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In-cellulo chemical cross-linking to visualize protein-protein interactions

Shirsha Saha, Ashutosh Ranjan, Monika Godara, and Arun K. Shukla*

Department of Biological Sciences and Bioengineering, Indian Institute of Technology, Kanpur, India

**Corresponding author: e-mail address: arshukla@iitk.ac.in*

Chapter outline

Abstract

Reversible protein-protein interaction in cells is an integral and central aspect of intracellular signaling mechanisms. This allows distinct signaling cascades to become active upon stimulation with external signal resulting in cellular and physiological responses. Several distinct methods are currently available and utilized routinely to monitor protein-protein interactions including co-immunoprecipitation (co-IP). An inherent limitation associated with co-IP assay

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however is the inability to efficiently capture transient and short-lived interactions in cells. Chemical cross-linking of such transient interactions in cellular context using cell permeable reagents followed by co-IP overcomes this limitation, and allows a simplified approach without requiring any sophisticated instrumentation. In this chapter, we present a step-by-step protocol for monitoring protein-protein interaction by combining chemical cross-linking and co-immunoprecipitation using GPCR-β-arrestin complex as a case example. This protocol is based on previously validated method that can potentially be adapted to capture and visualize transient protein-protein interactions in general.

1 Introduction

Nearly every signaling pathway in eukaryotic cells depends on non-covalent protein-protein interactions and formation of multi-protein complexes (Jones & Thornton, 1996; Wodak, Vlasblom, Turinsky, & Pu, 2013). While some of these interactions are relatively stable, others are rather transient in nature, occurring in response to specific stimuli or perturbation of cellular milieu followed by rapid dissociation (McDowall, Scott, & Barton, 2009; Pagel et al., 2005; Slater, Miller, & Kontoyianni, 2020). There are numerous approaches currently available to measure protein-protein interactions in cellular context, both, under basal condition and upon exposure to specific ligand or stimuli (Rao, Srinivas, Sujini, & Kumar, 2014; Slater et al., 2020; Titeca, Lemmens, Tavernier, & Eyckerman, 2019; Westermarck, Ivaska, & Corthals, 2013). Some of these methods such as those based on resonance energy transfer often require genetic fusion of large fluorophores to the interaction partners, and therefore, have a potential caveat of influencing the spatio-temporal dynamics of interactions (Eidne, Kroeger, & Hanyaloglu, 2002; Jares-Erijman & Jovin, 2006; Matoulkova & Vojtesek, 2014). Assays based on enzyme-complementation also involve an analogous strategy of engineering enzyme fragments fused to the interaction partners and therefore, present similar caveats (Dixon et al., 2016; Morell, Ventura, & Aviles, 2009; Wehrman, Kleaveland, Her, Balint, & Blau, 2002).

Co-immunoprecipitation (co-IP) assays, where the interaction of two proteins can be measured by pulling down one of the interaction partners using an epitope tag or an antibody, followed by monitoring the co-elution of the partner protein using Western blotting, offers a robust alternative approach. However, a potential limitation of this assay may arise from the affinity of the interaction partners and temporal pattern of interaction, which may restrict the utility of this approach in detecting transient and low-affinity interactions. Integrating a chemical cross-linking step using cell permeable bifunctional cross-linkers that can potentially stabilize even transient interactions based on proximity of the partner proteins overcomes this limitation of co-IP approach to a significant extent (Fig. 1)

One of the key areas where chemical cross-linking combined with co-IP assay has been utilized extensively is to measure agonist-induced interaction of G protein-coupled receptors (GPCRs) with their regulatory proteins called β-arrestins

