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Photo-cross-linking interacting proteins with a genetically encoded benzophenone

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A major challenge in understanding the networks of interactions that control cell and organism function is the definition of protein interactions[1–4]. Solid-phase peptide synthesis has allowed the photo-cross-linkable amino acid *p*-benzoyl-L-phenylalanine (*p*Bpa; Fig. 1a) to be site-specifically incorporated into peptide chains, to facilitate the definition of peptide-ligand complexes[5,6]. This method, however, is limited to the *in vitro* study of peptides and small proteins. An innovative development allows the incorporation of a site-specific photo-cross-linker into virtually any protein that can be expressed in *Escherichia coli*, thereby promoting *in vivo* or *in vitro* cross-linking of proteins[7–9]. The method relies on an orthogonal aminoacyl tRNA synthetase–$tRNA_{CUA}$ pair that incorporates *p*Bpa at the position encoded by the amber codon (UAG) in any gene transformed into *E. coli*[7] (Fig. 1b). The system described in this protocol uses two plasmids: a p15A-based plasmid to express the orthogonal tRNA and synthetase pair (pDULE) and a second plasmid containing an amber mutant of the gene of interest. To produce the photo-cross-linker–containing protein, cultures of *E. coli* carrying both plasmids are grown in the presence of the unnatural amino acid. To photo-cross-link the protein to its binding partner *in vivo* or *in vitro*, cells or purified proteins, respectively, are exposed to UV light (Fig. 2).

MATERIALS

REAGENTS

Talon metal affinity resin (BD Biosciences)

Coomassie (Bradford) Protein Assay kit (Pierce)

E. coli strain DH10B, prepared as electrocompetent cells

Elution buffer: 50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole (pH 7)

Heavy metal–containing glycerol minimal medium[10] prepared as described in the text (Step 5)

InVision His-tag In-gel stain (Invitrogen)

Lysis buffer: 1 mg/ml lysozyme, 50 mM sodium phosphate, 300 mM sodium chloride (pH 7)

PAGEr Gold precast polyacrylamide gels, 4–12% Tris-glycine, 9 × 10 cm, 1-mm thick (Cambrex)

Phosphate-buffered saline (PBS): 10 mM sodium phosphate, 140 mM sodium chloride (pH 7.2)

*p*Bpa (**Fig. 1a**; Bachem) solublized in 1 M sodium hydroxide solution (Step 6)

Petri dish (polystyrene, tight lid, 47-mm; Fisher)

Plasmid vectors: pTrcHis (Invitrogen); pDULE-*p*Bpa (**Fig. 1b**),

pDULE-Tyr (**Fig. 1b**; available on request from Peter Schultz (Schultz@scripps.edu)

Poly-Prep chromatography columns (0.8 × 4 cm; Bio-Rad)

Polystyrene 96-well plate (flat, low evaporation, 0.1–2 µl; Costar)

Selective medium: 2×YT

SOC medium

Wash buffer: 50 mM sodium phosphate, 300 mM sodium chloride (pH 7)

Isopropyl-β-D-thiogalactoside (IPTG)

PD10 columns (Amersham)

EQUIPMENT

Light panel, camera and Digigenius (SYNGENE) software for imaging and quantifying silver-stained SDS-PAGE gels

Sonic dismembrator (model 100; Fisher)

UV filters: Pyrex recrystallization dish (3-mm thick) or Petri dish (polystyrene, tight lid, 47-mm; Fisher)

UV lamp: Rayonet RPR-100 chamber photoreactor, equipped with 16 350-nm bulbs (preferred) or handheld long-wavelength UV lamp such as Entela UVG1-58 at 366 nm (acceptable)

Preparation of the mutant construct and transformation

PROCEDURE

1 | To introduce the photo-cross-linkable amino acid *p*Bpa into your protein of interest (your favorite protein; YFP), clone the gene encoding this protein (your favorite gene; *YFG*) into a standard IPTG-inducible overexpression vector (for example, pTrcHis) to create the plasmid pTrc-YFG. The proteins should be His-tagged for purification on the Talon resin.

