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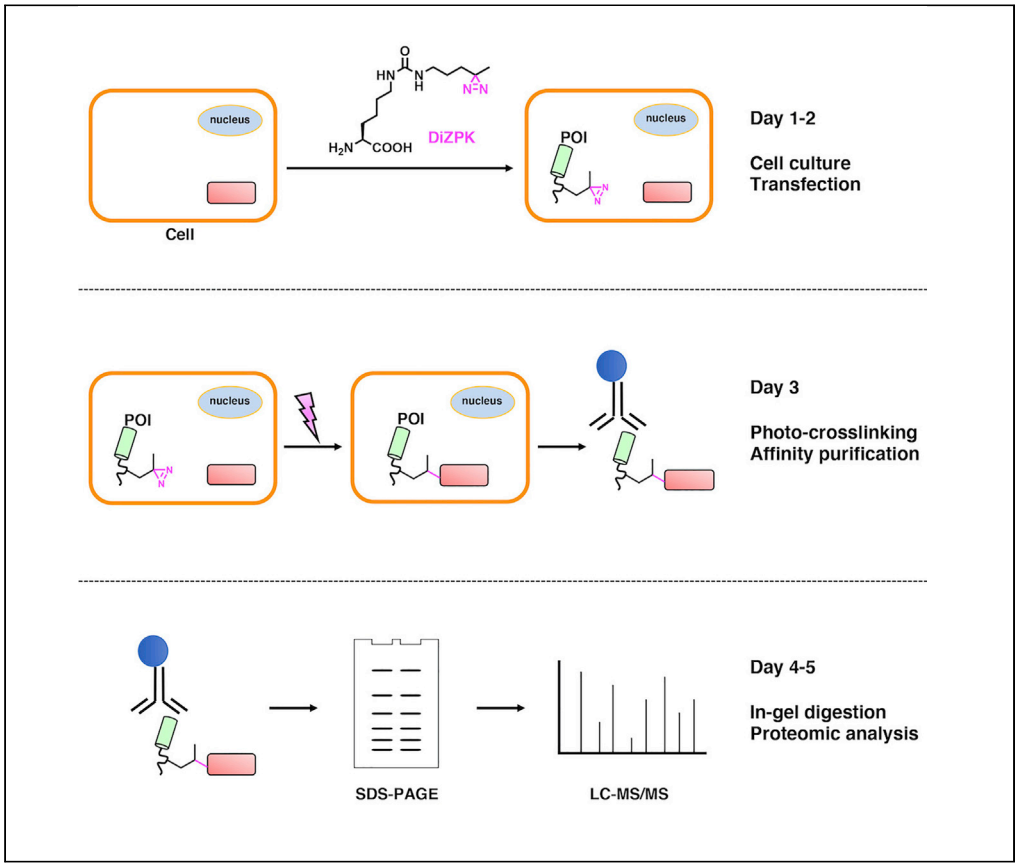
Protocol for Site-Specific Photo-Crosslinking Proteomics to Identify Protein-Protein Interactions in Mammalian Cells



Protein-protein interactions (PPIs) play essential roles in almost all aspects of cellular processes. However, PPIs remain challenging to study due to their substoichiometry, low affinity, dynamic nature, and context dependence. Here, we present a protocol for the capture and identification of PPIs in live mammalian cells, which relies on site-specific photo-crosslinking in live cells, affinity purification, and quantitative proteomics. The protocol facilitates efficient and reliable identification of the interacting proteins of a given protein of interest in live cells.

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HIGHLIGHTS

Site-specific incorporation of photo-crosslinkable amino acid into protein of interest

Site-specific photo-crosslinking for capture of protein interactions in live cells

Quantitative proteomics for reliable identification of photo-crosslinked proteins

Validation of protein interactions by site-specific photo-crosslinking

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Protocol for Site-Specific Photo-Crosslinking Proteomics to Identify Protein-Protein Interactions in Mammalian Cells

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SUMMARY

Protein-protein interactions (PPIs) play essential roles in almost all aspects of cellular processes. However, PPIs remain challenging to study due to their sub-stoichiometry, low affinity, dynamic nature, and context dependence. Here, we present a protocol for the capture and identification of PPIs in live mammalian cells, which relies on site-specific photo-crosslinking in live cells, affinity purification, and quantitative proteomics. The protocol facilitates efficient and reliable identification of the interacting proteins of a given protein of interest in live cells. For complete details on the use and execution of this protocol, please refer to Wu et al. (2020).

BEFORE YOU BEGIN

The protocol for identifying protein-protein interactions in mammalian cells integrates site-specific incorporation of a photo-crosslinkable unnatural amino acid (UAA), DiZPK (Zhang et al., 2011), into the protein of interest (POI) via genetic code expansion technology (Wang et al., 2006; Chin, 2017; Young and Schultz, 2018) and stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative proteomic analysis (Ong et al., 2002) (Figure 1). To initiate the experiment, the genetic code expansion system including DiZPK and plasmids encoding a DiZPK-aminoacyl-tRNA synthetase/tRNA pair and the POI should be prepared. For SILAC quantitative proteomics, stable isotope-labeled mammalian cells should be cultured.

Preparation of the Genetic Code Expansion System for Site-Specific Incorporation of DiZPK

1. DiZPK, a lysine-derived UAA that contains a photo-activatable diazirine functionality and facilitates covalent photo-crosslinking of protein-protein interactions in mammalian cells (Zhang et al., 2011), is utilized in this protocol. DiZPK can be chemically synthesized in an organic chemistry laboratory according to published procedures (Zhang et al., 2011). Alternatively, DiZPK is also commercially available from many vendors such as MedChemExpress.
2. For site-specific incorporation of DiZPK via genetic code expansion, the plasmid encoding a DiZPK-aminoacyl-tRNA synthetase/tRNA pair, i.e., pCMV-DiZPK-PylRS (Zhang et al., 2011), is required and can be obtained from Addgene.

Preparation of Amber Codon-Containing Plasmids of POI

Timing: 3–4 days

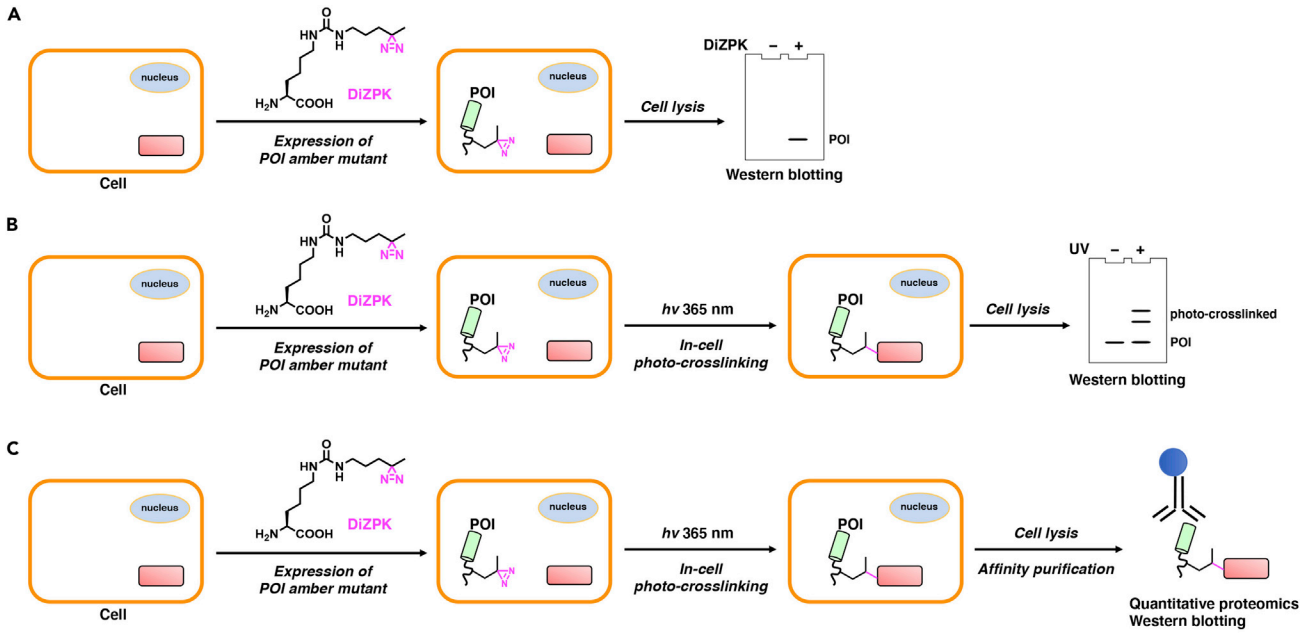

Figure 1. Workflow for Site-Specific Photo-Crosslinking Proteomics to Identify PPIs in Mammalian Cells

(A) Schematic for evaluating site-specific incorporation of DiZPK into POI in mammalian cells.

