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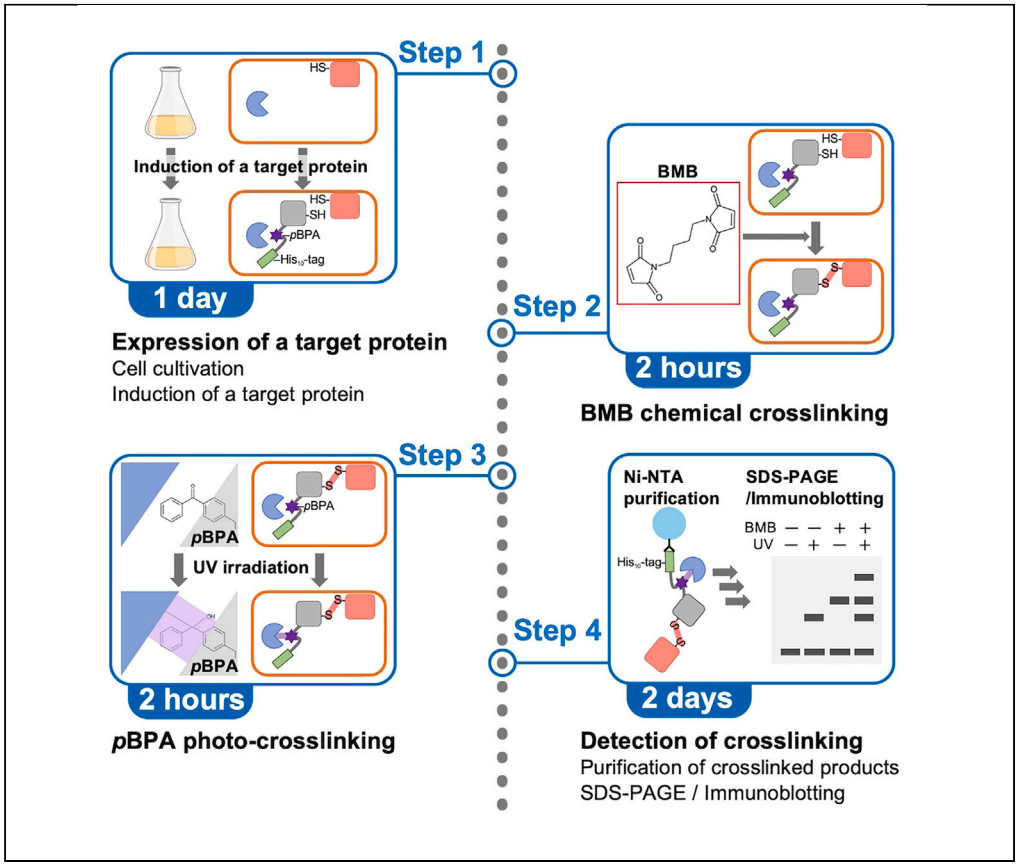

Information on protein-protein interactions is crucial in understanding protein-mediated cellular processes; however, analyzing transient and unstable interactions in living cells is challenging. Here, we present a protocol capturing the interaction between an assembly intermediate form of a bacterial outer membrane protein and β-barrel assembly machinery complex components. We describe steps for expression of a protein target, chemical crosslinking combined with *in vivo* photo-crosslinking and crosslinking detection procedures including immunoblotting. This protocol can be adapted to analyze interprotein interactions in other processes.

Ryoji Miyazaki,
Yoshinori Akiyama

m.ryoji@bs.naist.jp (R.M.)
yakiyama@infront.kyoto-u.ac.jp (Y.A.)

Highlights

Analyze protein assembly intermediates in living *E. coli* cells

Combine chemical and photo-crosslinking to capture a protein ternary complex

Applicable for analysis of protein intermediates in other organisms

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Analyzing protein intermediate interactions in living *E. coli* cells using site-specific photo-crosslinking combined with chemical crosslinking

Ryoji Miyazaki[1,3,*] and Yoshinori Akiyama[2,4,*]

[1]Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan

[2]Institute for Life and Medical Sciences, Kyoto University, Kyoto 606-8507, Japan

[3]Technical contact

[4]Lead contact

*Correspondence: m.ryoji@bs.naist.jp (R.M.), yakiyama@infront.kyoto-u.ac.jp (Y.A.)
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SUMMARY

Information on protein-protein interactions is crucial in understanding protein-mediated cellular processes; however, analyzing transient and unstable interactions in living cells is challenging. Here, we present a protocol capturing the interaction between an assembly intermediate form of a bacterial outer membrane protein and β-barrel assembly machinery complex components. We describe steps for expression of a protein target, chemical crosslinking combined with *in vivo* photo-crosslinking and crosslinking detection procedures including immunoblotting. This protocol can be adapted to analyze interprotein interactions in other processes.

For complete details on the use and execution of this protocol, please refer to Miyazaki et al. (2021).[1]

BEFORE YOU BEGIN

This approach revealed a detailed investigation of the protein-protein interactions in the membrane assembly process of *Escherichia coli* LptD (a β-barrel outer membrane protein). LptD forms a complex with lipoprotein LptE to form the lipopolysaccharide (LPS) translocon that inserts LPS into the outer membrane.[1,2] The membrane integration of LptD and the complex formation with LptE are mediated by the BAM complex (BamA/B/C/D/E), the cellular machinery for outer membrane protein biogenesis located in the outer membrane.[3–5] Periplasmic protease BepA is a dual-functional protein acting on the BAM complex to promote the normal outer membrane assembly of LptD or to degrade it when the assembly of LptD is compromised.[6] During an LptD membrane assembly event, an intermediate form of LptD simultaneously interacts with the BAM complex and BepA to form the BAM-LptD-BepA ternary complex. We investigated the interactions of the components and detected the ternary complex by using a combination of chemical- and photo-crosslinking. Crosslinkers, plasmids, and bacteria strains were prepared for this protocol.

