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Running head: Monitoring partner interactions with β-arrestins

Summary

Beta-arrestin 1 and 2 (β-arr1 and β-arr2) are ubiquitous proteins with common and distinct functions. They were initially identified as proteins recruited to stimulated G protein-coupled receptors (GPCRs), regulating their desensitization and internalization. The discovery that β-arrs also could interact with more than 400 non-GPCR protein partners brought to light their central roles as multifunctional scaffold proteins regulating multiple signalling pathways from the plasma membrane to the nucleus, downstream of GPCRs or independently from these receptors. Through the regulation of the activities and subcellular localization of their binding partners, β-arrs control various cell processes such as proliferation, cytoskeletal rearrangement, cell motility and apoptosis. Thus, the identification of β-arrs binding partners and the characterization of their mode of interaction in cells is central to the understanding of their function. Here we provide methods to explore the molecular interaction of β-arrs with other proteins *in cellulo*.

Keywords: β-arrestin, scaffold, GST pull-down, co-immunoprecipitation, Yeast two hybrid, Bioluminescence Resonance Energy Transfer (BRET)

1. Introduction

The two non-visual arrestins, β-arrestin 1 and 2 (β-arr1 and β-arr2) are ubiquitously expressed scaffold proteins with no catalytic activity that regulate a vast array of cellular processes from the cell surface to the nucleus [1]. β-arrs were originally identified as negative regulators of GPCR function [2]. Indeed the binding of β-arrs to the phosphorylated active form of these receptors functionally uncouple them from cognate G proteins in a process called desensitization [3], and β-arrs simultaneous interaction with both clathrin and the Adapter Protein-2 (AP-2) [4] promotes the internalization of activated GPCRs, decreasing their concentration at the cell surface [5]. Besides clathrin and AP-2, many others non-GPCR protein partners of β-arrs were identified in subsequent studies using various approaches [6,7]. Immunoprecipitations of epitope-tagged β-arrs combined with high throughput mass spectrometry revealed a few hundred potential partners depending on experimental conditions [8], and new ones are also regularly identified [9-15]. These β-arr partners, localized in different cell compartments, include protein kinases such as Src, PI3K-Akt, MAP kinases and components of their activating modules, as well as other enzymes such as phosphatases, E3 ubiquitin ligases and deubiquitinases, regulators of small GTPases, ion exchangers and other types of proteins like cytoskeletal proteins, nuclear proteins and transcription factors [16,17,8]. Although some of these proteins remain to be fully confirmed as β-arr protein partners and their mode of interaction (e.g. direct/indirect, regulated) with β-arr1 and/or β-arr2 further characterized [16], it is nonetheless established that β-arrs are essential scaffold proteins that organize signalling networks in different location within the cell, dynamically regulating the activities and/or the subcellular localization of their binding partners. Thus, through their central roles in signal transduction, β-arrs control a broad range of cellular functions such as proliferation, cytoskeletal rearrangement, cell motility and apoptosis in a GPCR–dependent or –independent manner [18,6]. Importantly, changes in β-arr expression and localization have been correlated with various pathological situations and diseases such as inflammation [19], addiction [20] and cancer [1], attesting for their critical physiological roles.

As cellular functions regulated by β-arrs depend on their association with other protein partners, it is critical to be able to study these interactions in cells. In this chapter we provide detailed biochemical and biophysical protocols to

investigate interactions of proteins with β-arrs in cells using yeast two-hybrid, co-immunoprecipitation, GST-β-arr pull-down and Bioluminescence Resonance Energy Transfer (BRET) assays.

2. Materials

2.1 Cell lines and yeast strain

1. Human Embryonic Kidney 293T (HEK-293T) cell line (ATCC).
2. *Saccharomyces cerevisiae* HF7c yeast strain (Feilotter 1994) (*see* **Note 1**).

2.2 Plasmids

1. Plasmids used for the Yeast two-hybrid assay: pGBT9 (Tryptophan, Trp, auxotrophic marker) and pGAD-GH (Leucine, Leu, auxotrophic marker) coding for the GAL4 DNA binding domain (GAL4-BD) and the GAL4 transactivation domain (Gal4-AD), respectively. The cDNAs of β-arr (β-arr1 and/or β-arr2) are cloned in pGBT9 and putative-binding partner in pGAD-GH plasmid (or *vice versa*), so that the GAL4 DNA binding or transactivation domains are at the N-terminal ends.
2. Plasmids encoding human β-arr2 and Src, used for the co-immunoprecipitation experiment: pCMVTag2B-Flag-β-arr2, pcDNA3.1-Src, pcDNA3.1 and pCMVTag2B (*see* **Note 2**).
3. Plasmids used for the GST-β-arr pull-down assay: cDNAs of β-arr1 or β-arr2 have been cloned in frame, downstream of the GST coding sequence, in pGEX-4T3 expression vector (pGEX-4T3-β-arr), to produce GST-β-arr fusion protein (*see* **Note 3**). The empty pGEX-4T3 vector is used to produce GST protein.
4. Plasmids used for the BRET saturation assays: GFP10 fusion protein (acceptor) and Rluc2 fusion protein (donor) inserted into pcDNA3.1 plasmids (*see* **Note 4**).