(B) Schematic for examining photo-crosslinking of DiZPK-modified POI in mammalian cells.

(C) Schematic for profiling and validating POI-interacting proteins with site-specific photo-crosslinking in mammalian cells.

For site-specific incorporation of DiZPK into POI, a plasmid encoding the POI with an affinity tag is also required, and an in-frame TAG mutation should be introduced into a selected position via site-directed mutagenesis.

3. Clone the gene of POI into a mammalian expression vector containing an affinity tag (e.g., HA tag) and generate the wild-type POI expression plasmid pCMV-POI-HA. Mutate the original stop codon TAG to TAA or TGA or 3xTAA if needed.

⚠ CRITICAL: For western blotting detection and affinity purification of the target protein, a C-terminal tag is preferred, as truncated proteins may be detected or isolated with an N-terminal tag especially when the amber codon position is near the C terminus. It should be checked whether the affinity tag would affect the function and expression level of the POI. If a C-terminal tag is not applicable, an N-terminal tag should be considered.

⚠ CRITICAL: It is recommended to change the intrinsic amber stop codon (i.e., TAG) of POI into ochre or opal stop codon (i.e., TAA or TGA) in order to avoid read-through in the amber suppression system.

Note: In addition to HA tag, other affinity tags such as FLAG and myc can also be used.

4. Introduce an exogenous TAG codon at a desired position of the POI using site-directed mutagenesis and generate the plasmid pCMV-POI-X-TAG-HA (X refers to the mutation site). The photo-crosslinkable DiZPK is site-specifically incorporated into the exogenous TAG position of the POI during translation.

⚠ CRITICAL: The amber codon position on POI affects the incorporation efficiency of DiZPK. Therefore, it is recommended to generate a series of mutants with varying amber codon

positions that are situated in different domains of the POI and evaluate them for optimal incorporation efficiency. Moreover, photo-crosslinking only occurs between protein domains in close proximity. Therefore, amber codon position determines the proteins to be photo-crosslinked and should be carefully chosen within the specific region of interest. Selection of the amber codon position is empirical. In general, the residues to be mutated should be inessential for the function of the POI, free of post-translational modifications, and accessible for protein-protein interactions.

Alternatives: This protocol uses QuikChange II Site-directed mutagenesis kit (Agilent Technologies) to introduce TAG codons. Alternatives are other commercial site-directed mutagenesis kits such as Q5 (NEB) and Phusion (Thermo Fisher Scientific).

Culture of SILAC Cells for Quantitative Proteomics

Timing: 2–3 weeks

SILAC-based quantitative proteomic analysis is used to identify photo-crosslinked PPIs in this protocol. In this regard, mammalian cells, such as HEK293T cells, are cultured in SILAC media according to published procedures (Ong and Mann, 2006).

5. Culture HEK293T cells in arginine and lysine-deficient DMEM media supplemented with 10% dialyzed FBS, $^{12}C_6$-L-lysine (Lys0; 0.80 mM), and $^{12}C_6$-L-arginine (Arg0; 0.40 mM) for at least five passages, referred to as light SILAC cells. Meanwhile, culture HEK293T cells in arginine and lysine-deficient DMEM media supplemented with 10% dialyzed FBS, $^{13}C_6$,$^{15}N_2$-L-lysine (Lys8; 0.80 mM), and $^{13}C_6$,$^{15}N_4$-L-arginine (Arg10; 0.80 mM) for at least five passages, referred to as heavy SILAC cells.

⚠ CRITICAL: After five cell doublings, the incorporation efficiency of heavy amino acids should be more than 97% as determined by LC-MS/MS analysis (Ong and Mann, 2006).

Note: The SILAC cells can be cryopreserved in SILAC culture media supplemented with 5% DMSO for future use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-GAPDH antibody HRP-conjugated	Proteintech	Cat#HRP-60004; RRID: AB_2737588
Anti-HA-Peroxidase; Rat monoclonal antibody (clone 3F10) conjugated with peroxidase	Roche	Cat#12013819001; RRID: AB_390917
Anti-c-myc-Peroxidase; Mouse monoclonal antibody (clone 9E10) conjugated to peroxidase	Roche	Cat#11814150001; RRID: AB_390910
Chemicals, Peptides, and Recombinant Proteins		
DiZPK	(Zhang et al., 2011)	N/A
DMSO	Sigma-Aldrich	Cat#D8418

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Benzonase	Sigma-Aldrich	Cat#E1014
EDTA-free Protease Inhibitor Cocktail	Roche	Cat#11873580001
$^{12}C_6$-L-lysine	Sigma-Aldrich	Cat#L8662
$^{12}C_6$-L-arginine	Sigma-Aldrich	Cat#A6969
$^{13}C_6$,$^{15}N_2$-L-lysine	Cambridge Isotope	Cat#CNLM-291-H-PK
$^{13}C_6$,$^{15}N_4$-L-arginine	Cambridge Isotope	Cat#CNLM-539-H-PK
DMEM	Thermo Fisher Scientific	Cat#11995065
DMEM for SILAC	Thermo Fisher Scientific	Cat#88364
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific	Cat#26140079
Fetal Bovine Serum (FBS), dialyzed	Thermo Fisher Scientific	Cat#26400044
Opti-MEM	Thermo Fisher Scientific	Cat#31985070
Xtremegene 9	Roche	Cat#6365809001
Jetprime	Polyplus transfection	Cat#114-15
Clarity Western ECL Substrate	Bio-Rad	Cat#1705060
Sequencing Grade Modified Trypsin	Promega	Cat#V5111
GelCode Blue Stain Reagent	Thermo Fisher Scientific	Cat#24590
Sodium chloride (NaCl)	Sigma-Aldrich	Cat#S3014
Potassium chloride (KCl)	Sigma-Aldrich	Cat#P9541
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich	Cat#S3264
Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich	Cat#P9791
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	Cat#L3771
Trizma hydrochloride (Tris-HCl)	Sigma-Aldrich	Cat#T3253
Hydrochloric acid (HCl)	Sigma-Aldrich	Cat#258148
Glycerol	Sigma-Aldrich	Cat#G5516
Bromophenol blue	Sigma-Aldrich	Cat#B8026
2-Mercaptoethanol (2-ME)	Sigma-Aldrich	Cat#M6250
Triton X-100	Sigma-Aldrich	Cat#T8787
Tween-20	Sigma-Aldrich	Cat#655205
Sodium deoxycholate	Sigma-Aldrich	Cat#D6750
DL-Dithiothreitol (DTT)	Sigma-Aldrich	Cat#D9779
Iodoacetamide (IAA)	Sigma-Aldrich	Cat#I6125
Ammonium hydroxide (NH_4OH)	Sigma-Aldrich	Cat#338818
Ammonium bicarbonate	Sigma-Aldrich	Cat#09830
Acetonitrile	Fisher Chemical	Cat#A955-4
Formic acid	Fisher Chemical	Cat#A117-50
Critical Commercial Assays		
BCA assay	Thermo Fisher Scientific	Cat#23228
QuikChange II Site-Directed Mutagenesis Kit	Agilent Technologies	Cat#200523
Experimental Models: Cell Lines		
HEK293T	ATCC	Cat#CRL-11268

