desired reaction products.

For selective simultaneous labeling to be successful, the two reaction pairs must be mutually orthogonal. This was a concern, as 1,2,4,5-tetrazines are known to react with cyclooctynes;[22,23] however, the tetrazines and alkynes that demonstrated good cycloaddition kinetics were some of the most highly reactive and unstable derivatives. Based on the wide range of reported reactivity of tetrazines with unsaturated compounds,[10,24] the probability of finding a tetrazine with suitable orthogonal properties to a cyclooctyne seemed plausible. The other potential cross reaction of azides with strained alkenes has also been reported;[25,26] however, this reaction leads to multiple products, some of which are not covalently stable, especially in water.[27]

To test for these potential cross-reactions, the cycloaddition kinetics of Alexa Fluor 647 azide (AF647-azide) with excess (*E*)-cyclooct-4-enol (TCO-OH) at 37 °C in phosphate-buffed saline (PBS), pH 7.4, was first investigated. Following the reaction by HPLC, new peaks formed with absorbance at 647 nm, indicating formation of reaction products. The reaction required three days to reach completion however, and was thus shown to have a second-order rate constant of $(0.0064 \pm 0.002) L mol^{-1} s^{-1}$ (Supporting Information, Figure S1). For the other potential undesired cross-reaction, [4-(1,2,4,5-tetrazin-3-yl)phenyl]methanamine, a tetrazine proven as a useful bioorthogonal reactant,[9,11,12,14] was first incubated with dibenzylcyclooctyne-PEG4-acid (DBCO-PEG4-acid) in PBS, pH 7.4 at 37 °C. However, the second-order rate constant for this reaction of $(0.06 \pm 0.01) L mol^{-1} s^{-1}$ was found to be tenfold greater than the corresponding azide–TCO-OH cross-reaction (Supporting Information, Figure S2). In an effort to minimize this undesired reactivity, a kinetically slower, but more stable and highly water-soluble tetrazine recently developed in our lab, 5-(6-methyl-1,2,4,5-tetrazin-3-yl)pentan-1-amine (Tz)[10] was tested with DBCO-PEG4-acid. No significant cyclo-

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addition reaction was observed with this reagent pair over a period of 48 h at 37°C in PBS, pH 7.4 (Supporting Information, Figure S2).

The cycloaddition kinetics of the desired reactions were also tested. This resulted in a k_2 of 210 L mol^{-1} s^{-1} for Tz and TCO-OH at 37°C in PBS, pH 7.4.[10] The azide–cyclooctyne pair of azide-PEG4-acid and DBCO-PEG4-acid was found to have a k_2 of (2.1 ± 0.2) L mol^{-1} s^{-1} as determined by stopped-flow spectrophotometry at 37°C in PBS, pH 7.4. Figure 1a depicts the four reactants and the corresponding products
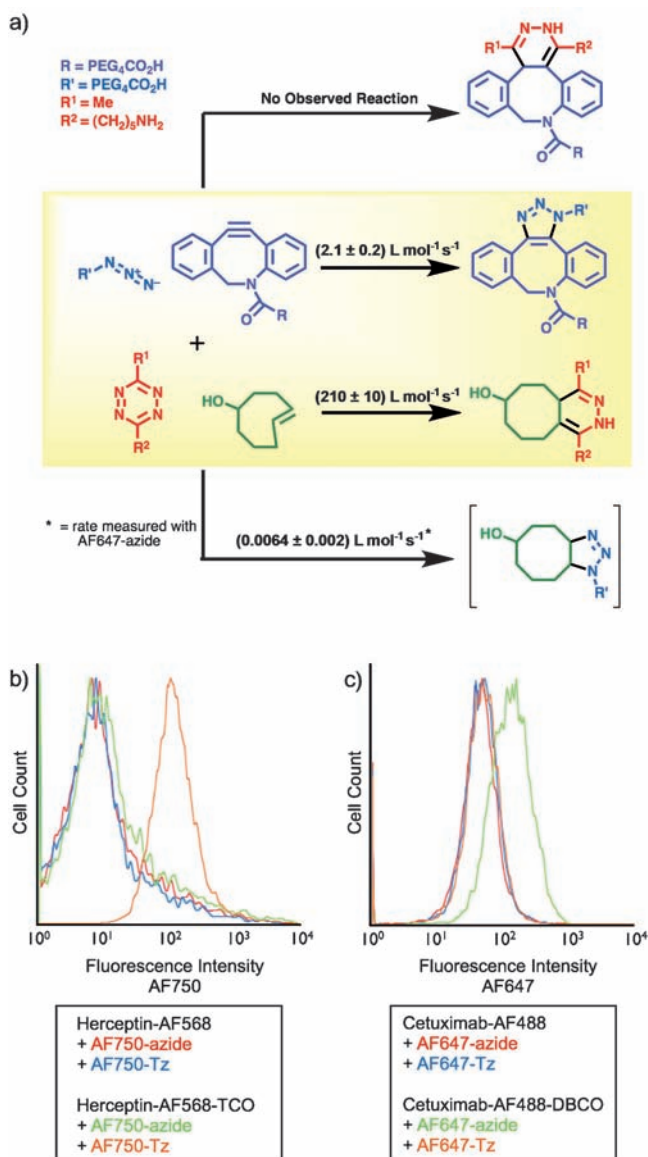
along with measured kinetic second-order rate constants for the individual reactions. These data suggest that the selected cycloaddition pairs show good mutual orthogonality and have the potential to be used simultaneously to yield only the desired reaction products.

