Chapter 12

Methods to Immobilize GPCR on the Surface of SPR Sensors

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Abstract

The G protein-coupled receptors (GPCRs) form one of the largest membrane receptor families. The nature of the ligands that interact with these receptors is highly diverse; they include light, peptides and hormones, neurotransmitters, and small molecular weight compounds. The GPCRs are involved in a wide variety of physiological processes and thus hold considerable therapeutic potential.

GPCR function is usually determined in cell-based assays, whose complexity nonetheless limits their use. The use of alternative, cell-free assays is hampered by the difficulties in purifying these seven-transmembrane domain receptors without altering their functional properties. Several methods have been proposed to immobilize GPCR on biosensor surfaces which use antibodies or avidin-/biotin-based capture procedures, alone or with reconstitution of the GPCR physiological microenvironment. Here we propose a method for GPCR immobilization in their native membrane microenvironment that requires no manipulation of the target receptor and maintains the many conformations GPCR can adopt in the cell membrane.

Key words G protein-coupled receptor (GPCR), Lentivirus, Surface plasmon resonance (SPR), Transfection, Packaging cell, Membrane protein, Human CXCR4, Oligomerization

1 Introduction

1.1 Surface Plasmon Resonance Sensors

Surface plasmon resonance (SPR) techniques offer great possibilities for studying and analyzing real-time interactions between molecules of biological interest [1]. These techniques are a clear alternative to more complex cell-based assays and can be used in high-throughput screening strategies. In the case of G protein-coupled receptors (GPCRs), SPR is particularly relevant for several reasons, as (1) this is the largest receptor family in the human genome, and includes receptors for hormones, neurotransmitters, chemokines, calcium ions, and light [2, 3], (2) it is probably the most clinically relevant receptor class [4, 5], and (3) due to their complex, seven-transmembrane-spanning hydrophobic α-helix

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structure, these receptors require a lipid environment to maintain their native conformation [6–8].

Proteins/molecules must be immobilized on an SPR biosensor surface to allow monitoring of interaction with a second protein/molecule in solution. Immobilization to the support should retain the native protein conformation and binding activity over the course of the binding assay [9]. Proteins are usually immobilized covalently to the substrate, which can lead to random receptor orientation or altered conformation. Chemical linker layers between the sensor surface and the biomolecule improve accessibility between the immobilized receptor and its ligands [10, 11]. Two basic strategies are used:

– *Covalent attachment* to an *N*-hydroxysuccinimide/(1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (NHS/EDC)-derivatized dextran matrix that allows coupling to amino groups. Low pH is sometimes required, which increases the possibility of receptor denaturation. Multiple amino groups on the receptor can result in random coupling and receptor orientation on the sensor surface [12, 13]. Other modified matrices can be used to allow more specific, oriented receptor coupling [9].
– *Non-covalent attachment* via histidine tag, avidin-biotin, or antibody-based approaches that allow unidirectional coupling of membrane proteins [14, 15].

These strategies require the use of detergents, as well as receptor purification and modification, to include epitope tags or biotin groups [16–18]. For receptors with many hydrophobic domains, these processes are complex and can alter receptor structure and ligand binding parameters; in addition, solubilization conditions must be optimized for each receptor. Many researchers use strategies that immobilize receptors derived from detergent-solubilized membranes or crude cell preparations, which are reconstituted in a lipid microenvironment to maintain receptor activity [19–23]. Lipid bilayer formation and reconstitution can nonetheless limit access to the receptor surface and modify the effect of the original lipid composition and/or other membrane proteins.

For proteins with complex structures, immobilization on the SPR surface might not completely retain their functional characteristics [9, 18]. In addition to their intrinsic structural complexity, GPCRs are found on the cell membrane in many conformations, which include preformed homo- and heterodimers and oligomers [24, 25]. These distinct conformations and the way a given ligand modulates these structures dictate the functional effect. GPCR can also be influenced by the lipid composition of specific membrane regions and/or other cell surface molecules [7, 26–29], which makes it difficult to establish reliable methods to reproduce native GPCR conformation(s) on the SPR biosensor surface.

Some years ago, Hoffman et al. described a technique to study ligand binding to complex transmembrane proteins using SPR, by incorporation of these proteins into murine leukemia virus (MLV) virions that are easily purified and immobilized on the sensor surface [30]. Here we describe an alternative method for membrane protein immobilization based on the generation of lentiviral particles.

1.2 Lentiviral Particles (LVP)

Viruses are biological agents that efficiently introduce their genetic material into target cells, on which they depend for replication [31]. This property has been used to obtain non-replicative vectors able to introduce genes of interest into a target cell [32]. Lentiviral vectors are one of the most commonly used gene-transfer systems in gene therapy, due to their low immunogenicity, the possibility of infecting unmanipulated cells, the high capacity for transgene integration independent of cell cycle status, and the long-term maintenance of transgene expression [33–36].

Lentiviruses are members of the *Retroviridae* family and can infect both dividing and nondividing cells [37]. Lentiviral vectors were originally designed to provide effective gene therapies [38–40]. Several accessory viral genes have been removed to generate minimal packaging systems; they are thus unable to replicate and are less immunogenic as they lack most genes that encode viral proteins [32, 41–43]. HIV-1 is the most studied of all lentiviruses, and HIV-1-based vectors are the most widely used for protein and siRNA delivery to target cells [32, 44, 45]. As for MLV and other viruses, lentivirus budding from the packaging cell will incorporate cell membrane fragments and the proteins they bear [46, 47].

