Published in final edited form as:

Methods Mol Biol. 2018 ; 1722: 151–164. doi:10.1007/978-1-4939-7553-2_10.

Detecting Cell Surface Expression of the G Protein-Coupled Receptor CXCR4

Amanda M. Nevins and Adriano Marchese

Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI, USA

Abstract

G protein-coupled receptors (GPCRs) are cell surface receptors that relay extracellular signals to the inside of the cells. C-X-C chemokine receptor 4 (CXCR4) is a GPCR that undergoes receptor internalization and recycling upon stimulation with its cognate ligand, C-X-C chemokine 12 (CXCL12). Using this receptor/ligand pair we describe the use of two techniques, enzyme-linked immunosorbent assay (ELISA) and flow cytometry, widely used to quantify GPCR internalization from the plasma membrane and its return to the cell surface by recycling.

Keywords

G Protein-coupled receptor (GPCR); C-X-C chemokine receptor 4 (CXCR4); C-X-C chemokine ligand 12 (CXCL12); Receptor internalization; ELISA; Flow cytometry

1 Introduction

G protein-coupled receptors (GPCRs) represent the largest class of cell surface receptors [1, 2]. Approximately 800 GPCRs have been identified in humans, the largest being the rhodopsin family, and their number within our genome is a reflection of their importance in human physiology [2]. GPCRs are often involved in disease, and as a consequence, are the targets of over 40% of drugs currently on the market [1, 3]. Comprised of an extracellular N-terminus, seven transmembrane spanning α -helices, alternating extracellular and intracellular loops, and an intracellular C-terminus, GPCRs function as complex signaling switchboards relaying information from the outside to the inside of cells [4]. Upon activation by a diverse range of stimuli, GPCRs transduce signals via conformational changes propagated through their transmembrane helices to intra-cellular molecules linked to various signaling cascades [3, 5, 6]. To ensure that signals are of the appropriate magnitude and duration, receptors are immediately uncoupled from intracellular signaling pathways, contributing to signal termination in a process known as homologous desensitization [2, 4]. Activated GPCRs are removed or internalized from the cell surface via endocytosis [7]. For most GPCRs, a large fraction of the internalized receptor is rapidly recycled to the plasma membrane leading to the resensitization of signaling [8–11].

The GPCR that we use here, as an example of internalization and recycling, is the C-X-C chemokine receptor 4 (CXCR4). Chemokine receptors constitute the largest branch of the γ

subfamily of rhodopsin-like GPCRs and, along with their ligands, chemotactic cytokines, are involved in the direction of leukocyte trafficking [3, 12–14]. Stimulation of CXCR4 by its cognate ligand, CXC-chemokine ligand 12 (CXCL12), plays a role in cancer metastasis and progression, HIV infection, and WHIM syndrome [3, 12, 15–18]. Monitoring the internalization and recycling of CXCR4 following CXCL12 stimulation using techniques targeted at the quantification of cell surface receptor expression is one way to assess the role of receptor trafficking and recycling in cellular fate. An enzyme-linked immunosorbent assay (ELISA) is a plate-based technique that employs an enzyme, like alkaline phosphatase, to detect an immobilized antigen after incubation with a substrate, yielding a measurable product [19]. Flow cytometry detects fluorescence emitted from cell-bound fluorophores upon excitation as they pass in front of a light source [20]. In this chapter, these techniques, ELISA (1.1 and 1.3) and flow cytometry (1.2), are described for the quantification of CXCL12-stimulated surface-CXCR4 internalization and recycling.

2 Materials

2.1 Cell Lines

1. Human embryonic kidney cells (HEK293) (Microbix, Toronto, CA) stably expressing tagged CXCR4 [9].
2. Human cervical cancer cells with high levels of endogenous CXCR4 expression (HeLa, ATCC, Manassas, VA, USA).

2.2 Cell Culture Materials and Reagents

1. Fetal Bovine Serum (FBS).
2. Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4500 mg/mL) supplemented with L-glutamine, and NaHCO_3 .
3. Complete DMEM: 10% FBS-supplemented DMEM.
4. 4-(2-hydroxyethyl)-piperazine ethane sulfonic acid (HEPES).
5. Incomplete DMEM: DMEM supplemented with 20 mM HEPES.
6. 0.05% Trypsin—EDTA.
7. Phosphate-Buffered Saline (PBS) without calcium and magnesium ($\text{Ca}^{+2}/\text{Mg}^{+2}$ -free PBS).
8. Bovine Serum Albumin (BSA).
9. Poly-L-Lysine (PLL).
10. Tissue culture dishes—10- and 6-cm (Falcon), 6-well plates (Eppendorf), 24-well plates (Falcon).

