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Cell Type-Selective Imaging and Profiling of Newly Synthesized Proteomes by Using Puromycin Analogues

Shubo Du,[a] Danyang Wang,[a] Jun-Seok Lee,[b] Bo Peng,[a] Jingyan Ge[c]* and Shao Q. Yao[a]*

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We have developed a versatile antibody-assisted strategy for imaging and profiling of newly synthesized proteomes in a cell-specific manner. This strategy remained highly selective even in heterogeneous co-cultured cells, thus enabling labeling and enrichment of nascent proteomes from targeted cells without the need for physical separation.

Global proteome analyses have provided important insights into diverse biological processes. Metabolic labelling of proteins with unnatural amino acids permits the analysis of the spatiotemporal regulation of protein synthesis.[1] However, metabolic incorporation strategies are generally nonspecific with respect to cell identity, rendering them of limited use in heterogeneous cellular systems.[2-5] Differentiating the various proteomes of individual cell subpopulations from co-cultures is highly important in cell-cell communications, e.g. host-pathogen interaction and intercellular signal transduction.[2,6] Therefore it is highly desirable that cell-selective methods are developed to resolve proteome changes with sufficient spatiotemporal resolutions. However, the study of cell-specific translational regulation *in vivo* is challenging, mainly due to the difficulty to ascertain the origin of the identified proteins in multi-cell cultures, especially for low-abundance cell populations.[7] Two major strategies have been exploited to address this issue. The first is the use of engineered aminoacyl-*t*RNA synthetases that specifically recognize and incorporate noncanonical amino acids into transgenic cells.[8-10] The need for genetic modifications, however, renders it to have limited applications in tissues or whole organisms in their native state. The second strategy involves the selective activation of a caged protein tag (e.g. puromycin) by removing the blocking group through a cell-specific enzyme.[7,11] Puromycin (**PO**) is an aminonucleoside antibiotic which could be used as a tag for synthesis of nascent polypeptides.[12] Recently, multiple **PO** analogues were developed to achieve multiplexed imaging and profiling of protein synthesis in live cells and neurons.[13] Since **PO** can function as a protein tag at a much lower concentration than noncanonical amino acids,[7,12,14] low-abundance enzymes might be employed as deblocking triggers. Moreover, **PO** provides a better temporal resolution than that of noncanonical amino acids.[12,15] In order to achieve cell-selective incorporation of **PO** into nascent polypeptides, a chemical genetic method was developed that involves an engineered enzyme to activate a clickable **PO** analogue.[7] However the deblocking enzyme was not endogenous, and the strategy was restricted to one specific enzyme. It is often the case that decaging triggers and blocking groups must be optimized for each new target cell population in order to maximize the **PO** release. Given the caveats of each of these methods, there remains a critical need to develop a more versatile and robust **PO**-based strategy to label newly synthesized proteomes from specific cells at a specific time.

Inspired by the success of antibody-drug conjugates (ADC), we turned to antibodies as potential decaging triggers in our cell-selective proteome labelling strategy (**Figure 1**).[16] In order to achieve the release of **PO** needed to tag nascent polypeptides, we took advantage of the inverse-electron-demand Diels–Alder (inv-DA) reaction, a "click-to-release" approach that affords instantaneous payload release upon conjugation between *trans*-cyclooctene (**TCO**) and tetrazine (**Tz**).[17-19] Instead of directly linking **PO** to the antibody, we modified the antibody with **Tz** to achieve easy antibody modification, maximize the number of covalent binding sites and minimize interference to the antigen-antibody interaction. **PO** was introduced as **TCO-PO** in the second step. The **TCO** group effectively blocks the free amino group on **PO**, rendering

[a] S. Du, D. Wang, B. Peng, Prof. Dr. S. Q. Yao
Department of Chemistry, National University of Singapore.
3 Science Drive 3, Singapore 117543 (Singapore)
E-mail: chmyaosq@nus.edu.sg
[b] Prof. Dr. J.-S. Lee
Molecular Recognition Research Center & Department of Biological Chemistry, KIST-School Korea Institute of Science & Technology (South Korea)
[c] Dr. J. Ge
Key Laboratory of Bioorganic Synthesis of Zhejiang Province, College of Biotechnology and Bioengineering, Zhejiang University of Technology (China).
E-mail: gejy@zjut.edu.cn