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
pCMV-DiZPK-PylRS	(Zhang et al., 2011)	Addgene Plasmid #91706
pCMV-HA-IFITM3	(Wu et al., 2020)	N/A
pCMV-HA-IFITM3-TAG mutants	(Wu et al., 2020)	N/A
pCMV-myc-VCP	(Wu et al., 2020)	N/A
Software and Algorithms		
ImageLab	Bio-Rad	http://www.bio-rad.com/en-us/product/image-lab-software?ID=KRE6P5E8Z
MaxQuant v1.6.3.4	Max Planck Institute of Biochemistry	https://www.maxquant.org/maxquant/
Andromeda	Max Planck Institute of Biochemistry	http://coxdocs.org/doku.php?id=maxquant:andromeda:start
Perseus	Max Planck Institute of Biochemistry	https://www.maxquant.org/perseus/
Other		
Spectrolinker XL-1000 UV crosslinker	Spectroline	Cat#XL-1000
Pierce™ anti-HA magnetic beads	Thermo Fisher Scientific	Cat#88837
DynaMag-2 Magnet	Thermo Fisher Scientific	Cat#12321D
Thermomixer	Eppendorf	Cat#5382000023
Heating Block	Thermo Fisher Scientific	Cat#88870005
1.5-mL LoBind Eppendorf Tube	Eppendorf	Cat#0030108051
PepMap™ 300 C18 HPLC Capillary Column	Thermo Fisher Scientific	Cat#163574

MATERIALS AND EQUIPMENT

Buffers and Solutions

4% SDS Lysis Buffer

Reagent	Final Concentration	Amount
SDS	4% (w/v)	40 g
NaCl	150 mM	8.78 g
Tris-HCl pH 7.4	50 mM	50 mL of 1 M stock solution
MilliQ		950 mL
Total	n/a	1,000 mL

Triton X-100 Buffer

Reagent	Final Concentration	Amount
Triton X-100	1% (v/v)	10 mL
NaCl	150 mM	8.78 g
Tris-HCl pH 7.4	50 mM	50 mL of 1 M stock solution
MilliQ		940 mL
Total	n/a	1,000 mL

RIPA Buffer

Reagent	Final Concentration	Amount
Triton X-100	1% (v/v)	10 mL
sodium deoxycholate	1% (w/v)	10 g
SDS	0.1% (w/v)	1 g
NaCl	150 mM	8.78 g
Tris-HCl pH 7.4	50 mM	50 mL of 1 M stock solution
MilliQ		940 mL
Total	n/a	1,000 mL

4 × SDS-PAGE Loading Buffer

Reagent	Final Concentration	Amount
SDS	8% (w/v)	8 g
Tris-HCl pH 6.8	200 mM	20 mL of 1 M stock solution
Glycerol	40% (v/v)	40 mL
Bromophenol blue	0.4% (w/v)	0.4 g
MilliQ		40 mL
Total	n/a	100 mL

1 × SDS-PAGE Loading Buffer

Reagent	Final Concentration	Amount
SDS	2% (w/v)	2 g
Tris-HCl pH 6.8	50 mM	5 mL of 1 M stock solution
Glycerol	10% (v/v)	10 mL
Bromophenol blue	0.1% (w/v)	0.1 g
MilliQ		85 mL
Total	n/a	100 mL

PBS (Phosphate-buffered saline; pH adjusted to 7.3 using HCl)

Reagent	Final Concentration	Amount
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2HPO_4	10 mM	1.44 g
KH_2PO_4	1.8 mM	0.24 g
MilliQ		1,000 mL
Total	n/a	1,000 mL

Other Solutions

Name	Composition
DiZPK stock solution	500 mM in DMSO
PBST (PBS/0.02% Tween-20)	PBS containing 0.02% (v/v) Tween-20
Ammonium hydroxide (NH_4OH) solution	0.5 M NH_4OH in deionized water
DTT solution	400 mM DTT in deionized water
Iodoacetamide solution	1 M iodoacetamide in deionized water

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Name	Composition
Ammonium bicarbonate buffer	100 mM NH_4HCO_3 in deionized water
Gel destaining buffer	100 mM ammonium bicarbonate buffer/ acetonitrile (1:1, v/v)
Trypsin solution	10 ng/μL trypsin in 50 mM ammonium bicarbonate buffer
Gel extraction buffer	5% formic acid/acetonitrile (1:2, v/v)

⚠ CRITICAL: Ammonium hydroxide solution is irritating and should be handled in a fume hood.

LC Settings

Use a PepMap™ 300 C18 HPLC capillary column (Thermo Fisher Scientific; 75 μm diameter, 15 cm length) for LC. The gradients for LC are as follows: 10–45% solvent B over 45 min, 45–95% solvent B over 5 min and then 95% solvent B for 20 min. Solvent A is water with 0.1% formic acid, and solvent B is 80% acetonitrile in water with 0.1% formic acid.

Mass Spectrometer Settings

In the proteomic analysis, the parameters for Orbitrap XL mass spectrometer are listed as below. These parameters may need to be adjusted for measurements using alternative instruments.

Parameter	Setting
Polarity	Positive
Full MS	
Resolution	60,000
Automatic gain control target	1×10^6 ion counts
Scan range	300–1,800 m/z
dd-MS2	
Resolution	15,000
Automatic gain control target	3×10^4 ion counts
Isolation window	2.0
Normalized collision energy	35
DD settings	
Charge exclusion	Unassigned, 1, 5, 6, 7
Exclude isotopes	On
Dynamic exclusion	60

Alternatives: This protocol uses an Orbitrap XL mass spectrometer for proteomic analysis. Alternatives are other Orbitrap series of mass spectrometers (Thermo Fisher Scientific).

Parameters for MS Data Searching

Search mass spectrometry data with MaxQuant against Uniprot complete human database concatenated with common contaminants. Set trypsin as the digestion enzyme with maximal two missed cleavages allowed. Set carbamidomethylation of cysteine as a fixed modification. Set methionine oxidation and N-terminal acetylation as variable modifications. Set mass deviation for MS/MS peaks at a maximum of 0.5 m/z. Set maximum false discovery rates (FDR) to 0.01 both at the peptide and at the protein levels. Use only unique and razor peptides for quantification with minimal two ratio counts.

Alternatives: This protocol uses MaxQuant for analysis of mass spectrometry data. Alternatives are for example Mascot (Matrix Science) and Proteome Discoverer (Thermo Fisher Scientific).

STEP-BY-STEP METHOD DETAILS

Site-Specific Incorporation of DiZPK into POI in HEK293T Cells

Timing: 3 days

Transfect HEK293T cells with plasmids encoding the DiZPK-aminoacyl-tRNA synthetase/tRNA pair and the amber mutant of POI (i.e., pCMV-DiZPK-PylRS and pCMV-POI-X-TAG-HA, respectively) to evaluate the incorporation efficiency of DiZPK (Figure 1A). Full-length POI is only expressed in the presence of membrane-permeable DiZPK, but not in the absence of DiZPK.

1. Seed HEK293T cells in 6-well plates with normal growth medium (i.e., DMEM supplemented with 10% FBS) and incubate the cells at 37°C/5% CO_2 for 12–18 h.

   Note: For cells to be transfected, growth medium containing 10% FBS and no antibiotics should be used.

   Note: For experimental design, a positive control experiment with wild-type POI and a negative control experiment without DiZPK in the media should be performed in parallel to evaluate specific incorporation of DiZPK into the POI.

   Note: In general, HEK293T cells are ideal for expressing amber mutants of POI because of their high transfection efficiency. Alternatively, other cell lines that are readily transfected, such as HeLa and HCT116 cells, can also be used.

2. Check cell confluency the next day and transfect the cells with Xtremegene 9 at ~80% confluency. Prepare transfection complexes for wild-type POI and amber mutants as follows.
   a. Transfection of wild-type POI: (i) Dilute 2 μg of plasmid encoding wild-type POI (e.g., pCMV-POI-HA) in 200 μL of Opti-MEM for two wells to be transfected in a microcentrifuge tube. (ii) Add 6 μL of Xtremegene 9 to the diluted DNA. (iii) Mix the solution by gently pipetting and incubate the solution for 20–30 min at room temperature (20°C–28°C).
   b. Transfection of amber mutants of POI: (i) Dilute 2 μg of plasmid encoding an amber mutant of POI (e.g., pCMV-POI-X-TAG-HA) and 2 μg of pCMV-DiZPK-PylRS in 400 μL of Opti-MEM for two wells to be transfected in a microcentrifuge tube. (ii) Add 12 μL of Xtremegene 9 to the diluted DNA. (iii) Mix the solution by gently pipetting and incubate the solution for 20–30 min at room temperature (20°C–28°C).