2.3 ELISA Materials and Reagents

1. Assay medium: DMEM, 0.1% BSA, 20 mM HEPES, 1 mM Ca^{+2} .
2. 100 mM Ca^{+2} stock.

3. $\text{Ca}^{+2}/\text{Mg}^{+2}$ -free PBS.
4. PBS supplemented with 1 mM Ca^{+2} .
5. Monoclonal mouse M1 anti-FLAG® antibody (Sigma-Aldrich, St. Louis, MO, USA).
6. Ethylenediaminetetraacetic acid (EDTA).
7. 10 μM stock of CXCL12 (CXCR4 ligand/agonist) (Protein Foundry, Milwaukee, WI, USA) (*see* Note 1).
8. AMD3100—CAS 155148–31–5 (CXCR4 antagonist) (Sigma-Aldrich).
9. Fixing solution: 3.7% paraformaldehyde (PFA) in PBS.
10. Alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma-Aldrich).
11. *p*-Nitrophenyl phosphate (developing solution) (Bio-Rad Laboratories, Hercules, CA, USA).
12. Diethanolamine buffer (Bio-Rad Laboratories).
13. 0.4 M NaOH.
14. Plate reader to measure absorbance. We use the FlexStation 3 multi-mode plate reader from Molecular Devices (Sunnyvale, CA, USA).

2.4 Flow Cytometry-Specific Material and Reagents

1. CellStripper® (Fisher Scientific Co., Pittsburgh, PA, USA).
2. Trypan Blue.
3. Flow buffer: PBS supplemented with 0.1% BSA.
4. PE-conjugated anti-CXCR4 (Catalog No: #306506 (clone 12G5); BioLegend, San Diego, CA, USA).
5. IgG_{2a} κ -isotype control (Catalog No: #400212; BioLegend, San Diego, CA, USA).
6. Fixing solution: 3.7% paraformaldehyde (PFA) in PBS.
7. 10 μM stock of CXCL12 (CXCR4 ligand/agonist) (*see* Note 1).
8. Round bottom test tubes suitable for flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA).
9. Our flow cytometry facility is equipped with a FACSCalibur flow cytometer and FlowJo software (BD Biosciences, San Jose, CA, USA).

¹CXCL12 was purchased from Protein Foundry (proteinfoundry.com). Protein Foundry produces recombinant chemokines using rigorous production and quality control methods [24] to ensure the highest standards of product quality and reproducibility in all research uses.

3 Methods

3.1 CXCR4 Internalization: ELISA

1. HEK293 cells stably expressing FLAG-tagged CXCR4 are maintained in 10-cm dishes containing 10 mL complete DMEM prepared as described in Subheading 2.2, at 37 °C in a humidified atmosphere composed of 5% CO₂ to a confluency of 80–90% (*see* Note 2).
2. Passage cells into PLL (Poly-L-Lysine) coated 24-well plates (or dishes) (*see* Note 3). Incubate at 37 °C for an additional 24 h allowing cells to reach 100% confluency. Assessment of CXCR4-internalization requires the following four conditions: (a) time ($t = 0$ (T_0); (b) $t = 0$, isotype control antibody only as background control (T_{BG}), and (c/d) internalization (I) for 45 min (I_{45}) for vehicle and CXCL12 treated cells. Each condition should be performed in triplicate for 18 total wells (*see* Note 4).
3. Wash cells once with 500 μ L warm with incomplete DMEM. Replace with 500 μ L warm, incomplete DMEM for at least 3 h (*see* Note 5).
4. Following serum starvation, place the 24-well plate(s) on ice and aspirate the incomplete DMEM. Wash cells twice with 500 μ L ice-cold (4 °C) assay medium prepared as specified in Subheading 2.3. Replace with 500 μ L fresh, ice-cold (4 °C) assay medium and incubate on ice for 15 min (*see* Note 6).
5. Cell surface CXCR4 is labeled with the calcium-dependent M1 anti-FLAG® monoclonal antibody. Aspirate medium and replace with the newly prepared medium containing the antibody. Add the antibody in a dilution of 1:100–250 μ L of ice-cold (4 °C) assay medium. Incubate on ice for 1 h (*see* Note 7).
6. Aspirate the medium containing the M1 anti-FLAG® monoclonal antibody and wash twice with 500 μ L ice-cold (4 °C) assay medium.
7. Aspirate medium from the I_{45} wells and apply warm assay medium containing vehicle (PBS + 0.1% BSA) or 50 nM CXCL12 (CXCR4 agonist) (*see* Note 8). Incubate treated cells at 37 °C for 45 min.

