Supplementary Information

Temporal control of protein labeling by photo-caged benzaldehyde motif and discovery of host cell factors of avian influenza virus infection

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1. Synthesis and characterization of compounds

General: All reagents used in chemical synthesis and biological work were procured from obtained commercially.

1.1 Chemical Synthesis of Suc-NPE

2-nitrophenyl)-1,3-propanediol (200 mg, 0.81 mmol) (following previously reported procedure: Tetrahedron Lett., 2020, 61,151709 ), aldehyde (319 mg, 1.62 mmol) (following previously reported procedure: Nat. Mater., 2015, 14, 523–531), BF$_3$·OEt$_2$ (100 µL, 0.81 mmol) and MgSO$_4$ in 6 mL of MC were stirred at −15 °C for 90 min, then saturated NaHCO$_3$ solution (20 mL) was added rapidly, extracted with dichloromethane (3×10 mL). The organic layer was dried with magnesium sulfate and then condensed. The product was purified by flash chromatography. (Yield = 54%).

$^1$H NMR spectroscopy: (CDCl$_3$, 400 MHz): 8.16 (d, $^3$J$_{H,H}$ = 8 Hz, 2H), 7.84-7.81 (m, 1H), 7.68 (d, $^3$J$_{H,H}$ = 8 Hz, 2H), 7.61-7.58 (m, 1H), 7.46-7.42 (m, 2H), 5.67 (s, 1H), 4.47-4.43 (m, 2H), 4.10 (t, $^3$J$_{H,H}$ = 12 Hz, 2H), 3.89-3.82 (m, 1H), 2.91 (Br, 4H). $^{13}$C NMR spectroscopy: 169.41, 161.67, 149.36, 144.86, 137.19, 133.15, 131.12, 130.59, 127.68, 126.73, 125.57, 126.73, 125.57, 124.49, 100.43, 70.79, 33.70, 25.71. ESI-MS: [M+H]$^+$ 427.1141 (cal.), 427.1149 (exp.).

1.2 Chemical synthesis of Cou-NPE

Suc-NPE (100 mg, 0.33 mmol) was suspended in DMF (3 mL) and heated until all solids were dissolved. Cou-amine (141 mg, 0.33 mmol) (following previously reported procedure: Eur. J. Med. Chem., 2019, 170 (2019) 45-54) was added, followed by TEA (238 µL, 1.65 mmol) and the reaction was stirred at room
temperature for 2 hours. Diluted with ethyl acetate and washed 5 times with water. Solvent was removed and the crude product was purified by column chromatography. (Yield = 75%)

$^1$H NMR spectroscopy: (CDCl$_3$, 400 MHz): 9.22-9.21 (m, 1H), 8.68 (s, 1H), 8.43 (d, $^3$J$_{(H,H)}$ = 8 Hz, 1H), 7.91-7.85 (m, 4H), 7.63-7.58 (m, 3H), 7.43-7.38 (m, 2H), 6.64 (d, $^3$J$_{(H,H)}$ = 8 Hz, 1H), 6.48 (s, 1H), 5.68 (s, 1H), 4.49-4.41 (m, 4H), 4.37-3.66 (m, 4H), 3.45 (q, $^3$J$_{(H,H)}$ = 8 Hz, 4H), 3.32 (s, 1H), 1.23 (t, $^3$J$_{(H,H)}$ = 8 Hz, 6H)

$^{13}$C NMR spectroscopy: 171.17, 167.16, 165.43, 162.68, 157.77, 152.83, 149.39, 148.33, 141.01, 137.31, 134.96, 133.15, 131.30, 131.27, 127.57, 127.26, 126.26, 124.40, 110.12, 109.43, 108.30, 101.23, 95.56, 70.80, 60.40, 53.47, 45.12, 42.52, 39.18, 33.74, 29.69, 22.69, 21.04, 14.20, 12.42. ESI-MS: [M+H]$^+$ 615.2455 (cal.), 615.2546 (exp.).