   ⚠ CRITICAL: The transfection mixtures are prepared for two wells of a 6-well plate, one well for "+DiZPK" sample and the other for "-DiZPK" control. The transfection mixtures are split equally into two wells in step 4.

   ⚠ CRITICAL: It is recommended to optimize the cell confluency for optimal transfection efficiency. Other transfection reagents that are applicable for HEK293T cells can also be used, such as Jetprime.

   ⚠ CRITICAL: The concentrations of plasmid stocks used for transfection are normally around 500 ng/μL.

3. During incubation, replace the cell culture media with 2 mL of fresh cell growth media supplemented with or without 1 mM DiZPK per well. For the negative control without DiZPK in the media, cell growth media containing same volume of DMSO should be used.

⚠ CRITICAL: DiZPK is dissolved in DMSO at 500 mM as the stock solution, which is diluted with fresh cell growth media before the experiment. The DiZPK stock solution can be stored at −20°C for three months.

⚠ CRITICAL: Fresh cell growth media should be added into the cells carefully to avoid cell detachment.

4. Split the transfection mixtures equally for the wells to be transfected. Add the transfection mixtures prepared in step 2 dropwise into the cell media.
5. Incubate the cells at 37°C/5% CO_2 for additional 16–24 h.
6. Remove the media and add 1 mL of PBS into the wells. Collect the cells by repeated pipetting with 1 mL tips and transfer to microcentrifuge tubes. Centrifuge the cells at 1,000 × *g* and 4°C for 3 min.

Note: The "+DiZPK" sample and "-DiZPK" control should be processed in parallel in the following steps. For cell lines that adhere firmly to culture dishes (e.g., HeLa cells), use 0.25% trypsin-EDTA solution or cell scrapers for collecting the cells.

7. Discard the supernatant and wash the cells with PBS twice and finally remove the supernatant. Use centrifugation at 1,000 × *g* and 4°C for 3 min to collect the cells between washes.

Pause Point: The cell pellets can be stored at −80°C for at least several months.

8. Lyse the cells by adding 50 μL of 4% SDS lysis buffer supplemented with EDTA-free protease inhibitor cocktail and 0.2 μL benzonase (50 U) to the cell pellets. Vortex at room temperature (20°C–28°C) until the lysates become not sticky.

Note: Lysis of cells with SDS-containing buffer maximally solubilizes the proteins. Alternatively, cells can also be lysed with other detergent-containing lysis buffers.

9. Clarify cell lysates by centrifugation at 12,000 × *g* for 20 min at room temperature (20°C–28°C).
10. Use a standard BCA assay to quantify the protein concentrations.

Note: The protein concentrations of cell lysates should be around 10 mg/mL.

11. Dispense aliquots of equal amounts of cell lysates (e.g., 100 μg) for each sample into microcentrifuge tubes and bring the volumes to 70 μL with 4% SDS lysis buffer supplemented with EDTA-free protease inhibitors. Add 25 μL of 4 × SDS loading buffer and 5 μL of 2-mercaptoethanol (2-ME; 5% final concentration) to each sample. The final concentration of each sample should be around 1 mg/mL.
12. Vortex and heat the samples at 95°C for 10 min in a heating block to denature the proteins.
13. Centrifuge the samples at 5,000 × *g* for 3 min at room temperature (20°C–28°C).
14. Load 15–20 μL of each sample onto a 4%–20% Tris-HCl gel and run electrophoresis. Transfer proteins from the gel onto a nitrocellulose membrane and probe for the POI using the antibody against the affinity tag (e.g., HA tag) with standard immunoblotting techniques.

Note: Western blotting analysis is used to assess the incorporation of DiZPK and expression of DiZPK-modified POI. As an example, the result for incorporation of DiZPK into an antiviral protein IFITM3 is shown in Figure 2A.

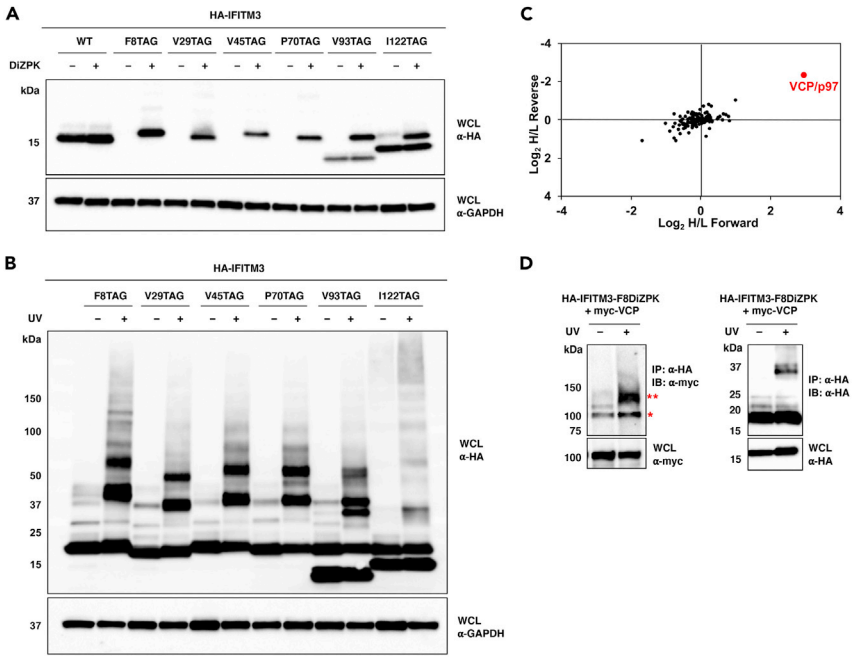

Figure 2. Results for Site-Specific Photo-Crosslinking of IFITM3 in HEK293T Cells

(A) Western blot analysis of HEK293T cells expressing HA-IFITM3-TAG mutants in the presence or absence of DiZPK.

(B) Western blot analysis of photo-crosslinking complexes in HEK293T cells expressing HA-IFITM3-TAG mutants with or without UV irradiation.

(C) Quantitative proteomic analysis for identification of photo-crosslinked IFITM3-interacting proteins. VCP, highly enriched in photo-crosslinking samples, is marked in red.

(D) Western blot analysis for validation of IFITM3-VCP photo-crosslinking complex. The red asterisk and double asterisks indicate co-immunoprecipitated VCP and photo-crosslinked IFITM3-VCP complex, respectively. Note that N-terminally HA-tagged IFITM3 constructs were used in these experiments.

Note: The incorporation efficiency of DiZPK is evaluated by comparing the expression levels of DiZPK-modified POI with that of wild-type POI. DiZPK-modified POI is generally expressed at a lower level relative to wild-type, but should be readily detected by western blot in order for successful photo-crosslinking experiments.

Photo-Crosslinking of DiZPK-modified POI in HEK293T Cells

Timing: 3 days

Cells expressing DiZPK-modified POI are irradiated with or without 365-nm UV light and lysed for western blot analysis to examine DiZPK-induced protein photo-crosslinking of POI in live cells (Figure 1B). Upon protein photo-crosslinking of the POI, a series of slower-migrating bands indicative of higher-molecular-weight photo-crosslinked complexes are observed in western blot.

15. Seed HEK293T cells in 6-well plates with normal growth medium (i.e., DMEM supplemented with 10% FBS) and incubate the cells at 37°C/5% CO_2 for 12–18 h.

⚠ CRITICAL: Generally, cells are seeded into two plates, one plate for UV irradiation ("+UV") and the other for non-irradiation ("-UV").

Note: It is recommended to survey a series of highly expressed amber mutants of POI for optimal photo-crosslinking efficiency and site-dependent photo-crosslinking.

16. Check cell confluency the next day and transfect the cells with Xtremegene 9 at ~80% confluency. Prepare transfection complexes for POI amber mutants as follows: (i) Dilute 2 μg of plasmid encoding an amber mutant of POI (e.g., pCMV-POI-X-TAG-HA) and 2 μg of pCMV-DiZPK-PylRS in 400 μL of Opti-MEM for two wells to be transfected in a microcentrifuge tube. (ii) Add 12 μL of Xtremegene 9 to the diluted DNA. (iii) Mix the solution by gently pipetting and incubate the solution for 20–30 min at room temperature (20°C–28°C).