To validate the orthogonality of these two reaction pairs in a biological setting, pre-targeted cancer cell labeling studies were performed. SKBR-3 human breast cancer cells were chosen based on their over-expression of HER2/neu receptors as well as their lower abundance (about 25-fold less) of EGFR receptors.[17] In order to exploit these receptors to test the orthogonality of our reaction pairs, the HER2/neu antibody Herceptin was labeled with AF568 and TCO, whereas the EGFR antibody Cetuximab was labeled with AF488 and DBCO. For cycloaddition reaction partners, AF647-Tz, AF750-Tz, AF647-azide, and AF750-azide were employed (for synthetic details, see the Supporting Information). Cells were incubated with labeled antibody for 30 min (fluorophore-only labeled antibodies were used as controls) and then washed before incubating for another 30 min with one of the dye modified reaction partners (for antibody binding histograms, see the Supporting Information, Figure S4). Either AF647-azide or AF647-Tz was added to Cetuximab incubated cells and AF750-azide or AF750-Tz to Herceptin incubated cells before they were washed and analyzed by flow cytometry. In Figure 1B,C, SKBR-3 cells pre-targeted with Herceptin-AF568-TCO are shown to react with AF750-Tz, but not AF750-azide. Furthermore, when EGFR, which is less abundant than HER2/neu on the SKBR-3 cells, is targeted with Cetuximab-AF488-DBCO further fluorescent labeling is only observed after addition of AF647-azide and not AF647-Tz. This set of experiments confirms the mutual orthogonality of the tetrazine–TCO and azide–DBCO reaction pairs in a biological environment.

To further explore the utility of the two orthogonal reaction pairs, a labeling experiment using two different cell types in a simultaneous double-click experiment was undertaken (Figure 2). For this study, SKBR-3 (expressing 2.00×10^6 HER2/neu and 0.08×10^6 EGFR receptors per cell) and A431 (expressing 0.02×10^6 HER2/neu and 1.23×10^6 EGFR receptors per cell) cell lines were chosen.[17] The labeled antibodies from the flow cytometry experiment were used along with AF750-Tz and AF647-azide as the cycloaddition partners. The co-cultured A431 and SKBR-3 cells were incubated with the Herceptin-AF568-TCO and Cetuximab-AF488-DBCO antibodies together for 30 minutes, followed by washing and another 30 min incubation with AF750-Tz and AF647-azide simultaneously. The cells were then fixed and imaged (Figure 2B–G). These data show that the A431 cells have strong fluorescence signal from Cetuximab-AF488-DBCO and AF647-azide, whereas the SKBR-3 cells have strong signal from Herceptin-AF568-TCO and AF750-Tz (dye-only labeled control antibodies showed no signal in the 650 or 750 nm imaging channels; Supporting Information, Figure S5). These results suggest AF750-Tz and AF647-azide are reacting selectively with TCO and DBCO, respectively. A weaker fluorescence signal can be seen on the SKBR-3 cells in the 488 and 650 channels. This is consistent with the flow cytometry studies showing Cetuximab-AF488-DBCO label-