2.3 Cell culture and transfection

1. Complete Dulbecco's modified Eagle's medium (DMEM), 4.5 g/L glucose, 4 mM glutamine, 1 mM Pyruvate, supplemented with 10% fetal bovine serum, 100U/mL penicillin and 0.1 mg/mL streptomycin.
2. Trypsin-EDTA solution (0.05%).
3. OptiMEM.
4. 6-well and 10 cm plates for cell culture, white opaque 96-well Microplates: sterile and tissue culture treated (PerkinElmer OptiplateTM-96HB or equivalent).
5. GeneJuice tranfection agent (Novagen) is used as transfecting reagent for HEK-293T cells.

2.4 Yeast two-hybrid assay

1. Dextrose solution, 10% (5X solution): Prepare in dH_2O, filter sterilize and store at 4°C.
2. YPD Media: make YP media (2% w/v Peptone and 1% w/v yeast extract) in dH_2O, autoclave. Add 1 volume of filtered sterilized 5X Dextrose solution to 4 volume of YP media to make up the YPD media containing 2% Dextrose. Store at 4°C.
3. Dropout base synthetic defined medium (DOB) and double or triple dropout Complete Supplement Mixture (CSM), CSM-Leu-Trp and CSM-Leu-Trp-His (MP Biomedicals).
4. Agar plates:
   - YPD Plates: YPD supplemented with 2% (w/v) agar.
   - CSM-Leu-Trp and CSM-Leu-Trp-His Selective Plates: 2.7% (v/v) DOB Medium, 0.064% CSM-Leu-Trp or 0.062% CSM-Leu-Trp-His, and 3% Agar in dH_2O.

Autoclave and pour plates once the temperature comes down to 55°C.

5. Salmon sperm DNA (10 mg/mL) as carrier DNA.
6. PEG-4000, 50%: dissolve 50 g PEG-4000 in 50 mL dH_2O, add dH_2O to 100 mL. Filter sterilize and store at room temperature.
7. Lithium Buffer : 0.1 M Lithium Acetate (LiAc), 10 mM Tris-Base, 1 mM EDTA, pH 7.5. Sterilize and store at room temperature.
8. Lithium Acetate/PEG-4000: to 1 mL of Lithium Buffer, pH 7.5, add 9 mL of 50% PEG-4000 and vortex to mix properly. Prepare fresh for each experiment.
9. DMSO: cell-culture grade.

2.5 Mammalian cell lysis

1. Phosphate Buffered Saline 1x without calcium (DPBS).
2. Lysis buffer, pH 7.4: 1% Triton X-100, 150 mM NaCl, 20 mM Tris-Base, 0.05% (v/v) Tween20, 1 mM sodium orthovanadate, 10 mM NaF and Protease Inhibitors cocktail (Roche) (*see* **Note 5-6**).
3. Pierce BCA Protein Assay Kit.
4. Laemlli Buffer, 5x: 250 mM Tris-Base, pH 6.8, 50% Glycerol, 10% SDS, 0.1 g/mL Bromophenol Blue, 10% β-Mercaptoethanol. Store aliquots at -20°C.

2.6 Co-immunoprecipitation and western blots

1. EZview Red anti-Flag M2 affinity gel (Sigma-Aldrich).
2. Lysis buffer (as described in section 2.5.2) supplemented with 2% BSA.
3. High-salt lysis buffer: lysis buffer (section 2.5.2) supplemented with 150 mM NaCl (300 mM final).
4. Page Ruler Plus Prestained Protein Ladder.
5. Protran Nitrocellulose membranes.
6. Primary Antibodies: rabbit antibodies against the Flag peptide (Cell Signalling) and Src (Santa Cruz Biotechnology).
7. Secondary antibody: rabbit True blot IgG-HRP (Rockland).
8. Pierce ECL western Blotting substrate.
9. Antibody stripping buffer.
10. Chemiluminescent Imager LAS-3000 (Fuji Lifesciences).

2.7 GST-β-arr Pull Down from cell lysate

1. One Shot BL21(DE3)pLysS bacteria.
2. Luria-Bertani (LB) liquid media: 0.5% (w/v) Yeast extract, 1% NaCl, 1% Tryptone in dH_2O.
3. Isopropyl β-D-1-thiogalactopyranoside (IPTG, 100 mM), dioxane-free in dH_2O, filter sterilize. Store aliquots at -20°C.
4. Phosphate Buffered Saline (PBS, 1x): sterile.
5. Glutathione Sepharose-4B Beads.
6. Bacteria lysis buffer, pH 7.4: 20 mM Tris-Base, 1M NaCl, 0.2 mM Ethylene diaminetetraacetic acid (EDTA), 0.2 mM Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 1 mM Dithiothreitol (DTT), Protease Inhibitors cocktail (Roche). The lysis buffer is prepared without DTT, filter sterilized and stored at 4°C. DTT should be added fresh before the start of the procedure.
7. NaCl, 1 M with protease inhibitors.
8. PBS 1x with protease inhibitors.

2.8 BRET2 Saturation assay

1. Poly-L-Ornithine. Dissolve Poly-L-Ornithine in dH_2O to a final concentration of 1.5 mg/mL (50X). Filter sterilize, aliquot and freeze at -20°C.

2. 6-well plates for cell culture, white opaque sterile and tissue culture treated 96-well Microplates for BRET (PerkinElmer OptiplateTM-96HB or equivalent).
3. Phosphate Buffered Saline (PBS, 1X) with $CaCl_2$ and $MgCl_2$.
4. Hanks Buffered Salt Solution (HBSS, 1X) containing $CaCl_2$ and $MgCl_2$.
5. Coelenterazine 400a (DeepBlueC^{TM}), 1 mM: dissolve 500 µg of Coelenterazine 400a (Interchim) in 1.27 mL of ethanol. Store at -20°C in opaque microcentrifuge tubes.
6. Multimode microplate reader, Mithras LB943 (Berthold) or equivalent.