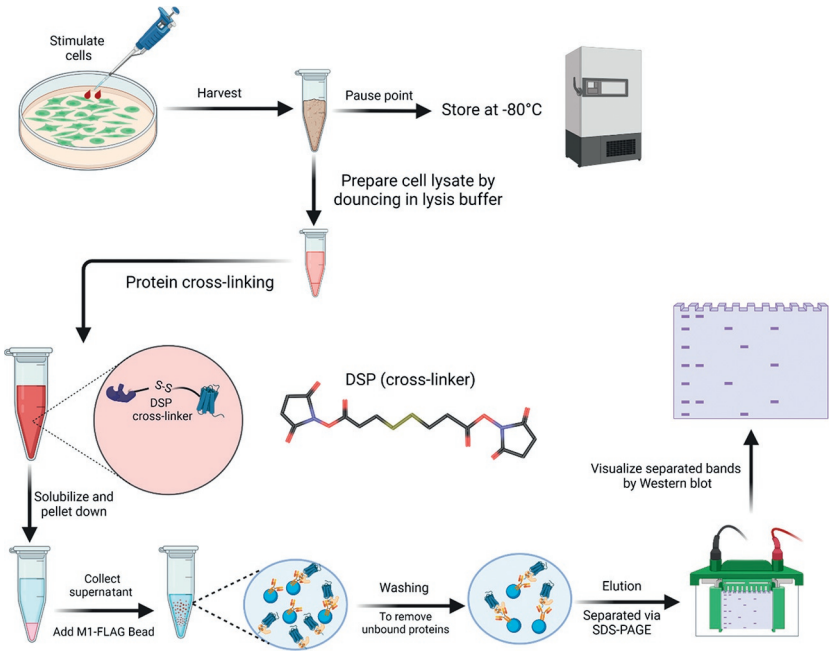

FIG. 1

A schematic representation of chemical cross-linking and co-immunoprecipitation (co-IP) assay. Cultured cells expressing the proteins of interest are stimulated with appropriate concentration of agonist followed by addition of cell permeable chemical cross-linker. Subsequently, the cross-linking reaction is terminated using appropriate quencher reagent, cells are lysed and one of the interaction partners is immunoprecipitated using either an antibody or epitope tag. Afterward, co-elution of the second protein, i.e., the potential interaction partner is detected using Western blot.

(Ghosh et al., 2019; Baidya et al., 2020b; Dwivedi-Agnihotri et al., 2020; Pandey et al., 2021, 2019; Shenoy et al., 2006). GPCRs, the largest family of cell surface proteins are localized primarily in the plasma membrane while β-arrestins are expressed as cytosolic proteins (DeWire, Ahn, Lefkowitz, & Shenoy, 2007). Upon agonist-stimulation, β-arrestins translocate to the plasma membrane to interact with activated GPCRs, and subsequently, either dissociate from the receptors or facilitate their endocytosis through stable interaction (Oakley, Laporte, Holt, Caron, & Barak, 2000). Agonist-induced receptor phosphorylation is a key determinant of β-arrestin binding and spatial pattern of receptor phosphorylation often drives subsequent functional outcomes (Gurevich & Gurevich, 2004, 2013; Ranjan, Dwivedi, Baidya, Kumar, & Shukla, 2017; Reiter & Lefkowitz, 2006). Monitoring GPCR-β-arrestin interaction using co-IP assay involves detergent solubilization of the receptor from the plasma membrane, which typically results in dissociation of β-arrestins.

To overcome this limitation, chemical cross-linking of the GPCR-β-arrestin complexes formed in cells upon agonist-stimulation has been developed and optimized (Ghosh et al., 2019; Baidya et al., 2020a, 2020b; Dwivedi-Agnihotri et al., 2020; Pandey et al., 2021, 2019; Shenoy et al., 2006). Using cell permeable bifunctional cross-linkers allows the stabilization of GPCR-β-arrestin complexes in cells that can subsequently be captured by co-IP and detected by Western blotting. This approach of chemical cross-linking combined with co-IP has been utilized in a large number of research publications, and it continues to serve as a robust method to detect the interaction of β-arrestin with GPCRs without requiring any sophisticated instrumentation. This approach has been successfully utilized previously to qualitatively compare the efficacy of different ligands to promote GPCR-β-arrestin interaction and for comparing the profile of β-arrestin interaction of receptor mutants with the wild-type receptor (Ghosh et al., 2019; Baidya et al., 2020a, 2020b; Dwivedi-Agnihotri et al., 2020; Pandey et al., 2021, 2019; Shenoy et al., 2006).

In this chapter, we present a step-by-step protocol for chemical cross-linking combined with co-IP to visualize agonist-induced GPCR-β-arrestin interaction as a case example. We also discuss the potential problems that may be encountered while employing this method and provide troubleshooting guidelines to address these problems. While we present the protocol in the context of measuring GPCR-β-arrestin interaction, it should be directly adaptable for protein-protein interactions in general with relatively minor modifications and optimization.