▲ **CRITICAL STEP**

2 | In the sequence of YFP select amino acids for replacement with *p*Bpa. Mutate the codon for the selected amino acid in *YFG* to the amber codon (TAG) using a standard oligonucleotide-directed mutagenesis method to create the plasmid pTrc-*YFG*-TAG.

We used the QuikChange method (Stratagene), but other methods are acceptable.

▲ **CRITICAL STEP**

3 | Prepare four different protein production strains by transforming *E. coli* (DH10B) with the following plasmid or pair of plasmids:

1. pTrc-*YFG* (Amp^R)
2. the amber mutant, pTrc-*YFG*-TAG (Amp^R)
3. pDULE-Tyr (Tet^R) and pTrc-*YFG*-TAG (Amp^R)
4. pDULE-*p*Bpa (Tet^R) and pTrc-*YFG*-TAG (Amp^R)

To prepare each expression strain, mix 0.2 µg of total plasmid DNA with 50 µl electrocompetent *E. coli* DH10B cells and transform by electroporation (in a 2-mm cuvette at 2.5 kV). Rescue the cells in 1 ml SOC medium at 37 °C for 1 h with shaking at 250 r.p.m. Select cells carrying appropriate plasmid(s) by plating onto selective LB agar medium containing 100 mg/l ampicillin and 25 mg/l tetracycline, as appropriate (if the expression vector has a marker other than the ampicillin resistance gene, substitute with the appropriate antibiotic).

For delivering two plasmids, we find it most convenient to perform a cotransformation into DH10B electrocompetent cells and select for transformants containing both plasmids on rich-medium agar plates with both antibiotics.

➨ **TROUBLESHOOTING**

4 | For each transformed strain, select a fresh colony grown on a rich-medium agar plate containing the appropriate antibiotic(s) and grow at 37 °C to saturation in 5 ml of 2× YT medium with 100 mg/l ampicillin and 25 mg/l tetracycline, as appropriate.

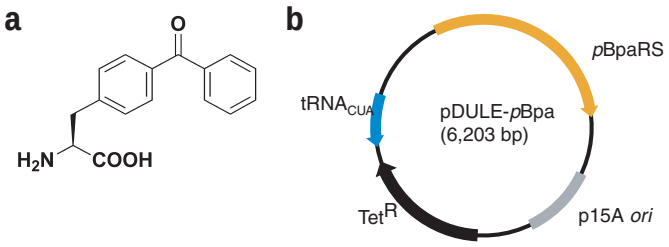

Figure 1 | Structure of *p*Bpa and a schematic of the pDULE plasmid. (**a**) The chemical structure of *p*Bpa. C-H bonds within 3 Å of the carbonyl oxygen are targets for cross-linking. (**b**) pDULE-pBpa plasmid. The pBpa-specific aminoacyl-tRNA synthetase gene is located between the *lpp* promoter and *rrnB* terminator. The $tRNA_{CUA}$ gene is located between an *lpp* promoter and an *rrnC* terminator. The plasmid contains a p15A origin and a tetracycline resistance marker. Vector map of pDULE-Tyr and sequence details are available (see **Supplementary Figure 1** online and **Supplementary Note 1** online).