⚠ CRITICAL: The transfection mixtures are prepared for two wells of a 6-well plate, one well for "+UV" sample and the other for "-UV" control. The transfection mixtures are split equally into two wells in step 18.

⚠ CRITICAL: It is recommended to optimize the cell confluency for optimal transfection efficiency. Other transfection reagents that are applicable for HEK293T cells can also be used, such as Jetprime.

⚠ CRITICAL: The concentrations of plasmid stocks used for transfection are normally around 500 ng/μL.

17. During incubation, replace the cell culture media with 2 mL of fresh cell growth media supplemented with 1 mM DiZPK per well.

⚠ CRITICAL: DiZPK is dissolved in DMSO at 500 mM as the stock solution, which is diluted with fresh cell growth media before the experiment. The DiZPK stock solution can be stored at −20°C for three months.

⚠ CRITICAL: Fresh cell growth media should be added into the cells carefully to avoid cell detachment.

18. Split the transfection mixtures equally for the wells to be transfected. Add the transfection mixtures prepared in step 16 dropwise into the cell media.
19. Incubate the cells at 37°C/5% CO_2 for additional 16–24 h.
20. Remove the media and add 1 mL of sterilized PBS carefully into each well.
21. Subject the "+UV" sample plate to UV irradiation at 365 nm on ice for 10 min using a Spectrolinker XL-1000 UV crosslinker at a distance of ~3 cm for in-cell photo-crosslinking. Meanwhile, preserve cells of the "-UV" group in the dark.
22. Collect the cells into microcentrifuge tubes by repeated pipetting with 1 mL tips. Also collect the cells in "-UV" control plate into microcentrifuge tubes. Wash the cells with PBS twice as described in steps 6 and 7 and finally remove the supernatant.

Pause Point: The cell pellets can be stored at −80°C for at least several months.

23. Lyse the cells and analyze the lysates with western blotting as described in steps 8–13.

Note: Western blotting analysis is used to evaluate protein photo-crosslinking of DiZPK-modified POI. High-molecular weight bands that are only present in UV-irradiated samples indicate putative photo-crosslinked interacting proteins of POI. As an example, the result for photo-crosslinking of DiZPK-modified IFITM3 is shown in Figure 2B.

Photo-Crosslinking Proteomics for Identification of POI-Interacting Proteins in HEK293T Cells

Timing: 4–5 days

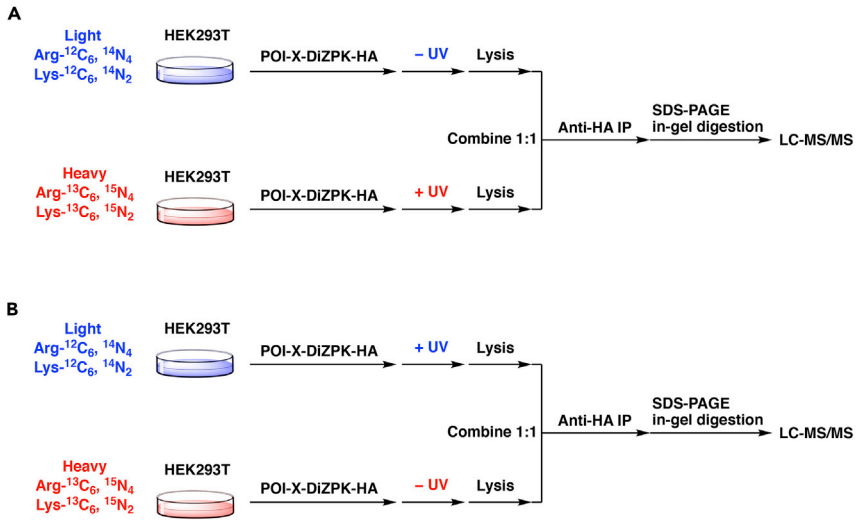

Figure 3. Experimental Design of the Quantitative SILAC Proteomic Analysis

(A) Schematic for forward SILAC experiment. Heavy SILAC cells are UV-irradiated, while light SILAC cells are not UV-irradiated.

(B) Schematic for forward SILAC experiment. Light SILAC cells are UV-irradiated, while heavy SILAC cells are not UV-irradiated.

Cells expressing DiZPK-modified POI are photo-crosslinked and lysed for affinity purification and quantitative SILAC proteomic identification (Figure 1C). Candidate interacting proteins (CIPs) of the POI are identified and quantified in the proteomic analysis.

24. Cell Preparation.

    a. Culture two 150-mm dishes of light SILAC HEK293T cells and two 150-mm dishes of heavy SILAC HEK293T cells until ~80% confluency.

⚠ CRITICAL: It is recommended to perform two sets of proteomic experiments for one amber mutant of POI by switching the SILAC labels and UV treatment conditions (termed "forward" and "reverse" SILAC experiments) (Figure 3) in order to eliminate potential false-positives and increase identification reliability. Therefore, for proteomic analysis of one amber mutant, two dishes of light and heavy SILAC cells should be prepared respectively.

    b. Transfect the cells with Jetprime. Prepare the transfection mixture of a POI amber mutant for each dish as follows: (i) Dilute 10 μg of plasmid encoding an amber mutant of POI (e.g., pCMV-POI-X-TAG-HA) and 10 μg of pCMV-DiZPK-PylRS in 1 mL of Jetprime buffer for one 150-mm dish in a microcentrifuge tube. (ii) Add 60 μL of Jetprime to the diluted DNA. (iii) Mix the solution by gently pipetting and incubate the solution for 20–30 min at room temperature (20°C–28°C).

⚠ CRITICAL: The concentrations of plasmid stocks used for transfection are normally around 500 ng/μL.

⚠ CRITICAL: Jetprime should be used for transfection of SILAC cells, as the Jetprime buffer does not contain amino acids. By contrast, the amino acids in Opti-MEM may decrease the isotope labeling levels in heavy SILAC cells.

    c. During incubation, replace the cell culture media respectively with 20 mL of fresh light and heavy SILAC media supplemented with 1 mM DiZPK per dish.

⚠ CRITICAL: DiZPK is dissolved in DMSO at 500 mM as the stock solution, which is diluted with fresh cell growth media before the experiment. The DiZPK stock solution can be stored at −20°C for three months.

⚠ CRITICAL: Fresh cell media should be added into the cells carefully to avoid cell detachment.

d. Add the transfection mixtures prepared in step 24b dropwise into the cell media.

Note: All dishes are transfected in the presence of DiZPK.

e. Incubate the cells at 37°C/5% CO_2 for additional 20 h.
f. Remove the media and add 10 mL of sterilized PBS carefully into each dish.
g. Treat one dish of light SILAC cells and one dish of heavy SILAC cells with UV irradiation at 365 nm on ice at a distance of ~3 cm for 10 min using a Spectrolinker XL-1000 UV crosslinker for in-cell photo-crosslinking.

Note: The rest dishes are not treated with UV irradiation and serve as non-photo-crosslinking controls.

⚠ CRITICAL: In "forward" SILAC experiment, heavy-labeled cells are irradiated with UV while light-labeled cells are not. Alternatively, in "reverse" SILAC experiment, light-labeled cells are irradiated with UV while heavy-labeled cells are not (Figure 3).

h. Harvest the cells by repeated pipetting into 15-mL centrifuge tubes. Also harvest the cells that are not UV-irradiated. Centrifuge to collect the cells at 1,000 × *g* for 3 min at 4°C.
i. Remove the supernatant and wash the cells with PBS twice. Use centrifugation with 1,000 × *g* at 4°C for 3 min to collect the cells between washes. In the last wash, transfer the cells into microcentrifuge tubes. Discard the supernatant after the last wash.

Pause Point: The cell pellets can be stored at −80°C for at least several months.

25. Cell Lysis.
    a. For cells harvested from one 150-mm dish, add 200 μL of 4% SDS lysis buffer supplemented with Roche EDTA-free protease inhibitor cocktail and benzonase.
    b. Lyse cells with vigorous vortex and sonication. Leave the lysates on bench for ~30 min.