Lentiviral vectors are thus based on modified HIV-1 virus and are usually generated by transient cell transfection of several plasmids:

1. The packaging unit (e.g., pCMV-dR8.91, pCMV-dR8.74, psPAX2) contains the genes that encode structural proteins and enzymes (gag, pol, tat, rev) needed to generate the lentiviral particle.
2. The transfer vector (pLVTHM) bears the genetic material to be transduced in the target cell.
3. The envelope plasmid (pMD2G) is derived from a heterologous virus, usually vesicular stomatitis virus (VSV), and determines vector tropism.

In the packaging unit, genes that code for viral proteins crucial for virulence (env, vif, vpr, vpu, nef) have been eliminated.

As commented above, transient transfection of a producer cell such as human embryonic kidney 293T cells (HEK-293T) with these plasmids generates LVP that include membrane fragments bearing proteins, among them is GPCR. LVP can thus act as vehicles that express membrane proteins in their native context and can be immobilized on a biosensor surface [48–50]. Receptor expression

can be evaluated before LVP generation, and control LVP can be made using mock-transfected packaging cells. A gene of interest can be included in the transfer plasmid (pLVTHM) to increase receptor levels at the cell surface and thus at the surface of the LVP. The GPCR-expressing LVP can be attached to the surface of SPR biosensors by standard immobilization strategies. This method eliminates GPCR solubilization, stabilization, and purification, as well as the need to purify and reconstitute membrane proteins; it does not require membrane regeneration, specific capture antibodies, or alternative capture reagents. In summary, LVP immobilization for use in SPR techniques is a simple, broadly applicable means to present GPCR and other membrane proteins on a biosensor surface.

2 Materials

Always use sterile materials and solutions.

2.1 Generation of Lentiviral Particles

1. Plasmids: pLVTHM, psPAX2, and pMD2G were obtained from Prof. Didier Trono (http://tronolab.epfl.ch Tronolab, Lausanne, Switzerland) and can be ordered from Addgene. pGPCR contains the GPCR of interest.
2. Transfection reagents: jetPEI 150 mM NaCl, sterile solution.
3. Packaging cells: HEK-293T cells (ATCC CRL-11268). The HEK-293T cell line is a highly transfectable derivative of the human embryonic kidney 293 (HEK-293) cell line [51, 52].
4. Tissue culture media: HEK-293T are cultured in DMEM supplemented with 10 % fetal calf serum (FCS), 2 mM L-glutamine, and 1 mM sodium pyruvate (complete DMEM) in the absence of antibiotics. When required, 0.05 % trypsin/EDTA solution is used to detach cells.
5. 150×20 mm, 150 cm^2 culture area, cell culture Petri dishes (p150).
6. Sucrose solution (20 %) for LVP purification.
7. BCA protein assay kit.
8. SW55 Beckman-type rotor. Optima L-100 XP ultracentrifuge.
9. 24-well, flat bottom tissue culture plates, 2 cm^2 culture area (p24).

2.2 Characterization of Lentiviral Particles

1. All techniques described for the characterization of LVP (ELISA, flow cytometry, and electron microscopy) require the use of adequate primary antibodies that recognize specifically the GPCR of interest or other proteins present in the LVP that can be used as control (in our case GFP and CXCR4). Although there are commercially available antibodies against most GPCR, the availability of the correct antibody for the different

applications should be confirmed in advance. Secondary antibodies labeled with appropriate molecules will also be required. We employ goat anti-mouse IgG HRPO-labeled secondary antibodies for ELISA, FITC- or PE-labeled rabbit anti-mouse antibodies for flow cytometry, and colloidal gold-conjugated anti-mouse IgG + IgM antibody for electron microscopy.

2. Latex beads (4 μm) in 4 % w/v aldehyde/sulfate.
3. Glycine 1 M buffer (7.53 g glycine in 100 mL PBS, pH 8.0).
4. 96-well ELISA plates specially formulated and treated to provide protein coating capacity.
5. ELISA blocking solution: PBS (10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), 0.5 % BSA, 2 % normal goat serum.
6. SIGMAFAST OPD tablets (*o*-phenylenediamine).
7. Carbon grids for electron microscopy (EM) (Gilder Grids).
8. 10-nm gold-conjugated goat $F(ab')_2$ anti-mouse IgG + IgM antibody (15 min, RT). Supplied in 20 mM Tris (*tris-hydroxymethyl-aminomethane*); 20 mM sodium azide; 154 mM NaCl; 1 % BSA; 20 % glycerol; pH 8.2. To make 100 mL: 0.242 g (20 mM) Tris + 0.9 g (154 mM) NaCl + ultrapure water. Adjust pH from 7.2 to 8.2 with 1 N HCl or 1 N NaOH.
9. Wash buffer for EM: PBS, 1 % BSA, 1 % normal goat serum, 0.02 % sodium azide.
10. Uranyl acetate 4 % (w/v) solution (dissolve 4 g of uranyl acetate in 100 mL of boiling bidistilled water, filtrate, and maintain at 4 °C protected from light).
11. Transmission electron microscope (1200-EX II, JEOL, Tokyo).
12. ^{35}S-Methionine (50 mCi/mL, PerkinElmer).
13. 12 % SDS-PAGE polyacrylamide gels.
14. 3-MM filter paper.
15. Fluorescence imager (Phosphorimager Storm 869, Molecular Dynamics, GE Healthcare).
16. Quantity One software (Bio-Rad).

2.3 Immobilization of Lentiviral Particles

1. CM5 sensor chip (GE Healthcare).
2. Reagents for cleaning gold surfaces: trichloroethylene, acetone, ethanol, and H_2O.
3. Cleaned gold surfaces.
4. Biacore 3000 (GE Healthcare).
5. Piranha solution: H_2SO_4-H_2O_2 (3:1).
6. 11-Mercaptoundecanoic acid (0.05 mM; 1.092 mg in 100 mL H_2O).