5 | For each protein studied, four control expression trials should be conducted in parallel with the expression of the protein of interest as described in **Table 1.** For each expression trial, prepare one flask containing 200 ml of glycerol minimal medium supplemented with heavy metals as follows:

1.25% glycerol	160 ml	(autoclaved)
5× M9 salts	40 ml	(autoclaved)
Leucine (4 mg/ml)	2 ml	(autoclaved)
$CaCl_2 \bullet 2H_2O$ (20 mg/ml)	200 µl	(autoclaved)
$MgSO_4$ (0.12 g/ml)	200 µl	(autoclaved)
D-biotin (5 mg/ml)	40 µl	(sterile-filtered)
Thiamine-HCl (5 mg/ml)	40 µl	(sterile-filtered)
Heavy-metal stock solution	200 µl	(see recipe below)
Water	to 200 ml	(autoclaved)

To prepare 1 l of 1,000× heavy-metal stock solution, combine the following metals in 1 M HCl, stir overnight at 15–25 °C, and then filter (through a 0.2-µm filter) to remove insoluble ingredients[10]. This stock solution is stable at 15–25 °C.

$MoNa_2SO_4 \bullet 2H_2O$	500 mg
$CoCl_2$	250 mg
$CuSO_4 \bullet 5H_2O$	175 mg
$MnSO_4 \bullet H_2O$	1 g
$MgSO_4 \bullet 7H_2O$	8.75 g
$ZnSO_4 \bullet 7H_2O$	1.25 g
$FeCl_2 \bullet 4H_2O$	1.25 g
$CaCl_2 \bullet 2H_2O$	2.5 g
H_3BO_3	1 g
1 M HCl	to 1 l

6 | Prewarm each flask of medium at 37 °C for 1 h with shaking at 250 r.p.m. Add the antibiotics and *p*Bpa as described in **Table 1.** To obtain a final concentration of 1 mM *p*Bpa (in flask #5), dissolve 54 mg of *p*Bpa in 220 µl of 1 M sodium hydroxide, then add to 200 ml of prewarmed medium to give a final concentration of 1 mM.
Note that the concentration of antibiotics is reduced in these cultures to maximize protein expression (50 µg/ml ampicillin and 2.5 µg/ml tetracycline).
▲ CRITICAL STEP

7 | Centrifuge 1 ml of each of the saturated cell cultures (from Step 4) for 10 min at 1,000*g* and remove 0.75 ml of the supernatant. Resuspend the cells in the remaining medium (~0.25 ml) and add each concentrated culture to the appropriate flask as described in **Table 1.** Prepare two 1-ml aliquots of concentrated cultures of the fourth strain (transformed with both pDULE-*p*Bpa plasmid and pTrc-YFG-TAG) for trials 4 and 5; one of these should be added to the fourth flask, the other to the fifth flask containing 1 mM *p*Bpa (**Table 1**).

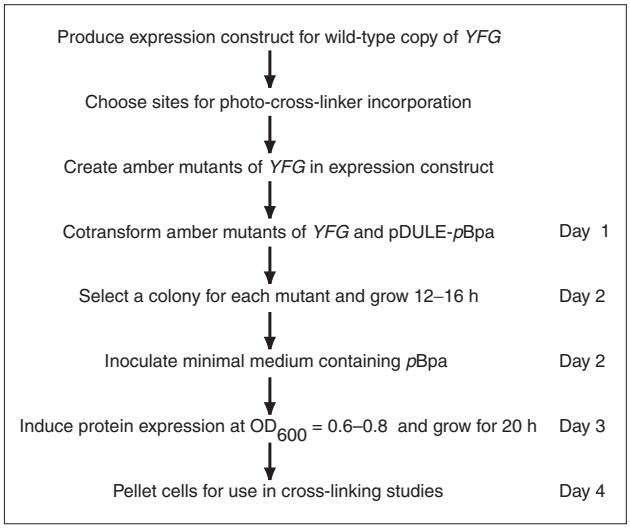

Figure 2 | The steps to express a protein with a site-specifically incorporated *p*Bpa in *E. coli*. A timeline is included to the right of the flowchart.