3. Methods

3.1 Yeast two-hybrid assay

Two-hybrid assays use transcription as a read-out to study protein-protein interactions in eukaryotic cells. They take advantage of the fact that transcription factors are composed of two distinct domains, the DNA binding domain (BD) and the transcription-activating domain (AD), which are independently non–functional but can reconstitute transcription activity when in close proximity to one another even if not covalently associated. In the yeast two-hybrid system, the use of independent fusion proteins including these separate domains (BD and AD) generates an active transcription factor initiating transcription of reporter genes integrated in the yeast genome, if the proteins fused to each domain interact. Nutritional markers allowing yeast to grow on medium lacking histidine and enzymatic reporter (β-galactosidase) are used for such analysis [21]. Yeast two-hybrid assays have been used to identify and characterize new β-arr protein partners [12,22]. We describe here such an approach successfully used in this context.

3.1.1 Yeast Transformation

1. Inoculate 50 mL of complete YPD medium with HF7c yeast from a YPD agar plate (*see* **Note 7**) and incubate overnight at 30°C with agitation (180 rpm).
2. Dilute the saturated overnight culture to an OD_{600} of 0.2 in 100 mL of complete YPD medium (*see* **Note 8**).
3. Incubate the diluted culture at 30°C (180 rpm) until the OD_{600} is 0.5-0.6 (usually 2-3 hours) indicating log-phase growth, which is critical for successful yeast transformation.
4. Transfer the culture into 50 mL tubes and centrifuge at 700 xg for 5 minutes (min) at room temperature (RT).
5. Discard supernatants and wash the yeast pellets with 50 mL dH_2O.
6. Centrifuge at 700 xg for 5 min at RT to remove dH_2O.
7. Resuspend/pool the yeast pellet in 2 mL Lithium buffer.
8. Incubate the yeast at 30°C (180 rpm) for 60 min; the competent cells should be used for transformation within an hour.
9. Boil Salmon sperm DNA for 5 min at 100°C and quickly chill in ice. Denaturation of DNA promotes transformation efficiency.

10. In a 2 mL eppendorf tube assemble the transformation mix in the following order: Add 500 ng of pGBT9 and 500 ng of pGAD-GH plasmids, 100 µL of competent yeast cells, 100 µg of denatured salmon sperm DNA (10 µL of 10 mg/mL stock) and 600 µL Lithium Acetate/PEG-4000 solution. Multiple transformations can be carried in parallel using different combination of the pGBT9 and pGAD-GH control plasmids and pGBT9 and pGAD-GH plasmids encoding the fused proteins, to test for specificity.
11. Vortex the Eppendorf tube twice 5 seconds (s) and incubate at 30°C for 30 min.
12. Add DMSO to a final concentration of 10%, and mix immediately by inverting the tube 2-3 times.
13. Heat shock cells at 42°C for 15 min.
14. Place cells on ice for 2 min.
15. Centrifuge the tube at 12,000 rpm in a microcentrifuge for 20 s at RT.
16. Discard the supernatant and wash the cells with 500 µL YPD medium.
17. Centrifuge the cells at 12,000 rpm in a microcentrifuge for 20 s at RT.
18. Discard the supernatant, resuspend cells in 100 µL YPD medium and spread the whole content on a CSM-Leu-Trp Selective Plate.
19. Incubate plates at 30°C until colonies appear (2-3 days).

3.1.2 Histidine auxothrophy to test for interaction

1. Pick 3-4 colonies per transformation using a sterile pipette tip or tooth-pick and streak a patch (1.5cm x 0.5cm) for each one onto a fresh CSM-Leu-Trp Selective Plate. Incubate the plate at 30°C for 2 days (*see* **Note 9**).
2. For the replica plating, place a sterile velveteen cloth on a replica block and secure it using the lock to create an even surface (*see* **Note 10**).
3. Place the CSM-Leu-Trp selective plate with the yeast patches on the velveteen square and press gently but firmly. An impression of the yeast patches should be observed (*see* **Note 11**).
4. In the following order, inoculate a CSM-Leu-Trp-His Selective Plate, and a CSM-Leu-Trp selective plate onto the velvet cloth, and incubate the plates at 30°C.
5. Monitor yeast growth over the next couple of days. Growth of yeast transformed with pGBT9 and pGAD-GH plasmids expressing β-arr and its putative binding partner fused to GAL4-BD and Gal4-AD on the CSM-Leu-Trp-His Selective Plate,

and no growth of yeast transformed with the other plasmid combinations indicate specific interaction between β-arr and the other protein (*see* **Note 12-13**).