    Note: The lysates should be clear and not sticky.

    Note: Harsh denaturing conditions (i.e., 4% SDS) are used for cell lysis to break non-covalent protein-protein interactions of POI.

    c. Centrifugate cell lysates at 12,000 × *g* for 20 min at room temperature (20°C–28°C) to remove cell debris.
    d. Use a standard BCA assay to quantify the protein concentrations.

    Note: For one 150-mm dish, 5–10 mg protein should be obtained.

    Optional: Take 50 μg of each sample for SDS-PAGE and western blot analysis to confirm expression and photo-crosslinking of POI as described in steps 11–14.

26. Affinity Purification.

a. Take equal amounts (e.g., 7.5 mg) of light and heavy cell lysates based on protein concentrations, and mix them in 15-mL centrifuge tubes. Dilute the mixtures with 4% SDS lysis buffer to total 400 μL.

⚠ CRITICAL: In "forward" SILAC experiment, UV-irradiated heavy cell lysates are equally mixed with non-irradiated light cell lysates. In "reverse" SILAC experiment, UV-irradiated light cell lysates are equally mixed with non-irradiated heavy cell lysates (Figure 3).

⚠ CRITICAL: The mixtures should be normalized to an equal volume with 4% SDS lysis buffer.

b. Add 7.6 mL of Triton X-100 buffer to each of the mixture. Mix by vortexing gently.

⚠ CRITICAL: Addition of Triton X-100 buffer dilutes the concentration of SDS for subsequent affinity purification.

c. Resuspend the anti-HA magnetic beads in the bottle with gentle vortex and transfer 1.5 mL of bead slurry (10 mg/mL) per sample from the bottle with a 1-mL pipette to a 15-mL centrifuge tube.

Note: The actual amount of anti-HA magnetic beads should be titrated to ensure depletion of over 80% of the POI.

d. Place the tubes on the magnet to separate the beads and remove the supernatant.
e. Wash the beads three times with 5 mL PBST by adding the PBST and gentle vortexing. In the last wash, place the tubes on the magnet to separate the beads and remove the supernatant.

⚠ CRITICAL: A magnet is recommended to separate the beads. Centrifugation should not be used for this purpose, as it may cause aggregation of the beads and impact antigen binding.

f. Transfer the cell lysates prepared in step 26b to the washed beads.
g. Incubate with end-over-end rotation at 4°C for 1.5 h.
h. Pulse spin the centrifuge tubes to bring down mixtures around the tubes and lids. Place the tubes on the magnet to separate the beads and remove the supernatant.
   i. Add 5 mL of pre-chilled RIPA buffer to the beads and incubate with end-over-end rotation at 4°C for 5 min.

⚠ CRITICAL: The RIPA buffer should be pre-chilled on ice.

j. Pulse spin the centrifuge tubes to bring down mixtures around the tubes and lids. Place the tubes on the magnet to separate the beads and remove the supernatant.
k. Wash the beads four more times as described in steps 26i and 26j and finally remove the supernatant.

Note: Multiple washes with RIPA buffer decrease non-specific binding proteins and non-covalent interacting proteins of POI. A total of five washes is recommended.

l. Add 500 μL of 0.5 M NH_4OH to the beads and vortex at 37°C for 30 min.

Note: Treatment with NH_4OH dissociates POI and its interacting proteins from the beads.

m. Place the tubes on the magnet to separate the beads and collect the eluates into 1.5-mL microcentrifuge tubes.
n. Repeat the elution as in steps 26l and 26m and pool the eluates together.
o. Dry the pooled eluates by Speedvac.

Pause Point: The protein samples can be stored at −80°C for at least several weeks.

27. In-gel digestion.
    a. Add 40 μL of 1 × SDS-PAGE loading buffer into each sample and vortex to dissolve the residues.
    b. Add 1 μL of DTT stock solution (400 mM) to a final concentration of 10 mM and incubate at 37°C for 30 min.

⚠ CRITICAL: DTT should be freshly prepared in deionized water.

c. Add 2 μL of iodoacetamide stock solution (1 M) into each sample and incubate in the dark at room temperature (20°C–28°C) for another 30 min.

⚠ CRITICAL: Iodoacetamide should be freshly prepared in deionized water.

d. Load 40 μL protein mixture for each sample onto a 4%–20% Tris-HCl gel and separate the samples by electrophoresis.

⚠ CRITICAL: It is recommended to use gels with large sample loading volumes. Alternatively, run the samples in multiple adjacent lanes and cut the gels across these lanes in steps 27f and 27g.

⚠ CRITICAL: For gels to be cut for proteomic analysis, blank lanes should be placed between samples to prevent cross-contamination during gel electrophoresis and slicing. Blanks should contain 1 × SDS-PAGE loading buffer.

⚠ CRITICAL: The gels can be run halfway down to save time.

⚠ CRITICAL: A fraction of the protein mixture for each sample can be saved for immunoblotting to confirm isolation of POI and photo-crosslinked complexes.

e. Stain the gel with GelCode Blue Stain reagent for 1 h and destain with deionized water for another 1 h.

Alternatives: Other gel staining reagents that are compatible with in-gel digestion and mass spectrometry such as Coomassie Brilliant Blue reagent can also be used.

⚠ CRITICAL: To minimize contamination, do all staining and destaining steps in a sterile 150-mm tissue culture dish.

Note: In-gel digestion is next performed as previously reported for LC-MS/MS analysis (Shevchenko et al., 2006).

Pause Point: The gel can be destained in deionized water with overnight (e.g., 10–12 h) incubation.

f. Transfer the gel to a clean 150-mm tissue culture dish and excise the sample lanes of interest from the gel with a fresh scalpel or razor blade.

g. Cut the lanes into 5–6 slices and cut each slice into 1 × 1 mm pieces. Transfer the gel pieces into labeled 1.5-mL Lobind Eppendorf tubes.
h. Add 300 μL of 100 mM ammonium bicarbonate buffer into each tube to wash the gel pieces. Remove the supernatant using a 1-mL tip with a 200-μL tip on the end.

⚠ CRITICAL: Make ammonium bicarbonate buffer freshly daily and discard after use.

⚠ CRITICAL: The gel pieces may be lost through 1-mL tips, so a 200-μL tip on the end should be used.

i. Add 500 μL of destaining buffer into each tube to destain the gel pieces. Shake the tubes for 15–30 min until gel pieces become colorless. Spin down the gel pieces at 1,000 × *g* for 1 min at room temperature (20°C–28°C) and discard the supernatant.

⚠ CRITICAL: If the gel pieces are still blue, repeat the destaining in step 27i.

⚠ CRITICAL: The supernatant should be removed using a 1-mL tip with a 200-μL tip on the end.

j. Add 500 μL of acetonitrile into each tube to dehydrate the gel pieces. Shake the tubes for 10 min at room temperature (20°C–28°C) until gel pieces shrink and become completely white. Spin down the gel pieces at 1,000 × *g* for 1 min and discard the supernatant.
k. Dry the gel pieces in a Speedvac for 1 h.

⚠ CRITICAL: The gel pieces are shrunken, white, and completely free of acetonitrile after Speedvac.

l. Add 100–200 μL of trypsin solution into each tube to cover the dry gel pieces. Leave the tube on ice for 30 min to allow rehydration of gel pieces.

⚠ CRITICAL: There should be enough trypsin solution to cover all gel pieces. If required, add more trypsin buffer to cover the gel pieces.

m. Incubate at 37°C overnight (e.g., 10–12 h) with gentle mixing in a Thermomixer for trypsin digestion.
n. Spin down the gel pieces at 1,000 × *g* for 1 min at room temperature (20°C–28°C).
o. Add 200–600 μL of extraction buffer into each tube depending on gel piece sizes and incubate at room temperature (20°C–28°C) for 30 min in a shaker.

⚠ CRITICAL: The volume of extraction buffer should be at least twice of the trypsin solution in step 27l. If too little extraction buffer is used, the peptides may not be extracted out of the gel pieces. If too much extraction buffer is used, the total volume of extraction buffer may be too large for a 1.5-mL microcentrifuge tube.

p. Transfer the supernatant to a new 1.5-mL Lobind Eppendorf tube.