Table 1 | Parallel overexpression series for each protein site studied

Expression trial	(1) Native protein (positive control)	(2) Background UAG (negative control)	(3) Tyr-containing protein (positive control)	(4) No *p*Bpa control (negative control)	(5) *p*Bpa-containing protein
YFP expression vector	pTrc-*YFG*	pTrc-*YFG*-TAG	pTrc-*YFG*-TAG	pTrc-*YFG*-TAG	pTrc-*YFG*-TAG
pDULE vector	None	None	pDULE-Tyr	pDULE-*p*Bpa	pDULE-*p*Bpa
*p*Bpa	None	None	None	None	1 mM
Antibiotics	Ampicillin (50 μg/ml)	Ampicillin (50 μg/ml)	Ampicillin (50 μg/ml) and tetracycline (2.5 μg/ml)	Ampicillin (50 μg/ml) and tetracycline (2.5 μg/ml)	Ampicillin (50 μg/ml) and tetracycline (2.5 μg/ml)

8 | Incubate the cultures at 37 °C with shaking at 250 r.p.m. until they reach OD_{600} = 0.6–0.8 (~18–20 h for the control strains); then induce protein expression by adding IPTG to a final concentration of 1 mM (48 mg of IPTG to each 200-ml culture). Continue incubating at 37 °C for an additional 20 h with shaking at 250 r.p.m.; pellet each culture in four 50-ml aliquots by centrifugation at 5,000*g*. *When growing multiple cultures in parallel, it is common for them to double at slightly different rates.*
■ **PAUSE POINT** These cell pellets may be stored at –80 °C for use in Steps 9, 14 and 18.

Testing protein expression

9 | Before setting up the cross-linking experiments, purify protein from each of the five cultures, using the BD Talon Affinity Resin Native Purification protocol VI B, which is summarized in Steps 9–12. Resuspend one pellet from 50 ml of cells of each culture (Step 8) in 1 ml of lysis buffer and place on ice for 20 min. Sonicate the samples at 4 °C until the viscosity is visibly reduced and then centrifuge at 20,000*g* at 4 °C for 30 min.

10 | Prepare the Talon resin by suspending 150 μl of the resin (bed volume) in 1.5 ml wash buffer (ten times the bed volume). Centrifuge the resin at 700*g*, remove and discard supernatant, and repeat the wash process twice. *Prepare five aliquots of resin, one for each lysate. Note that Steps 11 and 12 deal with purification of one sample.*

11 | Add the cleared lysate from Step 9 to 150 μl of washed Talon resin, and gently mix at 15–25 °C for 20 min. Centrifuge the samples at 700*g* to pellet the resin, and then carefully remove and discard the supernatant.

12 | Resuspend the resin in 1 ml of wash buffer, and transfer the sample to a Poly-Prep chromatography column. Allow the column to drain by gravity flow, and wash with 5 ml of wash buffer. Elute the protein in 2.5 ml of elution buffer.