2 Materials and methods

2.1 Reagents for cell culture and transfection

2.1.1 Cell culture related plastic ware and equipment

- 10 cm plates
- CO_2 incubator
- Barrier micro-tips
- Laminar flow cabinet
- Sterile pipettes

2.1.2 Cell culture media and associated consumables

- Dulbecco's Modified Eagle Medium, DMEM (Cellclone, Cat. no. CC3004)
- GIBCO Fetal Bovine Serum (Thermo Fisher Scientific, Cat. no. 10270-106FBS)
- Phosphate Buffer Saline (PBS) (Sigma Aldrich, Cat. no. D1283)
- GIBCO Penicillin-Streptomycin (Thermo Fisher Scientific, Cat. no. 15140122)
- Trypsin-EDTA

2.1.3 Reagents for transfection and subsequent harvesting of cells

- Polyethyleneimine (PEI) (Polysciences, Cat. no. 23966)
- Expression plasmid construct with the gene encoding GPCR of interest
- Expression plasmid construct with the gene encoding interacting partner of interest

- HEK-293T (ATCC, Cat. no. CRL-3216)
- Receptor ligands (for stimulation prior to harvesting)

2.2 Reagents for cross-linking co-immunoprecipitation and equipment

- Benzamidine hydrochloride (SRL, Cat. no. 93014 (0248255))
- Bovine Serum Albumin (BSA) (SRL, Cat. no. 83803 (0140105))
- Chemicals for different buffers (Tris-HCl, SDS, β-mercaptoethanol, Glycine, Methanol, Tween-20, Hydrochloric acid)
- Chemi-Doc imaging system
- Dimethyl sulfoxide (DMSO)
- DSP (3,3′-Dithiodipropionic acid di(N-hydroxysuccinimide ester); Sigma Aldrich, Cat. no. D3669)
- EDTA (Ethylenediaminetetraacetic acid; Merck, Cat. no. 6381-92-6)
- ECL reagents (Promega, Cat. no. W1015)
- FLAG peptide (Genscript)
- HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.4 (SRL, Cat. no. 7365-45-9)
- Horseradish peroxidase (HRP) coupled anti-FLAG M2 antibody (Sigma Aldrich, Cat. no. A8592)
- Horseradish peroxidase (HRP) coupled anti-rabbit IgG secondary antibody (Genscript, Cat. no. A00098)
- Lauryl Maltose Neopentyl Glycol (L-MNG) (Anatrace, Cat. no. NG310, CAS no.1257852-96-2)
- Microcentrifuge tubes
- Sodium Chloride (NaCl)
- Tris-HCl pH 8.0
- Phosphatase inhibitor cocktail (PhosStop; Roche, Cat. no. 4906837001)
- Phenylmethane Sulfonyl Fluoride (PMSF) (SRL, Cat. no. 84375)
- 4–20% precast gradient gel (Bio-Rad, Cat. no. 4561-093)
- Protein molecular weight marker (Qiagen, Cat. no. PG-PMT2922)
- Polyvinylidene fluoride (PVDF) membrane for protein transfer (BioRad, Cat. no. 1620177)
- Rabbit anti-β-arrestin antibody (CST, Cat. no. 4674)
- Regular micro-tips (1000, 200, 20 μL)
- Suitable power supply and SDS-PAGE apparatus
- Suitable power supply and Western blot apparatus for Semi-Dry transfer

2.3 Procedure

The method presented here is adapted based on our previous studies to measure the interaction of GPCRs with β-arrestins, however, it can be extended to measure physical association between GPCRs and other effector proteins, for instance, G-proteins or GRKs (GPCR kinases).

2.3.1 Preparing cell pellets co-expressing proteins of interest

2.3.1.1. The proteins of interest, i.e., the receptor and β-arrestins are expressed in HEK293T cells, which are cultured as per standard protocol. Briefly, cells are maintained in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) Penicillin/Streptomycin (10,000 U/mL) in 10 cm plates. Ideally, cells are sub-cultured at a confluency level of 70–80%.

Critical: Use media pre-warmed to room temperature for sub-culturing the cells.

! Caution: Maintain sterile condition while handling cell culture to avoid risk of contamination. Filter reagents using a 0.22 μm filter before use, wherever necessary.

2.3.1.2. For transfection, sub-culture cells 24 h ahead at a density of 3×10^6 cells per 10 cm plate. Aspirate media and gently add 2 mL 1XPBS and swirl the plate to wash away residual media.

! Caution: Be careful when adding PBS in order to avoid detachment of adhered cells.

Note: FBS present in complete media interferes with trypsinization.