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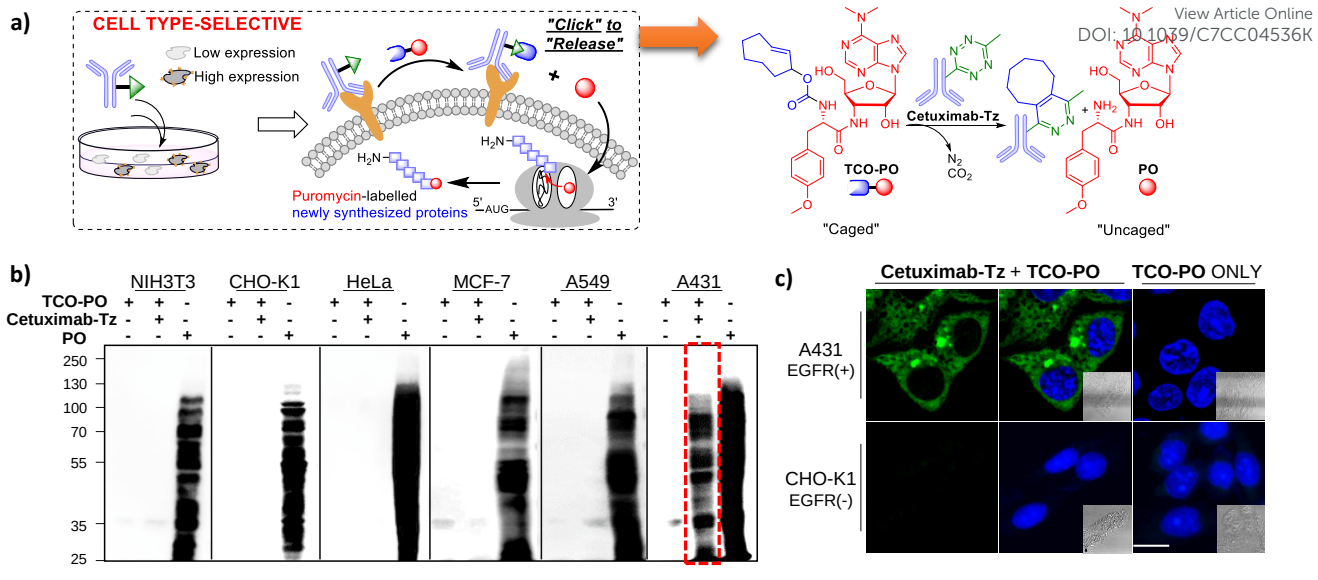

Figure 1. a) Antibody-assisted cell type-specific delivery and release of **PO** with "click-to-release" chemistry in a heterogeneous environment. b) Selective labelling of nascent polypeptides in EGFR-overexpressing A431 cells against a panel of other cell lines with no/low endogenous EGFR expression. Synthesis of nascent proteomes was detected by Western blotting (WB) using *anti*-puromycin antibody. EGFR expression was assessed by WB analysis with *anti*-EGFR antibody, and the total proteome was visualized by Ponceau S stain (**Figure S4**). c) A431 and CHO-K1 cells were sequentially treated with **Cetuximab-Tz** and **TCO-PO**. Labelled proteins were detected by immunofluorescence (IF; green channel) with *anti*-puromycin. Fluorescence microscopy revealed labelled proteins in A431 cells, whereas proteins were not labelled in the absence of **Cetuximab-Tz** or in CHO-K1 cells. (blue): DAPI. Scale bar = 15 μm.

it unable to be incorporated in protein synthesis.

As shown in **Figure 1a**, the **Tz**-loaded antibody (i.e. **Cetuximab-Tz**) binds to a tumour cell-specific membrane receptor. Subsequently **TCO-PO** is introduced. Upon ligation between **TCO** and tetrazine, the resulting adduct undergoes conversion into a conjugated pyridazine with the concomitant elimination of CO_2 and release of free **PO** (**Figure 1a** and **Figure S3**). Labelled proteins can be distinguished from the rest of the protein pool through either immunological staining or bioorthogonal conjugation that permits facile visualization and isolation of proteins in a cell specific manner (see **Figures 2** & **3**).

As an example to demonstrate the feasibility of our strategy, we chose to selectively label the newly synthesized proteomes in A431 cancer cells, which are known to endogenously overexpress epidermal growth factor receptor (EGFR).[20] The monoclonal antibody **Cetuximab**, which is used clinically for cancer treatment, was used to target EGFR.[21] The antibody was modified with **Tz-NHS** according to literature with minor modifications.[22] **Tz** modifications had little effect on binding affinity and did not affect the level of non-specific binding to control CHO-K1 cells (**Figure S2**). (*E*)-cyclooct-2-enol was synthesized according to published procedures[16] and subsequently linked to the α-amino group on **PO** through carbamate formation. The reaction between **Tz** and **TCO-PO** and subsequent release of free **PO** was confirmed by LC-MS analysis (**Figure S3**). The hydrolytic stabiltiy and cell permeability of **TCO-PO** was also confirmed (**Figure S4 & 5**).