²Ten-centimeter dishes work well when larger volumes of cells are required, and 6-well plates (Eppendorf) or 6-cm dishes are a good alternative for smaller scale analyses. For a 6-well plate use 2 mL of PBS and 2 mL of the appropriate medium per well.

³Twenty-four-well plates are coated with PLL in house. Briefly, 500 μ L of a 1 μ g/mL PLL stock is added to each well. After 15 min aspirate PLL from wells and let dry for 1 h. Before use wash three times with PBS and let dry.

⁴The number of conditions can change depending on alterations to the cells including knockdowns and transfections; however, the internalization and recycling portions of the experiment monitors CXCR4 cell surface expression as diagramed in Fig. 1. The amount of internalized receptor is expressed as a proportion of the initial surface labeling of the receptor ($t = 0$).

⁵Depending on the receptor type, cells are serum-starved to minimize basal receptor activity [25]. We prefer to serum-starve for 3–4 h. This can vary depending upon the cell type or mechanism of receptor internalization. The researcher should determine this empirically.

⁶Endocytosis does not occur at 4 °C. Keeping cells at this temperature ensures cell surface labeling of the receptor and results in synchronous activation of receptor internalization upon return to 37 °C [26]. Therefore, it is important that samples remain on ice at all times and only ice-cold (4 °C) solutions are used. Pelleting should be done in a centrifuge cooled to 4 °C.

⁷The M1 anti-FLAG® monoclonal antibody labels cell surface FLAG-CXCR4 in a calcium-dependent manner and requires the presence of at least 1 mM Ca²⁺ during all incubation and wash steps.

⁸Treatment with the agonist, in this case CXCL12, promotes the internalization of the receptor/antibody complex [17].

8. During this incubation aspirate medium from the T_0 and T_{BG} wells and wash cells twice with ice-cold (4 °C) assay medium. Treat cells with 500 μ L of 3.7% paraformaldehyde (PFA) in PBS (fixing solution). Incubate plates at room temperature (RT) for 5–10 min (*see* Note 9).
9. Following fixation, wash cells twice with 500 μ L PBS containing 1 mM Ca^{+2} . Replace with 500 μ L fresh PBS (+1 mM Ca^{+2}) after this wash, and leave plates on ice to process in parallel with the internalization (I_{45}) wells.
10. Following the 45 min incubation with either vehicle or CXCL12, aspirate medium from the internalization (I_{45}) wells. Fix and wash cells in the I_{45} wells as described for control wells in **steps 8 and 9**. Cover with aluminum foil to prevent exposure to light and leave on ice to process in parallel with the I_{45} wells.
11. After washes incubate each plate with 300 μ L of alkaline phosphatase-conjugated goat anti-mouse antibody (*see* Note 10) for 1 h at RT.
12. Post-incubation, wash cells three times with 500 μ L PBS (+1 mM Ca^{+2}). Incubate plates with 250 μ L developing solution that has been diluted in diethanolamine buffer for 5–15 min (*see* Note 11).
13. Quench the reactions of each well with 100 μ L of 0.4 M NaOH. Take 100 μ L aliquots of each condition and measure the absorbance at 405 nm using a plate reader.
14. Calculate the proportion of receptor internalized by dividing the amount of surface receptor by the total number of receptor present at the cell surface prior to treatment with CXCL12 according to the following formula:

$$\text{Proportion of receptor internalized} = 1 - \left[(I_{45} - T_{BG}) \div (T_0 - T_{BG}) \right],$$

where T_0 is equal to the total signal at time $t = 0$, T_{BG} is the isotype only background control and I_{45} is the surface signal remaining 45 min after either vehicle or CXCL12-stimulated internalization. The percentage of receptor internalization can be calculated by multiplying the result from the above formula by 100.

3.2 CXCR4 Surface Expression and Internalization: Flow Cytometry

1. Culture HeLa cells in 10-cm dishes as described in Subheading 3.1 until 90–95% confluent (*see* Note 2).

⁹. Fixing the control wells (T_0 and T_{BG}) during the 45 min incubation with vehicle/CXCL12 prevents constitutive receptor internalization or loss of antibody binding.

¹⁰. Prepare antibody in a 1:1000 dilution in PBS + 1% BSA. The antibody dilution will have to be determined empirically for each receptor and receptor expression system.

¹¹. The alkaline phosphatase conjugated to the antibody enzymatically processes *p*-nitrophenyl phosphate to *p*-nitrophenol in the presence of diethanolamine resulting in the color change monitored as the assay output [19]. The time required to obtain a strong signal will depend on the expression of the receptor and the efficiency of recycling. Care must be taken to ensure that the signals obtained fall within the linear range of your instrument to ensure accuracy.