7. *N*-Hydroxysuccinimide (NHS, 0.1 M; 1.15 g in 100 mL H_2O).
8. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 0.4 M; 7.67 g in 100 mL H_2O).
9. Phosphate-buffered saline (PBS), 0.05 % Tween 20, pH 7.4.
10. HEPES-buffered saline-P [10 mM HEPES, 0.15 M NaCl, 0.005 % polyoxyethylene sorbitan monolaurate (P20), pH 7.4].
11. Acetate buffer (10 mM, pH 4.0; 82 mg CH_3COONa in 100 mL H_2O).
12. Ethanolamine (1 M, pH 8.5; 6.108 g in 100 mL H_2O).

3 Methods

3.1 Transfection of Packaging Cells and Purification of LVP

LVP are generated by transfecting a packaging cell line (in our case, HEK-293T cells) with three plasmids, pLVTHM, psPAX2, and pMD2G (Fig. 1), using jetPEI (*see* **Note 1**):

1. Plate p150 dishes with HEK-293T cells (0.25×10^6 cells/mL) in complete DMEM 24 h before transfection (*see* **Note 2**).
2. Transfect cells with the GPCR of interest (*see* **Note 3**): For each p150 dish, prepare 1.5 mL NaCl (150 mM) containing 10–15 μg of pGPCR, the plasmid bearing the receptor of interest (Tube A). Vortex.
3. For each p150 dish, prepare 1.5 mL NaCl, 150 mM containing 20–30 μL jetPEI (Tube B). Vortex.
4. Add the preparation in Tube B to Tube A and vortex 30 s at room temperature (RT).
5. Incubate 30 min at RT.
6. Carefully eliminate the medium from the p150 dishes that contain the HEK-293T cells and add 17 mL/plate of fresh complete DMEM.
7. Add 3 mL/plate of Tube A + Tube B mixture.
8. Allow cells to express the transfected receptor by incubation for 24 h at 37 °C, 5 % CO_2 prior to transfection with the lentiviral plasmids.
9. Determine receptor expression by flow cytometry using specific antibodies.
10. Transfect cells with the lentiviral plasmids: For each p150 dish, prepare 1.5 mL NaCl, 150 mM containing 10 μg each of the three plasmids (pLVTHM, psPax2, and pMD2G; Tube A). Vortex (*see* **Note 4**).
11. For each p150 dish, prepare 1.5 mL NaCl, 150 mM containing 60 μL jetPEI (Tube B). Vortex.
12. Add the preparation in Tube B to Tube A and vortex 30 s at room temperature.

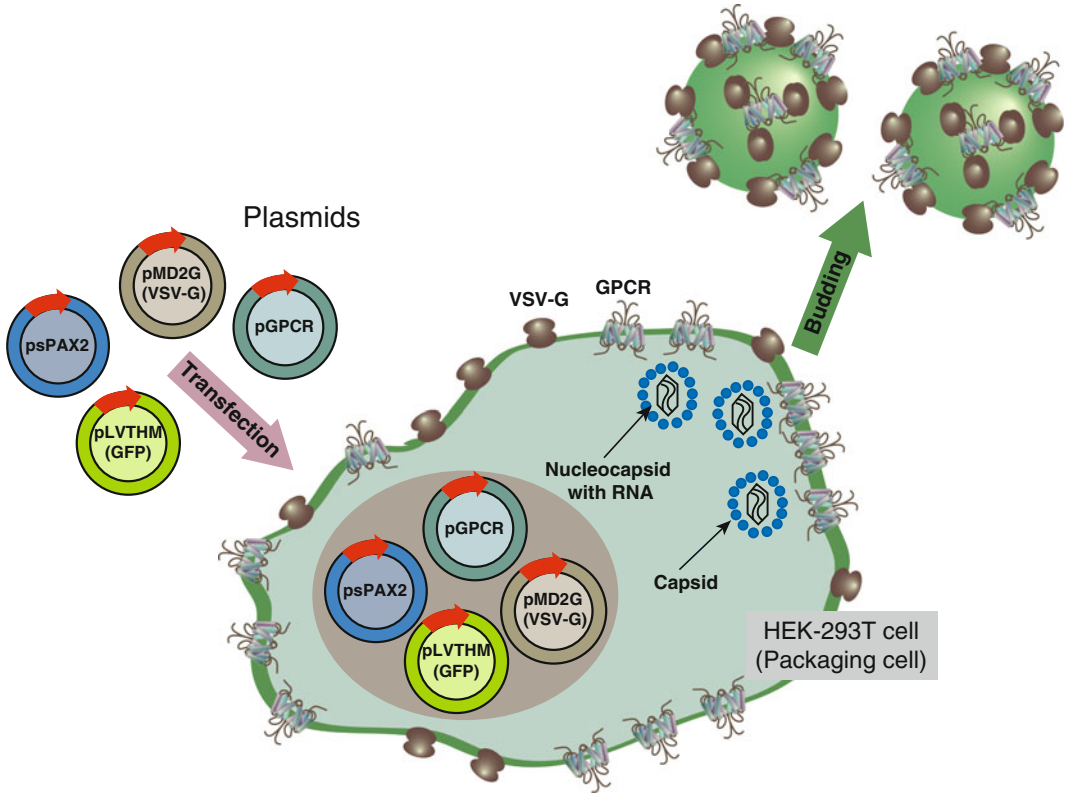

Fig. 1 Procedure followed to generate lentiviral particles (LVP). Cells are transfected with the GPCR of interest (pGPCR), followed by transfection with lentiviral plasmids (psPAX2, pLVTHM, pMD2G). During budding, LVP incorporate membrane fragments that include the transfected GPCR in its native context

