Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

Selective Analysis of Newly Synthesized Proteins by Combining Fluorescence Correlation Spectroscopy with Bioorthogonal Noncanonical Amino Acid Tagging

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Cell Culture. Triple negative breast cancer cells MDA-MB-468 (Cellbio, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose medium (Gibco, USA) with 10% (v/v) fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin sulphate (Life Technologies, USA) at 37 ℃ with 5% CO_2 gas atmosphere in a humidified incubator.

Incorporation of HPG. In a typical experiment, MDA-MB -468 cells are cultured in the complete medium (DMEM supplemented with 10% FBS, 1% penicillin / streptomycin) until the cells have reached about 70% confluency, then washed with PBS and replaced with serum-free medium (DMEM with 1% penicillin/streptomycin sulphate, no methionine, no FBS) for 30 min, to deplete the natural methionine stores.[1] Then the cells were cultured with a certain concentration~~s~~ of HPG in 2 mL conditional medium (DMEM containing 10% dialyzed FBS, 1% penicillin/streptomycin, Met-free, no Phenol Red) for a predefined time window. After that, the cells were washed with cold PBS three times and cultured further for 15 min in conditional medium to remove the intracellular free HPG, then the cells were counted by staining with trypan blue, lysed using RIPA buffer according to the product instructions: 1mL RIPA buffer was used for every 5×10^6 cells. Next, the whole cell lysate, containing HPG-labeled proteins, were tagged with freshly prepared click cocktail (50 μM TAMRA azide, 100 μM reduction, 10 μM ligand and 100 μM CuSO4) at 37 ℃ for 2 h. Subsequently, the mixed samples were analyzed by certain analytical techniques such as gel electrophoresis or our home-built fluorescence correlation spectroscopy (FCS) setup.

Gel Electrophoresis. The slab electrophoresis system (Bio-Rad, USA) was used to analyze newly synthesized proteins. The same amount of lysate samples tagged with click cocktail were resuspended in 1 × standard reducing SDS-loading buffer, then heated for 10 min at 95 ℃. Finally, the samples were loaded onto 10% SDS-PAGE gel, electrophoresis was carried out at 80 V for 30 min, then 120 V for 110 min. The gels were firstly scanned by BioRad imaging system, and then stained with coomassie Blue as loading control. The fluorescence intensity was analyzed with Image Lab software.

Extraction of Secreted Proteins. The serum-free conditional medium was firstly collected carefully, centrifuged at 1000 × g for 5 min, 10000 × g for another 10 min at 4 ℃ to pellet smaller debris and vesicles, respectively.[2] Finally, the supernatant was filtered through a 0.22 μm filter unit, and then used directly for next step.

Western Blot. The cells were harvested and lysed using RIPA Buffer with protease/phosphatase inhibitors. Both The total protein and the culture supernatants levels were quantified by bicinchoninic acid (BCA) assay. Equal amounts of proteins were separated by 10% SDS-PAGE, and then transferred to

PVDF membrane. The membrane was blocked with blocking buffer for 1h, incubated overnight with β-tubulin antibodies at 4 ℃, washed three times with PBST buffer, and then incubated with secondary antibodies labeled with Alexa Fluor 680 for 1 h at room temperature. The proteins were visualized using the Odyssey infrared imaging system.

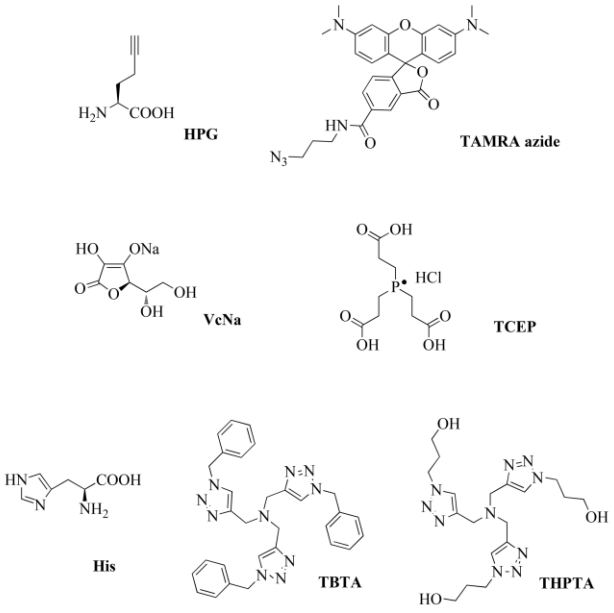

Figure S1. Structures for CuAAC reaction used in this study.