2. Wash cells once with 10 mL incomplete DMEM. Subsequently, serum-starve the cells in 10 mL of fresh incomplete DMEM for at least 3 h (*see* Note 5).
3. Wash cells twice with 10 mL PBS and detach cells from plates using a nonenzymatic dissociation buffer such as CellStripper® (Corning) (*see* Note 12). Aspirate medium, add 2 mL of CellStripper® and incubate cells at 37 °C for 10 min or until detached (*see* Note 13).
4. Add 8 mL cold (4 °C) (*see* Note 6) flow buffer prepared as specified in Subheading 2.4 and transfer cells to a conical centrifuge tube. Pellet cells by centrifugation at $800 \times g$ for 3 min (*see* Note 14).
5. Resuspend cells in 2 mL cold (4 °C) flow buffer. Count cells using trypan blue staining and a hemacytometer. We typically use an automated cell counter. Transfer approximately 5×10^5 cells per sample and condition [20] to a 5 mL round bottomed test tube. Wash cells twice with 500 μ L cold (4°C) flow buffer, pelleting as described in **step 4**.
6. Resuspend cells in 500 μ L warm (37 °C) flow buffer and incubate for 15 min at 37 °C (*see* Note 15).
7. During the wash steps and incubation with ligand, prepare antibody dilutions in cold (4 °C) flow buffer and keep on ice until use (*see* Note 16). Choosing the primary antibody is a crucial step in experimental design (*see* Note 17). There are multiple antibodies available that recognize several distinct epitopes on CXCR4. In this analysis a 1:100 dilution of PE-conjugated anti-CXCR4 was used (*see* Notes 18 and 19). The presence of post-translational modifications on the receptor including, but not limited to, sulfation (*see* Note 20) and glycosylation [21] must also be considered as these can prevent epitope

¹²The use of a nonenzymatic dissociation buffer, rather than an enzyme-based buffer prevents the digestion of the extracellular domains of CXCR4 [26]. Digestion of these domains can result in the removal of antibody epitopes and thereby impact antibody labeling of the receptor or in some cases alter receptor function in response to ligand stimulation. Enzymatic agents can be used when incubations are long enough to allow for new receptor synthesis to occur.

¹³For more difficult cell lines, tapping the side of the dishes may help to dislodge more cells. Gently break up clumps of cells by repeat pipetting; clumped cells will not give accurate readings by flow cytometry as they will count as a single event in the cytometer [20].

¹⁴Depending on the cell type, a lower RCF (relative centrifugal force) may result in less stress on the cells. Cells must remain intact, therefore, DO NOT exceed $1000 \times g$; greater speeds will exert sufficient force to damage cell membranes.

¹⁵Resuspend two samples in cold (4 °C) flow buffer to use as controls, with and without ligand treatment. Although receptor internalization is generally regarded as a ligand-dependent event, basal levels of constitutive internalization and recycling are possible [27, 28].

¹⁶NaN₃ is often added to the antibody dilutions prior to flow analysis. If further functional assays are planned using the sorted cells it should not be included in the primary antibody buffer as it is also known to irreversibly inhibit the electron transport chain [29].

¹⁷The antibodies chosen should recognize epitopes that are distinct from ligand binding sites, in order to ensure that only internalization is being monitored rather than other factors such as epitope occupancy or masking by the ligand or receptor activation [21].

¹⁸The 12G5 monoclonal anti-CXCR4 antibody recognizes residues in the second extracellular loop (ECL2) of the receptor [30]. ECL2 is a vital part of CXCL12 binding, recognition and activation [12] and is necessary for CXCR4 to function as an HIV coreceptor [16]. It has been shown that CXCL12 competes with 12G5 for receptor binding, while AMD3100 blocks antibody binding completely [22, 30].

¹⁹As an alternative to the 12G5 monoclonal anti-CXCR4 antibody, fluorescently conjugated versions of the 4G10 (Santa Cruz, sc-53,534) or 2B11 (BD Biosciences #551852) anti-CXCR4 antibodies may be used. Each of these antibodies recognizes the N-terminus of CXCR4 [17, 30] avoiding some of the problems seen with 12G5, including competition for CXCL12 binding [22].

²⁰Sulfation of the CXCR4 N-terminus is known to play a vital role in CXCL12 binding and recognition [31–33]. Using an antibody that obscures these moieties could inhibit CXCL12 binding and downstream CXCR4 activation. Alternatively this posttranslational modification could also prevent antibody binding and recognition [22].

recognition by monoclonal antibodies [21, 22]. Finally, the fluorochrome you choose should have both a high quantum yield and resistance to photobleaching (*see* Note 21).