13. Incubate 30 min at RT.
14. Carefully eliminate the medium from the p150 dishes that contain the HEK-293T cells and add 17 mL/plate of fresh complete DMEM.
15. Add 3 mL/plate of Tube A + Tube B mixture.
16. At 72 h post-transfection, collect the cell supernatant and centrifuge (2,100 × *g*, 30 min, RT) to eliminate cell debris.
17. Collect the transfected HEK-293T cells to analyze expression of transfected proteins by flow cytometry, immunocytochemistry (Fig. 2), and/or Western blot.
18. Place the supernatant on a 20 % sucrose cushion (17 mL supernatant on 4 mL sucrose solution) and centrifuge (247,000 × *g*, 120 min, 4 °C).
19. The resulting pellet will contain the purified LVP (*see* **Note 5**). Resuspend the pellet in a small volume of sterile PBS; aliquot and store at −80 °C. LVP should be stored in small aliquots to avoid repeated freeze/thaw cycles. LVP can be quantitated using a BCA protein assay kit.

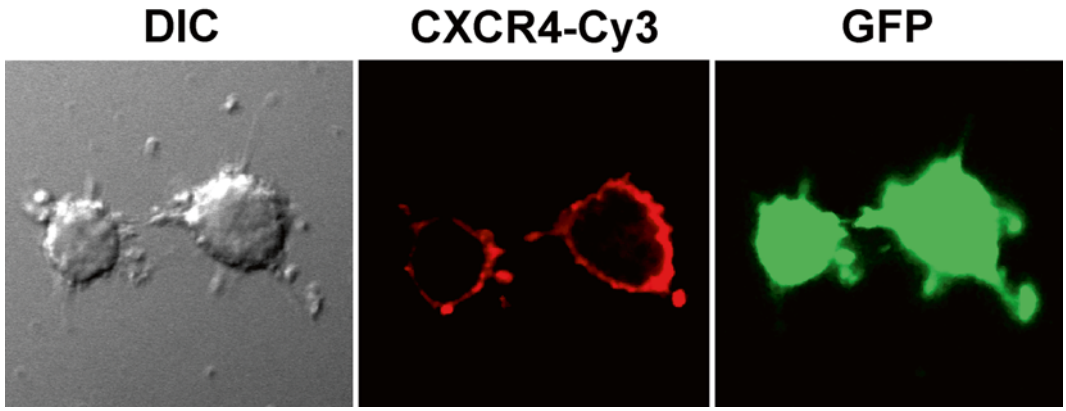

Fig. 2 Immunocytochemistry analysis of hCXCR4 and GFP expression on HEK-293T cells transfected with lentiviral vectors. hCXCR4 is constitutively expressed on HEK-293T cells and was visualized using specific antibodies. GFP is expressed by the pLVTHM plasmid and can easily be followed by immunocytochemistry. The figure shows an example of HEK-293T cells at 72 h post-transfection with the lentiviral plasmids

3.2 Titration of LVP

LVP is titrated by transducing HEK-293T cells with serial dilutions of the purified particles (*see* **Note 6**).

1. Plate HEK-293T cells (3×10^4 cells/well) in 1 mL complete DMEM using 24-well plates (day 1) and allow them to adhere (overnight, 37 °C, 5 % CO_2).
2. Prepare serial dilutions of the purified LVP in 250 μL complete DMEM.
3. On day 2, count cells in one well (should contain $6–8 \times 10^4$ cells) for use as reference.
4. Aspirate the medium from the 24-well plates and add the different LVP dilutions. Incubate (24 h) and add 1 mL/well complete DMEM.
5. At 96 h after LVP addition, detach the transduced cells using 0.05 % trypsin/EDTA. By flow cytometry, evaluate the percentage of transduced cells (*see* **Note 7**) by measuring GFP expression (included in the pLVTHM plasmid). Titer is expressed as transforming units per milliliter, which are calculated as the number of cells transduced by a given volume of LVP.

3.3 Characterization of LVP by Flow Cytometry

LVP expression of the protein of interest can be determined by many techniques, including flow cytometry, Western blot analysis, ELISA, and electron microscopy. The transfected HEK-293T cells should be included in this study as a control. Due to the small size of LVP, the proteins on their surface cannot be followed by conventional flow cytometry techniques. To allow their detection in flow cytometers, we therefore couple the LVP to latex beads.

Aldehyde/sulfate latex beads have numerous aldehyde groups grafted to the surface of the polymer particle. The high density of the aldehyde group allows easy coupling of proteins and other materials to the latex particles in a one-step process. The sulfate groups on the microsphere surface maintain stability during covalent coupling. The use of fluorescently labeled ligands or conformation-dependent antibodies allows detection of the target GPCR in its native conformation on the LVP surface:

1. Sonicate a 20–100 μL of latex beads (5 min, RT).
2. Add 15 μL LVP to 15 μL sonicated beads and incubate (15 min, RT).
3. Add 1 mL PBS and incubate (60 min, 4 °C, with continuous shaking).
4. Add 100 μL glycine buffer (100 mM final concentration) to block free reactive groups. Incubate (30 min, RT, with continuous shaking).
5. Centrifuge (1,950 × *g*, 3 min, RT).
6. Wash with 1 mL PBS-0.5 % BSA and centrifuge (1,950 × *g*, 3 min, RT).
7. Suspend the beads in PBS-0.5 % BSA; 15 μL of beads are usually sufficient for eight different staining conditions. GPCR on the surface of LVP bound to latex beads can now be stained with appropriate antibodies by standard techniques (*see* **Notes 8** and **9**) (Fig. 3) [53, 54].