3.2 Transfection and cell lysate preparation for co-immunoprecipitation and GST pull-down.

1. Seed HEK-293T cells at a density of 0.5-1x10^6 cells per 10 cm tissue culture plate. The cells are incubated overnight, in a cell culture incubator at 37°C in a humidified atmosphere of 95% air and 5% CO_2.
2. The next day, cells should be about 60% confluent and ready for transfection using the GeneJuice reagent (3 μL/μg of DNA). For the β-arr2/Src co-immunoprecipitation experiment described below (section 3.3), HEK-293T cells are transfected with 1 μg of plasmid encoding FLAG-tagged β-arr2 and/or 0.5 μg of plasmid encoding untagged Src, empty pcDNA3.1 and pCMVTag2B plasmids being used to keep the amount of transfected plasmid DNA constant in the control conditions (FLAG-β-arr2 alone, Src alone and no FLAG-β-arr2 or Src). The transfection conditions (amount of each plasmid DNA and ratio of GeneJuice/DNA) should be determined empirically for each new experimental setup and potential β-arr protein partner (*see* **Note 14**).
3. Transfected cells are incubated for 60 hours at 37°C in the cell incubator.
4. For lysis of transfected cells, place the 10 cm dishes on ice, aspirate the media and wash the dishes once with 10 mL of ice-cold PBS. For all subsequent steps, the plates and lysates should be kept on ice.
5. Add 750 μL of ice-cold lysis buffer per plate, swirl the plates so that the buffer cover the whole surface, scrape the cells on ice with a cell scraper and collect the lysates in Eppendorf tubes. For cells more difficult to lyse, the collected extracts can be incubated on a rotating wheel at 4°C for 15 min or the plates can be incubated with the lysis buffer on a rotating platform at 4°C for 15 min before the cells can be scraped and collected.
6. Centrifuge at 13,000 rpm in a microcentrifuge for 15 min at 4°C to pellet cell debris.
7. Transfer the supernatants to new Eppendorf tubes and determine the protein concentration using the Pierce BCA Protein Assay Kit.

3.3 Co-immunoprecipitation

Immunoprecipitation, which is the isolation of a target protein bound to an antibody from unbound proteins in a cell or tissue lysates, allows the identification of proteins engaged in a stable interaction with the immunoprecipitated target. This approach can be used to characterize molecular complexes with β-arrs [9-12,22,14,15]. Here, starting from the HEK-293T cell lysates prepared above, we use antibodies directed against the Flag epitope and immobilized on gel to show that transfected Src is co-immunoprecipitated with transfected Flag-β-arr2.

1. 15 µL of EZview Red anti-Flag M2 affinity gel (*see* **Note 15**) is used per IP. Calculate the amount of gel needed for the total number of IP +1 more (here 5). Wash the gel two times with a volume of ice-cold lysis buffer equivalent to three volumes of gel. Centrifuge the beads at 4,000 rpm in a microcentrifuge for 4 min at 4°C. Aspirate the supernatant between each wash.
2. After the second wash, centrifugation and aspiration of the supernatant, incubate the anti-Flag M2 affinity gel with 500 µL of ice-cold lysis buffer containing 2% BSA on a rotating wheel for 3 hours at 4°C to reduce non-specific binding.
3. During step 2, set aside one 50 µg cell lysate aliquot (15-20 µL) of each condition prepared in section 3.2, in a new Eppendorf tube. Add 10 µL of 5x Laemlli Buffer and adjust the volume to 50 µL with lysis buffer (*see* **Note 16**). Boil the sample for 5 min at 100°C, centrifuge it at 13,000 rpm in a microcentrifuge for 1 min at RT. The samples are ready to be loaded on a gel or stored at -20°C (*see* **Note 17**).
4. After 3 hours, centrifuge the anti-Flag M2 affinity gel at 4,000 rpm, 4 min (4°C) in a microcentrifuge. Aspirate the supernatant and wash the gel with ice-cold lysis buffer, centrifuge, aspirate the supernatant and add ice-cold lysis buffer again.
5. Before centrifugation, aliquot on ice the washed saturated gel into a number of Eppendorfs corresponding to the number of IP (for this experiment 4). Centrifuge 4 min at 4,000 rpm (4°C) in a microcentrifuge, and aspirate the supernatant.
6. Add 500 µg of each cell extract to different Eppendorf tubes containing the anti-Flag M2 affinity gel, adjust the volume to 500 µL with ice-cold lysis buffer and incubate overnight on a rotating wheel at 4°C to immunoprecipitate the FLAG-tagged β-arr.

7. The next day, centrifuge the samples 4 min at 4,000 rpm (4°C) in a microcentrifuge, and discard the cell lysate (*see* **Note 18**).
8. Wash samples twice with 750 µL of ice-cold high salt lysis buffer, followed by two washes with 750 µL ice-cold lysis buffer. For each wash, samples can be incubated at 4°C for 5 min on a rotating wheel to decrease non-specific binding. Centrifuge 4 min at 4,000 rpm (4°C) in a microcentrifuge after each wash and aspirate the supernatant. Transfer the third washes to new Eppendorf tubes before centrifugation.
9. After the last centrifugation, aspirate the supernatants and add 50 µL of 1,3X Laemlli buffer at RT (prepared by diluting the 5X stock with lysis buffer) to the anti-Flag M2 affinity gel. Vortex rapidly and boil immediately at 100°C for 5 min. Centrifuge the samples at 13,000 rpm in a microcentrifuge for 5 min at RT.
10. Load immunopurified samples on a 12% SDS-PAGE gel along with the whole cell extract prepared in section 3.3.3, and the molecular markers. Run the gel, and transfer the resolved proteins on a nitrocellulose membrane.
11. Western blots are performed with antibodies directed against the Flag epitope (2368, 1:1000) and Src (sc-18, 1:1000). The co-immunoprecipitated protein (here Src) is blotted first and the immunoprecipitated one (here β-arr2), after stripping of the membrane for 15 min with antibody stripping buffer. After incubation with secondary antibodies (*see* **Note 19**), develop the WB immunoreaction with ECL using films or an appropriate chemiluminescent imager.

