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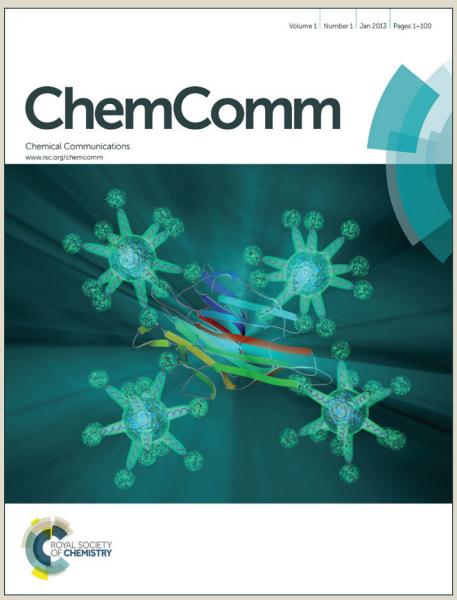

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A Cu-free clickable fluorescent probe for intracellular targeting of small biomolecules

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We synthesized a novel cyclooctyne-based clickable fluorescent probe with versatile properties such as high cell-membrane permeability and free diffusibility in the cell. Our probe "FC-DBCO" was conjugated to an azide-modified mannose *via* a Cu-free click reaction in living HeLa cells and displayed intracellular specific fluorescence imaging with low background signals.

Bioorthogonal reactions, such as click chemistry, is a powerful tool for specifically conjugating fluorescent chemical probes with target biomolecules inside living cells.[1] 1,3-Dipolar [3+2] cycloaddition with an azide and an alkyne (Huisgen cycloaddition[2]) in which a 1,2,3-triazole is formed in the presence of Cu(I) as a catalyst, termed the Cu-catalyzed azide–alkyne 1,3-dipolar cycloaddition (CuAAC[3-5]), is one of the most popular click chemistry reactions and has been widely used in biological research. Azides and alkynes are ideal partners for coupling due to the ease with which most organic compounds are tagged as well as their tolerance to various solvents including water.[6] These extremely energetic small moieties with a highly specific reactivity are also well-suited to biological applications.[7] Nevertheless, exogenous metals such as Cu(I), show cytotoxicity at low concentrations and can interfere with the metabolic balance of cellular systems.[8] This is a serious limitation for CuAAC in applications involving live cell imaging.

"Cu-free" click reactions (i.e., 1,3-dipolar cycloaddition between azides and cyclooctynes), developed by Bertozzi and coworkers,[9] enables specific conjugation between target biomolecules and fluorescent chemical probes in biological systems without using cytotoxic catalysts. Bertozzi *et al*. demonstrated the labeling of azide-modified *O*-glycan using difluorinated cyclooctyne (DIFO)-conjugated probes and visualized glycan trafficking under several conditions in living cells, tissues, and whole organisms.[10-12] Because these probes were designed to label azide modified glycans on the cell membrane, they are unsuitable for intracellular labeling due to their low membrane permeability.

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Commercially available cyclooctyne-based fluorescent probes, Alexa Fluor®-conjugated dibenzocyclooctyne (DIBO) dyes have also been used for intracellular azide labeling.[13] However, undesirable intracellular retention of the unbound probes caused high background fluorescence, leading to poor specific imaging of the target biomolecule. Cu-free click reaction between azide-modified biomolecules and cyclooctyne-based fluorescent probes has enabled live intracellular and *in vivo* biological imaging applications. Nonetheless, more specific imaging techniques are required that utilize cyclooctyne-based Cu-free clickable fluorescent probes possessing high membrane permeability and good cytosolic diffusibility.

The azide has been one of the most widely used chemical reporters due to its size and high stability (extremely low reactivity) against metabolic chemical reaction and natural degradation,[10] and already introduced into various bioactive small molecules such as glycans, lipids, and proteins in live cells.[14] A commercially available azide-modified mannose; tetraacylated *N*-α-azidoacetylmannosamine (Ac_4ManNAz, Click Chemistry Tools) can be detected after 72 hours incubation with extracellular imaging probes, when it is incorporated into the sugar chains of glycoproteins and transferred into the cell membrane.[15] According to a previous report,[16] monosaccharide derivatives modified with bioorthogonal reactive groups, such as an azide, are incorporated into cellular metabolic processes and integrated into cell-membrane sugar chain. Ac_4ManNAz is taken up by cells and efficiently hydrolyzed to *N*-α-azidoacetylmannosamine (ManNAz) by cytosolic esterases. ManNAz is converted to sialic acid *via* five enzymatic steps and then conjugated to the end of the