3.4 Characterization of LVP by Enzyme-Linked Immunoassay (ELISA)

GPCR on the LVP surface can be detected in ELISA assays [48, 49, 55]:

1. Coat 96-well ELISA plates directly with 1–5 μg/mL or 10^7 LVP particles in PBS (100 μL/well, overnight, 4 °C). LVP from mock-transfected HEK-293T cells should be used as control.
2. After washing 3× with 300 μL/well PBS, block plates with 200 μL/well ELISA blocking solution (60 min, 37 °C).
3. Wash 3× with PBS.
4. Incubate LVP with specific anti-GPCR antibodies or ligands diluted in PBS, 0.5 % BSA (100 μL/well, 90 min, 37 °C).
5. Wash 3× with PBS.
6. Add horseradish peroxidase (HRPO)-labeled secondary antibodies diluted in PBS, 0.5 % BSA (100 μL/well, 90 min, 37 °C).
7. Wash 3× with PBS.
8. Incubate with OPD or an alternative HRPO substrate and read absorbance using appropriate settings.

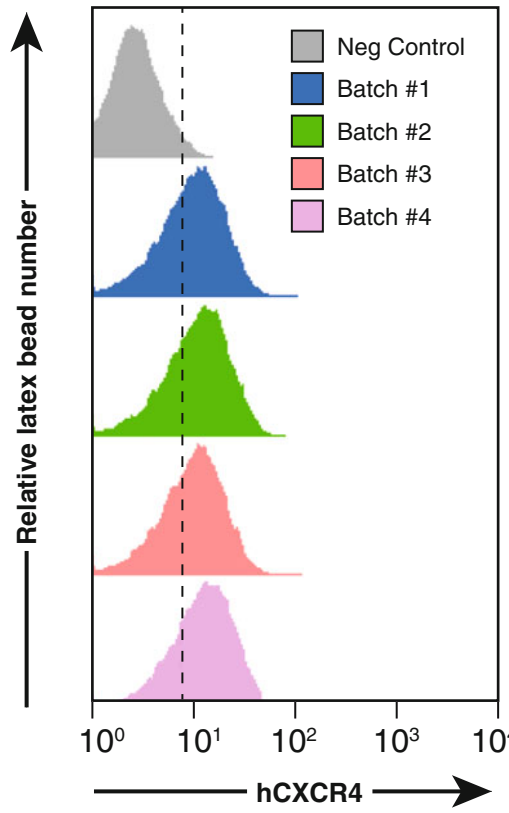

Fig. 3 Staining of hCXCR4 expressed LVP coupled to latex beads. LVP were generated from HEK-293T cells that constitutively express the GPCR hCXCR4. After LVP coupling to latex beads, CXCR4 was analyzed using specific anti-hCXCR4 antibodies by flow cytometry. The figure shows fluorescence intensity of hCXCR4 in different LVP batches

3.5 Characterization of LVP by Transmission Electron Microscopy

Receptor expression in LVP can be analyzed on carbon grids by negative staining using transmission electron microscopy [49]:

1. Incubate LVP samples (15 μL) on carbon grids with anti-GPCR antibody in PBS, 0.5 % BSA (15 min, RT).
2. Incubate with 50 μL of 10-nm gold-conjugated goat $F(ab')_2$ anti-mouse IgG + IgM antibody (10 μg/mL in EM wash buffer, 15 min, RT).
3. Add 200 μL EM wash buffer (5×, 1–2 min, RT).
4. Treat samples with 100 μL 2 % uranyl acetate (30 s, RT) and allow to dry.
5. Examine if the EM grids are in a transmission electron microscope.

3.6 Quantification of Receptor Number

Target protein expression can also be controlled by standard *SDS-PAGE* and *Western blot* techniques. GFP or VSV envelope glycoprotein (VSV-G) expression can be used as control (*see* **Note 10**):

1. Plate and transfect HEK-293T cells with lentiviral plasmids as indicated (Subheading 3.1).
2. Incubate in complete DMEM (24 h, 37 °C, 5 % CO_2).
3. Wash cells with methionine (Met)-free DMEM and maintain cells in this medium supplemented with ^{35}S-Met (50 mCi/mL) for 48 h, 37 °C, 5 % CO_2.
4. Collect ^{35}S-Met-labeled LVP and purify as indicated above (Subheading 3.1).
5. Load serial dilutions of the resulting LVP onto 12 % polyacrylamide gels and resolve by SDS-PAGE.
6. Dry the gels on 3-MM filter paper and visualize with the phosphorimager.
7. Calculate relative receptor stoichiometry using Quantity One software (Bio-Rad, Hercules, CA, USA) by normalizing to the number of Met residues for p24 and the receptor of interest and interpolating these values on a regression curve of p24 data used as internal standard. We assume 2,100 molecules of p24/LVP, as indicated [56].

3.7 Coupling LVP to Homemade Biosensors

We use two types of SPR biosensors for LVP coupling: a homemade biosensor [57, 58] and a commercial Biacore 3000. The coupling of LVP should be designed to optimize LVP density on the sensor surface and avoid nonspecific interactions, without altering the biological activity of the receptors on the LVP surface. LVP can be attached to the gold sensor surface through self-assembled monolayers (SAM) that allow orientated, reproducible, simple biomolecule immobilization, which reduces nonspecific adsorption of molecules (Fig. 4).

Covalent coupling of LVP to SAM

1. Clean gold surfaces by ultrasonication successively with trichloroethylene, followed by acetone, ethanol, and H_2O.
2. Immerse the chips in piranha solution for 15 s.
3. Rinse with water.
4. Dry under a N_2 stream.
5. Form alkanethiol SAM by injecting mercaptoundecanoic acid (0.05 mM) at a flow rate of 20 µL/min. This flow rate will be maintained throughout the immobilization process.
6. Rinse with ethanol and then with H_2O.
7. Activate the surface by injecting a mixture of equal volumes of NHS (0.1 M) and EDC (0.4 M) (*see* **Note 11**).