Essential crosslinkers, plasmids and bacterial strains

1. 1,4-bismaleimidobutane (BMB), a covalent irreversible maleimide crosslinker for sulfhydryl groups of cysteine residues with a 10.9Å-spacer arm, was used in this protocol. BMB can be commercially obtained from several vendors, such as Thermo Scientific™.
2. *p*-benzoyl-L-phenylalanine (*p*BPA, also called BPA), an unnatural amino acid containing in its side chain a photo-reactive benzophenone group that forms covalent crosslinking with neighboring cellular components such as a protein or a lipid in living cells,[7,8] was used in this protocol. *p*BPA was purchased from Bachem and Watanabe Chemical Co., Ltd.

3. pEVOL-pBpF is a plasmid encoding the evolved *Methanocaldococcus jannaschii* aminoacyl-tRNA synthetase/suppressor tRNA pair that allows the incorporation of *p*BPA into proteins (at the *amber* codon site) through the *amber* suppression mechanism in a living cell.[9] pEVOL-pBpF is available from Addgene.
4. pNB91 (pMW118-*bepA(E137Q)*), a plasmid encoding BepA carrying the proteolytic active site substitution, E137Q)[10]; the substitution would stabilize the interaction between BepA and LptD.[11]
5. pRM829 (pRM294-*lptD(E733C)-his*$_{10}$),[10] a plasmid encoding the E733C mutant form of LptD-His$_{10}$ (LptD with a C-terminal decahisitidine tag) for BMB-crosslinking with the chromosomally encoded BamA protein bearing an S439C substitution.

CRITICAL: The recovery of the crosslinked products was much lower with shorter His-tags such as the widely-used hexa-His tag because the binding affinity of the decahistidine tag to the Ni-NTA agarose is higher than that of the hexahistidine tag.

6. pRM831(pRM294-*lptD(Y331amb/E733C)-his*$_{10}$),[10] a plasmid encoding LptD-His$_{10}$ carrying both the E733C substitution for the BMB-crosslinking with chromosomally encoded BamA(S439C) and the Y331*amb* substitution for photo-crosslinking with BepA.
7. RM3655 (Δ*pro-lac thi*/F′ *lacI*^{q} *Z* Δ*M15 Y*$^{+}$ *pro*$^{+}$ Δ*bepA bamA(S439C) zae-502*::Tn*10*),[10] an *E. coli* strain carrying a chromosomal *bamA(S439C)* mutant gene for BMB-crosslinking with LptD. The native chromosomal *bepA* gene was deleted in this strain.

Preparation of *E. coli* cells for crosslinking analysis

⚠ Timing: 11 days

8. Inoculate a small amount of glycerol stock of *E. coli* strain RM3655 into 1.5 mL of L medium (medium composition: 10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 5 g/L NaCl; pH adjusted to 7.2 with NaOH) in a sterile test tube.
9. Shake it overnight (16–20 h) at 30°C.
10. Prepare chemical competent cells of RM3655 by a standard protocol,[12] and transform them with pNB91.
11. Select transformants by plating on an L-agar plate supplemented with 0.4% (w/v) Glucose and 50 μg/mL ampicillin (Amp).
12. Incubate overnight (16–20 h) at 30°C.
13. Streak a transformant on an L-agar plate supplemented with 0.4% (w/v) Glucose and 50 μg/mL Amp, and incubate the plate overnight (16–20 h) at 30°C to isolate single colonies.
14. Inoculate the isolated single colony of RM3655 carrying pNB91 into 1.5 mL of L media supplemented with 0.4% (w/v) Glucose and 50 μg/mL Amp in a sterile test tube.
15. Shake it overnight (16–20 h) at 30°C.
16. Prepare the chemical competent cells of RM3655 carrying pNB91 by a standard protocol, and transform them with pEVOL-pBpF.
17. Select transformants by plating on an L-agar plate supplemented with 0.4% (w/v) Glucose, 50 μg/mL Amp and 20 μg/mL chloramphenicol (Cm).
18. Incubate overnight (16–20 h) at 30°C.

Note: Introduction of pEVOL-pBpF may result in the formation of mucoid colonies. Avoid using these colonies.

19. Streak a transformant on an L-agar plate supplemented with 0.4% (w/v) Glucose, 50 μg/mL Amp and 20 μg/mL Cm, and incubate the plate overnight (16–20 h) at 30°C to isolate single colonies.
20. Inoculate the isolated single colony into 1.5 mL of L medium supplemented with 0.4% (w/v) Glucose, 50 μg/mL Amp and 20 μg/mL Cm in a sterile test tube.