1.3 Chemical synthesis of BD-NPE

Suc-NPE (100 mg, 0.33 mmol) was suspended in DMF (3 mL) and heated until all solids were dissolved. BD-amine (135 mg, 0.33 mmol) (was prepared by previously reported procedure: Anal. Biochem., 2008, 378, 166–170), and was added followed by TEA (238 µL, 1.65 mmol) and the reaction was stirred at room temperature for 2 hours. Diluted with ethyl acetate and washed 5 times with water. Solvent was removed and the crude product was purified by column chromatography. (Yield = 65%)

$^1$H NMR spectroscopy: (CDCl$_3$, 400 MHz): 8.38 (d, $^3$J$_{(H,H)}$ = 8 Hz, 4Hz, 1H), 7.97 (d, $^3$J$_{(H,H)}$ = 8 Hz, 2H), 7.89-7.85 (m, 3H), 7.67 (Br, 1H) 7.60-7.55 (m, 3H), 7.42-7.34 (m, 4H), 7.30, 5.96 (s, 2H), 5.68 (s, 1H), 4.48-4.40 (m, 4H ) 3.73 (Br, 4H), 3.32 (S, 1H), 2.54 (s, 6H), 1.31 (s, 6H). $^{13}$C NMR spectroscopy:172.01, 167.53, 155.95, 142.95, 141.78, 138.29, 137.21, 235.38, 134.20, 133.08, 131.11, 128.54, 128.05, 127.66,
1.4 Chemical synthesis of TCO-NPE

Suc-NPE (50 mg, 0.17 mmol) was suspended in DMF (3 mL) and heated until all solids were dissolved. TCO-diamine (44 mg, 0.17 mmol) was added, followed by TEA (119 µL, 0.83 mmol) and the reaction was stirred at room temperature for 2 hours. Diluted with ethyl acetate and washed 5 times with water. Solvent was removed and the crude product was purified by column chromatography. (Yield = 69%)

$^1$H NMR spectroscopy: (CDCl$_3$, 400 MHz): 8.34 (d, $^3$J$_{(H,H)} = 8$ Hz, 1H), 7.80 (t, $^3$J$_{(H,H)} = 8$ Hz, 3H), 7.53 (t, $^3$J$_{(H,H)} = 8$ Hz, 3H), 7.34 (t, $^3$J$_{(H,H)} = 8$ Hz, 1H), 5.62 (s, 1H), 5.43-5.49 (m, 2H), 4.34-4.42 (m, 4H), 4.28 (m, 1H), 3.39-3.44 (m, 2H), 3.25 (s, 1H), 3.18 (m, 3H), 2.23-2.30 (m, 3H), 1.83-1.97 (m, 4H), 1.60-1.68 (m, 4H), 1.43-1.53 (m, 2H), 1.13-1.21 (m, 2H), 0.76-0.79 (m, 1H). $^{13}$C NMR spectroscopy: 167.22, 157.22, 149.42, 141.25, 137.29, 135.06, 134.96, 133.10, 132.96, 131.22, 127.61, 127.10, 126.34, 124.45, 101.12, 80.82, 70.83, 67.09, 60.40, 41.13, 38.69, 37.45, 36.16, 34.28, 33.75, 32.53, 30.97, 30.17, 21.05, 14.20. ESI-MS: [M+H]$^+$ 538.2553 (cal.), 538.2564 (exp.).
2. General procedure for light decaging and peptide labeling using Cou-NPE.

The probes were added to 10% acetonitrile-supplemented PBS to a final concentration of 100 µM. The reaction was subjected to UV (365 nm) treatment on ice for 1 hour and the reaction mixture was analyzed with UPLC-MS (Ultimate 3000-Orbitrap-Velos, Waltham, MA, US). Peptides (100 µM) was dissolved in an aqueous solution of Na₂HPO₄ pH 4.5, followed by the addition of 100µM deprotected Cou-NPE. The reaction mixture was incubated at 37°C for 4 hours. The reaction products were then desalted and then analysed with LCMS to deduce the conversion rate.