    The Figure shows a co-immunoprecipitation experiment where non-tagged Src transfected in HEK-293T cells is specifically co-immunoprecipitated with FLAG-β-arr2 using the protocol described above.

3.4 GST-βarr Pull-down

GST pull-down assay is a method closely related to co-immunoprecipitation. The difference in this assay is that the GST-tagged bait protein replaces antibodies and the isolation of the complex is performed through affinity chromatography. In the case of the identification of protein partners of β-arrs [23,24,15], GST-β-arr recombinant proteins is prepared in bacteria before being used for the pull-down assay. A simple procedure for this approach is described below.

3.4.1 GST and GST-β-arr fusion protein induction in bacteria

1. Transform BL21(DE3)pLysS bacteria separately with pGEX-4T3 plasmid DNA encoding GST or with pGEX-4T3-β-arr vector encoding GST-β-arr. Plate each transformed bacteria on a LB agar plate containing ampicillin and incubate at 37°C overnight.
2. Inoculate a single bacterial colony of each transformation into 5 mL of LB (with ampicillin) and grow cells for 6 hours at 37°C (180 rpm).
3. Transfer 250 µL of each bacterial culture into 20 mL of fresh LB media (with ampicillin) in a conical flask and grow cells overnight at 37°C with agitation (180 rpm).
4. Transfer 10 mL of each saturated bacterial culture into 100 mL of fresh LB media with ampicillin (1:10) in a flask and agitate (180 rpm) at 37°C, until the OD_{600} reaches 0.6-0.8 (approximately 60 min).
5. For GST induction, add IPTG to a final concentration of 0.1 mM and agitate at 37°C for 4 hours. Add IPTG to a final concentration of 0.1 mM to induce the expression of GST-β-arr1, and 1 mM to induce GST-β-arr2. Agitate cells at 110 rpm for 16 hours at 20°C. (*see* **Note 20**).
6. Centrifuge the bacterial cultures in 50 mL-conical tubes at 2,000 xg for 15 min.
7. Wash the bacterial pellets with 10 mL of PBS and centrifuge at 2,000 xg for 10 min.
8. Discard the PBS and proceed on ice for the next steps. The bacterial pellets can also be frozen at this step, and stored at -80°C.

3.4.2 Preparation of Glutatione Sepharose beads

This part of the procedure can be performed during the centrifugation described in section 3.4.1.6 above, or during the one from section 3.4.3.3 below.

1. Prepare a 1:1 mix containing 200 µL of Glutathione Sepharose Beads and 200 µL sterile PBS per pull-down to be performed.
2. Wash the beads three times with 1 mL of ice-cold 1M NaCl containing protease inhibitors in a microcentrifuge at 7,000 rpm for 30 s at 4°C.
3. Wash the beads three times with 1 mL of ice-cold sterile PBS with protease inhibitors (centrifuge at 7,000 rpm for 30 s at 4°C).
4. Remove the supernatant and suspend the 200 µL beads (1:1) with 200 µL of sterile PBS.

5. Divide the beads equally into four Eppendorf tubes and keep them on ice. Two Eppendorf tubes with washed beads will be used for GST purification and the two others for GST-β-arr (see section 3.4.3.5 below).

3.4.3. Purification of GST and GST-tagged fusion protein

1. Resuspend each bacterial pellet in 2 mL of ice-cold bacterial lysis buffer and transfer to 15 mL-conical tubes.
2. Sonicate the bacterial lysates three times (15 s), with a 2 min incubation on ice between each sonication.
3. Transfer to 2 mL Eppendorf tubes and centrifuge the bacterial lysates for 20 min at 12,000 rpm (4°C) in a microcentrifuge.
4. Take a 4 µL aliquot of each clarified bacterial lysate, mix it with 16 µL of lysis buffer and 5 µL of 5x Lamelli Buffer, and boil the samples for 5 min at 100°C. Freeze the samples (*see* **Note 21**).
5. Divide each clarified bacterial lysate in two, and transfer to two Eppendorf tubes containing the Glutathione Sepharose beads prepared in section 3.4.2.
6. Incubate the tubes for 4 hours at 4°C on a rotating wheel.
7. Centrifuge the tubes at 7,000 rpm for 30 s at 4°C in a microcentrifuge and remove the lysates (supernatant) that can be kept separately at 4°C after removing 4 µL aliquots of each as in section 3.4.3.4 above (*see* **Note 21**).
8. Wash the beads three times with 1 mL of ice-cold 1M NaCl with protease inhibitors. Each wash is performed for 5 min at 4°C on a rotating wheel. Centrifuge the samples at 7,000 rpm for 30 s (4°C) in a microcentrifuge between each wash.
9. Wash the beads three times, as indicated in the previous step, with 1 mL of ice-cold sterile PBS containing protease inhibitors. Following the first wash, transfer the beads to new tubes.
10. After the final wash, resuspend the beads in 100 µL of sterile PBS and keep them at 4°C (*see* **Note 22**).
11. Take small aliquots of each set of beads and mix them with lysis buffer and Laemmli buffer (final concentration 1X). Boil samples for 5 min at 100°C.
12. Run these samples on a SDS-Page gel along with BSA standards ranging from 0.2 µg to 10 µg in 1x Laemlli Buffer.

13. By Coomassie Brilliant Blue staining of the gel, determine the concentration of GST and GST-β-arr proteins by comparing the intensity of the colored GST-β-arr to that of the different BSA samples. The GST-bound beads aliquots may need to be diluted to evaluate the GST concentration by comparison to the GST-β-arr bound to the beads.