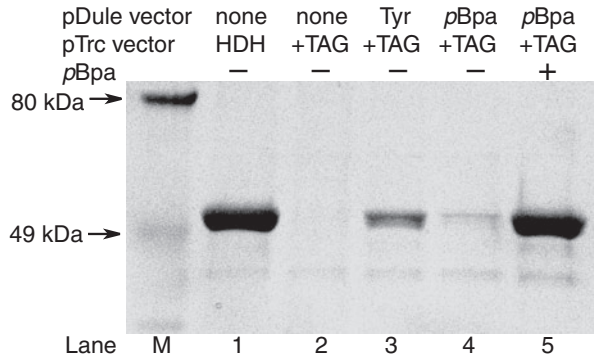

Figure 3 | Analysis of *p*Bpa-containing protein production. Positive and negative controls for protein production separated by SDS-PAGE and silver-stained. In this example the protein of interest was histidinol dehydrogenase (HDH). The lysate from cells carrying pTrc-HDH yields 28 mg/l protein, pTrc-HDH225-TAG with pDULE-pBpa yields 26 mg/l of pBpa-containing protein, and pTrc-HDH225-TAG with pDULE-Tyr yields 11 mg/l of tyrosine-containing protein (all protein samples are >95% pure). Lanes 1–5, protein expression results from the plasmids indicated and match the controls in **Table 1**. M, broad-range prestained markers (Bio-Rad). Lane 1, native protein from pTrc-*HDH* (pTrc-*YFG*). Lane 2, background negative control pTrc-*HDH*-225TAG (pTrc-*YFG*-TAG). Lane 3, tyrosine-containing protein from pTrc-*HDH*-225TAG with pDULE-Tyr. Lane 4, negative control from pTrc-*HDH*-225TAG with pDULE-*p*Bpa. Lane 5, *p*Bpa-containing protein from *HDH*-225TAG with pDULE-*p*Bpa and *p*Bpa in solution. For pTrc-*HDH* and pTrc-*HDH*-225TAG nucleotide sequences see **Supplementary Note 1**, and the plasmids are available on request from R.A.M. (rmehl@fandm.edu).

13 | Analyze an aliquot of each sample by SDS-PAGE.
The results of purification from cell lysates of strains carrying our control plasmids are shown in ***Figure 3****.*
➨ TROUBLESHOOTING
▲ CRITICAL STEP

14 | Resuspend a cell pellet from the third and the fifth cultures (Step 8), each in 30 ml of PBS. Centrifuge immediately at 3,000*g* and resuspend in 12 ml PBS. Place 3-ml aliquots into each of four disposable 47-mm polystyrene Petri dishes.

15 | Place the zero time point sample at 4 °C for 2 h and place the three remaining samples in a Rayonet RPR-100 chamber photoreactor at 4 °C with the cooling fan on. Irradiate the samples: one sample for 30 min, the second for 60 min and the third for 120 min, removing each sample to 4 °C after irradiation for the time remaining until each has remained at 4 °C for 2 h, including the irradiation period.
▲CRITICAL STEP

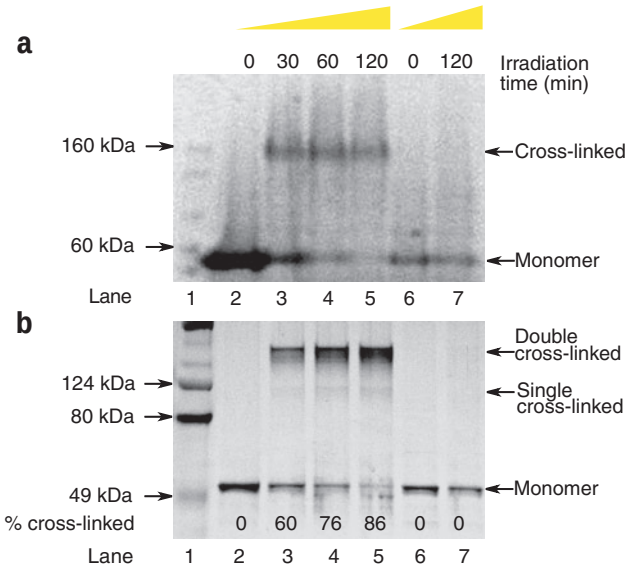

Figure 4 | Irradiation time courses of HDH-225*p*Bpa and HDH-225Tyr. (**a**) *In vivo* irradiation: SDS-PAGE separation and His-tag stain analysis. Lane 1, His-tag stain molecular markers. Lanes 2–5, whole-cell lysates from equal amounts of cells containing HDH-225*p*Bpa protein irradiated for the times indicated. Lanes 6 and 7, whole-cell lysates from equal amounts of cells containing HDH-225Tyr protein irradiated for the times indicated. (**b**) *In vitro* irradiation: Identical concentration of purified pBpa-HDH and Tyr-HDH protein were used in the experiment; the products were silver-stained after separation by SDS-PAGE. Lane 1, broad-range prestained markers (Bio-Rad). Lanes 2–5, equal amounts of pure HDH-225*p*Bpa protein irradiated for the times indicated, and lanes 6 and 7, the HDH-225Tyr protein. A single cross-linked dimer migrates at dimer molecular weight (120 kDa), whereas a double cross-linked dimer migrates with an apparent molecular weight of 150 kDa.