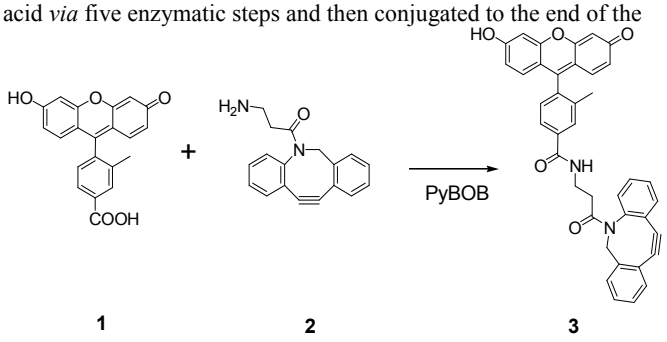

Fig. 1 Synthetic procedure for the preparation of FC-DBCO **3**.

sugar chains.[17] The intracellular dynamics of glycoproteins before transportation to the membrane, as well as that of mannose and sialic acid before incorporation into glycoproteins, has not been observed using small chemical fluorescent probes because there is few suitable probes available for specific intracellular target imaging.

Here, we designed a novel cyclooctyne-contained fluorescent probe for specific intracellular azide labeling. The probe, named Fluorescein-Conjugated Dibenzocyclooctyne (FC-DBCO) **3**, was designed and synthesized from a reported fluorescent dye (4-Carboxy-2-Me TokyoGreen **1** by Nagano *et al*.[18,19]) and a commercially available cyclooctyne derivative (DBCO-amine **2**) (**Fig. 1**). Fluorescein derivatives have been widely used as a platform for various fluorescence probes because of its high fluorescence quantum yield and good cytosolic diffusibility.[20] We anticipated that replacement of the phenyl-carboxyl group of fluorescein with a methyl group would increase the molecule's hydrophobicity leading to enhanced cell membrane permeability.[21,22] Moreover, conjugation with **2** would further increase hydrophobicity of the probe.

Compound **1** was synthesized according to a previously reported method.[17,18] Condensation between **1** and **2** to afford **3** was performed using a condensing reagent, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), which is a derivative of BOP reagents. Other coupling reagents were found to be unsuitable for this reaction. For example, BOP gave an inseparable byproduct, hexamethylphosphoric triamide (HMPA).

The excitation and emission spectra of FC-DBCO (see ESI†) were similar to those of fluorescein.[23] The reactivity of FC-DBCO against an azide compound *in vitro* was evaluated using Ac_4ManNAz. FC-DBCO **3** (final concentration: 10 μM) and Ac_4ManNAz **4** (final concentration: 40 μM) were mixed in HEPES solution at pH 7.4 (**Fig. 2a**) and the reaction was monitored by observing the change in the UV-vis spectrum of the mixture. The reaction mixture was prepared

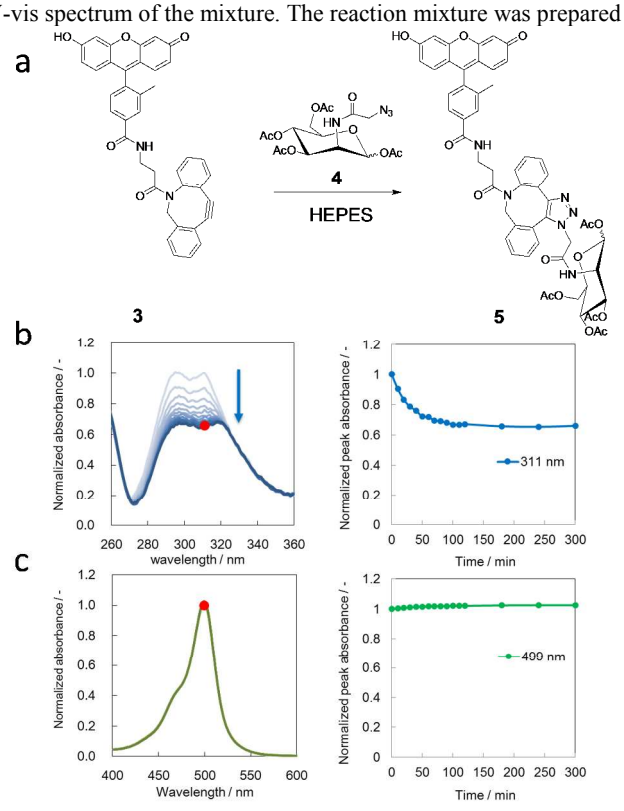

Fig. 2 *In vitro* evaluation of FC-DBCO; Cu-free click reaction between FC-DBCO 3 (final concentration: 10 μM) and azide-modified mannose (Ac_4ManNAz 4) (final concentration: 40 μM) in HEPES buffer **(a)**, and the change in UV-vis absorbance at and 311 nm **(b)** and 499 nm **(c)** during the reaction. The red dots are values for 24 hour- reaction in DMSO.