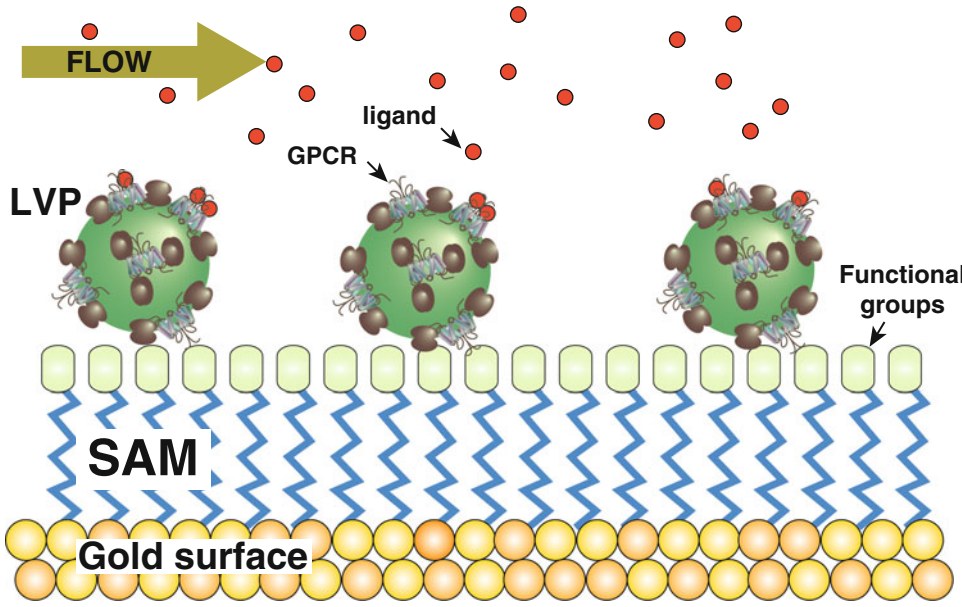

Fig. 4 Scheme showing LVP immobilization to the biosensor surface self-assembled monolayers (SAM). GPCRs expressed on the LVP surface are immobilized to the sensor surface through interaction with functional groups on SAM

8. Inject the LVP in acetate buffer (*see* **Note 12**).
9. Block with ethanolamine (1 M, pH 8.5).
10. Inject a continuous flow (60 μL/min) of PBS Tween 20 0.05 %, pH 7.4 (*see* **Note 13**).

3.8 Coupling LVP to Biacore 3000

1. Activate the carboxymethylated dextran surface of a CM5 sensor chip by mixing equal volumes of 0.1 M NHS and 0.4 M EDC and injecting the mixture (5 μL/min, 7 min, RT) over the sensor chip.
2. Use HEPES-buffered saline-P as immobilization running buffer.
3. Inject LVP (10^7/mL) diluted in acetate buffer (10 mM, pH 4.0) over the activated surface (5 μL/min, 7 min, RT).
4. Inject ethanolamine (1 M, pH 8.5, 5 μL/min, 7 min, RT) to deactivate remaining active carboxyl groups.
5. Correct LVP coupling to the biosensor surface should be determined for each receptor using specific antibodies. If HEK-293T cells are used as packaging cells, the chemokine receptor CXCR4 that is constitutively expressed by this cell line can be used as control. Specific anti-CXCR4 antibodies, ligands, and antagonists are commercially available.

Other commercial or homemade sensor chips with different surfaces can be used. LVP coupling to the biosensor surface does not appear to be a major problem.

4 Notes

1. Cells can also be transfected using calcium phosphate, Lipofectamine, or any other method that yields good transfection efficiencies.
2. Always use fresh HEK-293T cells. Avoid repeated passages.
3. Always generate LVP from untransfected HEK-293T cells for use as negative control in all experiments. When using the pLVTHM plasmid, the control particles will express GFP, which can be used to control the correct LVP expression. If HEK-293T cells express the receptor of interest (e.g., the chemokine receptor CXCR4 is constitutively expressed in this cell line) [49], or if you have stable transfectants of the target GPCR, lentiviral plasmids can be transfected without the transient transfections with pGPCR described in Subheading 3.1, **step 2**.
4. In most cases, we do not include the pMD2-G plasmid, as it is not needed for GPCR expression on the LVP surface. LVP lacking pMD2-G will be unable to infect target cells, but are fully functional for SPR immobilization and GPCR expression. When an increase in receptor levels is needed, the receptor DNA can be incorporated into the pLVTHM plasmid.
5. There is increasing evidence that some viruses bud selectively from the cell surface through specific detergent-insoluble lipid rafts, which affects expression of some receptors in the LVP. Incorporation efficiency of the transfected GPCR in the LVP probably correlates with expression level.
6. LVP can also be titrated by PCR using primers specific for lentiviral genes in your plasmids [59].
7. LVP that lack VSV-G or alternative envelope plasmids cannot be titrated by transducing HEK-293T cells, as the resulting LVP are neither infective nor replicative.
8. As negative control for flow cytometry, include uncoupled beads or beads coupled to LVP obtained from mock-transfected HEK-293T cells.
9. Using the original pLVTHM, GFP will be expressed in the LVP; secondary antibodies should therefore be selected that do not interfere with GFP fluorescence.
10. Western blot detects denatured proteins; therefore, it cannot be assumed that the receptor of interest retains its native conformation in the LVP. Detection by these methods depends on the availability of specific antibodies that recognize the denatured protein. Receptor numbers in the LVP can be estimated by comparing the target receptor expression with the known number of expressed structural viral protein p24 molecules.