Photo-cross-linking of proteins *in vivo*

16 | Remove each sample from the dish completely using 1 ml of PBS to aid in washing. Centrifuge 1 ml of each sample for analysis by SDS-PAGE.
The remaining 3 ml can be centrifuged and stored at –80 °C for purification and analysis.

17 | Resuspend the pellets from the 1-ml sample of irradiated cells in 50 µl of PBS and mix with 50 µl of 2× SDS load buffer. Heat the samples at 100 °C for 10 min (mixing intermittently); remove cellular debris by centrifugation for 5 min at 16,000 r.c.f. Load 25-µl samples on a 4–12% Tris-glycine gel and run the electrophoresis at 120 V until the loading dye migrates out of the gel. Stain the gel with InVision His-tag In-gel stain by incubating the gel in the staining solution for 12 h. Visualize cross-linked and not cross-linked His-tagged protein with a standard DNA light box and camera as indicated by the manufacturer.
Example results using the control constructs are shown in ***Figure 4a****.*
➨ TROUBLESHOOTING
▲ CRITICAL STEP

18 | Using pelleted cells from Step 8, purify protein as described in Steps 9–12. Exchange the buffer of the eluted protein (2.5 ml) to PBS, using a PD10 column. Measure the protein concentration using the Coomassie Protein Assay kit and dilute the protein samples for cross-linking.
In the example shown in ***Figure 4b****, we cross-linked at 60 µg/ml protein.*

Photo-cross-linking protein containing *p*Bpa *in vitro*

19 | For each time point, aliquot 200 µl of protein sample into the wells of a polystyrene 96-well plate and cover with the tight polystyrene lid. Place the plate and filter into a Rayonet RPR-100 photoreactor at 4 °C and irradiate as described in Step 15 with a cooling fan. After each time point (0, 30, 60 and 120 min), remove each sample to 4 °C after irradiation for the time remaining until each has remained at 4 °C for 2 h.
➨ TROUBLESHOOTING
▲ CRITICAL STEP

20 | Analyze the extent of cross-linking by SDS-PAGE, which resolves the slowly migrating covalently cross-linked products from the starting proteins. Prepare samples for SDS-PAGE by combining 20 µl of each with 20 µl of 2× SDS load dye and heat at 100 °C for 10 min. Cool the samples and load 25 µl directly onto a SDS-PAGE gel. Run the electrophoresis at 120 V until the loading dye migrates out of the gel, and analyze by silver staining the gel.
Example results using the control constructs are shown in ***Figure 4b***.
➨ **TROUBLESHOOTING**

21 | To determine the percentage of cross-linking, integrate the band density corresponding to the cross-linked and not cross-linked material (we used Digigenius). Then calculate the fraction cross-linked using the equation

$$\frac{\text{number of counts cross-linked}}{\text{number of counts cross-linked + number of counts not cross-linked}} \times 100 = \text{percent cross-linking efficiency}$$