21. Shake it overnight (16–20 h) at 30°C.
22. Prepare chemical competent cells of RM3655 carrying pNB91 and pEVOL-pBpF by a standard protocol, and transform them with pRM829 or pRM831.
23. Select transformants by plating on an L-agar plate supplemented with 0.4% (w/v) Glucose, 50 μg/mL Amp, 20 μg/mL Cm, and 50 μg/mL spectinomycin (Spc).
24. Incubate overnight (16–20 h) at 30°C.
25. Streak each transformant on an L-agar plate supplemented with 0.4% (w/v) Glucose, 50 μg/mL Amp, 20 μg/mL Cm and 50 μg/mL Spc, and incubate the plate overnight (16–20 h) at 30°C to isolate single colonies.
26. Inoculate the isolated single colony into 1.5 mL of L media supplemented with 0.4% (w/v) Glucose, 50 μg/mL Amp, 20 μg/mL Cm, and 50 μg/mL Spc in a sterile test tube.
27. Shake it overnight (16–20 h) at 30°C.
28. Mix 800 μL of overnight cultures with 200 μL of sterile 80% (v/v) Glycerol in sterile cryotubes.
29. After freezing in liquid Nitrogen, store the samples at −80°C.

CRITICAL: Some strains carrying the three plasmids may grow very slowly and/or express proteins at very low levels. Cell growth and protein expression levels should be assessed before conducting crosslinking experiments.

Note: Because pEVOL-pBpF is a derivative of pACYC184 carrying the p15 replicon, the other plasmids should have a compatible replicon, such as pSC101 and pMB1(pBR322). pNB91 used in this protocol is a derivative of pSC101, while pRM829 and pRM831 are pBR322 derivatives.

Preparation of culture media containing *p*BPA

Timing: ≈ 2 h

30. Dissolve 10 g bacto-tryptone, 5 g bacto-yeast extract, and 5 g NaCl in 900 mL of MilliQ water. Adjust the pH to 7.2 with NaOH and dilute the solution to 1,000 mL to prepare the L medium.
31. Add the powder of 13.5 mg of *p*BPA (H-p-Bz-Phe-OH; Bachem) directly into 100 mL of the L medium (to make a final concentration of 0.5 mM).

Note: A higher concentration of *p*BPA (1 mM) did not significantly affect the results.

32. Homogenize *p*BPA by sonicating the medium for 1 min (or more) using a desk-top bath sonicator (W103T, Honda Electronics. Co., Ltd).

CRITICAL: The sonication step is important because *p*BPA forms large lumps that are not completely dissolved even after autoclaving without prior homogenization by sonication.

Alternatives: *p*BPA can be dissolved in 1 M NaOH solution to obtain 100−1,000 mM *p*BPA solution, which can be added to the L medium to prepare the L medium containing *p*BPA. The L- *p*BPA medium prepared by either of the methods will work well and provides essentially the same result.

33. Sterilize the solution by autoclaving.
34. Store at room temperature (20°C–25°C) away from light.

Note: The *p*BPA-containing L medium can be stored at room temperature (20°C–25°C) for several months based on our experience.

Preparation of BMB solution

⚠ Timing: ≈ 10 min

35. Dissolve 2.5 mg of BMB (Thermo Scientific™) in 500 μL of DMSO (Dimethyl sulfoxide).
36. Divide the solution into 20 μL portions, and store them in 1.5-mL tubes at −25°C.

CRITICAL: Use only once for each BMB solution to avoid freezing/thawing, which could decrease crosslinking efficiency.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-BepA antibody	Narita et al.[6]	N/A
Anti-BamA antibody	Gunasinghe et al.[13]	N/A
Goat Anti-Rabbit IgG (H + L)-HRP Conjugate	Bio-Rad Laboratories	Cat# 1706515
Bacterial and virus strains		
Escherichia coli RM3655	Miyazaki et al.[10]	N/A
Chemicals, peptides, and recombinant proteins		
BMB	Thermo Scientific™	Cat# 22331
*p*BPA	Bachem	Cat# F2800
Ni-NTA Agarose	QIAGEN	Cat# 30250
Bacto™ Tryptone	Gibco™	Cat# 211705
Bacto™ Yeast Extract	Gibco™	Cat# 212750
NaCl	Nacalai Tesque	Cat# 31320-05
NaOH	Nacalai Tesque	Cat# 315-11
Spectinomycin	Sigma	Cat# S9007-5G
Chloramphenicol	Wako	Cat# 034-10572
Ampicillin	Meiji Seika	N/A
Glycerol	Nacalai Tesque	Cat# 17018-25
Glucose	Nacalai Tesque	Cat# 16806-25
L-arabinose	Nacalai Tesque	Cat# 03306-62
IPTG (Isopropyl β-D-1-thiogalactopyranoside)	Nacalai Tesque	Cat# 19742-07
$Na_2HPO_4 \cdot 7H_2O$	Nacalai Tesque	Cat# 31725-15
Na_2HPO_4	Nacalai Tesque	Cat# 31801-05
NaH_2PO_4	Nacalai Tesque	Cat# 31720-65
TCEP-HCl (Tris(2-carboxyethyl)phosphine Hydrochloride)	Fluka	Cat# 93284
DMSO (dimethyl sulfoxide)	Cica Reagent	Cat# 2950-1B
L-Cysteine	Nacalai Tesque	Cat# 10309-12
NEM (N-ethylmaleimide)	Nacalai Tesque	Cat# 15512-24
TCA (trichloroacetic acid)	Nacalai Tesque	Cat# 34637-85
Acetone	Kishida	Cat# 000-00305
Tris(hydroxymethyl)aminomethane	Nacalai Tesque	Cat# 35434-34
HCl	Nacalai Tesque	Cat# 18321-05
EDTA (ethylenediaminetetraacetic acid)	DOJINDO	Cat# 345-01865
Triton X-100	Nacalai Tesque	Cat# 12969-25
SDS (sodium dodecyl sulfate)	Nacalai Tesque	Cat# 31607-65
Bromophenol blue	Nacalai Tesque	Cat# 05808-61
2-Mercaptoethanol	Nacalai Tesque	Cat# 21418-42
NaN_3	Nacalai Tesque	Cat# 31208-82
Skim milk	MEGMILK SNOW BRAND	N/A
Antifoam A concentrate	Sigma-Aldrich	Cat# A5633-25G
KCl	Nacalai Tesque	Cat# 28514-75

