Bioorthogonal Chemistry

DOI: 10.1002/ange.201408015

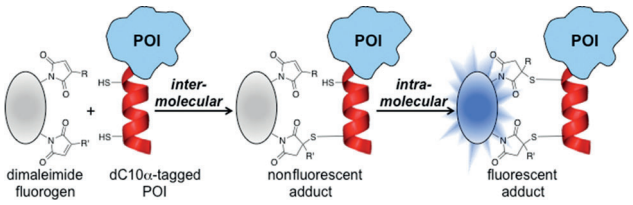
Coumarin-Based Fluorogenic Probes for No-Wash Protein Labeling**

*Yingche Chen, Christopher M. Clouthier, Kelvin Tsao, Miroslava Strmiskova, Hugo Lachance, and Jeffrey W. Keillor**

Abstract: *A fluorescent protein-labeling strategy was developed in which a protein of interest (POI) is genetically tagged with a short peptide sequence presenting two Cys residues that can selectively react with synthetic fluorogenic reagents. These fluorogens comprise a fluorophore and two maleimide groups that quench fluorescence until they both undergo thiol addition during the labeling reaction. Novel fluorogens were prepared and kinetically characterized to demonstrate the importance of a methoxy substituent on the maleimide in suppressing reactivity with glutathione, an intracellular thiol, while maintaining reactivity with the dithiol tag. This system allows the rapid and specific labeling of intracellular POIs.*

Visualizing and monitoring specific proteins with minimal disruption of their biological function and distribution is one of the foremost challenges in chemical biology. Fluorescent labeling of a specific protein of interest (POI) is a widely used method for studying expression, localization, and trafficking. The genetic encoding of fluorescent proteins (FPs) such as green fluorescent protein (GFP) is the most broadly applied approach for protein labeling because of its intrinsic specificity.[1] However, there are some limitations of this method, including the large size of GFP (ca. 30 kDa), which can perturb the function and the localization of the POI.[2] Alternative labeling methods have been developed based on the fusion of the POI to an enzyme tag. These include the HaloTag, which involves fusion to haloalkane dehalogenase,[3] the SNAP-tag, which involves fusion to O6-alkylguanine-DNA-alkyltransferase,[4] and the related CLIP-tag.[5] These enzymes can then be labeled with excellent specificity by using functionalized irreversible inhibitors. However, these 'tags' are also of considerable size (ca. 18–30 kDa) and thus pose the same risk for steric perturbation of the POI. In one of the first attempts to reduce the size of this tag, Tsien and coworkers designed a short β-hairpin tag, the four Cys residues of which react with fluorogenic bisarsenic agents such as FlAsH and ReAsH.[6] This small-molecule method has been used to label specific proteins in living cells, but its toxicity and reversibility represent limitations of its application.[7] More recent work includes the expression of proteins incorporating unnatural amino acids[8] that can subsequently undergo bioorthogonal reactions to allow covalent labeling.[9] However, unnatural amino acid mutagenesis is not yet widely applicable and is highly dependent on host cell type.

We have developed a complementary strategy for covalent, fluorogenic protein labeling in which the POI is genetically fused to a short peptide sequence (dC10α tag) that presents two Cys residues separated by two turns of an α-helix (ca. 10 Å); the POI is fluorescently labeled upon covalent reaction of these two Cys residues with complementary synthetic fluorogenic reagents[10] (Scheme 1). These



Scheme 1. Protein labeling with dimaleimide fluorogens.

fluorogenic reagents comprise a fluorophore and a dimaleimide moiety, such that their latent fluorescence is quenched by photoinduced electron transfer (PeT) until both maleimide groups undergo specific thiol addition reactions.[11] A study of the effect of spacer length and conformation on the fluorescence quenching confirmed that direct linkage of the fluorophore and the dimaleimide scaffold is critical to the quenching efficiency. Recently, we have reported "spacerless" dansyl-based probes, the fluorescence of which increases by approximately 350-fold after reaction with our target peptide tag.[12]