11. Covalent immobilization allows formation of stable, reproducible surfaces with control LVP density.
12. Correct LVP coupling to the biosensor surface should be determined for each receptor using specific antibodies. If HEK-293T cells are used as packaging cells, the chemokine receptor CXCR4 that is constitutively expressed by this cell line can be used as control. Specific anti-CXCR4 antibodies, ligands, and antagonists are commercially available.
13. Appropriate assay buffer can greatly reduce nonspecific binding to the matrix. Evaluate a range of pH and ionic strength during immobilization and regeneration processes.

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References

1. Rich RL, Myszka DG (2007) Higher-throughput, label-free, real-time molecular interaction analysis. Anal Biochem 361:1–6
2. Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol 63:1256–1272
3. Vassilatis DK, Hohmann JG, Zeng H, Li F, Ranchalis JE, Mortrud MT et al (2003) The G protein-coupled receptor repertoires of human and mouse. Proc Natl Acad Sci U S A 100: 4903–4908
4. Overington JP, Al-Lazikani B, Hopkins AL (2006) How many drug targets are there? Nat Rev Drug Discov 5:993–996
5. Wise A, Gearing K, Rees S (2002) Target validation of G-protein coupled receptors. Drug Discov Today 7:235–246
6. Chini B, Parenti M (2004) G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there? J Mol Endocrinol 32:325–338
7. Drake MT, Shenoy SK, Lefkowitz RJ (2006) Trafficking of G protein-coupled receptors. Circ Res 99:570–582
8. Tan CM, Brady AE, Nickols HH, Wang Q, Limbird LE (2004) Membrane trafficking of G protein-coupled receptors. Annu Rev Pharmacol Toxicol 44:559–609
9. Alves ID, Park CK, Hruby VJ (2005) Plasmon resonance methods in GPCR signaling and other membrane events. Curr Protein Pept Sci 6:293–312
10. Wink T, van Zuilen SJ, Bult A, van Bennkom WP (1997) Self-assembled monolayers for biosensors. Analyst 122:43R–50R
11. Fruh V, IJzerman AP, Siegal G (2011) How to catch a membrane protein in action: a review of functional membrane protein immobilization strategies and their applications. Chem Rev 111:640–656
12. O'Shannessy DJ, Brigham-Burke M, Peck K (1992) Immobilization chemistries suitable for use in the BIAcore surface plasmon resonance detector. Anal Biochem 205:132–136

13. Stein T, Gerisch G (1996) Oriented binding of a lipid-anchored cell adhesion protein onto a biosensor surface using hydrophobic immobilization and photoactive crosslinking. Anal Biochem 237:252–259
14. Gottschalk I, Lagerquist C, Zuo SS, Lundqvist A, Lundahl P (2002) Immobilized-biomembrane affinity chromatography for binding studies of membrane proteins. J Chromatogr B Analyt Technol Biomed Life Sci 768:31–40
15. Civjan NR, Bayburt TH, Schuler MA, Sligar SG (2003) Direct solubilization of heterologously expressed membrane proteins by incorporation into nanoscale lipid bilayers. Biotechniques 35:556–563
16. Charvolin D, Perez JB, Rouviere F, Giusti F, Bazzacco P, Abdine A et al (2009) The use of amphipols as universal molecular adapters to immobilize membrane proteins onto solid supports. Proc Natl Acad Sci U S A 106:405–410
17. Harding PJ, Hadingham TC, McDonnell JM, Watts A (2006) Direct analysis of a GPCR-agonist interaction by surface plasmon resonance. Eur Biophys J 35:709–712
18. Neumann L, Wohland T, Whelan RJ, Zare RN, Kobilka BK (2002) Functional immobilization of a ligand-activated G-protein-coupled receptor. Chembiochem 3:993–998
19. Karlsson OP, Lofas S (2002) Flow-mediated on-surface reconstitution of G-protein coupled receptors for applications in surface plasmon resonance biosensors. Anal Biochem 300: 132–138
20. Navratilova I, Dioszegi M, Myszka DG (2006) Analyzing ligand and small molecule binding activity of solubilized GPCRs using biosensor technology. Anal Biochem 355:132–139
21. Navratilova I, Sodroski J, Myszka DG (2005) Solubilization, stabilization, and purification of chemokine receptors using biosensor technology. Anal Biochem 339:271–281
22. Stenlund P, Babcock GJ, Sodroski J, Myszka DG (2003) Capture and reconstitution of G protein-coupled receptors on a biosensor surface. Anal Biochem 316:243–250
23. Salamon Z, Wang Y, Brown MF, Macleod HA, Tollin G (1994) Conformational changes in rhodopsin probed by surface plasmon resonance spectroscopy. Biochemistry 33:13706–13711
24. Munoz LM, Holgado BL, Martinez AC, Rodriguez-Frade JM, Mellado M (2012) Chemokine receptor oligomerization: a further step toward chemokine function. Immunol Lett 145:23–29
25. Thelen M, Munoz LM, Rodriguez-Frade JM, Mellado M (2010) Chemokine receptor oligomerization: functional considerations. Curr Opin Pharmacol 10:38–43
26. Fallahi-Sichani M, Linderman JJ (2009) Lipid raft-mediated regulation of G-protein coupled receptor signaling by ligands which influence receptor dimerization: a computational study. PLoS One 4:e6604
27. Ostrom RS, Insel PA (2004) The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. Br J Pharmacol 143:235–245
28. Xu W, Yoon SI, Huang P, Wang Y, Chen C, Chong PL et al (2006) Localization of the kappa opioid receptor in lipid rafts. J Pharmacol Exp Ther 317:1295–1306
29. Yoshiura C, Kofuku Y, Ueda T, Mase Y, Yokogawa M, Osawa M et al (2010) NMR analyses of the interaction between CCR5 and its ligand using functional reconstitution of CCR5 in lipid bilayers. J Am Chem Soc 132: 6768–6777
30. Hoffman TL, Canziani G, Jia L, Rucker J, Doms RW (2000) A biosensor assay for studying ligand-membrane receptor interactions: binding of antibodies and HIV-1 Env to chemokine receptors. Proc Natl Acad Sci U S A 97:11215–11220
31. Weiss RA (1987) Retroviruses and human disease. J Clin Pathol 40:1064–1069
32. Verma IM, Somia N (1997) Gene therapy – promises, problems and prospects. Nature 389: 239–242
33. Balliet JW, Bates P (1998) Efficient infection mediated by viral receptors incorporated into retroviral particles. J Virol 72:671–676
34. Walther W, Stein U (2000) Viral vectors for gene transfer: a review of their use in the treatment of human diseases. Drugs 60:249–271
35. Lewis PF, Emerman M (1994) Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. J Virol 68:510–516
36. Matrai J, Chuah MK, VandenDriessche T (2010) Recent advances in lentiviral vector development and applications. Mol Ther 18: 477–490
37. Vogt VM, Simon MN (1999) Mass determination of rous sarcoma virus virions by scanning transmission electron microscopy. J Virol 73: 7050–7055
38. Akkina RK, Walton RM, Chen ML, Li QX, Planelles V, Chen IS (1996) High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. J Virol 70: 2581–2585
39. Bartz SR, Rogel ME, Emerman M (1996) Human immunodeficiency virus type 1 cell cycle control: Vpr is cytostatic and mediates G2