8. Treat cells with 50 nM (final concentration) (*see* Note 20) of CXCL12 or vehicle (PBS + 0.1% BSA) for 2, 5, 10, 20, and 60 min (*see* Note 22).
9. After 60 min (or final time point) (*see* Note 23), wash cells twice with 4 mL of cold (4 °C) flow buffer, pelleting as described in **step 4**. Resuspend cells in 500 µL of cold (4 °C) flow buffer containing antibody dilutions. Cover the samples with aluminum foil in order to protect fluorochromes from light and any subsequent photobleaching. Incubate samples on ice for 20 min.
10. Following the incubation, wash cells twice with 4 mL of cold (4 °C) flow buffer, pelleting as described in **step 4** while limiting exposure to light. Resuspend cells in 500 µL of 3.7% PFA (*see* Note 24) maintaining a concentration of 1×10^6 cells/mL in each sample.
11. After fixation, wash samples in 500 µL of cold (4 °C) flow buffer and run cell-associated fluorescence analysis immediately (*see* Note 25) or store samples at 4 °C until use (*see* Note 26).
12. Raw data is analyzed using a software package such as FlowJo. The percent of internalized receptor is calculated using the geometric mean of PE fluorescence intensity [10, 23].

3.3 CXCR4 Receptor Recycling: ELISA

1. Maintain HEK293 cells stably expressing FLAG-tagged CXCR4 in 10-cm dishes containing 10 mL complete DMEM prepared as described in Subheading 2.2, at 37 °C in a humidified atmosphere composed of 5% CO₂ to a confluency of 90–95% (*see* Note 2).
2. Passage cells into PLL coated 24-well plates (*see* Note 3). Incubate at 37 °C for an additional 24 h such that cells reach 90–95% confluency. Assessment of CXCR4-recycling requires the following eight conditions: (a) time ($t = 0$ (T_0); (b) $t = 0$, isotype only as background control (T_{BG}); (c/d) internalization (I) for 45 min (I_{45}), for vehicle and CXCL12 treated cells (*see* Note 27); (e/f) recycling (R) at $t = 0$ post-internalization (R_0), for vehicle and CXCL12; and (g/h)

²¹The most commonly available fluorescently labeled anti-CXCR4 antibodies are phycoerythrin (PE) conjugated. PE has a maximum excitation at 565 nm, however, if the cytometer used is equipped with a blue laser (488 nM) rather than yellow/green (561 nM) this fluorophore can be excited at a lower wavelength resulting in reduced signal brightness at the emission wavelength of 578 nM. If this is a concern, Alexa or Brilliant Violet dyes could be employed instead [21, 22].

²²Using 500 µL sample volume described, a final concentration of 50 nM CXCL12 is acquired by adding 2.5 µL of a 10 µM stock directly to the appropriate tube. The required concentration of chemokine may need optimization. Some chemokines induce internalization more readily at higher concentrations (100–200 nM) [21].

²³Have individual time points prepared to end at the same time (longest to shortest) so that samples can be processed in parallel.

²⁴Returning the cells to 4 °C stops any further internalization.

²⁵Set up the cytometer to count 10,000 events per condition [26].

²⁶Samples can be stored in the dark at 4 °C for 2–3 days after fixing. If desired, prior to analysis transfer samples to mini FACS (Fluorescence-activated cell sorting) tubes (BD Bioscience).

²⁷ I_{45} refers to wells that will be treated the same as in section 3.1 for the ELISA measuring CXCR4 internalization only. This is because in order to calculate the percentage of receptor recycling the initial amount of receptor internalization needs to be known.

recycling after 60 min (R_{60}), for vehicle and CXCL12 treated cells (*see* Note 28). Each condition should be performed in triplicate for 24 total wells (*see* Note 4).