TROUBLESHOOTING TABLE

PROBLEM	SOLUTION
Step 3 Colonies do not grow after cotransformation.	Cotransformation with both plasmids and direct selection for colonies that are both ampicillin and tetracycline resistant will have a lower transformation efficiency than transformation with single plasmids. It is therefore advisable to perform sequential transformation. First transform with pDULE plasmids and select on rich-medium agar plates containing tetracycline; then make competent cells, and transform with the pTrc-*YFG*-TAG plasmid into the cells containing the pDULE plasmid.
Step 13 Little or no mutant protein is produced.	This may have several causes. Analyze the controls to determine which of the following applies.
The mutant protein may be toxic.	If this is the case, the culture may not reach an OD_{600} of 0.6. Grow cells in medium containing 2% glucose instead of glycerol, pellet when culture reaches an OD_{600} of 0.6 and resuspend in glycerol-based medium containing IPTG. Take aliquots at 1-h time intervals and visualize protein expression as described (Step 9). If toxicity is still a problem, use another p15A-compatible expression vector such as the arabinose-inducible pBAD system (Invitrogen) for producing the mutant protein.
The mutant *p*Bpa- and tyrosine-containing mutant proteins are poorly expressed.	Suppression may decrease protein yield relative to the wild-type protein. The nucleotides proximal to the amber codon can have an effect on suppression efficiency[11]. If a C or T directly follows the amber codon in the gene, change this nucleotide to A or G, and repeat the expression experiment with the new mutant protein.
The mutant *p*Bpa-containing protein is poorly expressed, but the tyrosine-containing protein expresses well.	There is a structural problem with incorporating *p*Bpa. Try incorporating *p*-azido-L-phenylalanine at the same sites using pDULE-pAz. Alternatively, substitute another amino acid in the protein with *p*Bpa.
Step 17 No *in vivo* cross-linking is observed by In-gel stain.	Protein (monomer or cross-linked protein complexes) may be at too low of a concentration to be easily visualized by In-gel stain in cell lysate. Purify the protein to concentrate it and assay cross-linking by silver-staining of SDS-PAGE gel. Denatured protein purification and gel analysis can be used if protein insolubility is suspected.
	Incorrect lamp or filter setup. Check that your lamp and filter setup allows reproduction of the *in vivo* cross-linking experiment shown in **Figure 4a**.

TROUBLESHOOTING TABLE (cont.)

PROBLEM	SOLUTION
Step 19 No *in vitro* cross-linking is observed.	Increase the irradiation time with UV light. Increase the concentration of protein. Check that your lamp and filter setup allows reproduction of the *in vitro* cross-linking shown in Figure 4b. The stability or solubility of your protein when cross-linked may be compromised. Make sure to mix irradiated samples well before SDS-PAGE.
Step 20 Several low-mobility bands detected by SDS-PAGE.	If you are cross-linking a homodimer, the singly cross-linked dimer is likely to migrate differently than the doubly cross-linked protein. Multiple bands may also reflect the interaction of your protein in several orientations or conformations.

CRITICAL STEPS

Step 1 The overexpression vector does not have to be a pTrc vector. But the vector you choose must not use a tetracycline resistance selection marker and should contain an origin of replication compatible with the p15A origin of replication. Most common expression vectors meet these criteria; common examples of compatible vectors include those derived from pUC or pBR322, such as pET vectors (Novagen).

Step 2 Ideal sites for mutagenesis will be amenable to substitution: that is, mutagenesis to *p*Bpa will not alter protein function or expression. As *p*Bpa is an aromatic hydrophobic amino acid, is likely that tyrosine, phenylalanine or tryptophan residues will be the most amenable to replacement with the photo-cross-linker. Because it is often not clear *a priori* whether an amino acid will be amenable to substitution, it is worth constructing several mutants in parallel. We recommend that the interface site also be replaced with tyrosine using pDULE-TyrRS via suppression. We have seen in several cases that this serves as very good positive control for protein production and interface disruption.

Step 6 Use freshly prepared amino acid stock. The medium must be prewarmed; otherwise the amino acids may come out of solution. We occasionally observe amino acids precipitating out of solution on addition, but this has no effect on protein expression.

Step 13 The yield of proteins containing *p*Bpa produced with the pDULE plasmid may vary. Expression of protein containing *p*Bpa at several sites in the protein interface of three different homodimers gave yields of 6–26 mg/l pure *p*Bpa-containing protein from the medium described (I.S.F. and R.A.M., unpublished observations). The protein yield normally reflects the production levels of native protein in this medium. Tyrosine-containing protein yields from pDULE-Tyr are often lower than both the native and *p*Bpa-containing protein yields.

