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Monitoring Chemokine Receptor Trafficking by Confocal Immunofluorescence Microscopy

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Abstract

Here, we describe a protocol to detect chemokine receptor CXCR4 by confocal immunofluorescence microscopy in HeLa cells treated with its chemokine ligand CXCL12. Typically, ligand-activated chemokine receptors undergo a multistep process of desensitization and/or internalization from the plasma membrane in order to terminate signaling. Once internalized to endosomes, chemokine receptors readily enter the recycling pathway and return to the cell surface, giving rise to resensitization of signaling. The chemokine receptor CXCR4, when activated by CXCL12 is also internalized to endosomes, but in contrast to many chemokine receptors it is mainly sorted to the degradative pathway, contributing to a loss in the cellular complement of CXCR4 and long-term downregulation of signaling. The trafficking of CXCR4 from early endosomes to lysosomes can be easily detected by confocal immunofluorescence microscopy by immunostaining fixed cells for the receptor and with markers of these vesicular compartments. This approach is advantageous because it can be used to identify factors that regulate the trafficking of CXCR4 from early endosomes to lysosomes. The protocol described here focuses on CXCR4, but it can be easily adapted to other chemokine receptors.

1. INTRODUCTION

The chemokine ligand CXCL12 and its chemokine receptor CXCR4 have important biological functions (Balkwill, 2004; Domanska et al., 2013; Karpova & Bonig, 2015; Zlotnik & Yoshie, 2012). In addition, CXCR4 has been linked to several diseases, including cancer (Balkwill, 2012). CXCR4 expression is upregulated in at least 20 solid tumors and also in some hematological cancers (Balkwill, 2004; Domanska et al., 2013). CXCR4 expression correlates with poor prognosis, mainly due to its role in metastasis (Muller et al., 2001; Oskarsson, Batlle, & Massague, 2014). In cancer cells, CXCR4 expression is regulated by multiple mechanisms, including at the level of transcription (Vanharanta et al., 2013), translation (Li et al., 2004), and protein (Li et al., 2004). The protocol described here will focus on examining CXCR4 expression at the level of the protein.

CXCR4 expression is directly regulated by several posttranslational modifications, including phosphorylation and ubiquitination (Marchese, 2014). These posttranslational modifications act in concert to target CXCR4 for lysosomal degradation upon activation by CXCL12

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(Marchese, 2014). As with most chemokine receptors, CXCR4 is rapidly internalized from the cell surface following agonist binding (Orsini, Parent, Mundell, Marchese, & Benovic, 1999). CXCL12 binding to CXCR4 also promotes rapid phosphorylation of CXCR4 at specific residues within the C-tail, leading to an interaction with the E3 ubiquitin ligase AIP4 (Bhandari, Robia, & Marchese, 2009). AIP4 mediates ubiquitination of the receptor on nearby lysine residues located within the C-tail (Marchese & Benovic, 2001). The ubiquitin moieties serve as a signal for targeting CXCR4 into the degradative pathway (Marchese et al., 2003). This is consistent with fact that upon internalization from the plasma membrane ubiquitinated CXCR4 traffics to early endosomes and then to lysosomes, the terminal degradative compartment (Slagsvold, Marchese, Brech, & Stenmark, 2006). A small fraction of CXCR4 also enters the recycling pathway (Malik & Marchese, 2010), which may be because not all internalized receptors are ubiquitinated or ubiquitin moieties are removed by deubiquitinases thereby facilitating receptor recycling (Marchese, 2014).