accumulation by a mechanism which differs from DNA damage checkpoint control. J Virol 70:2324–2331

40. Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH et al (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272:263–267
41. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol 15:871–875
42. Sachdeva G, D'Costa J, Cho JE, Kachapati K, Choudhry V, Arya SK (2007) Chimeric HIV-1 and HIV-2 lentiviral vectors with added safety insurance. J Med Virol 79:118–126
43. Gruber A, Kan-Mitchell J, Kuhen KL, Mukai T, Wong-Staal F (2000) Dendritic cells transduced by multiply deleted HIV-1 vectors exhibit normal phenotypes and functions and elicit an HIV-specific cytotoxic T-lymphocyte response in vitro. Blood 96:1327–1333
44. Amado RG, Chen IS (1999) Lentiviral vectors–the promise of gene therapy within reach? Science 285:674–676
45. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D et al (1998) A third-generation lentivirus vector with a conditional packaging system. J Virol 72:8463–8471
46. Nguyen DH, Taub DD (2003) Inhibition of chemokine receptor function by membrane cholesterol oxidation. Exp Cell Res 291:36–45
47. Zhang J, Pekosz A, Lamb RA (2000) Influenza virus assembly and lipid raft microdomains: a role for the cytoplasmic tails of the spike glycoproteins. J Virol 4:4634–4644
48. Vega B, Calle A, Sanchez A, Lechuga LM, Ortiz AM, Armelles G et al (2013) Real-time detection of the chemokine CXCL12 in urine samples by surface plasmon resonance. Talanta 109:209–215
49. Vega B, Munoz LM, Holgado BL, Lucas P, Rodriguez-Frade JM, Calle A et al (2011) Technical advance: surface plasmon resonance-based analysis of CXCL12 binding using immobilized lentiviral particles. J Leukoc Biol 90:399–408
50. Barroso R, Martinez Munoz L, Barrondo S, Vega B, Holgado BL, Lucas P et al (2012) EBI2 regulates CXCL13-mediated responses by heterodimerization with CXCR5. FASEB J 26:4841–4854
51. Gasmi M, Glynn J, Jin MJ, Jolly DJ, Yee JK, Chen ST (1999) Requirements for efficient production and transduction of human immunodeficiency virus type 1-based vectors. J Virol 73:1828–1834
52. Naldini L, Blomer U, Gage FH, Trono D, Verma IM (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc Natl Acad Sci U S A 93: 11382–11388
53. Frade JM, Mellado M, del Real G, Gutierrez-Ramos JC, Lind P, Martinez-A C (1997) Characterization of the CCR2 chemokine receptor: functional CCR2 receptor expression in B cells. J Immunol 159:5576–5584
54. D'Hautcourt JL (2002) Quantitative flow cytometric analysis of membrane antigen expression. In: Robinson JP (ed) Current protocols in cytometry. Wiley, New York, Chapter 6: Unit 6 12
55. Mellado M, Kremer L, Manes S, Martinez-A C, Rodriguez-Frade JM (1997) Characterization of antigen-antibody and ligand-receptor interactions. In: Lefkovits I (ed) Immunology methods manual: . Academic, London, pp 1145–1162
56. Briggs JA, Wilk T, Welker R, Krausslich HG, Fuller SD (2003) Structural organization of authentic, mature HIV-1 virions and cores. EMBO J 22:1707–1715
57. Mauriz E, Calle A, Abad A, Montoya A, Hildebrandt A, Barcelo D et al (2006) Determination of carbaryl in natural water samples by a surface plasmon resonance flow-through immunosensor. Biosens Bioelectron 21:2129–2136
58. Mauriz E, Calle A, Montoya A, Lechuga LM (2006) Determination of environmental organic pollutants with a portable optical immunosensor. Talanta 69:359–364
59. Scherr M, Battmer K, Blomer U, Ganser A, Grez M (2001) Quantitative determination of lentiviral vector particle numbers by real-time PCR. Biotechniques 31:520–524