3.4.4. GST-Pull-Down assay.

1. To reduce non-specific binding to the beads, the cell lysate to be used for the pull down can be pre-cleared. Fresh Glutathione beads are equilibrated by two successive washes with four volumes of mammalian cell lysis buffer (see section 2.5.) and centrifugation at 4,000 rpm for 4 min at 4°C in a microcentrifuge. After the second wash, the beads are suspended 1:1 with lysis buffer.
2. Pre-clear two 100-500 µg lysate aliquots from cells transfected with DNA plasmid encoding the potential β-arr protein partner to be tested and prepared as in section 3.2 (*see* **Note 23**), by adding 50 µL of equilibrated Glutathione Sepharose Beads to each aliquot.
3. Rotate the Eppendorf tubes for 2 hours at 4°C on a rotating wheel.
4. Centrifuge at 13,000 rpm for 5 min at 4°C in a microcentrifuge, and transfer the cell lysates to new tubes.
5. Add 5 µg of GST bound beads to one pre-cleared sample (negative control) and 5 µg of GST-β-arr1 or GST-β-arr2 bound beads to the other one. Rotate the tubes from 2 hours to overnight at 4°C on a rotating wheel (*see* **Note 24**).
6. Wash the beads twice with High-salt cold mammalian cell lysis buffer (section 2.6, same volume as cell lysate), and centrifuge them at 4,000 rpm for 4 min at 4°C in a microcentrifuge after each wash.
7. Wash the beads twice with ice-cold lysis buffer (section 2.5, same volume as cell lysate), and centrifuge again at 4,000 rpm for 4 min at 4°C in a microcentrifuge.
8. Add ice-cold lysis buffer to the beads and transfer the mix to a new Eppendorf tubes before centrifuging them at 4,000 rpm for 4 min at 4°C in a microcentrifuge.
9. After removing the supernatant, add 50 µL of 2x Laemlli Buffer to the beads.
10. Boil the samples at 100°C for 5 min and centrifuge the beads at 13,000 rpm for 5 min in a microcentrifuge.
11. Transfer the supernatant to fresh Eppendorf tubes and proceed to load the samples on SDS-PAGE gels. Perform a western blot with appropriate antibodies.

Specificity of interactions is demonstrated by the pull-down of the protein partner with GST-β-arr, but not with GST.

3.5 BRET2 Saturation assay

Bioluminescence Resonance Energy Transfer (BRET), a non-radiative energy transfer, measures the dynamics of protein-protein interactions in intact live cells when the two protein of interest are fused to a luminescent energy donor and to a fluorescent acceptor molecule. Resonance energy transfer depends both on the proximity between the two proteins (less than 10 nm) and on the spatial orientation of the donor and acceptor in fusion with each protein [25]. BRET monitors changes in the light emission ratios between acceptor and donor. In the presence of its substrate, *Renilla* luciferase (Rluc, the energy donor) emits light that excites a Green Fluorescent Protein (GFP) variant (the energy acceptor), which in turn emits light. The Rluc substrate is cell permeable and can be directly added to the cell culture supernatant, hence no excitation of the donor is required. Different types of BRET technique can be used depending on donor/acceptor pair combinations and the Rluc substrate [26-28]. BRET has been used to study β-arrs interactions with GPCRs and other types of proteins [29-31]. To characterize β-arr interaction with other proteins, we have recently used BRET2. In this approach there is large separation between the emission peak of the energy donor RLuc2 (~400-410nm), a variant of Rluc with increased bioluminescence [26,32], and the one of the energy acceptor GFP10 (~515nm) [28] ensuring a high signal-to-noise ratio and detection of small BRET changes. In BRET saturation assays, constant amounts of donor-fused proteins are transfected with increasing concentrations of acceptor-fused protein. In case of specific interaction, BRET ratios will increase with GFP10 concentration up to a plateau when all donor molecules (Rluc2), are saturated by excess GFP10 acceptor molecules and a hyperbolic curve with an asymptote is observed. In the case of non-specific interactions, BRET ratios will continue to increase with increasing concentration of acceptor proteins resulting in a linear curve.

3.5.1. Cell preparation and data acquisition

1. HEK-293T cells are seeded in 6-well plates at a density of $2.5x10^5$ cells per well and incubate for 24 hours in a cell culture incubator at 37°C in a humidified atmosphere of 95% air and 5% CO_2.
2. Transfect cells with constant amount of plasmid DNA comprising a fixed quantity of plasmid encoding the Rluc2-fusion protein (donor) in the presence or absence of increasing concentrations of GFP10-fusion protein (acceptor) plasmid in different wells (*see* **Note 25**).
3. After 24 hours, aspirate media and wash the cells once with PBS.
4. Add 200 µL of Trypsin-EDTA to each 6-well. Transfer plates to the incubator for 30-60 s, allowing the cells to detach.
5. Add 2 mL of pre-warm complete DMEM media in each well and re-suspend the cells with a pipette.
6. Seed 20,000 cells (100-150 µL) per well on poly-L-ornithine coated 96-well microplates (*see* **Note 26**) and allow the cells to attach overnight in a 37°C incubator. Cells from each donor/acceptor plasmid transfection ratio should be distributed into 3 separate wells for multiple readings of the same condition.
7. Aspirate the media and wash the 96-well microplate once with 100 µL HBSS per well and add 90 µL of the same buffer (*see* **Note 27**).
8. The lid from the microplate is removed and the microplate transferred into a microplate reader. The fluorescence of the acceptor-fusion protein is measured using a 405+/-5nm excitation filter to verify that it is expressed in increasing quantities.
9. Add 10 µL of 25 µM Coelenterazine 400a working solution, prepared in HBSS, to each well (final concentration 2.5 µM) using a repeating pipette. Incubate the plate in the dark at room temperature for 30-45 s (*see* **Note 28**).
10. Rluc2 luminescence and GFP10 fluorescence measurements are performed sequentially for each well using 410+/-40nm and 515+/-20nm emission filters, respectively. Measurements are repeated three to six times for each well in order to get average values.