Ubiquitinated CXCR4 is targeted to the endosomal sorting complex required for transport (ESCRT) pathway (Marchese et al., 2003). This pathway is comprised of five major protein complexes that act at the limiting membrane of endosomes to selectively target ubiquitinated transmembrane proteins into the lumen of endosomes and multivesicular bodies, which subsequently fuse with lysosomes where the luminal contents are degraded (Henne, Buchkovich, & Emr, 2011). Several factors have been identified that regulate the ability of ESCRTs to target ubiquitinated CXCR4 into lysosomes (Bhandari, Trejo, Benovic, & Marchese, 2007; Holleman & Marchese, 2014; Malik & Marchese, 2010). One of these factors is β-arrestin1, which appears to interact with specific ESCRT proteins on early endosomes to regulate their sorting activity (Malik & Marchese, 2010). In HeLa cells depleted of β-arrestin1 by siRNA, CXCR4 internalizes normally upon ligand binding, but fails to traffic from early endosomes to lysosomes and is therefore not degraded (Bhandari et al., 2007). This defect in trafficking can be easily detected by fixed cell confocal immunofluorescence microscopy. In cells treated with β-arrestin1 siRNA, CXCR4 accumulates on EEA1-positive early endosomes, while in control siRNA treated cells, CXCR4 accumulates on LAMP2-positive late endosomes/lysosomes (Bhandari et al., 2007), suggesting that β-arrestin1 regulates endosome to lysosome trafficking of CXCR4. Although these experiments were performed in HEK293 cells, expressing YFP-tagged CXCR4, similar experiments were performed in HeLa cells that express CXCR4 endogenously (Holleman & Marchese, 2014). Using this approach, it was successfully determined that the E3 ubiquitin ligase DTX3L, also regulates CXCR4 trafficking from early endosomes to late endosomes/lysosomes (Holleman & Marchese, 2014). In the protocol here, we use HeLa cells to examine CXCR4 trafficking from early endosomes to lysosomes in β-arrestin1 depleted cells by fixed cell confocal immunofluorescence microscopy.

Fixed cell immunofluorescence microscopy is advantageous to study receptor trafficking because it provides spatial and temporal resolution of receptor localization to a level that cannot be achieved by other techniques, such as Western blotting. For example, Western blotting clearly shows that in β-arrestin1 depleted cells, agonist promoted degradation of CXCR4 is inhibited as compared to control, confirming that β-arrestin1 is necessary for targeting CXCR4 to the degradative pathway (Bhandari et al., 2007). However, a limitation of the Western blotting approach is that it does not specify where in the trafficking pathway

a particular factor actually works. This is an important point because β-arrestins generally mediate internalization of GPCRs from the plasma membrane, and inhibiting internalization will also inhibit receptor degradation, as internalization is a prerequisite for degradation (Marchese & Benovic, 2001). In the case of β-arrestin1, images of cells taken by fixed cell confocal immunofluorescence microscopy showed that β-arrestin1 depletion did not impact agonist promoted internalization of CXCR4, as most of the receptor was found to be internalized to endosomes in cells treated with CXCL12, similar to control (Bhandari et al., 2007). It is important to note that although fixed cell immunofluorescence microscopy was essential to define a role for β-arrestin1 in CXCR4 trafficking from endosomes to lysosomes, follow-up studies were required to understand the mechanism by which this occurs, which culminated in defining a novel noncanonical role for β-arrestin1 in receptor trafficking (Malik & Marchese, 2010).

The protocol here examines intracellular trafficking of CXCR4 in HeLa cells transfected with control siRNA or siRNA targeting β-arrestin1 by fixed cell confocal immunofluoresence microscopy. HeLa cells are an adherent, epithelial cell line derived from cervical cancer and expresses high levels of CXCR4 that can be detected by several techniques using commercially available antibodies (Marchese et al., 2003). The protocol described here can be applied to any adherent cell type that expresses CXCR4, including other cancer cells that express CXCR4 to high levels (Fischer, Nagel, Jacobs, Stumm, & Schulz, 2008).

2. METHODS

2.1 Required Materials

HeLa cells; can be obtained from American Type Culture Collection (ATCC)

DMEM (Hyclone #SH3002201)

FBS (Fisher Scientific #03600511)

0.05% Trypsin–EDTA 1× (Life Technologies #25300054)

Phosphate-buffered saline (PBS) 1× (Hyclone #SH30256.01)

Mounting medium with DAPI (Vector Laboratories #H1200; Life Technologies Prolong Gold #P36931)

Microcentrifuge tubes (Dot Scientific #010)

Coverslips, No. 1.5 (Dot Scientific #MC22-15)

Poly-L-lysine (Sigma #P1399)

Formaldehyde (Sigma #X100)

Triton X-100 (Sigma #F1635)