3. Wash cells once with 500 μ L warm, incomplete DMEM. Replace with 500 μ L warm, incomplete DMEM for at least 3 h (*see* Note 5).
4. Following serum starvation, place the 24-well plate(s) on ice and aspirate the incomplete DMEM. Wash cells twice with 500 μ L ice-cold (4 °C) assay medium prepared as specified in Subheading 2.3. Replace with 500 μ L fresh, ice-cold (4 °C) assay medium and incubate on ice for 15 min (*see* Note 6).
5. Cell surface CXCR4 is labeled with the calcium-dependent M1 anti-FLAG® monoclonal antibody. Aspirate medium and replace with the newly prepared medium containing the antibody. Add the antibody in a dilution of 1:100 to 250 μ L of ice-cold (4 °C) assay medium. Incubate on ice for 1 h (*see* Note 7).
6. Aspirate the medium containing the M1 anti-FLAG® monoclonal antibody and wash twice with 500 μ L ice-cold (4 °C) assay medium.
7. Aspirate medium from the I_{45} , R_0 , and R_{60} wells, and then apply warm assay medium containing vehicle (PBS + 0.1% BSA) or 50 nM CXCL12 (CXCR4 agonist) (*see* Note 8). Incubate treated cells at 37 °C for 45 min.
8. During this incubation, aspirate medium from the T_0 and T_{BG} wells and wash cells twice with ice-cold (4 °C) assay medium. Treat cells with 500 μ L of 3.7% fixing solution. Incubate plates at room temperature for 5–10 min (*see* Note 9).
9. Aspirate the fixing solution, wash cells twice with 500 μ L PBS containing 1 mM Ca^{+2} . Replace with 500 μ L fresh PBS (+1 mM Ca^{+2}) after this wash, cover plates with aluminum foil to prevent light exposure and leave plates on ice to process in parallel with the internalization (I^{45}) and recycling (R_0 and R_{60}) wells.
10. Following the 45 min incubation with either vehicle or CXCL12, aspirate medium from I_{45} . Fix and wash cells in the I_{45} wells as described for control wells in **steps 8 and 9**. Cover with aluminum foil to prevent exposure to light and leave on ice to process in parallel with the recycling (R_0 and R_{60}) wells.
11. For the R_0 and R_{60} wells, the remaining surface antibody bound to uninternalized receptor must be removed. Wash each plate three times with 500 μ L $\text{Ca}^{+2}/\text{Mg}^{+2}$ -free PBS containing 0.04% EDTA (*see* Note 29).
12. The R_0 wells should be processed as described above in **steps 8 and 9**.
13. In order to monitor receptor recycling, treat cells in the R_{60} wells with 500 μ L DMEM supplemented with 1 mM Ca^{+2} and 10 μ M AMD3100 (*see* Note 30). Incubate cells in this medium for 60 min at 37 °C.

²⁸Recycling rates can differ between receptors, however, for CXCR4 maximum receptor recycling was found to occur after 60 minutes [9].

²⁹EDTA chelates metal ions; in this case Ca^{+2} , resulting in the uncoupling of the calcium-dependent M1 antibody from any remaining surface receptors. Successive washes should be sufficient to remove all bound M1 antibody. However, this must be determined empirically. It is possible that prolonged incubations may be necessary to completely remove bound antibody, which is essential to accurately calculate receptor recycling.

14. Wash R_{60} wells with 500 μL PBS (+1 mM Ca^{+2}), then apply fixing solution for 5 min on ice. Following fixation, wash all plates three times with 500 μL PBS (+1 mM Ca^{+2}).
15. After washes, incubate all wells with 300 μL of alkaline phosphatase-conjugated goat anti-mouse antibody (*see* Note 10) for 1 h at RT.
16. Post-incubation, wash cells three times with 500 μL PBS (+1 mM Ca^{+2}). Incubate plates with 250 μL developing solution that has been diluted in diethanolamine buffer for 5–15 min (*see* Note 11).
17. Quench reactions with 100 μL of 0.4 M NaOH. Take 100 μL aliquots of each condition and measure the absorbance at 405 nm using the “well scan” setting of a FlexStation 3.
18. Calculate the proportion receptor internalized by dividing the amount of remaining surface receptor by the total number of receptor present at the cell surface prior to treatment with CXCL12 according to the following formula:

$$\text{Proportion of receptor internalized} = 1 - \left[(I_{45} - T_{\text{BG}}) \div (T_0 - T_{\text{BG}}) \right],$$

where T_0 is equal to the total signal at time $t = 0$, T_{BG} is the 2°-only background control and I_{45} is the surface signal remaining 45 minutes after either vehicle or CXCL12-stimulated internalization.

19. Using the determined proportion of internalized receptor, calculate the percentage of receptor recycling by dividing the proportion of internalized receptor by the amount surface receptor recovered post-incubation according to the following formula:

$$\% \text{Receptor Recycling} = (R_{60} - R_0) \div \left\{ 1 - \left[(I_{45} - T_{\text{BG}}) \div (T_0 - T_{\text{BG}}) \right] \right\},$$

where R_{60} is equal to the total signal recovered 105 min (45 min stimulation + 60 min recovery) after the initiation of either vehicle or CXCL12-stimulated internalization, R_0 is equal to the signal remaining on $t = 0$ after removal of the Ca^{+2} -dependent 1° antibody. This is a percentage of the internalized receptor that has recycled to the cell surface.

Acknowledgment

This work was supported by NIH grant GM106727 (A.M.).