The chemistry underpinning this fluorogenic addition reaction (FlARe) technology is based on two properties of the maleimide group. First, they are known to undergo specific thiol addition reactions[13] and are widely applied (although typically nonspecifically) in protein labeling. Second, they are also known to quench fluorescence in their conjugated form but not as their thiol adduct products.[10a,11] Thus, a dimaleimide fluorogen must undergo two thiol addition reactions before its latent fluorescence is restored. This fluorogenic response is selective for a POI tagged with our dC10α tag because vanishingly few native proteins present two free Cys residues on their surfaces, approximately 10 Å apart. However, intracellular labeling could pose a challenge for the FlARe method since the intracellular concentration of the tripeptide thiol glutathione (GSH) is in the millimolar range. In this environment, we were concerned that our dimaleimide

[*] Y. Chen, C. M. Clouthier, K. Tsao, M. Strmiskova, H. Lachance, Prof. J. W. Keillor
Department of Chemistry, University of Ottawa
10 Marie-Curie, Ottawa, ON K1N 6N5 (Canada)
E-mail: jkeillor@uottawa.ca

[**] We acknowledge the financial support of the Canadian Institutes of Health Research (CIHR), the Natural Sciences and Engineering Research Council (NSERC), and the Ottawa Technology Transfer Network (OTTN). We also thank Prof. R. E. Campbell (University of Alberta) and Prof. A. E. Pelling (University of Ottawa) for expression plasmids.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201408015.

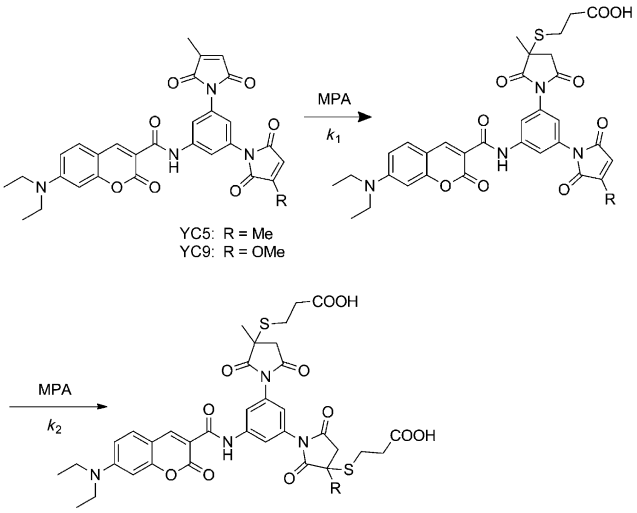
fluorogen could conceivably react with one Cys residue of an adventitious protein, followed by a reaction with one equivalent of the ubiquitous GSH, thus leading to a non-specific fluorescent labeling reaction. Herein, we report successful efforts to tune the reactivity of our probes; the resulting improved selectivity makes them suitable for intracellular application.

Our first attempt was based on the design of an asymmetric dimaleimide moiety, wherein the intrinsic reactivity of one maleimide is greatly reduced, thereby effectively suppressing its intermolecular reaction. However, in the reaction with an appropriate dithiol, the reaction of the less reactive maleimide is intramolecular and is thus efficiently accelerated by the high effective molarity[14] of the adjacent second thiol (Scheme 1). Previous work in our group[10a] suggested that the reactivity of a maleimide group bearing an electron-donating substituent may be suitably attenuated. Herein, we report the synthesis and characterization of a coumarin-based labeling reagent bearing such an asymmetric dimaleimide moiety.