Note: The supernatant contains the peptides to be analyzed.

q. Add another 200–600 μL of extraction buffer into each tube depending on gel piece sizes and incubate at room temperature (20°C–28°C) for 30 min in a shaker.

⚠ CRITICAL: The volume of extraction buffer should be at least twice of the trypsin solution in step 27l.

r. Transfer the supernatant to the tube in step 27p and pool the peptide samples.
s. Dry the peptide samples in a Speedvac for several hours.

Pause Point: The peptide samples can be stored at −20°C for a few weeks.

28. Proteomic Analysis.
    a. Resuspend the tryptic peptides in 20 μL of 1% formic acid.
    b. Load the samples onto a 75 μm × 15 cm C18 reverse-phase column. Spray the eluted peptides into an Orbitrap XL mass spectrometer with a nano-ESI source. The setting parameters for nano-LC and mass spectrometer are described in Materials and Equipment.
    c. Search and analyze the acquired tandem MS spectra against Uniprot complete human database concatenated with common known contaminants using MaxQuant software (Cox and Mann, 2008). Typical parameters used for searching data are listed in Materials and Equipment.
    d. Analyze the search results with Perseus (Tyanova et al., 2016). Remove the proteins identified as known contaminants or reverse hits in Perseus.

Note: Known contaminants and reverse hits are identified and marked by MaxQuant software (Cox and Mann, 2008).

    e. Transform the SILAC ratios in forward and reverse experiments with log2 transformation. Plot the SILAC ratios in forward and reverse experiments with Excel.

Note: The SILAC ratios in forward and reverse experiments are used to filter out the CIPs. Protein hits identified to have high SILAC ratios in the forward experiment and meanwhile have low SILAC ratios in the reverse experiment are considered to be CIPs of the POI. As an example, the result for photo-crosslinking proteomic analysis of DiZPK-modified IFITM3 is shown in Figure 2C, which suggests VCP (Meyer et al., 2012) as a primary CIP of IFITM3.

Validation of Photo-Crosslinking Complexes of POI

Timing: 3 days

Cells co-expressing DiZPK-modified POI and a CIP identified in proteomic analysis are photo-crosslinked and lysed for affinity purification and western blotting to validate the interaction and formation of the photo-crosslinking complex (Figure 1C). Upon photo-crosslinking, a high-molecular-weight band corresponding to the covalent complex of POI and CIP is observed in western blot.

29. Clone the gene of CIP into a mammalian expression vector containing an affinity tag (e.g., myc tag) and generate the expression plasmid for CIP, e.g., pCMV-myc-CIP. Mutate the original stop codon TAG to TAA or TGA or 3xTAA if needed.

⚠ CRITICAL: The affinity tag on CIP should be orthogonal to that on POI. For example, FLAG or myc tag can be used.

⚠ CRITICAL: It is recommended to change the intrinsic amber stop codon (i.e., TAG) of CIP into ochre or opal stop codon (i.e., TAA or TGA) in order to avoid read-through in the amber suppression system.

30. Seed HEK293T cells in 60-mm dishes with normal growth medium and incubate the cells at 37°C/5% CO_2 for 12–18 h.

31. Check cell confluency the next day and transfect the cells with Xtremegene 9 at ~80% confluency. Prepare transfection complexes as follows: (i) Dilute 4 μg of plasmid encoding an amber mutant of POI (e.g., pCMV-POI-X-TAG-HA), 4 μg of plasmid encoding pCMV-myc-CIP, and 4 μg of pCMV-DiZPK-PylRS in 1 mL of Opti-MEM for two dishes to be transfected in a microcentrifuge tube. (ii) Add 36 μL of Xtremegene 9 to the diluted DNA. (iii) Mix the solution by gently pipetting and incubate the solution for 20–30 min at room temperature (20°C–28°C).

⚠ CRITICAL: The concentrations of plasmid stocks used for transfection are normally around 500 ng/μL.

⚠ CRITICAL: The transfection mixtures are prepared for two 60-mm dishes, one dish for "+UV" sample and the other for "-UV" control. The transfection mixtures are split equally into two dishes in step 33.

⚠ CRITICAL: Other transfection reagents that are applicable for HEK293T cells can also be used, such as Jetprime.

32. During incubation, replace the cell culture media with 3 mL of fresh cell growth media supplemented with 1 mM DiZPK per dish.

⚠ CRITICAL: DiZPK is dissolved in DMSO at 500 mM as the stock solution, which is diluted with fresh cell growth media before the experiment. The DiZPK stock solution can be stored at −20°C for three months.

⚠ CRITICAL: Fresh cell growth media should be added into the cells carefully to avoid cell detachment.

33. Split the transfection mixtures equally for the dishes to be transfected. Add the transfection mixtures prepared in step 31 dropwise into the cell media.
34. Incubate the cells at 37°C/5% CO_2 for additional 16–24 h.
35. Remove the media and add 2 mL of sterilized PBS carefully into each dish.
36. Subject the "+UV" sample dish to UV irradiation at 365 nm on ice for 10 min using a Spectrolinker XL-1000 UV crosslinker at a distance of ~3 cm for in-cell photo-crosslinking. Meanwhile, preserve cells of the "-UV" control in the dark.
37. Harvest the cells into 15-mL centrifuge tubes by repeated pipetting with 1-mL tips. Also harvest the cells in "-UV" control plate into 15-mL centrifuge tubes. Centrifuge the cells with 1,000 × *g* at 4°C for 3 min and remove the supernatant.
38. Add 1 mL of PBS into the tubes and transfer the cells into 1.5-mL microcentrifuge tubes. Wash the cells with PBS twice as described in steps 6 and 7 and finally remove the supernatant.

Pause Point: The cell pellets can be stored at −80°C for at least several months.

39. For cells harvested from one 60-mm dish, add 50 μL 4% SDS lysis buffer supplemented with EDTA-free protease inhibitor cocktail and benzonase.
40. Lyse cells with vigorous vortex and sonication. Leave the lysates on bench for ~30 min.

Note: The lysates should be clear and not sticky.

Note: Harsh denaturing conditions (i.e., 4% SDS) are used for cell lysis to break non-covalent protein-protein interactions of POI.

41. Centrifugate cell lysates at 12,000 × *g* for 20 min at room temperature (20°C–28°C) to remove cell debris.

42. Use a standard BCA assay to quantify the protein concentrations.
43. Dispense equal amount of proteins for each sample into 1.5-mL microcentrifuge tubes. Dilute each protein sample with 4% SDS lysis buffer to total 50 μL.

⚠ CRITICAL: Take 50 μg of each sample for SDS-PAGE and western blot analysis to confirm the expression of POI and CIP.

⚠ CRITICAL: The mixtures should be normalized to an equal volume with 4% SDS lysis buffer.

44. Add 950 μL of Triton X-100 buffer to each sample. Mix by vortexing gently.

⚠ CRITICAL: Addition of Triton X-100 buffer dilutes the concentration of SDS for subsequent affinity purification.

45. Carry out anti-HA affinity purification with anti-HA magnetic beads (~0.3 mL bead slurry) as described in steps 26c–26k, and wash the beads with pre-chilled RIPA buffer five times.
46. Add 25 μL 1× SDS loading buffer supplemented with 2-ME (5% final concentration) into each sample. Vortex vigorously and heat the samples at 95°C for 5 min using a heating block.
47. Place the microcentrifuge tubes on the magnet to separate the beads.
48. Load 20 μL of each sample onto a 4%–20% Tris-HCl gel and separate the proteins by electrophoresis. Transfer proteins from the gel onto a nitrocellulose membrane and probe for the CIP using the antibody against the affinity tag (e.g., myc tag) with standard immunoblotting techniques.

Note: Western blotting analysis for CIP is used to assess the co-immunoprecipitation of CIP and formation of the slower-migrating photo-crosslinking complex between CIP and POI. As the CIP and photo-crosslinking complex are present in low abundance, majority of the eluent in steps 46 and 47 should be analyzed using the antibody against CIP. As an example, the result for validation of IFITM3-VCP interaction and photo-crosslinking is shown in Figure 2D.