Slides (Fisher Scientific #125442)

10 cm dishes (Falcon #353003)

6-well dishes (Falcon #353046)

Primary antibodies against CXCR4 (BD Transduction Laboratories #BDB551852); LAMP2 (Developmental Studies Hybridoma Bank #H4B4), EEA1 (BD Transduction Laboratories #BDB610456), β-arrestin1 (kindly provided by Jeffrey L. Benovic, Thomas Jefferson University, Philadelphia, PA)

Secondary antibodies (Molecular Probes); goat anti-rat IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor 649) #A21094; goat anti-mouse IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor 488 conjugate) #A11029

siRNA (Dharmacon) against Luciferase (P-002099), β-arrestin1 (siGenome SMARTpool #M-011971)

Lipofectamine 3000 Transfection Reagent (Life Technologies #L3000008)

Opti-MEM (Life Technologies #31985062)

Hemacytometer (Fisher Scientific #0267154)

Tissue paper (Fisher Scientific #06666A)

Tweezers

Lens paper

Whatman filter paper

Parafilm

Confocal microscope equipped with 60× objective

2.2 Cell Culture and Transfection

2.2.1 Passaging and Maintaining HeLa Cells

1. HeLa cells are grown on 10 cm culture dishes at 37 °C, 5% CO_2.
2. Passage cells at 85–95% confluence; approximately every 2 days.
3. Aspirate medium and wash cells 1× with 10 mL PBS.
4. Add 2 mL trypsin and incubate at 37 °C, 5%CO_2 for 5 min. Cells should be detached from the surface of the culture dish. If many cells are still attached, incubate for an additional minute at 37 °C, 5% CO_2.
5. Add 4 mL DMEM supplemented with 10% FBS.
6. Pipette the entire contents up and down at least 3×.
7. Ensure cells are dispersed into a single cell suspension by observing cells under the microscope using a 4× or 10× objective.
8. Add 2 mL of equilibrated cell suspension to a 10-cm culture dish containing 10 mL DMEM supplemented with 10% FBS. This is referred to as a 1:3 split.
9. Incubate cells at 37 °C, 5% CO_2 until cells reach 90–95% confluency;

this typically takes 48 h.

2.2.2 Cell Counting and Plating

1. Aspirate medium and wash cells 1× with 10 mL PBS.
2. Add 2 mL trypsin and incubate at 37 °C, 5% CO_2 for 5 min.
3. Add 4 mL DMEM containing 10% FBS.
4. Pipette the entire contents up and down at least 3×.
5. Transfer cells to a 50-mL conical tube.
6. Count cells using a Hemacytometer.
7. Seed approximately 1.5×10^5 cells per well of a 6-well culture dish in 2 mL DMEM containing 10% FBS.
8. Incubate cells at 37 °C, 5% CO_2 overnight or approximately for 15–18 h.
9. Cells should be approximately 70–80% confluent and ready for transfection.

2.2.3 Transfection with siRNA

1. Prepare the siRNA/Opti-MEM mixture:
   a. 5 μL of each siRNA (20 μ*M* stock) to a microcentrifuge tube.
   b. 50 μL Opti-MEM.
   c. Incubate at room temperature for 5 min.
2. Prepare transfection reagent/Opti-MEM mixture:
   a. 50 μL Opti-MEM
   b. 6 μL Lipofectamine 3000 transfection reagent
   c. Incubate at room temperature for 5 min
3. Add the transfection reagent/Opti-MEM mixture dropwise to the tube containing the siRNA/Opti-MEM mixture. A single well should be treated with a single siRNA.
4. Tap tube gently to mix.
5. Incubate at room temperature for 20 min.
6. In the mean time, replace media in 6-well culture dish with 2 mL DMEM supplemented with 10% FBS.
7. Add siRNA/transfection reagent mixture to cells dropwise.
8. Incubate cells overnight at 37 °C, 5% CO_2.