To determine whether **Cetuximab-Tz** can remove the **TCO** blocking group on **TCO-PO** in EGFR-overexpressing A431 cells, we monitored the incorporation of **PO** into nascent polypeptides by using Western blotting (WB) with *anti*-puromycin antibody. Treatment of A431 cells with **Cetuximab-Tz** (10 μg/mL) followed by **TCO-PO** (3 μM) for 24 h resulted in extensive **PO** labelling across a spectrum of molecular weights (boxed lane 2 in **Figure 1b**); in the absence of **Cetuximab-Tz**, cells were essentially devoid of **PO** incorporation (lane 1). Importantly, no labelling was observed in similarly treated cells known to have no/low EGFR expression (i.e. NIH3T3/CHO-K1/HeLa/MCF-7/A549; **Figure 1b and Figure S6**). In contrast, robust **PO** incorporation was observed across the entire panel of cells directly treated with **PO** (lanes 3 in **Figure 1b**). We further observed a concentration-dependent increase in **PO** incorporation from A431 cells with an increasing amount of **TCO-PO** (**Figure S7**). Under these conditions, protein labelling was equivalent to a low dose of **PO** incorporation previously shown to cause little or no disruption to synthesis of newly synthesized proteomes.[13,23] XTT cell viability assay with (**Cetuximab-Tz** + **TCO-PO**)-treated A431 cells showed no obvious difference in cell vitality when compared with cells treated with **Cetuximab-Tz** or **TCO-PO** individually, indicating that **PO** treatment under our conditions did not have significant cell toxicity (**Figure S8**). A431 cells treated with increasing doses of **Cetuximab-Tz** for 30 min showed a significant increase in **PO** incorporation, and the labelling can be partially blocked by pre-incubation with unmodified **Cetuximab** (**Figure S9**), indicating that **PO** release was dependent on the endogenous EGFR expression level. To further demonstrate the deblocking of **TCO-PO** occurred only in cells expressing a high level of EGFR, we monitored **PO** incorporation into nascent polypeptides in single cells by using IF with *anti*-puromycin antibody (**Figure 1c**). In these experiments, the cells were similarly incubated with **Cetuximab-Tz** followed by **TCO-PO**. Strong fluorescence signals were detected in A431 cells, with a uniform distribution in the cytoplasm. These results are similar to those in cells directly treated with **PO** (**Figure S10**). In contrast, no fluorescence was observed when **Cetuximab-Tz** was omitted, or with CHO-K1 cells. Taken together, our results unequivocally demonstrate

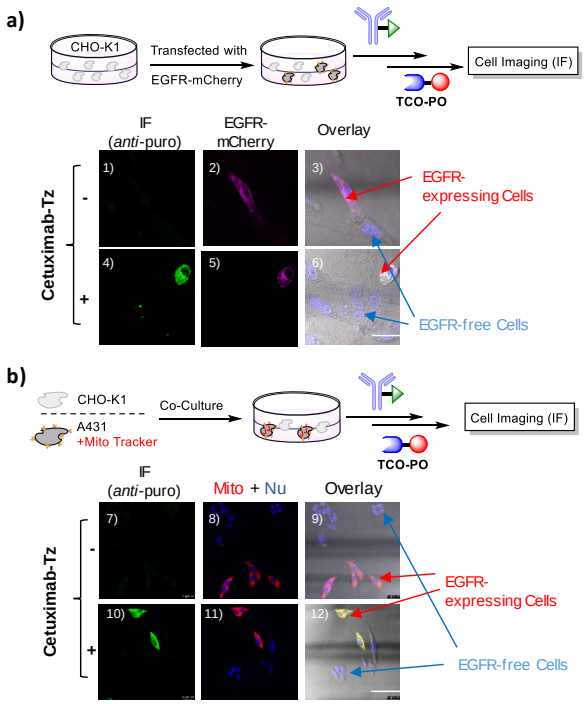

Figure 2. Selective labelling of nascent polypeptides in heterogeneous environments, in (a) CHO-K1 cells transfected with EGFR-mCherry, and (b) A431/CHO-K1 co-cultures. Cells were treated with **Cetuximab-Tz** (10 μg/mL, where applicable) for 30 min then **TCO-PO** (3 μM) for 24 h. Cells were fixed with cold methanol and IF by using *anti*-puromycin antibody. Total cells were detected by DAPI (blue). Scale bar = 25 μm.