49. Load 5 μL of each sample onto another 4%–20% Tris-HCl gel and separate the proteins by electrophoresis. Transfer proteins from the gel onto a nitrocellulose membrane and probe for the POI using the antibody against the affinity tag (e.g., HA tag) with standard immunoblotting techniques.

Note: Western blotting analysis for POI is used to assess the expression and photo-crosslinking of DiZPK-modified POI. As the POI are enriched and present in very high abundance, a small quantity of the eluent in steps 46 and 47 should be analyzed using the antibody against POI. As an example, the result for IFITM3 expression and photo-crosslinking in validation of IFITM3-VCP interaction is shown in Figure 2D.

⚠ CRITICAL: The photo-crosslinking complex of CIP and POI may not be observed on the western blot against POI, due to its relatively low abundance compared to POI.

EXPECTED OUTCOMES

With the protocol described here, site-specific incorporation of photo-activatable DiZPK into POI is achieved in mammalian cells (e.g., HEK293T cells) that are co-transfected with plasmids encoding DiZPK-aminoacyl-tRNA synthetase/tRNA pair and the amber mutant of POI (i.e., pCMV-DiZPK-PylRS and pCMV-POI-X-TAG-HA, respectively). Full-length POI is only expressed in the presence of DiZPK. For example, as shown in western blot analysis (Figure 2A), DiZPK is site-specifically incorporated

into varying positions of IFITM3, an antiviral protein as the model, in HEK293T cells, which is dependent on the presence of DiZPK.

Upon photo-irradiation, crosslinking of DiZPK-modified POI with its interacting proteins is achieved in live HEK293T cells. A series of slower-migrating bands indicative of higher-molecular-weight photo-crosslinked complexes are only observed in UV-irradiated samples, but not in non-UV-irradiated controls. For instance, western blot analysis shows that DiZPK-modified IFITM3 is photo-crosslinked to generate high-molecular-weight covalent complexes that migrate slowly on SDS-PAGE (Figure 2B).

By following the protocols for photo-crosslinking, affinity purification, and quantitative SILAC proteomic analysis, the potential interacting partners are photo-crosslinked with POI, isolated, and identified. Only protein hits that exhibit high SILAC ratios in the forward experiment and low SILAC ratios in the reverse experiment are considered to be interacting partners of POI. For example, the photo-crosslinking proteomic analysis for DiZPK-modified IFITM3 enables robust identification of VCP that is selectively more enriched in the photo-crosslinking samples versus the non-photo-crosslinking controls in both forward and reverse experiments (Figure 2C).

With the candidate interacting partners of POI in hand, the interaction of POI with CIP and formation of photo-crosslinked complex are validated using affinity purification and western blotting analysis. Taking the validation results for IFITM3-VCP interaction as an example (Figure 2D), the co-immunoprecipitated VCP band confirms the interaction between VCP and IFITM3. Moreover, a slower-migrating band with molecular weight equal to the summed mass of VCP and IFITM3 is only observed for DiZPK-modified IFITM3 upon UV irradiation. By contrast, no such protein band is observed without UV irradiation, thus confirming the formation of photo-crosslinked IFITM3-VCP complex.

LIMITATIONS

The protocol for site-specific photo-crosslinking proteomics should be generally applicable for profiling PPIs occurring on a specific region of a POI. As photo-crosslinking only occurs between parties in close proximity because of the short half-life of photo-generated reactive species (Peng et al., 2014), interacting proteins of the POI that are far away from the photo-crosslinking functionality may not be crosslinked and identified. As a result, photo-crosslinking efficiency and pattern are highly dependent on the position of photo-crosslinking functionality (e.g., DiZPK). The site-specific photo-crosslinking proteomic analysis is thus only suitable for studying PPIs of the region of interest in a given POI. Nevertheless, photo-crosslinking proteomics on multiple positions in different regions of the POI should enable characterization of the interactome of the POI.

In addition, transient transfection used in the protocol may result in heterogeneous and high expression levels of the POI, which may introduce non-specific binding and false-positive identification. Therefore, it is recommended to evaluate whether the expression level of POI is comparable to endogenous level and the POI is functional. To solve this limitation, cell lines stably expressing DiZPK-modified POI can be generated and utilized (Elsässer et al., 2016). With the current protocol, it is still difficult to directly identify the photo-crosslinked peptides and map the interaction interfaces on CIP, due to the relatively low abundance of photo-crosslinked peptides and uncertainty of the photo-crosslinking amino acid on CIP. Recently, cleavable photo-crosslinkers have been developed (Lin et al., 2014; Yang et al., 2016; He et al., 2017) to allow bait-prey separation after isolation of photo-crosslinked complexes and facilitate identification of crosslinking sites by mass spectrometry analysis.

The in-gel digestion used in the protocol is time-consuming and not amenable for processing multiple samples. In this regard, in-solution or on-bead digestion can be used as an alternative to in-gel

digestion. It is, however, worth mentioning that the detergents should be completely removed before in-solution or on-bead digestion.

TROUBLESHOOTING

Problem 1

Detachment of HEK293T cells during replenishment of fresh media (step 3)

Potential Solution

Add the media slowly near the edge of the dish to minimize perturbation to the cell monolayer. It is recommended to use an electronic pipette aid with a gravity drain feature. Alternatively, use tissue culture dishes coated with poly-D-lysine for attachment of HEK293T cells.

Problem 2

Cells unhealthy after transfection (step 5)

Potential Solution

Make sure that cell confluency is ~80% before transfection. Adjust the amount ratio of transfection reagent and plasmid. Reduce the transfection time. Change to fresh media 6 h after transfection.

Meanwhile, check whether overexpression of POI is toxic to cells.

Problem 3

Low expression level of DiZPK-modified POI (step 14)

Potential Solution

Evaluate multiple residues for DiZPK incorporation. The position of amber codon significantly affects the expression of POI.

Optimize the ratio of plasmids expressing POI and DiZPK-PylRS. Increase the relative amount of POI-expressing plasmid.

Check the quality of plasmids used for transfection. For instance, the plasmids should be free of salts, proteins, and endotoxins. Purify the plasmids with an endotoxin-free plasmid isolation kit, for example, the EndoFree Plasmid Maxi Kit from Qiagen. Change to a highly efficient transfection reagent depending on the cell types if needed, such as Lipofectamine 3000 (Thermo Fisher Scientific) and Viafect (Promega).

Increase the transfection time to 36–48 h.

Problem 4

Expression of truncated POI in the absence of DiZPK (step 14)

Potential Solution

Choose another residue for DiZPK incorporation. Truncated proteins may be observed when the amber codon is near the protein C terminus. In addition, N-terminal truncation is also possible because of translation from an in-frame start codon downstream of the amber codon.

Problem 5

Low photo-crosslinking efficiency in the UV-irradiated sample (step 23)

Potential Solution

Evaluate multiple amber codon positions. The photo-crosslinking efficiency and pattern are highly dependent on DiZPK position.

Stimulate the cells appropriately to functionally activate the POI.

Increase the UV irradiation time to 20 min in step 21.

Problem 6
Non-specific bands detected in the non-UV-irradiated sample (step 23)

Potential Solution
Check the specificity of the antibody used for immunoblotting. Isolate the photo-crosslinking complexes with affinity purification before immunoblotting analysis.

Problem 7
Low number of total proteins identified in proteomic analysis (step 28c)

Potential Solution
Increase the lysate amount for affinity purification. Use a new vial of trypsin. Increase the sample loading volume for LC-MS/MS analysis.

Problem 8
Poor reproducibility of the forward and reverse experiments (step 28e)

Potential Solution
Check the incorporation efficiency of heavy isotope-labeled amino acids before the experiment. Make sure that the light and heavy cell lysates are equally mixed.

RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Tao Peng (tpeng@pku.edu.cn).

Materials Availability
All unique/stable reagents generated in this study are available from the Lead Contact, Tao Peng (tpeng@pku.edu.cn).

Data and Code Availability
No original data is generated in this protocol.

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AUTHOR CONTRIBUTIONS
Conceptualization, T.P.; Investigation, T.P.; Writing – Original Draft, C.C.; Writing – Review & Editing, T.P.; Funding Acquisition, T.P.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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