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
KH_2PO_4	Nacalai Tesque	Cat# 28721-55
Tween 20	Nacalai Tesque	Cat# 35624-15
Critical commercial assays		
ECL™ Prime Western Blotting Detection Reagents	GE Healthcare	Cat# RPN2232
Can Get Signal®	TOYOBO	Cat# NKB101
Recombinant DNA		
pEVOL-pBpF	Young et al.[9]	Addgene Plasmid #31190
pNB91 (pMW118-*bepA(E137Q)*)	Miyazaki et al.[10]	N/A
pRM829 (pRM294-*lptD(E733C)*)	Miyazaki et al.[10]	N/A
pRM831 (pRM294-*lptD(Y331amb, E733C)*)	Miyazaki et al.[10]	N/A
Other		
Image Qaunt LAS 4000 mini	Fujifilm/GE Healthcare	N/A
Blak-Ray® High Intensity Lamp, B-100AP	UVP	Cat# 95-0127-01
Easy operation OD Monitor miniphoto518R	TAITEC Co.	Cat# 0040889-000
Sonicator	Honda Electronics	Cat# W-103T
High Speed Mixer CM-1000	EYELA	Cat# 188140
Split Petri Dish 4 Sections	VWR	Cat# 4-418-03

MATERIALS AND EQUIPMENT

L medium		
Reagent	Final concentration	Amount
bacto-tryptone	1% (w/v)	10 g
Bacto-yeast extract	0.5% (w/v)	5 g
NaCl	0.5% (w/v)	5 g
1 M NaOH	N/A	1.7 mL
ddH_2O	N/A	Adjust to 1,000 mL
Total	**N/A**	**1,000 mL**

Note: Sterilize the solution by autoclaving.

Note: Add 1.2 g of agar to 100 mL of L medium (final concentration 1.2% (w/v)) to prepare an L agar plate.

Phosphate Buffer		
Reagent	Final concentration	Amount
$Na_2HPO_4 \cdot 7H_2O$	15.1 mM	4.04 g
NaH_2PO_4	4.9 mM	0.58 g
NaCl	150 mM	8.76 g
ddH_2O	N/A	Adjust to 1,000 mL
Total	**N/A**	**1,000 mL**

Note: At least one year storage at 4°C is assured.

Triton Buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl (pH 8.1)	50 mM	25 mL
Triton X-100	2% (v/v)	10 mL
NaCl	150 mM	4.38 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
100 mM EDTA (pH 7.0)	0.1 mM	0.5 mL
ddH_2O	N/A	Adjust to 500 mL
Total	**N/A**	**500 mL**

Note: At least one year storage at 4°C is assured.

SDS Buffer		
Reagent	Final concentration	Amount
1 M Tris-HCl (pH 8.1)	50 mM	5 mL
10% (w/v) SDS	1% (w/v)	10 mL
100 mM EDTA	1 mM	1 mL
ddH_2O	N/A	84 mL
Total	**N/A**	**100 mL**

Note: At least one year storage at 4°C is assured.

Note: Warm the solution at 37°C to dissolve SDS before use.

SDS Sample Buffer		
Reagent	Final concentration	Amount
0.5 M Tris-HCl (pH 6.8)	62.5 mM	6.25 mL
10% (w/v) SDS	2% (w/v)	10 mL
Glycerol	10% (v/v)	5 mL
Bromophenol blue	0.05% (w/v)	25 mg
2-mercaptoethanol	10% (v/v)	5 mL
ddH_2O	N/A	Adjust to 50 mL
Total	**N/A**	**50 mL**

Note: At least one month storage at −25°C is assured.

Note: Warm the solution at 37°C to dissolve SDS before use.

10 × PBS-Tween Buffer		
Reagent	Final concentration	Amount
NaCl	1.4 M	400 g
KCl	27 mM	10 g
Na_2HPO_4	81 mM	57.5 g
KH_2PO_4	15 mM	10 g
Tween 20	1% (v/v)	50 mL
ddH_2O	N/A	Adjust to 5 L
Total	**N/A**	**5 L**

Note: At least one year storage at room temperature (20°C–25°C) is assured.

Note: Dilute 10 ×PBS-Tween Buffer with MilliQ H_2O to prepare 1 × PBS-Tween Buffer.

BLOTTO Solution		
Reagent	Final concentration	Amount
Skim milk	5% (w/v)	50 g
10 × PBS-Tween Buffer	N/A	100 mL
2% (w/v) NaN_3	0.02% (w/v)	10 mL
Antifoam A Concentrate	N/A	Trace amount
ddH_2O	N/A	Adjust to 1,000 mL
Total	**N/A**	**1,000 mL**

Note: At least a few months storage at 4°C is assured.