Our synthetic approach was shaped by our long-term goal of diversification through the coupling of several different dimaleimide moieties to several different fluorophores. To that end, the building blocks of our fluorogens consist of the fluorophore, the aromatic amine scaffold, and the maleic anhydride. The fluorophore was designed to present a carboxylic acid group, since this is readily available or synthetically accessible for most fluorophores and enables facile conjugation to an aromatic amine. For this study, we chose coumarin as a fluorophore because of its high quantum yield, photostability, and hydrophilicity. Moreover, the excitation wavelength of the 7-diethylamino derivative is in the visible spectrum, thus offering the potential to be useful in two-photon excitation experiments. The maleic anhydrides required for the desired labeling agents are either commercially available (citraconic anhydride) or synthetically accessible (methoxymaleimide).[15] Finally, for the aromatic scaffold of the dimaleimide moiety, we developed a selective reduction strategy to reduce two nitro groups, thereby allowing successive functionalization of the resulting amine without the requirement for protecting groups. Detailed synthesis procedures can be found in the Supporting Information.

In addition to the asymmetric labeling agent YC9, we also prepared the symmetric analogue YC5 for comparison purposes. Maltose-binding protein (MBP) was chosen as a highly soluble test protein and the dC10α tag was fused to the C terminus (MBP–dC10α) as described previously.[10b] Both YC5 and YC9 were found to exhibit negligible background fluorescence, thus indicating that their dimaleimide moieties are both capable of effectively quenching coumarin fluorescence, regardless of the substituents on the pendant maleimide groups. Furthermore, both fluorogenic reagents became strongly fluorescent after reaction with the test protein MBP–dC10α, with acceptable fluorescence enhancement (FE) ratios of 69 for YC5 and 62 for YC9 (see the Supporting Information).

Kinetics studies were then performed for the thiol addition reactions of YC5 and YC9 with 3-mercaptopropionic acid (MPA) as a water-soluble, nucleophilic model thiol (Scheme 2). Reactions were run in the presence of a large excess of MPA, thereby making them pseudo-first-order with respect to the fluorogen. The resulting time-dependent increase in fluorescence was fit to a model for consecutive first-order reactions (see the Supporting Information), thereby providing pseudo-first-order rate constants for both addition steps. The concentration of MPA was then varied to allow second-order rate constants to be calculated for both steps (see the Supporting Information). For YC5, the first thiol addition is 7 times faster than the second addition, possibly owing to a steric effect; however, for YC9, the second addition is 63 times slower than the first (Table 1). This confirms our assumption that for an asymmetric dimaleimide moiety with a less reactive maleimide, the second intermolecular thiol addition is significantly slower.



Scheme 2. Kinetics studies of reactions of YC5 and YC9 with test thiol MPA.

Table 1: Second order rate constants measured for the consecutive thiol addition reactions of MPA with fluorogens YC5 and YC9 (see Scheme 2).

Fluorogen	k_1 [$M^{-1} s^{-1}$]	k_2 [$M^{-1} s^{-1}$]
YC5	1.208 ± 0.249	0.1571 ± 0.0510
YC9	2.480 ± 0.216	0.0388 ± 0.0133

We then tested the selectivity of our fluorogens by measuring the rate of their fluorogenic reactions with our test protein MBP–dC10α and GSH. As shown in Figure 1a, YC5 reacted faster with one equivalent of MBP–dC10α than with two equivalents of GSH, but the latter reaction was still appreciable; for YC9 (Figure 1b), the reaction with two equivalents of GSH was negligible but the reaction with MBP–dC10α was still rapid. However, when the GSH concentration was increased to the millimolar range (i.e., 40 equivalents) to mimic the intracellular environment, the reaction of YC9 with GSH was still apparent.

Although YC9 did not provide the degree of specificity we were hoping for, a comparison of the results obtained with

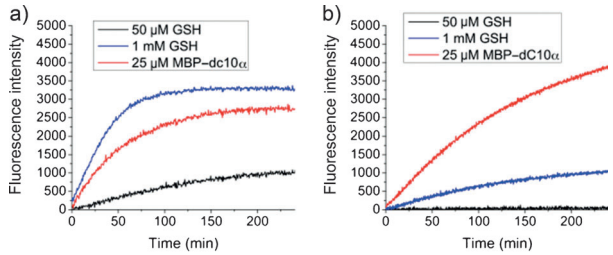


Figure 1. The time-dependent fluorescence increase of 25 μM YC5 (a) or YC9 (b) treated with one equivalent of test protein MBP–dC10α (red) or two equivalents of tripeptide thiol GSH (black), or with a large excess (40 equiv) of GSH (blue). $\lambda_{ex} = 440$ nm, $\lambda_{em} = 485$ nm.