that, in EGFR-expressing mammalian cells, **Cetuximab-Tz** binds to the cell surface-bound EGFR and removes **TCO** group on **TCO-PO** to generate free **PO**, which can be subsequently incorporated into nascent polypeptides during ribosomal protein synthesis. Importantly, this sequential incubation with **Cetuximab-Tz** and **TCO-PO** is inert to cells that have no or low endogenous EGFR expression levels.

In principle, in a heterogeneous cell environment, the unmasked **PO** from this antibody-assisted strategy might diffuse extracellularly to nearby cells lacking the target protein (e.g. EGFR), thus potentially confounding cell-specific labelling. To determine whether this was the case, we carried out **PO** labelling in heterogeneous cell cultures with two different model systems (**Figure 2**): 1) CHO-K1 cells transfected with EGFR-mCherry; and 2) co-culture of A431 and CHO-K1 cells. As shown in **Figure 2a**, transient transfection of CHO-K1 cells with an EGFR-mCherry plasmid led to the creation of a mixed culture of cells with those EGFR-expressing cells indicated by the mCherry fluorescence. Since the mCherry protein was fused to the *C*-terminus of EGFR, it did not hinder the antibody binding site (**Figure S11**); **Cetuximab-Tz** was found to selectively bind to the surface of transfected CHO-K1 cells with fluorescent signals colocalized to those from mCherry. Sequential treatments with **Cetuximab-Tz** and **TCO-PO** followed by IF resulted in the labelling of nascent polypeptides only in successfully transfected cells (**Figure 2a**; arrowed in red). Importantly, no labelling of nascent polypeptides was detected in cells not transfected with EGFR (arrowed in blue). We next investigated whether the above conclusion also holds true for co-culture of A431 and CHO-K1 cells. A431 cells were pre-stained with Mitotracker before being mixed with an equal number of unstained CHO-K1 cells. This resulting co-culture of A431 and CHO-K1 cells were further seeded in glass-bottom dishes, and cell labelling and IF imaging experiments were finally carried out (**Figure 2b**); to our delight, the antibody binding and **PO** incorporation were both restricted to EGFR-expressing A431 cells (arrowed in red), and no labelling was observed in the co-cultured CHO-K1 cells (arrowed in blue). The high cell type-specificity could be due to two reasons. The EGFR-Cetuximab complex was constantly internalized into the cells (**Figure S2c**), therefore the decaging process partially occurred intracellularly. Moreover, even when the decaging occurred on the cell surface, the decaged **PO** can be rapidly taken up by cells. Due to the relatively low expression of cell-surface protein receptors, the decaged **PO** was effectively maintained at a low concentration, thus limiting its diffusion to the medium and neighbouring cells.

Having demonstrated the successful labeling of nascent polypeptides with **PO** exclusively in EGFR-expressing cells, we next sought to identify newly synthesized proteomes from target cells by using our strategy. We first synthesized an alkyne-bearing **PO** analogue, **TCO-AY** (**Figures 3a**,**b** & **S12**); the release and incorporation of **TCO-AY** was confirmed by gel-based profiling and imaging experiments with **Cetuximab-Tz**-treated A431 and in transfected CHO-K1 cells. We next combined this antibody-assisted proteome labelling strategy with stable-isotope labelling by amino acids in cell culture (SILAC) and liquid chromatography–tandem mass spectrometry (LC-MS/MS). As shown in **Figure 3c**, isotopically "Light" cells served as a static control and were treated with **TCO-AY** only. Isotopically "Heavy" cells served as comparison groups and were treated with both **Cetuximab-Tz** and **TCO-AY**. The experiments were termed as "Forward". To account for differences in protein abundance between two isotopically labelled cells, we furthermore carried out "Reverse" experiments in which the probe treatment was reversed. The SILAC experiments were performed in both A431 and CHO-K1 cell lines. After 24-h incubation, CuAAC conjugation with TMR-biotin-azide was performed in cell lysates, and biotinylated proteins were enriched using NeutrAvidin beads.[24] Eluted proteins were SDS-PAGE-separated, followed by in-gel digestion and finally LC-MS/MS. Upon data analysis, proteins with a SILAC Heavy/Light ratio of ≥ 3 in Forward experiments or ≤ 0.3 in Reverse experiments were identified as hits. As shown in **Figure 3d**, we were able to identify significantly more proteins in (**Cetuximab-Tz** + **TCO-AY**)-treated A431 cells than in similarly treated CHO-K1 cells. Taking into consideration of the non-specific background labeling and intrinsic variability of large-scale proteomic profiling experiments, the significant difference in the numbers of quantified proteins identified from these two cell lines indicates that our newly developed strategy is indeed applicable for selective labelling and enrichment of newly synthesized proteomes in cell-specific manner. The high-confidence hits identified from labelled A431 cells were found to be distributed across various cellular components (**Figure 3e**), which is in accordance with our previous profiling and imaging results.[13]