The reaction was monitored using LC-MS. LC was configured with a binary-pump (Acquity UPLC system (Waters, Massachusetts, United States) fitted with a reverse phase Luna Omega Phenomenex C18 100Å column (30 x 2.1 mm, 1.6µm particle size (Phenomenex, CA, USA)) and a quadruple wavelength absorbance detector (220, 254nm, 280nm, 420nm). Flow rate was maintained at 0.2 ml/min, column temperature was kept at 28°C. Solvents used included solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Throughout the LC analysis, elution conditions were as follows: 5% solvent B for 0.5 min, followed by a linear gradient of up to 99% solvent B for 5.5 minutes. It was maintained for 2 minutes, followed by a steady decrease in the concentration of solvent B back to 5% for 2 more minutes. The MS (LTQ-XL, Thermofisher Scientific) was set to positive ESI mode with a scan range from 50–2,000. The capillary voltage was 3.44 kV. The sampling cone was set to 30 and the extraction cone to 3.44. Source temperature was set to 301.32°C and desolvation temperature to 312°C. The flow of the cone gas was 50 l/h and of the desolvation gas 800 l/h.
3. In vitro and in situ photo-activated protein labelling

Huh7 cells were obtained from Korean Cell Line Bank at Seoul National University Hospital. Huh7 cells were cultured in RPMI 1640 (HyClone, Cat#: SH30027.01) supplemented with 10% FBS (Gibco, Cat#: 16000-044), 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco, Cat#: 15140122) in humidified atmosphere containing 5% CO$_2$ at 37 °C. H1N1 virus (A/NSW/33) was obtained from Konkuk University (College of Veterinary Medicine). The subtypes of virus were propagated and titrated using 9-11 days old specific pathogen-free (SPF) embryonated chicken eggs. The 50% egg infective dose (EID50) was calculated by using the Reed–Muench method (H1N1 Log (EID50): 8.3; Reed and Muench, 1938).

3.1 Purified protein labelings: For in vitro purified protein labeling, 30 µg of purified human serum albumin (HSA), bovine serum albumin (BSA), and formylglycine generating enzyme (FGE) was dissolved in PBS (pH 7.4) buffer and BD-NPE (20 µM) was mixed. The reaction mixture irradiated under 365 nm UV for 30 min. After UV exposure, the laemmli sample buffer was added and quench the reaction. The samples were resolved on a 10 % SDS-PAGE gel and visualized using fluorescence gel imaging (excitation: blue LED, emission: 530/28nm).

3.2 in situ live cell labeling: For in situ protein labelling, Huh7 cells were plated in a 6-well plate for 24 h. Then, 0.8 µM, 3.9 µM, 7.7 µM and 15.4 µM of BD-NPE were treated to the Huh7 for 30 min (Final DMSO concentration was 1%). Then, the cells were irradiated with 365 nm UV light (100W) for 30 min on ice under dark conditions. Cells were washed with PBS and collected by scraping and centrifugation. Cells were resuspended in PBS with protease inhibitors cocktail (Thermo Fisher, Cat#: 78429), lysed by sonication on ice. The 15 µg of proteins were separated in a 10% SDS-PAGE, followed by in-gel fluorescence analysis to detect the probe-binding proteins pattern before and after the UV irradiation. The photo-labeled proteins were visualized by fluorescence gel imaging (excitation: blue LED, emission: 530/28nm).

For in situ protein labeling, 200 µL of Huh7 cell lysates (0.36 mg/mL) were incubated with 2 µl of the 2 mM or 100 µM Flu-NHS stock solutions at 23 °C for 30 min. Then 15 µg of proteins were separated in a 10% SDS-PAGE. The photo-labeled proteins were visualized by fluorescence gel imaging (excitation: blue LED, emission: 530/28nm).
For *in situ* protein labeling upon avian influenza infection, Huh7 cells were infected with the H1N1 viruses in 4 serial concentrations (Log (EID50): 7.3, 6.3, 5.3, 4.3). After 1-day incubation, the infected cells were treated with 15.4 μM of **BD-NPE** for 30 min. Then the cells were irradiated with 365 nm UV light (100W) for 30 min on ice under dark conditions. Cells were washed with PBS and collected by scraping and centrifugation. Cells were resuspended in PBS with protease inhibitors cocktail, lysed by sonication on ice. Then 12 μg of proteins were loaded to 10% SDS-PAGE. The photo-labeled proteins were visualized by fluorescence gel imaging (excitation: blue LED, emission: 530/28nm).