YC9 and YC5 showed that the methoxymaleimide moiety reacted much more slowly with GSH without losing much reactivity with the dC10α tag. This result suggests the Cys residues of our dC10α tag may be highly nucleophilic and relatively insensitive to the attenuated electrophilicity of the methoxymaleimide. Inspired by these results, we then designed YC20 (Figure 2a), a fluorogenic labeling agent

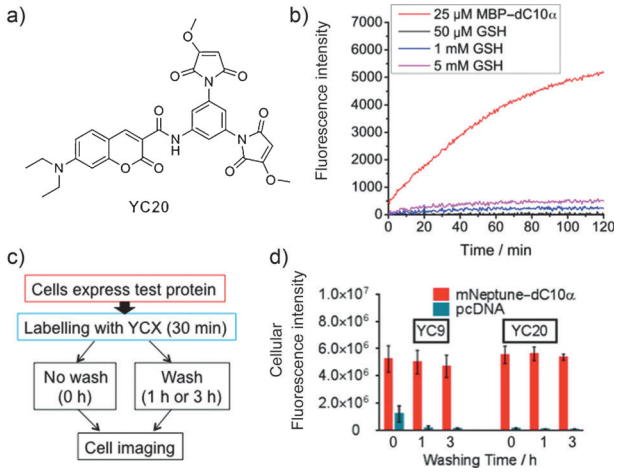


Figure 2. a) Structure of YC20. b) The time-dependent fluorescence increase of 25 μM YC20 upon reaction with 25 μM test protein MBP–dC10α (red), 50 μM GSH (black), 1 mM GSH (blue), or 5 mM GSH (pink). $\lambda_{ex} = 440$ nm, $\lambda_{em} = 485$ nm. c) Labeling and washing protocol. d) Fluorescence intensity (mean ± SD) of intracellular labeling of test protein mNeptune–dC10α with fluorogen YC20 in HEK293T cells ($n = 10$, triplicates). The red bars show cells transfected with the test protein and the blue bars show cells transfected with empty (mock) vector.

bearing two methoxymaleimide groups, in an effort to completely suppress the reaction with GSH, while relying on the ideal geometry and enhanced nucleophilicity of the dC10α tag to maintain reactivity with the POI. The synthesis of YC20 followed a synthetic scheme similar to that for YC5 (see the Supporting Information).

The novel dimethoxymaleimide fluorogen YC20 exhibited negligible background fluorescence and became strongly fluorescent after reaction with the test protein MBP–dC10α (see the Supporting Information). The selectivity of YC20 was then tested by comparing the rate of its fluorescence increase upon reaction with the test protein MBP–dC10α and with GSH. To closely mimic the intracellular environment, up to 5 mM GSH was used to probe the specificity. The reaction of YC20 with GSH was dramatically suppressed even at this high concentration of GSH, while the reaction with the more reactive MBP–dC10α was still rapid (Figure 2b).