In conclusion, we have developed an antibody-assisted strategy for cell type-specific labelling of newly synthesized proteomes. Compared to existing strategies for cell-specific proteomic studies, our approach provides the following advantages: (1) this strategy is highly versatile and can be built upon the vast array of available monoclonal antibodies that

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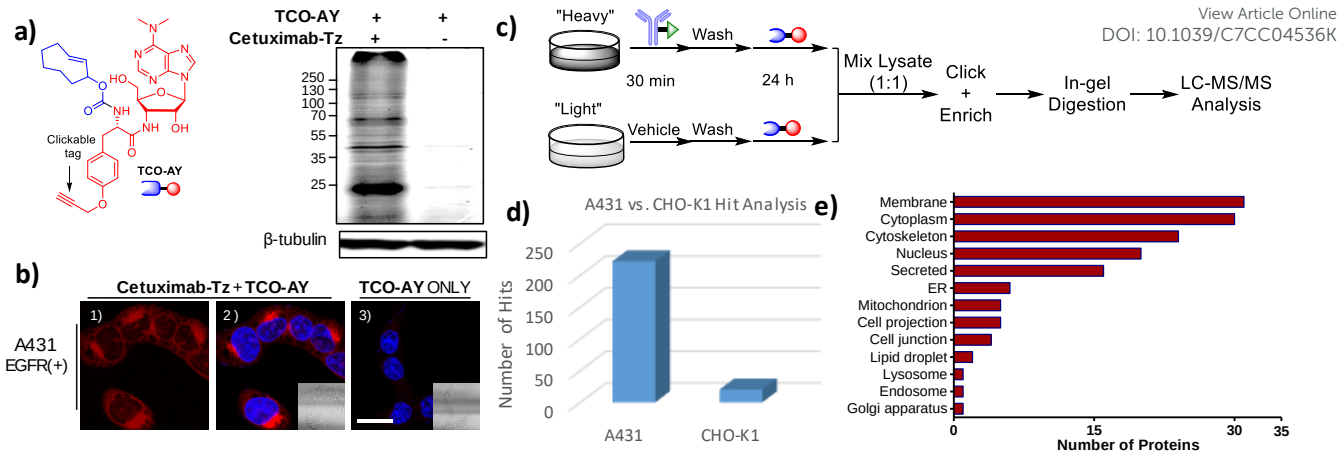

Figure 3. Antibody-assisted incorporation of **AY** for mapping newly synthesized proteomes from A431 cells. a) **TCO-AY** structure. (right) Incorporation of **TCO-AY** in nascent polypeptides. Cells were treated with **Cetuximab-Tz** (10 μg/mL, where applicable) for 30 min then **TCO-AY** (3 μM) for 24 h. Labelled proteins were "click" with **TER-azide**, followed by SDS-PAGE and in-gel fluorescence scanning.[13] β-tubulin was used as the loading control. b) Confocal images of A431 cells treated with **Cetuximab-Tz** and **TCO-AY**. Labelled proteins were visualized by click chemistry with **TER-azide** (red). (blue): DAPI. Scale bar = 15 μm. c) Scheme showing **TCO-AY** incorporation in newly synthesized proteomes by the antibody-assisted strategy to identify labelled proteins by quantitative MS-based proteomics (SILAC). d) Graphical comparison of high-confidence proteins enriched in treated A431 and CHO-K1 cells. More than 1200 proteins were identified from A431 cells, of which 222 were considered high-confidence hits (plotted). e) Subcellular distribution analysis of high-confidence proteins identified from treated A431 cells.

require minimal optimization; (2) the system does not require genetic modification and Is thus more suitable for tissue and organism studies; (3) in future, other similarly "caged" metabolites in addition to puromycin may be delivered to targeted cells, to achieve cell type-specific labelling of other biomolecules.

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