4. Chemoproteomics analysis

**Click reaction & sample preparation:** Huh7 cells were cultured in RPMI 1640 (HyClone, Cat#: SH30027.01) supplemented with 10% FBS (Gibco, Cat#: 16000-044), 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco, Cat#: 15140122) in humidified atmosphere containing 5% CO₂ at 37 °C. Huh7 cells were infected with the H1N1 viruses in 4 serial concentrations (Log (EID50): 7.3, 6.3, 5.3, 4.3). After 1-day incubation, the infected cells were treated with 20 μM of TCO-NPE for 30 min. Then the cells were irradiated with 365 nm UV light (100W) for 30 min on ice under dark conditions. Cells were washed with PBS and collected by scraping and centrifugation. Cells were resuspended in PBS with protease inhibitors cocktail (Thermo Fisher, Cat#: 78429), lysed by sonication on ice. For click chemistry, 200 μg of each lysates reacted with 20 μM Biotin benzyl tetrazine (Sigma Aldrich, Cat#: 793329) at 23 °C for 1 h. Then, Cell lysates were transferred to new tube and mixed with 50 μL of pre-washed streptavidin magnetic beads (Thermo Fisher, Cat#: 88816). The sample was rotated for 1 h at room temperature and washed with 50 mM Tris-HCl (pH 7.5). After removing the 50 mM Tris-HCl, incubate the beads with 80 μL of 2 M urea in 50 mM Tris-HCL containing 1 mM DTT and 0.5 μg trypsin at 23 °C for 1 h while shaking. After 1 h, the beads were separated magnetically, and the supernatant was saved for protein digestion. Reduce the disulfide bonds in the eluent by adding DTT to a final concentration of 4 mM and incubate at 23 °C for 30 min with shaking. Then, Alkylate the eluent by adding iodoacetamide to a final concentration of 10 mM and incubate at 23 °C for 45 min in the dark while shaking. The samples were digested with 0.5 μg trypsin for overnight at 37 °C. The trypsin digestion was terminated with 1% formic acid, and the samples were desalted using C18 Ziptip (Merck Millipore, Cat#: ZTC18S096) as the manufacture provided protocol. Briefly, the C18 Ziptip
were conditioned with 10 volumes of acetonitrile followed by additional equilibration step with 10 volumes of 0.1% formic acid. After equilibration step, the digested peptides were loaded to the C18 Ziptip, washed with 10 volumes of 0.1% formic acid, and eluted by elution buffer (70% acetonitrile, 0.1% formic acid). Eluted samples were dried in vacuum centrifuge.

**TMT-labeling:** Digested peptides were resuspended in 100 μL of 100 mM Triethylammonium bicarbonate (Thermo Fisher, Cat#: 90114). Each sample was mixed with a separate TMT label reconstituted in 41 μL of acetonitrile. Samples were incubated at 23 °C for 1 h. Labeling was quenched with 8 μL of 5% hydroxylamine for 15 min. Samples were combined and dried in vacuum centrifuge. Then, labeled peptides were resuspended in 300 μL of 0.1 % formic acid and desalting procedure was performed. The C18 Cartridges were conditioned with acetonitrile and then equilibrated with 0.1% formic acid. The peptides were bound to the C18 Cartridges, washed with 0.1% formic acid, and eluted by elution buffer (70% acetonitrile, 0.1% formic acid) for LC-MS/MS analysis.

**LC-MS/MS/MS analysis:** All LC-MS/MS/MS data was collected by an UltiMateTM 3000 RSLCnano System (Thermo Scientific, 5200.0356) equipped with a 50-cm C18 analytical column (Thermo Scientific, ES903), a 2-cm trap column (Thermo Scientific, 164535), and an Orbitrap Eclipse Tribrid mass spectrometer (Thermo Scientific, FSN04-10000). The analytical column temperature was maintained at 50 °C. Each fraction was eluted by using a 210 min method over a gradient from 5% to 80% acetonitrile in 0.1% formic acid. Full-scan MS spectra (400–2,000 m/z) were acquired in the FT-Orbitrap at a resolution of 120,000, followed by the selection of the twenty most intense ions for HCD-MS2 fragmentation. Directly after each MS2 experiment, the ten most intense fragment ions of the precursor m/z were selected for HCD-MS3. The resulting MS/MS/MS data were searched using Uniprot (Taxonomy: Homo sapiens, 2021.12.10), and analyzed by Proteome Discoverer software (v.2.4). TMT-plex (K) and Carbamidomethylation (C) were set as static modifications, and oxidation (M) and acetyl (N-terminus) were set as variable modifications. The precursor and fragment mass tolerances were set to 10 ppm and 0.9 Da, respectively. The maximum peptide and site false discovery rates were specified as 0.01 using Percolator. All protein list and quantitation data listed in Table S1, and LC-MS/MS data was deposited in MassIVE (Accession: MSV00089314, doi:10.25345/C5G44HV24). Comparison of enzyme class of identified proteins from huh7
cells were compared with our pull-down data, and publicly available data set from ProteomeXchange Dataset (Accession: PXD028693).