Aspirate PBS and add 2 mL trypsin. Place the plates inside CO_2 incubator for 2–3 min to allow efficient trypsinization (or cell detachment). Quench the reaction by adding 2 mL serum-supplemented DMEM. Count the cells using Hemocytometer and seed at a density of 3×10^6 cells per 10 cm plate. Place the plates inside CO_2 incubator maintained at 37 °C under 5% CO_2. Plates at a confluency level of 60–70% are suitable for transfection.

! Caution: Avoid over-trypsinization as this would impede the ability of cells to adhere.

2.3.1.3. To prepare the transfection mixture, add plasmid DNA encoding the proteins of interest in 300 μL of serum-free DMEM. We typically use 3.5 μg of each plasmid (GPCR and β-arrestin), however, it can be adjusted depending on the expression level of the GPCR being tested. Subsequently, add 21 μL of PEI (3 μL per 1 μg of DNA) to this mixture and vortex briefly. Allow the mixture to incubate at room temperature for 10 min. In the meantime, replace the serum-supplemented media in the cell culture plates with serum-free DMEM. Post-incubation, add the mixture to the plates dropwise and gently swirl the plate to ensure even distribution. Incubate the plates in a CO_2 incubator. 6 h post-transfection, replace the serum-free DMEM with serum-supplemented media.

! Caution: Ensure that plasmid DNA is of high quality with minimal contaminants and appropriate 260 nm/280 nm ratio. It is also advisable to validate the integrity of the plasmid DNA by agarose gel electrophoresis.

Note

(A) Typically, we add a total amount of 7 μg of DNA per 10 cm plate for transfection. In case the amount of DNA added is less than 7 μg, make it up to 7 μg with empty plasmid vector (e.g., pcDNA3.1). For transfection

mixtures containing more than 7 μg of total DNA, adjust the amount of PEI accordingly in order to maintain the DNA:PEI ratio of 1:3.

(B) For comparing the interaction between multiple GPCRs and β-arrestins, or, GPCR mutants with the wild-type receptor, normalize the amount of DNA to be added for each receptor by surface ELISA in order to ensure comparable cell surface expression of all the receptors constructs. This ensures a direct comparison of interaction across different receptors or receptor mutants as only the receptor population expressed on the cell surface is expected to be phosphorylated and therefore competent to interact with β-arrestins.

2.3.1.4. 48 h post-transfection, serum starve the cells in DMEM for at least 6 h followed by stimulation with ligand at appropriate concentration for required time duration

Note: Serum starvation is required in order to synchronize the cells and bring them to the same stage of the cell cycle.

After stimulation, remove the media by aspiration. Add 1 mL of 1XPBS to each 10 cm plate. Detach cells from the plates by scraping and collect in 1.5 mL microcentrifuge tubes. Centrifuge at 5000 rpm at 4 °C for 10–15 min to pellet the cells. Remove supernatant by aspiration.

Pause point: At this step, either flash freeze the pellets and store at −80 °C or proceed with the next step.

2.3.2 Crosslinking and co-immunoprecipitation

2.3.2.1. Resuspend each pellet in approximately 200 μL of lysis buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM PMSF, 2 mM benzamidine hydrochloride, 1XPhosStop).

Note: PhosStop is a commercially available cocktail of phosphatase inhibitors. It is added to ensure that the phosphate groups at the carboxyl-terminus of the receptor are protected from dephosphorylation since they play an essential role in mediating GPCR interaction with β-arrestins.

2.3.2.2. Transfer the resuspended pellet to a glass douncer and homogenize the mixture by implementing approximately 200 strokes. Collect the homogenized mixture in 1.5 mL microcentrifuge tubes.

2.3.2.3. For crosslinking, we typically use commercially available lipophilic and hydrophobic amine reactive cross-linker, DSP at a final concentration of 1 mM. Incubate the tubes at room temperature for 40–45 min under tumbling condition to allow chemical crosslinking of interacting proteins by targeting available primary amines in the protein.

Note

DSP is a homobifunctional amine-reactive crosslinker, and it is also known as Lomant's reagent (Swami et al., 2009). The reactive group is an NHS ester that forms an amide bond with the free amine groups present in proteins. The NHS

ester reaction occurs with highest efficiency at a pH range of 7–9. It contains a cleavable disulfide linkage which allows crosslinked proteins to be separated when treated with any reducing agent. Due to its hydrophobic nature, DSP can permeate through cell membranes and therefore can be used to crosslink intracellular and membrane proteins.