STEP-BY-STEP METHOD DETAILS

Cell growth and expression of the target proteins

⚠ Timing: 1 day

In this step (Figure 1,step 1), the cells for the crosslinking analysis were grown, induced for the expression of the target proteins (LptD and BepA) from the plasmids, and harvested; the chromosomal BamA is constitutively expressed.

1. Inoculate a small amount of glycerol cell stock of RM3655/pNB91/pEVOL-pBpF carrying pRM829 or pRM831 into 1.5 mL of L medium supplemented with 0.4% (w/v) Glucose, 50 μg/mL Amp, 20 μg/mL Cm, and 50 μg/mL Spc in a sterile test tube.
2. Shake it overnight (≈16 h) at 30°C.
3. Inoculate 50 μL of the overnight culture into 5 mL of L medium supplemented with 50 μg/mL Amp, 20 μg/mL Cm, 50 μg/mL Spc, 0.5 mM *p*BPA, and 0.02% arabinose.
4. Shake at 30°C for 105 min until the optical density reached ≈0.16 (corresponding to about 1.5 × 10^8 cells/mL) with TAITEC OD Monitor.

Note: Arabinose is included to induce the expression of the evolved aminoacyl-tRNA synthetase/suppressor tRNA pair from pEVOL-pBpF.

5. Add 5 μL of 1 M IPTG in MilliQ H_2O (final concentration 1 mM) to express LptD and BepA, and shake for 3 h at 30°C. The final optical density of the cultures after induction would be ≈0.8–0.85 (corresponding to about 7.5–8.0 × 10^8 cells/mL) with TAITEC OD Monitor.
6. Put test tubes on ice to cool the cultures.

BMB-crosslinking

⚠ Timing: 2 h

In this step (Figure 1, step 2), cells expressing the target protein are treated with BMB to crosslink the two proteins (LptD and BamA).

7. Take an appropriate volume of the culture to a 5 mL centrifuge tube (Take 5 mL when final turbidity is ≈0.8 with TAITEC OD Monitor, which corresponds to about 7.5 × 10^8 cells/mL).
8. Centrifuge the tube (2,300 × g for 5 min at 4°C) and discard the supernatant.
9. Suspend the cells in 500 μL of Phosphate Buffer.
10. Centrifuged the tube (2,300 × g for 5 min at 4°C) and discard the supernatant.
11. Suspend the cells in 500 μL of Phosphate Buffer.
12. Add 10 μL of 100 mM TCEP-HCl (Tris(2-carboxyethyl)phosphine Hydrochloride dissolved in MilliQ H_2O) at a final concentration of 2 mM.

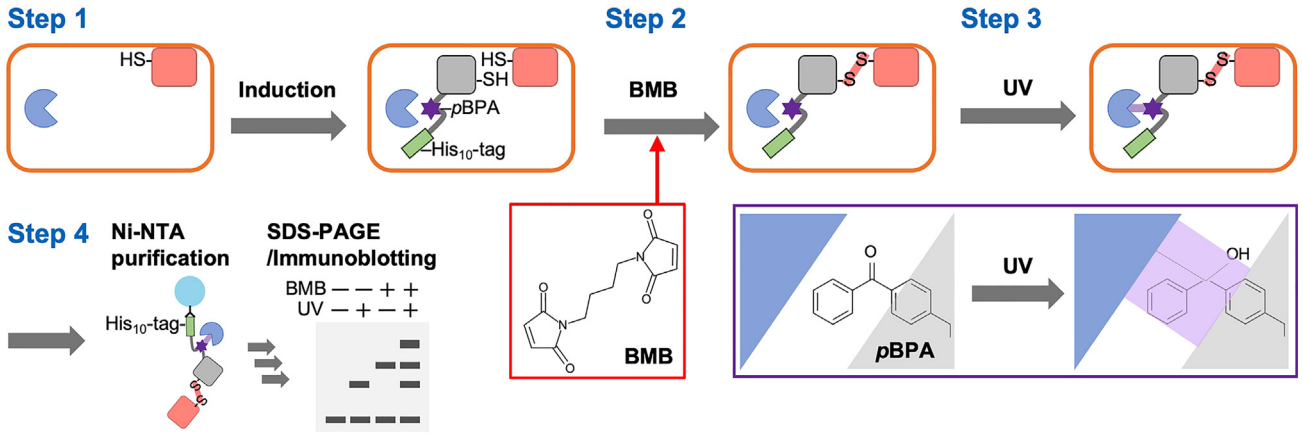

Figure 1. Workflow for site-specific *in vivo* photo-crosslinking combined with BMB-crosslinking

13. Mix vigorously at room temperature (20°C–25°C) for 20 min using High Speed Mixer CM-1000 (EYELA) to reduce the introduced Cys residues in LptD and BamA.

CRITICAL: This step is important to make the Cys residues for BMB-crosslinking in a reduced form.

14. Centrifuge the tube (2,300 × g for 5 min at 4°C) and discard the supernatant.
15. Suspend the cells in 500 μL of Phosphate Buffer.
16. Centrifuge the tube (2,300 × g for 5 min at 4°C) and discard the supernatant.
17. Suspend the cells in 500 μL of Phosphate Buffer.
18. Divide the solution into 125 μL portions in four 1.5 mL tubes. Two tubes are used for the BMB reaction and the rest for the DMSO control reaction.
19. Add 3.1 μL of DMSO (control) or 20 mM BMB in DMSO (final concentration 0.5 mM).
20. Mix vigorously at room temperature (20°C–25°C) for 40 min with High Speed Mixer CM-1000.
21. Add 12.5 μL of 100 mM L-Cysteine (final concentration 10 mM), and mix well.