2.3 Passaging Cells onto Cover Slips and Stimulation

2.3.1 Coverslip Preparation

1. Dip No. 1.5 coverslips in 100% ethanol and dry by blotting on a clean tissue.
2. Place a single coverslip into a well of a 6-well plate. We typically use 22 × 22 mm coverslips. Other sizes are available.
3. Coat coverslip with 0.1 mg/mL poly-L-lysine (PLL). Pipette approximately 500 μL directly onto the glass surface.
4. Incubate for approximately 1–5 min.
5. Aspirate PLL; save and reuse up to 3×. Store at −20 °C.
6. Let coverslips dry in flow hood with lids ajar for approximately 30 min.

2.3.2 Passaging Cells onto Coverslips

1. Aspirate media from each well.
2. Gently wash cells 1× with 2 mL PBS.
3. Aspirate PBS and add 500 μL trypsin, and incubate at 37 °C, 5% CO_2 for 5 min.
4. Add 2 mL DMEM supplemented with 10% FBS.
5. Pipette entire contents up and down at least 3× to mix and to disperse cells into a single cell suspension.
6. Pass 1 mL cells onto well with PLL coated coverslip. Each transfection condition should have two parallel wells: one for vehicle and one for agonist treatment. In addition, a third well is recommended to generate a cell lysate to confirm silencing of β-arrestin1 by Western blotting. Ideally, it would be advantageous to costain cells for β-arrestin1, but in our hands we have not found antibodies that can reliably detect endogenous β-arrestin1 by fixed cell immunofluorescence.
7. Incubate overnight at 37 °C, 5% CO_2.

2.3.3 Stimulating Cells

1. The next day, cells should be approximately 80–90% confluent.
2. Aspirate medium from each well and wash 1× with 2 mL PBS.
3. Add 1 mL complete DMEM in parallel wells containing vehicle or 10 n*M* stromal cell-derived factor (SDF).
    a. Optional: add leupeptin (20 m*M*) and pepstatin A (2 μ*M*) to each well to inhibit lysosomal proteases at least 1 h before and during the stimulation. This is advantageous

because it will enhance receptor stability and thereby facilitate detection by immunostaining.

4. Incubate at 37 °C, 5% CO_2 for 3 h. We have observed that at this time point CXCR4 is mainly localized to late endosome/lysosomes. At shorter time points, such as 30 min to 1 h of agonist treatment, CXCR4 is mainly localized to early endosomes in HeLa cells. Time-course studies should be performed for different cell types to determine the time it takes for CXCR4 to accumulate on early endosomes or lysosomes. It is also important to access the extent to which CXCR4 enters the recycling pathway at each time point.

2.4 Cell Preparation

2.4.1 Fixation and Permeabilization

1. Place plate on ice and wash 2× with 2 mL ice-cold PBS.
2. To fix cells, add 1 mL 3.7% formaldehyde in PBS, 10 min at room temperature.
3. Rinse cells 3× with 1 mL PBS at room temperature
4. To permeabilize cells, add 0.1% Triton X-100 in PBS, 10 min at room temperature.

2.4.2 Blocking and Antibody Incubation

1. Incubate cells with 1 mL 5% normal goat serum in 0.1% Triton X-100/PBS, 60 min at 37 °C.
2. Dilute primary antibody in 1% BSA in 0.1% Triton X-100/PBS. Rat anti-CXCR4 (1:100) in combination with mouse anti-EEA1 (1:1000) or anti-LAMP1 (1:1000). EEA1 is used as a marker for early endosomes and LAMP2 is used as a marker for late endosomes/lysosomes. Primary antibody titrations should be performed to identify optimal antibody dilutions to use for staining.
3. Place filter paper inside a sealable container. Moisten filter paper with water in order to maintain a humidified atmosphere during the incubation period.
4. Place a piece of parafilm on top of the moistened filter paper.
5. Add 40 μL antibody solution to parafilm, appropriately spaced to accommodate the desired amount of coverslips.
6. Carefully remove coverslip from the 6-well plate with forceps. We use an 18-gauge needle with a bent tip to help lift the coverslip from the well.
7. Remove excess buffer by touching the edge of the coverslip on a clean tissue.

8. Carefully invert the coverslip and place on drop of antibody solution. Performing the antibody incubations in this manner limits the amount of reagents, especially antibodies, used in an experiment.
9. Seal the container with a lid or parafilm and incubate at 37 °C, 30 min.