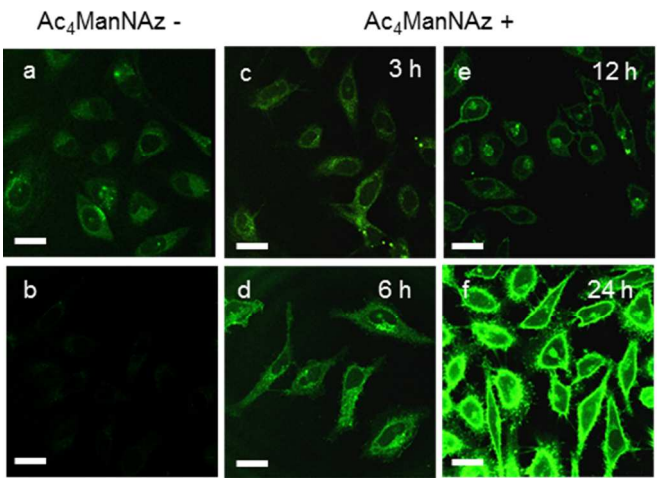

Fig. 3 Confocal images of HeLa cells stained with FC-DBCO in the absence/presence of target azide-mannose. HeLa cells were incubated with FC-DBCO-containing medium (final concentration: 10 μM) for 2 hours and images were then obtained after replacement of the medium **(a)** and after 30 min incubation with new medium **(b)**. HeLa cells were incubated with Ac_4ManNAz-containing medium (final concentration: 40 μM) for 3 **(c)**, 6 **(d)**, 12 **(e)** or 24 hours **(f)** and subsequently labeled with FC-DBCO. Images were obtained after incubation with fresh medium (30 min). The scale bar is 30 μm.

as shown in Table S1. The mixture was kept at 37˚C and UV-vis absorbance was measured every 10 min (from 0 to 120 min) or 60 min (from 120 to 300 min). The peak absorbance at 311 nm gradually decreased as the reaction between **3** and **4** proceeded (**Fig. 2b**) due to the structural change of a cyclooctyne of **3** into a triazole. The absorbance at 311 nm plateaued after 2 hours, but did not reach zero because the triazole product **5** retained some absorbance.

For the characterization of **5**, it was synthesized from **3** and **4** in DMSO solution (final concentration: 1 mM and 2 mM, respectively) with Cu-free click reaction. After 24 hours reaction, the triazole compound **5** was detected by LC/high resolution ESI-MS (LC/HR-ESI-MS) (see ESI†) with concomitant disappearance of the peak corresponding to starting compound **3**. The normalized absorbance at 311 nm and 499 nm of the reaction mixture was plotted in **Fig. 2b** and **c**. The excitation and emission spectra of the reaction mixture was almost the same as those of unreacted FC-DBCO (see ESI†). The results indicate that the Cu-free click reaction between **3** and **4** appeared to be completed within 2 hours and the reaction did not interfere with the fluorescence property of FC-DBCO.

In order to evaluate the cell-membrane permeability of FC-DBCO, we first introduced this compound into HeLa cells in the absence of azide target molecules. We found that the probe spread throughout the cytosol (**Fig. 3a**) within 2 hours of addition. After 30 min incubation in fresh medium, the probe was almost completely removed from the cells with very few fluorescence spots remaining (**Fig. 3b**). These results indicate that FC-DBCO possesses good cell-membrane permeability. Furthermore, unbound probe is easily removed from the cells by a simple process (incubation with fresh medium for 30 min), leading to very low background fluorescence, thereby facilitating specific target imaging in the living cells.

Conjugation of FC-DBCO against azide-modified small molecules in living HeLa cells was evaluated using a cell-permeable azide-modified mannose (Ac_4ManNAz). In order to obtain intracellular images of azide-mannose and azide-glycoproteins, cells were incubated with Ac_4ManNAz for different periods of time (3, 6, 12 or 24 hours) and then labeled by FC-DBCO (loading time: 2 hours). In the early incubation period (3 and 6 hours) fluorescence signals were only detected in the cytosolic space of the cell (**Fig. 3c, d**). FC-DBCO-conjugated ManNAz (four acetyl groups were

removed by cytosolic esterases) was detected by LC/HR-ESI-MS (see ESI†) from the cell extract. This observation suggested that acetylated azide-mannose was firstly taken up into the cytosol before incorporation into glycoproteins, which is performed in the endoplasmic reticulum (ER) and/or Golgi apparatus.[24] Fluorescence signals were observed on the membrane of some cells after 12 hours (**Fig. 3e**) and most of cells after 24 hours (**Fig. 3f**), suggesting the glycoproteins were transferred into the cell membrane during this period. These images clearly indicate that FC-DBCO were conjugated to azide-mannose and azide-sialic acid contained mannoglycoproteins inside of the living cell as well as on the cell membrane by the Cu-free click reaction.