3.5.2. BRET calculation

1. BRET ratios are calculated as the ratio of GFP10 fluorescence over Rluc2 luminescence signals.

2. For each well the average fluorescence and luminescence signals are calculated from the 3-6 measurements and the corresponding BRET ratio is established.
3. The average background BRET ratio from the triplicates of cells transfected with only the donor DNA plasmid is calculated.
4. Subtract the background BRET value from the BRET ratio of each well and calculate the average net BRET ratio for triplicates corresponding to each donor/acceptor plasmid transfection ratio.
5. The net BRET ratios are multiplied by 1000 and expressed as mBRET values, which is an arbitrary value.
6. Subtract background GFP10 fluorescence, the average fluorescence measurements of triplicate cells transfected with only the donor plasmid, from all other samples to obtain the net GFP10 fluorescence values.
7. Calculate the average of the net GFP10 fluorescence values over Rluc2 luminescence signals for each sample.
8. Saturation assay curves are obtained by plotting the net BRET ratios as a function of the average net GFP10 fluorescence over Rluc2 luminescence.
9. If specific interactions, hyperbolic BRET-donor saturation curves are expected. The $Bret_{max}$ is the maximal BRET value corresponding to the saturation of BRET donors by BRET acceptors. The curves are fitted using a non-linear regression equation assuming a single binding site using GraphPad Prism to calculate $BRET_{50}$ values, which is the BRET value corresponding to 50% saturation of BRET donors, it reflects the relative affinity of β-arr for its protein partner.

4. Notes

1. In this strain either the intact GAL1 Upstream Activating Sequence (UAS), containing four GAL4-binding sites, or an artificially constructed UAS consisting of three copies of the consensus binding site, drives the expression of two reporter genes, *HIS3* and *LacZ* respectively [33]. HF7c is thus auxotrophic for histidine. The replacement of the HIS3 promoter by the GAL1 promoter is responsible for a tighter regulation of the HIS3 reporter gene. The low number of GAL4-binding sites (as opposed to 8 in the L410 yeast strain for example) also decreases the possibility of false positive signals.

2. Plasmids coding for β-arr and/or the potential protein partner fused to other tag peptide (myc, HA) or protein (GFP) can be used for transfection and co-immunoprecipitation experiments.
3. A recognition site for a protease is encoded by the linker sequence between the cDNAs coding for the GST tag and the protein of interest. It can be used to cleave GST from the GST-fusion protein, if needed. pGEX-4T3-β-arr vector has a thrombin cleavage site between the GST and β-arr proteins. Various pGEX vectors containing different protease recognition sites (thrombin, Factor Xa, PreScission Protease) are available (GE Healthcare Life Sciences).
4. The plasmids encoding N- and C-terminal fusion of β-arr1, β-arr2 and β-arrs putative partners with GFP10 or Rluc2, should be engineered and initially tested to find the most efficient pair for BRET.
5. 2x lysis Buffer without sodium orthovanadate, 10 mM NaF and Protease Inhibitors can be prepared and stored at -20°C. On the day of the experiment, thaw the required amount of 2x lysis buffer, dilute it to 1X and supplement it with 1 mM sodium orthovanadate, 10 mM NaF and Protease Inhibitors.
6. Other lysis buffer can be used for cell lysis and β-arr co-immunoprecipitation experiments [12,22].
7. Streak HF7c yeast on a YPD agar plate and incubate cells at 30°C until colonies have grown and store the plate at 4°C. Pick a colony to inoculate YPD media. Yeast YPD agar plate should not be kept more than 2 weeks at 4°C.
8. Eighteen to twenty transformations can be carried out from 100 mL of yeast culture media. Scaling up or down the transformation can be done at this step depending on the number of transformations needed.
9. It is recommended not to make more than 14-16 patches per plate. A patching grid can be used to orient the plate and to prevent cross-contaminations.
10. The velveteen squares are re-usable. They should be washed and autoclaved between each use.
11. Label the top of the master and replica plates so you keep the same orientation.
12. Make sure that the last plate that is inoculated is the CSM-Leu-Trp selective plate in which all the patches will grow. This ensures that failure of growth on the CSM-Leu-Trp-His Selective Plate is not due to poor transfer from the velveteen cloth.