We then confirmed that fluorogens YC9 and YC20 were nontoxic by performing an MTT assay (see the Supporting Information) before testing both fluorogens in live-cell labeling studies. The red fluorescent protein mNeptune was chosen as a target protein because its intrinsic fluorescence serves as a control for the transfection and expression of the target protein; the target sequence dC10α was genetically fused to the C terminus (see the Supporting Information). Human embryonic kidney 293T (HEK293T) cells transfected with mNeptune–dC10α were incubated with YC9 or YC20 for 30 min and imaged either directly or after washing (Figure 2c). Cells transfected with pcDNA (empty protein expression vector) were labeled following the same protocol to provide a negative control. The fluorescence intensities of the labeled cells were measured after various washing times and compared (Figure 2d). Cells transfected with mNeptune–dC10α showed strong cyan fluorescence with both YC9 and YC20. For YC9, cells without target protein also showed slight cyan fluorescence from a nonspecific reaction, but this background fluorescence could be removed with one washing step. However, for YC20, no nonspecific labeling was observed. Cells that did not express the target protein showed negligible fluorescence, even without any washing steps. This result is consistent with those from our kinetics studies, which showed that YC9 still displays some reactivity with high concentrations of GSH, a reaction that could take place during an intracellular protein labeling experiment. However, YC20 is highly selective and as such can be used as a no-wash labeling agent in live cells. Furthermore, both fluorogenic reagents provided robust covalent labeling of the target proteins, the fluorescence of which was stable even after 3 h of washing (Figure 2d; red bars).

The no-wash fluorogenic labeling reagent YC20 was then used to label cellular proteins localized to different parts of the cell. Histone H2B, which localized to the nucleus, and actin, which is located in the cytosol as filaments, were chosen as test proteins. The target sequence dC10α was cloned to the C terminus of Histone H2B and to the N terminus of actin (see the Supporting Information) to demonstrate that the target sequence can be fused to either terminus of a POI. HEK293T cells transfected with a plasmid encoding histone-H2B–dC10α or dC10α–actin were then labeled with YC20 for 45 min and directly imaged by fluorescence microscopy without any washing steps (Figure 3). For cells expressing dC10α–actin, filament structures in the cytosol were clearly visible in the cyan channel of the microscope, thus indicating that the dC10α–actin was labeled by YC20. For cells expressing histone-H2B–dC10α, only the nuclei were fluorescently labeled and visible in the cyan channel, thus indicating that YC20 specifically labeled histone-H2B–dC10α in these cells. Cells transfected with pcDNA (empty vector) as a negative control showed negligible fluorescence.

To summarize, we have synthesized a novel protein-labeling reagent that contains a dimethoxymaleimide moiety and fluorescently labels POIs bearing our dC10α tag with

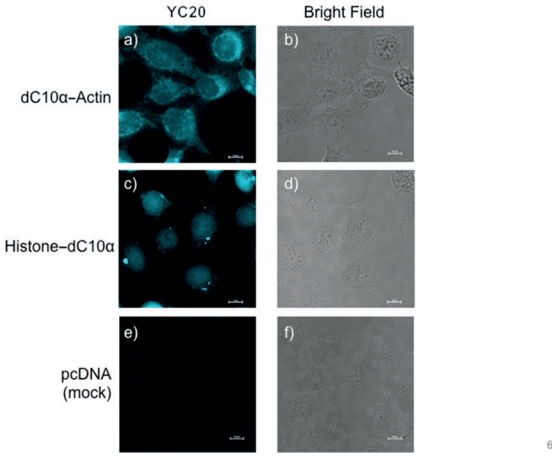


Figure 3. Fluorescence (left column) and corresponding bright-field (right column) confocal microscopy images for no-wash cell labeling with YC20: a, b) dC10α–actin-expressing cells treated with YC20. c, d) Histone-H2B–dC10α-expressing cells treated with YC20. e, f) pcDNA-transfected cells (negative control) treated with YC20. Scale bars = 10 μm. Cyan fluorescence conditions: Em 482 nm (bandwidth: 35 nm), dichromic mirror: 400–457 nm.

great selectivity in living cells. Kinetic studies clearly show that the improved selectivity for the dC10α tag over monothiol compounds is due to the attenuated reactivity of the maleimide groups, such that they react preferentially with the highly reactive thiols of the dC10α tag. This new labeling reagent is nontoxic and enables the highly specific labeling of a POI in live cells with no washing. It thus has significant potential for general application to labeling specific proteins in living cells.

Received: August 6, 2014
Published online: October 14, 2014

Keywords: bioorthogonal chemistry · fluorescent probes · glutathione · maleimide · protein labeling

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