Note: L-Cysteine quenches BMB and stops the crosslinking reaction.

22. Put the samples on ice for several minutes.
23. Combine the two separate BMB-treated and -untreated solutions into one tube, respectively.

*p*BPA mediated *in vivo* photo-crosslinking

⚠ Timing: 2 h

After the BMB-crosslinking, irradiate the cells with UV light to form *p*BPA-crosslinking and ternary crosslinked complexes (BamA-LptD-BepA; Figure 1, step 3).

24. Put a 150 μL portion of the cultures on Split Petri Dish 4 Sections (VWR) (Figure 2A).

Alternatives: The use of the above special dish enables simultaneous UV-irradiation to 4 different smaples without cross-contamination, but a normal petri dish can be used instead.

25. Irradiate the cells with UV light (365 nm, 22 mW/cm^2 at a distance of 5 cm) for 30 min at 4°C using B100-AP (UVP; Figure 2B). Skip this step if UV irradiation was not performed (for the control [no UV sample]).

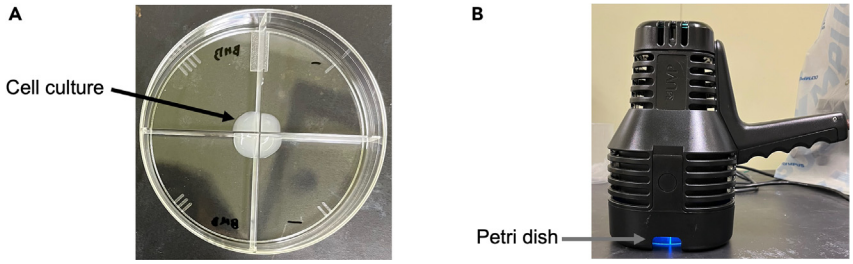

Figure 2. Detailed operations for *in vivo* photo-crosslinking experiments
(A) How to put the culture medium on Split Petri Dish 4 Sections (step 24).
(B) UV irradiation using B100-AP (step 25).

Note: UV irradiation for a long period would heat the cell culture and cause the denaturation of cellular proteins. Therefore, conducting UV-irradiation of the cells in the cold room is recommended.

26. After mixing the cultures by pipetting, take 100 μL and rapidly and thoroughly mix with 100 μL of 10% (v/v) TCA solution to denature proteins.
27. Put the tubes on ice for over 30 min.
28. Centrifuge the tube (20,400 × g for 5 min at 4°C) and discard the supernatant.
29. Add 600 μL of acetone to the pellet, and then sonicate with a desk-top bath sonicator and vortex to wash the protein precipitates.
30. Centrifuge the tube (20,400 × g for 5 min at 4°C) again and discard the supernatant.
31. Air-dry the pellets at room temperature (20°C–25°C).

Note: The pellets can be stably stored at least for a month at −25°C.

Purification and detection of crosslinked products

⚠ Timing: 2 days

In this step (Figure 1, step 4), the crosslinked product was isolated and concentrated by affinity isolation using the His_{10}-tag fused to the target protein, because the cellular levels of the ternary crosslinked products are often too low to be detected by SDS-PAGE and immunoblotting when total cell lysates are used. The purified proteins were analyzed by SDS-PAGE and Immunoblotting.

32. Add 99 μL of SDS buffer containing of 12.5 mM NEM (N-ethylmaleimide) to the pellet.

Note: NEM forms a covalent bond with free Cys residues and blocks the disulfide bond formation during subsequent processes.

33. Dissolve pellets by sonication, vortexing for 5 min and heating at 98°C for 5 min.
34. Then, put 57 μL of the SDS-solubilized proteins into a 2 mL tube containing 1,800 μL of Triton Buffer.
35. Mix by vortexing.
36. Centrifuge the tube (9,100 × g for 10 min at 4°C).
37. Transfer 1,800 μL of the supernatants to a new 2 mL tube.
38. Add 30 μL of Ni-NTA Agarose (QIAGEN) suspended in 10 mM Tris-HCl (pH 8.1).

Note: Be careful not to take the pellets.

Note: Before use, wash Ni-NTA agarose with 10 mM Tris-HCl (pH 8.1) as described below.

Pipet 100 μL of 50% Ni-NTA agarose suspension in 30% EtOH into a 1.5 mL tube containing 1 mL of 10 mM Tris-HCl (pH 8.1). Centrifuge the tube (9,100 × g for 20 s at 4°C), discard the supernatants, and suspend the Ni-NTA agarose in 10 mM Tris-HCl (pH 8.1) by adding 50 μL of 10 mM Tris-HCl (pH 8.1) and tapping.

39. Gently mix the samples overnight (12–16 h) at 4°C with vertical rotation of tubes at approximately 10 rpm.
40. Centrifuge the tubes (9,100 × g for 20 s at 4°C), discard the supernatants.
41. Wash the pellets by adding 1,000 μL of Triton Buffer and vortexing.
42. After centrifugation (9,100 × g for 20 s at 4°C), discard the supernatants.
43. Wash the pellets again by adding 1,000 μL of 10 mM Tris-HCl (pH 8.1) and vortexing.
44. Centrifuge the tube (9,100 × g for 20 s at 4°C), and completely discard the supernatants.
45. Add 80 μL of SDS Sample Buffer and mix well by tapping to elute the proteins (the His_{10}-tagged proteins are eluted by the SDS sample buffer as 2-mercaptoethanol reduces Ni in this buffer).
46. Heat the sample at 98°C for 5 min.