13. β-Gal assay can be run in parallel to confirm the results obtained with the auxotrophic assay as previously described [34].
14. We use 1 μg of plasmid encoding β-arr, the amount of plasmid encoding the putative partner can vary from 0.5 to 2 μg. Preliminary experiments should be performed by western blot of the cell lysates with the appropriate antibodies to ensure that the same amount of each protein is expressed in each condition.
15. The EZview Red anti-Flag M2 affinity gel is composed of agarose with a covalently attached mouse monoclonal antibody directed against the FLAG epitope. Its use shortens the immunoprecipitation procedure compared to the classical one using free primary antibody and binding to protein A and/or G beads. The red color of the gel also facilitates its visualization during the procedure. If primary antibodies are used instead of affinity gels, they can be added at a ratio of 1:100 (v/v) for co-immunoprecipitations. For primary antibodies targeting protein tags such as FLAG, HA and GFP, 1-2.5 μg of antibodies are used per IP.
16. Other final volume can be chosen depending on the protein concentration of the cell lysates and the type of volume that can be loaded on the SDS-PAGE gel used, the final Laemlli Buffer should in any case be 1X.
17. These samples are important to control the expression levels of the transfected proteins in the lysates.
18. After IP, the supernantants can be conserved to check by western blot for immunodepletion of FLAG-β-arr by comparison with cell extract before IP.
19. Because Src and β-arr2 run very close to immunoglobulin (IgG) heavy and light chains, HRP-conjugated secondary antibodies that do not recognize IgG (True-Blot anti rabbit-HRP, 18-8816-33, 1:1000) are used.
20. When purifying a new GST-fusion protein, a small-scale pilot experiment can be carried out with increasing concentration of IPTG from 0.1 mM to 1 mM to determine the best concentration to induce protein expression. The induction time is variable depending on the protein fused to GST. GST and most GST fusion protein induction requires only 4 hours at 37°C. For GST-β-arr1 and GST-β-arr2, the induction temperature is lowered to 20°C with a longer induction duration, as GST-β-arr localize to inclusion bodies at 37°C, resulting in low yield.

21. Aliquots of the clarified bacterial lysate can be collected before and after incubation with the beads and run on a SDS-PAGE gel to determine binding efficiency. The supernatants from the first incubation with beads can be conserved and incubated again with fresh glutathione beads, if the binding to the beads is low.
22. The beads with the immobilized GST or GST fusion proteins can be stored at 4°C and used during 7-10 days. Sodium azide can be added to a final concentration of 0.05% in the bead suspension to prevent bacterial growth.
23. If the protein to be pulled down is endogenously expressed, use 750µg-1mg of cell lysate.
24. When GST is used as a negative control for the GST-β-arr proteins, make sure that the bead volumes are the same. GST or GST-β-arr bound beads can be saturated with 5% BSA overnight to reduce background.
25. Genejuice (3 µL/µg DNA) is used to transfect the cells. The total amount of transfected DNA (around 1 µg) is maintained constant by adding appropriate amounts of the corresponding empty DNA vector (e.g. pCDNA3.1). The level of the BRET donor (Rluc2-fusion protein) used for saturation assays should correspond to the lowest amount of protein required to obtain a detectable and robust Rluc2 signal (e.g. a minimum of 100,000 light units with a 1 s integration time using a Mithras plate reader), ideally close to the endogenous expression of the protein fused to Rluc2. Preliminary transfection experiments with increasing concentrations of the BRET-donor DNA plasmid followed by Rluc2 measurements should be performed to determine the amount to be used for BRET (usually 1-100 ng). Western blot of the samples will also indicate its level of expression compared to the endogenous protein. The BRET-acceptor DNA plasmid is usually transfected within a range of 10 ng to 1 µg. The expression of the Rluc2 fusion protein must be equivalent in all conditions with a ±20% variability tolerated. Conditions with a negative acceptor-fusion protein should also be included in the saturation assay to demonstrate specificity of the generated BRET. This protein should be expressed in the same cell compartment as the protein of interest.
26. Microplates are coated with poly-L-Ornithine (a synthetic positively charged aminoacid) to enhance the adherence of HEK cells. Dilute Poly-L-Ornithine to a

final concentration of 30 µg/mL with dH_2O. Coat the 96-well microplate with 100 µL of this diluted poly-L-Ornithine per well. Leave the plate in the incubator at 37°C for 15 min-2 hours. Aspirate the Poly-L-Ornithine, wash once with 1x PBS and air-dry the plate. Poly-L-Ornithine-coated microplates can be stored at RT for a few days.

27. BRET measurements are carried in colorless HBSS or PBS buffer as phenol red interferes with the assay.
28. The 25 µM working solution of Coelenterazine 400a is always prepared fresh, as it is susceptible to oxidation.

Figure legend

Co-immunoprecipitation of Src and β-arr2. HEK-293T cells were transfected with Flag-β-arr2 and Src or the corresponding empty pcDNA3.1 vector as indicated. Immunoprecipitations of Flag-β-arr2 were performed with anti-Flag (IP: Flag) antibodies and immunoblotted with anti-Src and anti-Flag antibodies. Immunoblotting of the cell lysate used for the immunoprecipitation experiment is shown.

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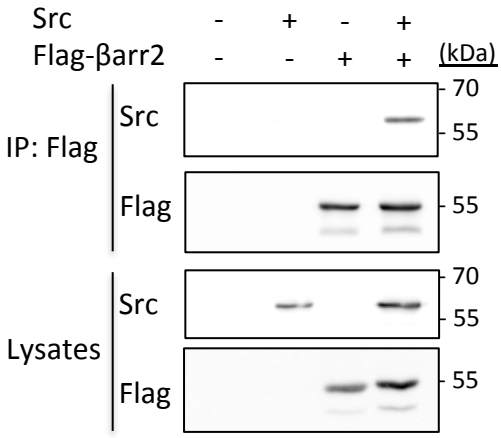
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