5. Gene Ontology analysis

The gene ontology enrichment analysis was conducted using web-based server (http://geneontology.org/). From isobaric TMT quantitation ratio, uniport accession codes of proteins that have significant fold change between vehicle and AI infected huh7 cell (criteria: \(|\log_2(\text{[AI infected (Log (EID50) =7.3)]/[vehicle]} | > 1\), number of proteins: 134) were uploaded to the server and statistically significant GO results were obtained. Enriched GO biological processes were listed in Table S2.

6. Photo-toxicity measurement

Hela cells were cultured to 80% confluence in DMEM (HyClone, Cat#: SH30243.01) supplemented with 10% FBS (Gibco, Cat#: 16000-044), 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco, Cat#: 15140122) in humidified atmosphere containing 5% CO₂ at 37 °C. Cells were treated with Cou-NBE (5.7 µM), TCO-NPE (10 µM) and BD-NPE (7.7 µM) incubated for 37 °C for 30 minutes. Cells were then washed twice with fresh medium to remove excess probes, UV treated for 30 minutes on ice and in dark conditions. Cells were then treated with Ez-Cytox (DoGenBio, Korea) for 30 minutes and absorbance measured at 450 nm using a spectrophotometer (Molecular Devices, USA). Each concentration was examined as duplicates.
Figure S1. Photophysical properties of **Cou-NPE** (a: in acetonitrile, b: in PBS buffer) and **BD-NPE** (c: in acetonitrile, d: in PBS buffer).
Figure S2. Fluorescence live cell imaging of **Cou-NPE** and **BD-NPE**. (a) **Cou-NPE** (5.7 μM) treated Hela cell, (b) **BD-NPE** (7.7 μM) treated cell. Images were obtained using EVOS-5000 microscope system using DAPI filter (λex 359nm, λem 459nm) and GFP filter (λex 475nm λem 509nm). Scale bar: 25 μm

Figure S3. Reaction kinetics of photo-cleavage reaction. (a) **Cou-NPE** (b) **BD-NPE** (c) **TCO-NPE**. Each experiment was conducted in triplicates
Figure S4. *In situ* photo-activated peptide crosslinking between Cou-NPE and LHAKPTD peptide.

LCMS profile of reaction mixture (a) before, (b) after 365 nm UV irradiation.
Figure S5. *In situ* photo-activated peptide crosslinking between BD-Aza and CLSWW peptide.

LCMS profile of reaction mixture (a) before, (b) after 365 nm UV irradiation.
Figure S6. Long-term fluorescence live Hela cell image after treatment of (a) BD for 0 to 2 hours without pre-irradiation of UV, (b) BD-NPE for 0 to 2 hours after pre-irradiation of 364 nm UV for 30 min. Images were obtained using EVOS-5000 microscope system using GFP filter (λex 475nm λem 509 nm). Scale bar: 25 μm
**Figure S7.** Fluorescence PAGE pattern of **BD-NPE** and fluorescein-NHS (**Flu-NHS**). Coomassie staining bands represent as loading control.

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**Figure S8.** Photo-toxicity of NPE probes with and without 365 nm UV irradiation. 10µM probes were treated to the Hela cell and 365 nm UV was irradiated for 30 min.
**Figure S9.** Full-size Coomassie staining images of Huh7 lysate upon H1N1 infection.
Figure S10. $^1$H – NMR spectrum of Suc-NPE.
Figure S11, $^{13}$C – NMR spectrum of Suc-NPE
**Fig S12.** $^1$H – NMR spectrum of Cou-NPE.
Figure S13. $^{13}$C – NMR spectrum of Cou-NPE.
Figure S14. $^1$H – NMR spectrum of BD-NPE.
Figure S15. $^{13}$C – NMR spectrum of BD-NPE.
Figure S16. H – NMR spectrum of TCO-NPE.
Figure S17. $^{13}$C – NMR spectrum of TCO-NPE.