! Caution: DSP is highly hygroscopic in nature. Bring the bottle of DSP to room temperature prior to opening it and re-seal it with parafilm after use. It is advisable to make small aliquots of the same and store them at −20 °C, preferably in presence of a desiccant. Take one aliquot out approximately 20–30 min before use and allow it to come to room temperature before opening it.

! Caution: DSP stock is prepared in DMSO. In order to ensure maximum crosslinking efficiency, prepare fresh stock solution each time.

Note: Other commercially available crosslinkers can also be used instead of DSP. It is advisable to determine the optimal concentration of crosslinking reagent to be used for obtaining maximal crosslinking and minimal aggregation prior to performing experiment.

2.3.2.4. Quench the reaction by adding 1/10th volume of 1 M Tris buffer, pH 8.0.

! Caution: It is imperative to quench the reaction in order to ensure that non-specific crosslinking or over crosslinking (that might ultimately lead to aggregation) does not occur. DSP being an amine-reactive crosslinker can be effectively quenched using any buffer containing primary amines.

2.3.2.5. For solubilization of the receptor-β-arrestin complex, we typically add 1% (v/v) L-MNG and incubate for 1 h at room temperature under tumbling conditions. Following this, centrifuge the mixtures at 15,000 rpm at 4 °C for 10–15 min to allow cell debris to pellet down. Collect the supernatant in fresh microcentrifuge tubes.

2.3.2.6. To capture the receptor-β-arrestin complex, add 20 µL of pre-equilibrated FLAG M1 antibody agarose bead slurry (beads:buffer ratio 1:1) and 2 mM $CaCl_2$. Incubate the reaction mixture under tumbling condition for 1.5 h at room temperature. Take out 20 µL of supernatant as loading control before adding beads.

! Caution: Addition of $CaCl_2$ is necessary for binding of FLAG-tag to M1-FLAG beads.

Note

(A) Typically GPCRs are expressed in mammalian cells with an N-terminal FLAG tag to allow detection. However, other affinity tags such as HA-tag should also work.

(B) Loading control is taken out to ensure that similar quantities of interacting protein partner(s) are expressed in all the reaction conditions.

2.3.2.7. Following bead binding, wash the beads in the following fashion to remove any unbound or non-specific protein: LSB-HSB-LSB-HSB-LSB, wherein

LSB refers to Low Salt Buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 0.01% (v/v) L-MNG) and HSB refers to High Salt Buffer (20 mM HEPES pH 7.4, 350 mM NaCl, 2 mM $CaCl_2$, 0.01%(v/v) L-MNG).

2.3.2.8. Elute bound protein by incubating with elution buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 0.01%(v/v) L-MNG, 250 μg/mL FLAG peptide, 2 mM EDTA) for 30 min at room temperature. Gently flick the tubes intermittently to allow efficient elution.

2.3.2.9. Spin the tubes at 2500 rpm at 4 °C for 2–3 min to allow beads to settle down. Gently collect the supernatant containing the eluted proteins while ensuring that the beads are not disturbed.

! Caution: Do not exceed 3000 rpm while centrifuging beads as this might lead to damage of the beads.

2.3.3 Western blotting to visualize co-immunoprecipitated proteins

2.3.3.1. Run both loading control and final eluted samples on SDS-PAGE gels to separate the co-immunoprecipitated proteins based on molecular size. This is followed by transfer of separated proteins to PVDF membrane.

2.3.3.2. Block non-specific sites on the PVDF membrane by incubating with 5% BSA (prepared in 1XTBST) for 1 h at room temperature.

2.3.3.3. Incubate with antibody against the interacting partner. For GPCR-β-arrestin interaction, we typically use rabbit anti-β-arrestin antibody for either 2 h at room temperature or overnight at 4 °C.

2.3.3.4. Wash the membrane three times with 1XTBST (10 min each) and incubate with HRP-coupled secondary antibody. We typically use HRP-coupled anti-rabbit IgG secondary antibody for 1 h at room temperature. Wash the membrane again for an additional three times.

2.3.3.5. Develop the blot by adding ECL substrate sufficient to cover the entire surface of the membrane and visualize using a standard CCD camera attached to the ChemiDoc imaging system. Signal quantification was done using ImageLab software (Bio-Rad) and appropriate statistical analysis was performed in GraphPad Prism software.