Note: Because BMB is an irreversible maleimide crosslinker, the BMB crosslinks are not cleaved by treatment with the 2-mercaptoethanol reducing agent, unlike disulfide bonds.

47. Centrifuge the tube (9,100 × g for 20 s at room temperature (20°C–25°C)) and collect the supernatant containing eluted proteins.
48. Separate the purified proteins by 7.5% Laemmli SDS-PAGE using a standard protocol.
49. Transfer the separated proteins on to PVDF (0.45 μm) membrane using a standard semi-dry blotting procedure.
50. Incubate the PVDF membrane in the BLOTTO solution for 30 min at 42°C for blocking.
51. Wash the PVDF membrane with PBS-Tween Buffer.
52. Incubate the PVDF membranes in Can Get Signal Solution 1 (TOYOBO) and primary antibody (anti-BepA antibody (1:10000 dilution) or anti-BamA antibody (1:20000 dilution)) at room temperature (20°C–25°C) overnight with gentle shaking (12–14 h).

CRITICAL: The time required for primary antibody treatment can often be shortened to a few hours. However, the intensity of the crosslinked bands was considerably weakened. Therefore, an overnight primary antibody treatment is strongly recommended to obtain clear results.

Note: Can Get Signal is a solution that improves the sensitivity and specificity in detection by immunoblotting.

53. Wash the PVDF membranes three times for 5–10 min with PBS-Tween buffer with gentle shaking.
54. Incubate the PVDF membranes in Can Get Signal Solution 2 (TOYOBO) with a secondary antibody (Goat Anti-Rabbit IgG (H + L)-HRP Conjugate; 1:5000 dilution) for 1 h with gentle shaking at room temperature (20°C–25°C).
55. Wash the PVDF membranes three times for 5–10 min with PBS-Tween buffer with gentle shaking.
56. Immerse the PVDF membrane in ECL prime solution for 1 min at room temperature (20°C–25°C).
57. Detect the chemiluminescence signals using the LAS4000 mini lumino-image analyzer.

EXPECTED OUTCOMES

BepA has been identified as a protease that maintains bacterial outer membrane integrity through the quality control of an essential outer membrane protein, LptD.[6] BepA directly interacts with the LptD assembly intermediate in the outer membrane on the BAM complex,[10,11] consequently promoting the maturation of normal LptD or degrading the stalled LptD on the BAM complex. The LptD intermediate was thought to form a ternary complex with the BAM complex and BepA (Figure 3A). However, the molecular mechanism of this ternary complex is indefinite.

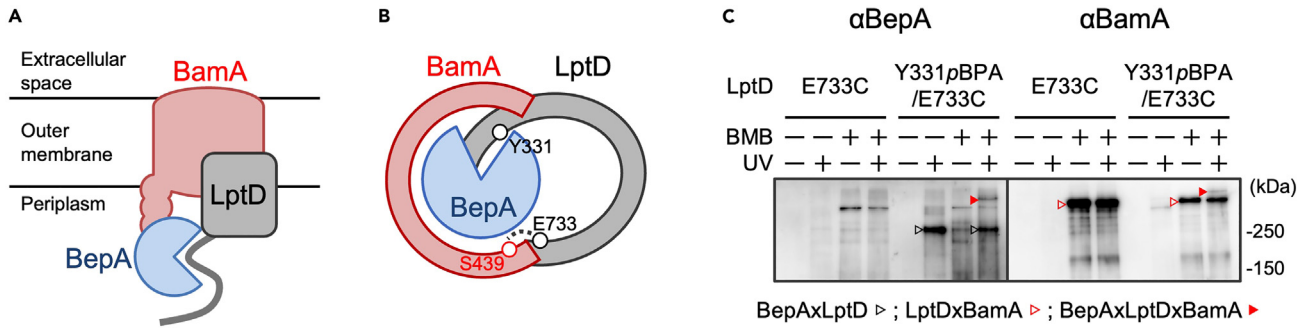

Figure 3. Results for site-specific *in vivo* photo-crosslinking combined with BMB-crosslinking

(A) A schematic cartoon of the BamA-LptD-BepA ternary complex.

(B) A schematic cartoon of the crosslinking sites of the LptD with BepA and BamA.

(C) Results for site-specific *in vivo* photo-crosslinking combined with BMB-crosslinking. The details of the operations are described in the main text.

A study revealed that a cysteine residue introduced near the N-terminal region of the BamA β-barrel (S439C) was crosslinked to a cysteine residue introduced near the C-terminal region of the LptD β-barrel (E733C) by BMB treatment (Figure 3B). On the other hand, we found that the LptD mutant harboring a *p*BPA at the position of the Y331 residue was crosslinked to BepA (Figure 3B). Therefore, as described in this protocol, we performed double crosslinking experiments by introducing *p*BPA at the position of the residue Y331 of the LptD(E733C) mutant and expressing it in the *bamA(S439C)* mutant strain. BamA and LptD were chemically crosslinked upon BMB treatment (Figure 3C, αBamA). In addition, LptD was photo-crosslinked to BepA according to the incorporation of *p*BPA at the Y331 position and UV irradiation (Figure 3C, αBepA). When cells were first treated with BMB and then UV irradiated, a band larger than the BamA-LptD and LptD-BepA crosslinked products was generated. This band represents the BamA-LptD-BepA ternary crosslinked product because it reacted with anti-BepA and anti-BamA antibodies. These results demonstrated that BepA interacts with an LptD assembly intermediate assembling on the BAM complex.