³⁰.AMD3100 is a CXCR4 antagonist, which is used to prevent the binding of any residual CXCL12 in the medium that may not have been removed by the washing step. It also serves to compete CXCL12 off of any receptors recycled with ligand (CXCL12) still bound. It should be noted that AMD3100 can bind to ACKR3, an atypical chemokine receptor that is also a receptor for CXCL12 [34]. AMD3100 could theoretically promote cointernalization of CXCR4-ACKR3 heterodimers and thereby limit the amount of CXCR4 that is recycled and available at the plasma membrane.

References

1. 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 [PubMed: 12761335]
2. Tesmer JJ (2016) Hitchhiking on the heptahelical highway: structure and function of 7TM receptor complexes. *Nat Rev Mol Cell Biol* 17:439–450 [PubMed: 27093944]
3. Bachelier F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ, Horuk R, Sparre-Ulrich AH et al. (2014) International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol Rev* 66:1–79 [PubMed: 24218476]
4. Van Rhee AM, Jacobson KA (1996) Molecular architecture of G protein-coupled receptors. *Drug Dev Res* 37(1):1–38 [PubMed: 21921973]
5. Rajagopal S, Ahn S, Rominger DH, Gowen-MacDonald W, Lam CM, Dewire SM, Violin JD, Lefkowitz RJ (2011) Quantifying ligand bias at seven-transmembrane receptors. *Mol Pharmacol* 80:367–377 [PubMed: 21610196]
6. Whalen EJ, Rajagopal S, Lefkowitz RJ (2011) Therapeutic potential of beta-arrestin- and G protein-biased agonists. *Trends Mol Med* 17:126–139 [PubMed: 21183406]
7. Neel NF, Schutyser E, Sai J, Fan GH, Richmond A (2005) Chemokine receptor internalization and intracellular trafficking. *Cytokine Growth Factor Rev* 16:637–658 [PubMed: 15998596]
8. Bhandari D, Trejo J, Benovic JL, Marchese A (2007) Arrestin-2 interacts with the ubiquitin-protein isopeptide ligase atrophin-interacting protein 4 and mediates endosomal sorting of the chemokine receptor CXCR4. *J Biol Chem* 282:36971–36979 [PubMed: 17947233]
9. Malik R, Marchese A (2010) Arrestin-2-interacts with the endosomal sorting complex required for transport machinery to modulate endosomal sorting of CXCR4. *Mol Biol Cell* 21:2529–2541 [PubMed: 20505072]
10. Malik R, Soh UJ, Trejo J, Marchese A (2012) Novel roles for the E3 ubiquitin ligase atrophin-interacting protein 4 and signal transduction adaptor molecule 1 in G protein-coupled receptor signaling. *J Biol Chem* 287:9013–9027 [PubMed: 22275353]
11. Marchese A, Benovic JL (2001) Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J Biol Chem* 276:45509–45512 [PubMed: 11641392]
12. Chevigne A, Fievez V, Szpakowska M, Fischer A, Counson M, Plessier JM, Schmit JC, Deroo S (2014) Neutralising properties of peptides derived from CXCR4 extracellular loops towards CXCL12 binding and HIV-1 infection. *Biochim Biophys Acta* 1843:1031–1041 [PubMed: 24480462]
13. Moser B, Willmann K (2004) Chemokines: role in inflammation and immune surveillance. *Ann Rheum Dis* 63(Suppl 2):ii84–ii89 [PubMed: 15479880]
14. Moser B, Wolf M, Walz A, Loetscher P (2004) Chemokines: multiple levels of leukocyte migration control. *Trends Immunol* 25:75–84 [PubMed: 15102366]
15. Balabanian K, Lagane B, Pablos JL, Laurent L, Planchenault T, Verola O, Lebbe C, Kerob D et al. (2005) WHIM syndromes with different genetic anomalies are accounted for by impaired CXCR4 desensitization to CXCL12. *Blood* 105:2449–2457 [PubMed: 15536153]
16. Brelot A, Heveker N, Adema K, Hosie MJ, Willett B, Alizon M (1999) Effect of mutations in the second extracellular loop of CXCR4 on its utilization by human and feline immunodeficiency viruses. *J Virol* 73:2576–2586 [PubMed: 10074102]
17. Forster R, Kremmer E, Schubel A, Breitfeld D, Kleinschmidt A, Nerl C, Bernhardt G, Lipp M (1998) Intracellular and surface expression of the HIV-1 coreceptor CXCR4/fusin on various leukocyte subsets: rapid internalization and recycling upon activation. *J Immunol* 160:1522–1531 [PubMed: 9570576]
18. Ramsey DM, McAlpine SR (2013) Halting metastasis through CXCR4 inhibition. *Bioorg Med Chem Lett* 23:20–25 [PubMed: 23211868]
19. Engvall E (1980) Enzyme immunoassay ELISA and EMIT. *Methods Enzymol* 70:419–439 [PubMed: 6999296]