2.3.3.6. To probe the presence of the FLAG-tagged receptor, strip the membranes by adding stripping buffer, heat for 10–15 s and place on a rocker at fast rocking speed for 10 min. Wash thrice with 1XTBST, one wash at fast speed, followed by two washes at slow speed. Block the non-specific sites on the PVDF membrane by incubating with 5%BSA, and probe with HRP-coupled anti-FLAG M2 antibody as mentioned above (steps 2.3.3.2–2.3.3.4).

2.3.3.7. Develop signal as described above (step 2.3.3.5). The results of a typical co-IP experiments are presented in Fig. 2.

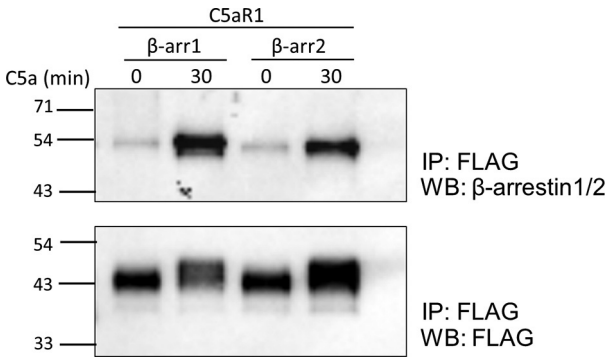

FIG. 2

A typical experimental data showing agonist-induced interaction of C5aR1 with β-arrestins using the co-IP assay. HEK-293T cells expressing N-terminally FLAG-tagged C5aR1 and β-arrestins were stimulated with C5a agonist followed by in-cellulo cross-linking using DSP. Subsequently, the receptor was immunoprecipitated using anti-FLAG M1 antibody agarose beads and co-elution of β-arrestins was detected using Western blot. These data are based on, and adapted from, our previously published study (Pandey et al., 2021).

3 Troubleshooting

Potential problem	Possible reason	Suggested solution
No signal is observed in blot	There might be a problem with either transfection or DNA quality	Confirm DNA quality and presence of insert by digesting DNA construct with appropriate restriction enzymes and running on 1% agarose gel. Also confirm the DNA sequence. Optimize transfection by varying the ratio of DNA to PEI used.
	Antibody used is old/not functional	Use freshly prepared antibody (in 1% BSA dissolved in 1XTBST) dilution.
	Crosslinking did not occur efficiently	Confirm that buffers used are free of primary amines since DSP is a primary amine reactive crosslinker. Confirm that DMSO is of high quality and functional.
Blot appears black	Blot was not washed properly prior to developing	Check the pH of 1XTBST buffer used for washing the blot. pH of the buffer should be approximately 7.4. Use freshly prepared 1XTBST buffer for washing the blot. Use freshly prepared 1% BSA for blocking.

4 Additional considerations

To evaluate the interaction of GPCRs with β-arrestins by co-IP, we typically use cell lysate from HEK293T cells co-expressing recombinant receptor and β-arrestin. As an alternate strategy, it is also possible to follow this co-IP protocol using cell lysate prepared from other cells co-expressing the receptor of interest, GRKs (if necessary) and β-arrestins. Alternatively, it is also feasible to add purified β-arrestins to the cellular lysate prepared from cells expressing the receptor, followed by cross-linking and co-IP, as we have demonstrated earlier (Ghosh et al., 2019). Protein-protein interactions that can be monitored using this method also depends on the spacer arm length of the crosslinker used in the assay. DSP has a spacer arm length of 12 Å and therefore, it may not be used if the potential sites for cross-linking between the two interaction partners are not within this distance. However, there are additional chemical crosslinkers commercially available having longer spacer arm length that can be used depending on the specific requirement. It is worth noting here that DSP contains a cleavable disulfide linkage that can be cleaved by adding reducing agents such as DTT or TCEP. Therefore, the cross-linked proteins can be separated on a reducing gel and they can be detected on the basis of their molecular weight by either Coomassie staining or using Western blot.

5 Concluding remarks

The step-by-step protocol described here should be potentially generic and adaptable to protein-protein interactions other than GPCR-β-arrestin system described here. The scope of this protocol can be further expanded by using other commercially available cross-linking reagents such as those which are heterobifunctional, have different spacer arm length, and are reactive to other functional groups in proteins. Although the protocol described here is focused on measuring the interaction of two proteins, i.e., a GPCR and β-arrestin, it should also be applicable for evaluating the formation of multi-protein complexes in cells. Finally, a combination of more than one chemical cross-linker may further enhance the utility and versatility of this approach to measure multi-protein interactions in cellular context.

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