Figure 1. a) The four cycloaddition reactants and representative cyclo-addition products/potential products. Second-order rate constants listed are from incubations of the two individual reactants in phosphate-buffered saline (PBS), pH 7.4 at 37°C. The desired azide–DBCO and tetrazine–TCO-OH cycloadditions are highlighted in yellow. b),c) Flow cytometry histograms of SKBR-3 cells. b) AF750 fluorescence c) AF647 fluorescence. Enhanced cell-associated NIR fluorescence is only observed with the Herceptin-AF568-TCO + AF750–tetrazine (b, orange) and Cetuximab-AF488-DBCO + AF647–azide (c, green) reaction pairs.

Herceptin-AF568-TCO = (red)
Cetuximab-AF488-DBCO = (green)
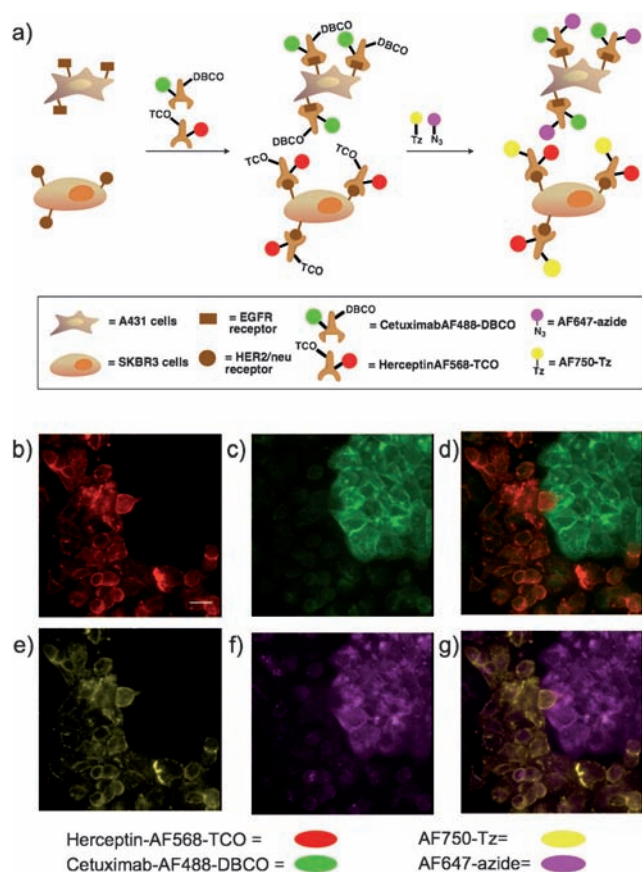AF750-Tz= (yellow)
AF647-azide= (magenta)

Figure 2. a) Simultaneous tetrazine–TCO and azide–DBCO pre-targeted cell-labeling, and b)–g) fluorescence microscopy images of co-cultured A431 (EGFR+) and SKBR-3 (HER2/neu+) cells. Cells were treated with Cetuximab-AF488-DBCO and Herceptin-AF568-TCO, followed by AF647-azide and AF750-tetrazine concurrently. Shown: fluorescence from b) AF568, c) AF488, d) AF488/AF568 merge, e) AF750, f) AF647, g) AF647/AF750 merge. Scale bar in (b): 50 µm. Panels (e) and (f) show the selective covalent labeling by AF647-azide and AF750-tetrazine of Cetuximab-AF488-DBCO bound to the EGFR receptors and Herceptin-AF568-TCO bound to the SKBR-3 cells, respectively.

ing of the lower-abundance EGFR receptors on the SKBR-3 cells and subsequent reaction with AF647-azide.

In conclusion, we describe the testing and in-cell validation of a tetrazine–TCO reaction pair that is orthogonal to azide–cyclooctyne cycloaddition chemistry. The chosen pairs were able to react concurrently in the same culture to fluorescently label two different cancer cell types. Our method uses only small molecule based reagents that also can be readily incorporated into a wide variety of systems and potentially may be used in conjunction with fluorogenic azide[28] and tetrazine substrates[12] to achieve additional improvements in specificity and sensitivity. The ability to perform multiple, rapid, simultaneous chemical reactions with a high degree of specificity in chemically complex environments should prove to be a useful tool in chemistry, biology, and medicine.

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