This chemical/photo double crosslinking protocol can be applied to analyze the mode of ternary interactions of other proteins in living cells.

LIMITATIONS

This method was established to analyze the ternary complex formation of the LptD intermediate with the BAM complex and BepA by combining BMB crosslinking and *in vivo* photo-crosslinking. BMB crosslinking and *in vivo* photo-crosslinking methods evaluated interactions among various proteins in many organisms. Therefore, the approach described in this protocol is potentially applicable to studying protein-protein interactions in many other biological processes. However, the application of this approach might be difficult in higher eukaryotes and some other organisms in which the use of the *in vivo* photo-crosslinking analysis is still limited compared with *E. coli* and yeast in which it has been widely used.

This crosslinking analysis utilizes Cys/*p*BPA-incorporated mutants of the target proteins that are BMB- and photo-crosslinked to other proteins. It is necessary to identify the crosslinking sites in the target proteins; however, this process is technically difficult and time-consuming. Suppose the experimentally-determined three-dimensional (3D) structures of the target proteins are available, we can focus on externally accessible residues as candidates for crosslinking residues and exclude residues buried inside the proteins in their structures. Recently, an AI system was developed that can predict 3D structures of monomeric proteins and even protein complexes with high accuracy.[14,15] The AI-predicted models would help to identify potential crosslinking residues. However, the structural prediction of proteins with flexible conformations and unstructured proteins such as

folding intermediates of proteins is still not sufficiently reliable. A systematic search for crosslinking residues may be required when targeting these proteins.

Furthermore, in analyzing assembly or folding intermediates using this protocol, it would be necessary to devise a method to stabilize the intermediate state. In some cases, stabilization may be attained using certain drugs (such as inhibitors) that bind to the relevant proteins or amino acid substitutions that retard the dissociation of the interacting proteins.

TROUBLESHOOTING

Problem 1

The cellular accumulation of target proteins is low.

Potential solution

This may be because of the low level of the *p*BPA incorporation into the *amber* site. The reason for variation between strains in the efficiency of *amber* suppression is unclear; it may be improved by using a different background strain as the host. In addition, since the efficiency depends on the culture medium, it may be improved by using the M9 minimal medium or some other appropriate media instead of the L medium used in this protocol. Although we used pEVOL-pBpF as a plasmid for *p*BPA-incorporation in this protocol, pSup-BpaRS-6TRN,[16] another plasmid for *p*BPA-incorporation, is also available. While higher amber suppression efficiency may be observed with pSup-BpaRS-6TRN, this plasmid generally causes slow cell growth.

Problem 2

The efficiency of BMB crosslinking is low.

Potential solution

One possible reason for this is the deterioration of the BMB solution. In this case, a fresh reagent should be prepared.

While BMB is appropriate for detecting the crosslinking between BamA and LptD, the use of other crosslinkers with different arm lengths, such as bismaleimidoethane (BMOE) and bismaleimidohexane (BMH) or the examination of a direct disulfide bond formation (without using a crosslinker) may be suitable to detect the crosslinking between cysteine residues in different proteins.

Problem 3

The efficiency of *p*BPA crosslinking is low.

Potential solution

Longer UV irradiation time will improve the crosslinking efficiency. The crosslinking inefficiency is often due to the low UV intensity of the irradiator used. Therefore, the use of a strong UV irradiator is recommended. In addition, the deterioration of the UV lamp of the irradiator can dramatically reduce the crosslinking efficiency. When the crosslinking efficiency appeared to be reduced, you should replace the UV lamp by a new one.

The positions of the incorporated *p*BPA in a protein may affect the crosslinking efficiency; introducing *p*BPA at other sites may improve the crosslinking efficiency. In addition, other photo-crosslinkable amino acids (such as *p*-azido-L-phenylalanine [*p*AzPA] or 3-(3-methyl-3H-diazirine-3-yl)-propamino-carbonyl-N^{ε}-L-lysine [DiZPK])[17] may be more suitable than *p*BPA in some cases.

Problem 4

Crosslinked products cannot be detected (step 57).

Potential solution

The inefficiency of BMB- and *p*BPA-crosslinking produces small amounts of crosslinked products that are hardly detected by a usual immunoblotting procedure; therefore, implement the above improvements (Problems 1 and 2). In addition, increase the antibody and/or the incubation time in the immunoblotting analysis and use a more efficient detection reagent. If these improvements do not work, the number of cells for immunoblotting analysis should be increased.

Problem 5

Many crosslinked products are detected.

Potential solution

The excessive overproduction of the *p*BPA-containing target proteins could cause artificial self-crosslinking to form a dimer or other multimers and/or crosslinking with molecular chaperones. In such cases, you should reconsider experimental conditions to reduce the expression level of target proteins.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yoshinori Akiyama (yakiyama@infront.kyoto-u.ac.jp).

Materials availability

All unique/stable reagents generated in this study are available from the lead contact, Yoshinori Akiyama (yakiyama@infront.kyoto-u.ac.jp).

Data and code availability

This study did not generate any unique datasets or code. No original data is generated in this protocol.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.M., Y.A.; methodology, R.M.; investigation, R.M., Y.A.; writing – original draft, R.M.; editing, R.M., Y.A.; funding acquisition, R.M., Y.A.; supervision, Y.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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