2.4.3 Washing

1. Transfer the coverslips back to the 6-well plate. Ensure that the cell side is oriented upward.
2. Rinse cells 3× with 1 mL 0.1% Triton X-100/PBS at room temperature.
3. Dilute secondary antibody in 1% BSA in 0.1% Triton X-100/PBS. Secondary antibody titration experiments should be performed to identify optimal secondary antibody dilutions to use for immunostaining.
4. Place coverslip with cell side onto 50 μL antibody solution.
5. Incubate at 37 °C, 30 min.
6. Wash 3× at room temperature with 1 mL 0.1% Triton X-100/PBS.
7. Leave last wash on as coverslips are being mounted. It is important to ensure that coverslips do not dry during the mounting step.

2.4.4 Mounting

1. Apply four small dots of nail polish to microscope slide and allow to dry. This is meant to preserve the thickness of the sample during confocal sectioning.
2. Place 60 μL mounting medium onto coverslip in the area delimited by the dots.
3. Carefully remove coverslip from the 6-well plate with forceps.
4. Remove excess buffer by touching the edge of the coverslip on a clean tissue.
5. Carefully invert the coverslip and place on drop of mounting medium.
6. If mounting medium seeps from the edges of the cover slip, carefully wipe with tissue.
7. Seal the edges of the coverslip to the slide with nail polish.
8. Samples can be viewed once the nail polish has set.

2.5 Microscope Image Acquisition

2.5.1 Sample Analysis

1. Image fixed samples by confocal microscopy. We mostly use a LSM 510 laser scanning confocal microscope for imaging (Carl Zeiss, Thornwood, NY) and a Plan-Apo 60×/1.4 NA oil lens objective.

2. Acquire images of cells in each channel and of multiple frames of view. We typically image using a 1.4-megapixel cooled extended spectra range RGB digital camera set at 512 × 512 resolution (Carl Zeiss, Thornwood, NY).

3. Adjust illumination and exposure times accordingly and be careful to remain within the linear range. Keep settings constant during image acquisition of parallel samples within an experiment.

4. Take z-series images. We typically set z-series to 0.2 μ*M*.

5. Analyze images at full bit depth using ImageJ software [(http://imagej.nih.gov/ij/), National Institutes of Health, Bethesda, MD].

6. Carefully perform puncta analysis. Manually scale images using linear transformations to discriminate between true puncta and background noise.

7. Set minimum and maximum threshold values for particle analysis separately for each channel; count particles.

8. Calculate colocalization between proteins using the Pearson coefficient Colocolization Finder plugin in ImageJ.

3. CONCLUDING REMARKS

The protocol described here examines the cellular distribution of the chemokine receptor CXCR4 in the absence or presence of its ligand CXCL12 by fixed cell confocal immunofluorescence microscopy. This protocol can be easily adapted to examine trafficking of other chemokine receptors, depending on the availability of suitable antibodies and cells. As we have shown, when this approach is coupled with gene silencing and other biochemical approaches it can be used to unequivocally identify factors that regulate receptor sorting from endosomes to lysosomes.

Understanding the mechanisms governing endosome to lysosome trafficking is important because this trafficking step can control the amount of receptors on the cell surface and therefore regulate chemokine responsiveness. Although most chemokine receptors are biased toward entering the recycling pathway, CXCR4 in contrast is biased toward entering the degradative pathway (Marchese, 2014). However, a small fraction of CXCR4 can also enter the recycling pathway and return to the plasma membrane, likely contributing to resensitization of signaling. The implication of this is that cellular responsiveness to CXCR4 is likely due in part to biased receptor sorting into the degradative pathway versus the recycling pathway. Dysregulation of this bias could lead to aberrant signaling and have deleterious consequences. For example, in a subset of breast cancer cells, high CXCR4 expression may in part be due to defective sorting of the receptor into the degradative pathway, therefore favoring receptor recycling and enhanced signaling, contributing to tumor metastasis (Li et al., 2004). In this regard, understanding the mechanisms controlling receptor sorting into the degradative pathway is important and may lead to innovative strategies to target receptor sorting therapeutically.

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