20. Givan AL (2011) Flow cytometry: an introduction. *Methods Mol Biol* 699:1–29 [PubMed: 21116976]
21. Anselmo A, Mazzon C, Borroni EM, Bonecchi R, Graham GJ, Locati M (2014) Flow cytometry applications for the analysis of chemokine receptor expression and function. *Cytometry A* 85:292–301 [PubMed: 24464630]
22. Sloane AJ, Raso V, Dimitrov DS, Xiao X, Deo S, Muljadi N, Restuccia D, Turville S et al. (2005) Marked structural and functional heterogeneity in CXCR4: separation of HIV-1 and SDF-1 α responses. *Immunol Cell Biol* 83:129–143 [PubMed: 15748209]
23. Alekhina O, Marchese A (2016) Beta-arrestin1 and signal-transducing adaptor molecule 1 (STAM1) cooperate to promote focal adhesion kinase autophosphorylation and chemo-taxis via the chemokine receptor CXCR4. *J Biol Chem* 2016:M116.757138
24. Veldkamp CT, Koplinski CA, Jensen DR, Peterson FC, Smits KM, Smith BL, Johnson SK, Lettieri C et al. (2016) Production of recombinant chemokines and validation of refolding. *Methods Enzymol* 570:539–565 [PubMed: 26921961]
25. Pinilla-Macua I, Sorkin A (2015) Methods to study endocytic trafficking of the EGF receptor. *Methods Cell Biol* 130:347–367 [PubMed: 26360045]
26. Kershaw T, Wavre-Shapton ST, Signoret N, Marsh M (2009) Analysis of chemokine receptor endocytosis and intracellular trafficking. *Methods Enzymol* 460:357–377 [PubMed: 19446735]
27. Bonecchi R, Savino B, Borroni EM, Mantovani A, Locati M (2010) Chemokine decoy receptors: structure-function and biological properties. *Curr Top Microbiol Immunol* 341:15–36 [PubMed: 20373092]
28. Borroni EM, Mantovani A, Locati M, Bonecchi R (2010) Chemokine receptors intracellular trafficking. *Pharmacol Ther* 127:1–8 [PubMed: 20451553]
29. Wilson DF, Chance B (1967) Azide inhibition of mitochondrial electron transport. I. The aerobic steady state of succinate oxidation. *Biochim Biophys Acta* 131:421–430 [PubMed: 4166781]
30. Carnec X, Quan L, Olson WC, Hazan U, Dragic T (2005) Anti-CXCR4 monoclonal antibodies recognizing overlapping epitopes differ significantly in their ability to inhibit entry of human immunodeficiency virus type 1. *J Virol* 79:1930–1933 [PubMed: 15650218]
31. Veldkamp CT, Seibert C, Peterson FC, De la Cruz NB, Haugner JC III, Basnet H, Sakmar TP, Volkman BF (2008) Structural basis of CXCR4 sulfotyrosine recognition by the chemokine SDF-1/CXCL12. *Sci Signal* 1:ra4 [PubMed: 18799424]
32. Veldkamp CT, Ziarek JJ, Peterson FC, Chen Y, Volkman BF (2010) Targeting SDF-1/CXCL12 with a ligand that prevents activation of CXCR4 through structure-based drug design. *J Am Chem Soc* 132:7242–7243 [PubMed: 20459090]
33. Ziarek JJ, Getschman AE, Butler SJ, Taleski D, Stephens B, Kufareva I, Handel TM, Payne RJ, Volkman BF (2013) Sulfopeptide probes of the CXCR4/CXCL12 interface reveal oligomerspecific contacts and chemokine allostery. *ACS Chem Biol* 8:1955–1963 [PubMed: 23802178]
34. Kalatskaya I, Berchiche YA, Gravel S, Limberg BJ, Rosenbaum JS, Heveker N (2009) AMD3100 is a CXCR7 ligand with allosteric agonist properties. *Mol Pharmacol* 75:1240–1247 [PubMed: 19255243]

Fig. 1.

Schematic representation of the conditions and experimental course for the recycling experiment. In this example, the *orange curve* represents data from the ELISA for internalization and the *blue curve* shows recycling of CXCR4. The asterisk represents the washes stripping the remaining primary antibody from the cell surface. The conditions denoted (T_0 , T_{BG} , I_{45} , R_0 , and R_{60}) are the minimum number of conditions monitored in the experiment