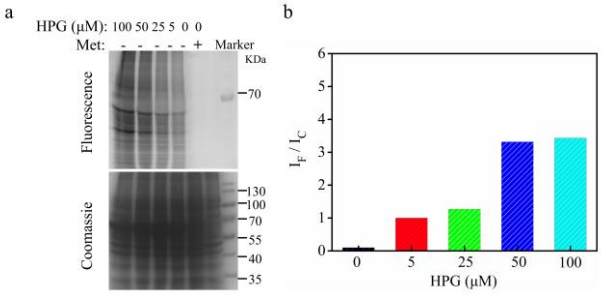

Figure S2. Optimization of HPG incorporation concentration. The cells were starved with SFM (serum-free and methionine-free medium) to deplete the intracellular methionine, and then an increase concentration of HPG up to 100 μM in 2 mL medium was added for a predefined time 8 h. Next, the cells were washed, counted, lysed and labeled with freshly prepared click cocktails, and the following was analyzed by examining the fluorescence intensity using SDS-PAGE technology. (a) Gel fluorescence scanning (above), the coomassie staining was used to show the equal loading of the protein samples (below). The fluorescence gels showed the newly synthesized proteins only, while the coomassie gels showed total proteins (including old and new). (b) Y-axis: The ratio of fluorescence intensity (I_F) to coomassie brilliant blue intensity (I_C) in each lane.

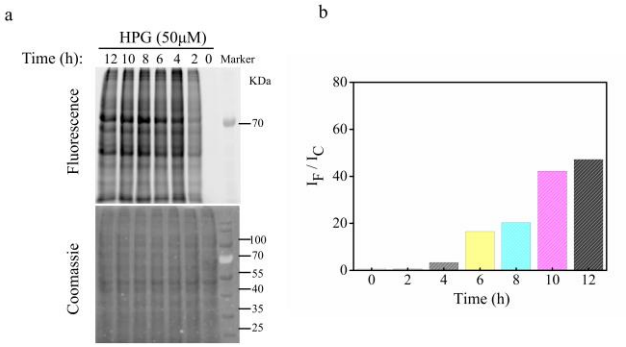

Figure S3. Optimization of HPG incorporation time. The cells were cultured in an optimized concentration of HPG and harvested at desired time points up to 12 h, then the following processes were the same as above. a) Gel fluorescence scanning (above), the coomassie staining (below). The fluorescence gels showed the newly synthesized proteins only, while the coomassie gels showed total proteins (including old and new). b) Y-axis: The ratio of fluorescence intensity (I_F) to coomassie brilliant blue intensity (I_C) in each lane.

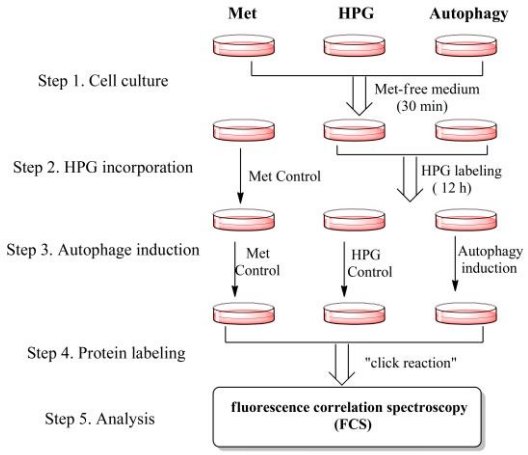

Figure S4. Workflow of autophagy protein degradation. Autophagy as the experimental group, Met as the negative control and HPG as the positive control.

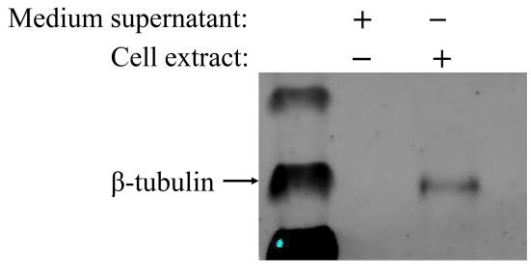

Figure S5. Verification of purity of secreted proteins. Proteins of the cell extracts and culture supernatants were analyzed with β-tubulin by western blot.

References

1. K. E. Beatty and D. A. Tirrell, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 5995-5999.
2. H. Xue, B. Lu and M. Lai, *J. Transl. Med.*, 2008, **6**, 52.