Finally, we investigated the localization of the target azide-molecules in the living cell at an organelle level. Ac_4ManNAz-incorporated cells were co-stained with FC-DBCO and one of four different organelle trackers; ER, lysosome, Golgi apparatus, and mitochondria at 6 or 12 hours incubation after the introduction of Ac_4ManNAz. Fluorescence signals of FC-DBCO primarily merged with those of ER-tracker and partly with Lyso-tracker after 6 hours incubation. We did not find any merged signals between FC-DBCO and Golgi-tracker or Mito-tracker (**Fig. 4a**). These observations suggested that fluorescence-labeled azide mannose was localized in the cytosol just after cell membrane permeation, ER during incorporation into glycoprotein and then lysosome-like acid vesicles during transportation from the ER to the Golgi apparatus. These observations also suggested that 6 hours incubation was insufficient time for azide mannose to reach the Golgi apparatus. The fluorescence signals of Mito-tracker were entirely independent from those of FC-DBCO, indicating that unbound free probe was totally removed by the washing procedure. After 12 hours incubation, fluorescence signals of not only ER- and Lyso- but also Golgi-tracker merged with those of FC-DBCO (**Fig. 4b**). Merged signals were mainly observed between FC-DBCO and Lyso-tracker. This observation suggests that azide-modified glycoproteins were gradually transported from the ER to the Golgi apparatus and from the Golgi to the cell membrane *via* lysosome-like acid vesicles. The results also suggested that it took 12 hours for some azide-mannose and/or azide-mannoglycoproteins to be transported to the Golgi apparatus and cell membrane. These observations clearly imply that FC-DBCO was conjugated with azide-mannose and/or azide-mannoglycoproteins localized in particular organelles at each transportation stage by an intracellular Cu-free click reaction and that the unbound probes were completely washed away from the cells.

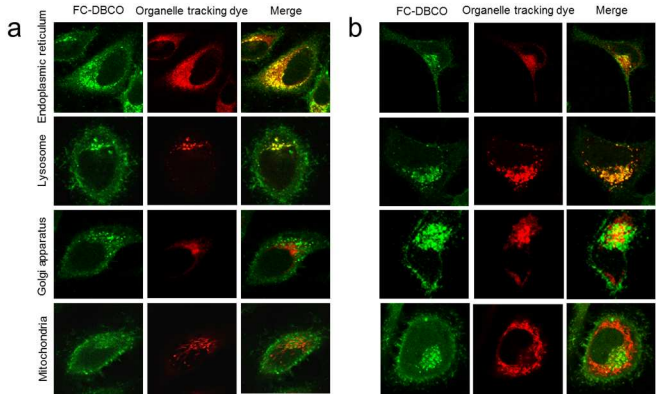

Fig. 4 Investigation of the localization of azide-mannose and azide-mannoglycoproteins in living HeLa cells. Confocal images of azide-mannose-introduced HeLa cells stained with FC-DBCO (left), each organelle tracker (middle) and merged images (right) are shown. Localization of azide-mannose after 6 **(a)** and 12 hours **(b)** incubation was observed using FC-DBCO (incubated for 2 hours) and organelle specific tracking dyes (0.5-1.0 hour incubation) after the wash process (30 min). Endoplasmic reticulum, lysosome (and lysosome-like vesicles), Golgi apparatus, and mitochondria (from top to bottom) were co-stained with FC-DBCO.

In conclusion, we synthesized a novel cyclooctyne-based Cu-free clickable fluorescent probe, FC-DBCO, and achieved the intracellular specific imaging of azide-modified biomolecules in living cells. FC-DBCO displayed good cell-membrane permeability and any unbound probe was efficiently removed from the cells by a simple washing step, leading to very low background fluorescence signals. In addition, the fluorescence properties of our probe were similar to those of fluorescein. As such, the probe is highly versatile for co-staining with other fluorescent dyes such as commercially available organelle specific trackers. In order to demonstrate the application of our probe, the intracellular dynamics of a commercially available azide mannose was visualized using FC-DBCO with several organelle trackers. FC-DBCO specifically labeled azide-mannose and/or azide-mannoglycoproteins at each transportation stage in the living HeLa cells. We believe FC-DBCO will be an invaluable tool not only for metabolism imaging in living cells by using a variety of azide-modified bioactive small molecules such as lipids, nucleic acids and other metabolites, but also monitoring intracellular dynamics of drugs.

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