Steps 15 and 19 Many long-wavelength UV lamps emit a considerable amount of light at shorter wavelengths, which can damage protein samples. To minimize the damaging effects of short-wavelength UV light when using high-power lamps, we recommend filtering the output to remove light with wavelengths <280 nm. Filtering light can reduce cross-linking efficiencies because filters rarely allow 100% transmittance of long-wavelength light required to activate the cross-linker; therefore, if filters are used, some protein systems may require longer irradiation time. We recommend checking the percentage of transmittance of your filter over a range of wavelengths using a UV-visible spectrometer. First, take a background scan with the door open from 200 to 450 nm. Then, place your filter in the light path and, with the door open, scan the same range. The transmittance trace will inform you what fraction of the light is transmitted to your sample at different wavelengths. The polystyrene Petri dish lids recommended above filter out all light below 280 nm but allow 30% and 65% transmittance at 300 nm and 350 nm, respectively. Pyrex and window glass filters are more effective at removing damaging light if photodamage is a concern (3-mm Pyrex glass: 15% and 90% transmittance at 300 nm and 350 nm, respectively; 3-mm window glass: 0% and 90% transmittance at 300 nm and 350 nm, respectively).

When irradiating many samples in parallel, the large chamber of the Rayonet RPR-100 photoreactor allows for each sample to receive the same amount of light. Handheld lamps are commonly used to cross-link benzophenones, but they do not deliver consistent spectral intensity over large surface areas. We have seen a consistent 20–30% decrease in cross-linking efficiencies when using the handheld Entela UVG1-58 UV lamp at 366 nm as compared to the Rayonet RPR-100 chamber photoreactor (I.S.F. and R.A.M., unpublished data). The sample size for irradiation can be varied considerably with consistent results as long as the surface area to sample size ratio is kept consistent. Be aware that long irradiations of small volumes can result in a decrease in sample volume. The type of filter does partly determine the irradiation time and amount of protein damage.

Note also that UV radiation can cause serious damage to eyes and skin. UV-protective face shield and gloves must be worn if in contact with irradiation sources for cross-linking and In-gel stain visualization.

Step 17 *In vivo* cross-linking can be monitored by many purification and detection methods. The method using InVision His-tag In-gel stain eliminates most variables that would prevent the detection of cross-linked protein. The incorporation of *p*Bpa into proteins, as with any substitution with natural amino acids, may alter the protein's stability, thereby affecting the ease of protein purification and analysis.

COMMENTS

The pDULE-*p*Bpa plasmid allows the *in vivo*, facile, site-specific incorporation of the photo-cross-linking amino acid, *p*Bpa, into proteins of almost any length. The amino acid is incorporated with high efficiency, fidelity and specificity. Because the pDULE-*p*Bpa plasmid is compatible with common high-copy protein production plasmids, excellent protein yields can be obtained.

With exposure of cells to light, protein-protein interactions can be cross-linked *in vivo* and rapidly visualized without the need for purification. Moreover, complementary studies can easily be performed *in vitro* (with crude cell extract or purified protein components). Site-specific cross-linking can help define a protein's binding partners and the topology of complexes. Expression of proteins containing *p*Bpa at physiologically relevant concentrations in *E. coli* or eukaryotic cells[12,13] may allow the definition of weak or transient protein interactions that can be irreversibly trapped by photo-cross-linking. Such interactions are probably not captured by the noncovalent affinity chromatography complex purification methods now used to map the protein interaction networks of cells[3,4].

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

J.W.C. and R.A.M. thank Peter Schultz for his support in the development of some of the vectors described in this protocol.

SOURCE

This protocol was provided directly by the authors listed on the title page. For further details on the composition of media, standard buffers and standard procedures, see Sambrook, J. & Russell, D.W., eds. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 2001).

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