Video Article

Detection of Ligand-activated G Protein-coupled Receptor Internalization by Confocal Microscopy

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Abstract

Confocal laser scanning microscopy (CLSM) is an optical imaging technique for high-contrast imaging. It is a powerful approach to visualize fluorescent fusion proteins, such as green fluorescent protein (GFP), to determine their expression, localization, and function. The subcellular localization of target proteins is important for identification, characterization, and functional analyses. Internalization is one of the predominant mechanisms controlling G protein-coupled receptor (GPCR) signaling to ensure the appropriate cellular responses to stimuli. Here, we describe an experimental method to detect the subcellular localization and internalization of GPCR in HEK293 cells with confocal microscopy. In addition, this experiment provides some details about cell culture and transfection. This protocol is compatible with a variety of widely available fluorescent markers and is applicable to the visualization of the subcellular localization of a majority of proteins, as well as of the internalization of GPCR. This technique should enable researchers to efficiently manipulate GPCR gene expression in mammalian cell lines and should facilitate studies on GPCR subcellular localization and internalization.

Video Link

The video component of this article can be found at https://www.jove.com/video/55514/

Introduction

Cells possess cargo trafficking machinery to transport extracellular materials-such as ligands, microorganisms, nutrients, and transmembrane proteins-into the cell for information, energy, and other purposes[1,2]. It is critical for cellular homeostasis, tissue function, and overall cell survival. The cell-based expression of fluorescent fusion proteins is a powerful way to study the localization and internalization of transmembrane receptors, such as G protein-coupled receptors (GPCR), in signaling pathways[3].

The expression of fusion proteins tagged with green fluorescent protein (GFP) from jellyfish *Aequorea victoria*, combined with direct fluorescence detection techniques, has gained wide acceptance in protein-targeting research[4,5,6]. GFP-based detection has the advantage of allowing the real-time imaging of living cells while avoiding fixation artifacts[7]. A series of versatile cloning vectors has been constructed to facilitate the expression of protein fusions to GFP in various cells[8,9]. The pEGFP-N1 vector is a widely used and commercialized plasmid vector encoding an enhanced green fluorescent protein (EGFP), which has been optimized from wild-type GFP for brighter fluorescence and higher expression in mammalian cells. To observe the fluorescent signal of EGFP expressed in mammalian cells, the fluorescence microscopy should be conducted with the excitation at 488 nm and the emission at 507 nm, preferably with confocal microscopy.

Monitoring the subcellular localization and ligand-mediated internalization of receptors is a common technique to investigate the signal transduction and function of GPCRs[10,11]. In most cases, internalization leads to desensitization of the GPCRs (the attenuation of the stimulus response in spite of the presence of agonists)[12,13]. The internalized receptors can be incorporated into the lysosome and degraded, or they can be recycled back to the cell surface, depending on the nature of the receptors and the cell lines used in the experiments.

The gonadotropin-releasing hormone receptors (GnRHRs) belong to the GPCR family and have a typical GPCR protein structure, with an extracellular amino terminal, an intracellular -COOH terminal, and seven transmembrane (TM) domains[14]. The transfection of recombinant plasmid *Sj*GnRHR-EGFP (with the *GnRHR* gene from *Sepiella japonica*) and the confocal microscopy detection of EGFP-tagged *Sj*GnRHR (expressed in HEK293 cells) was reported previously, and GFP fluorescence was established as a reporter for transmembrane protein localization assays[15]. Now, the internalization of *Sj*GnRHR, activated by *S. japonica* gonadotropin-releasing hormone (*Sj*GnRH), was demonstrated in time- and dose-dependent manners by confocal microscopy.

Protocol

1. Cell Preparation

1. **Cell recovery**
   1. Transfer the cryopreserved human embryonic kidney 293 (HEK293) cells from a liquid nitrogen tank to a 37 °C water bath and shake continuously for 1 - 2 min.
   2. Add 10 mL of equilibrated growth medium (Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin solution) to a 10-cm cell culture dish. Gently add the thawed cells to the equilibrated growth medium.
   3. Incubate the cells in a 37 °C water-jacketed CO_2 incubator with a humidified atmosphere containing 95% air and 5% CO_2 for 2 - 4 h.
   4. After 2 - 4 h, check that the cells are attached. Replace the equilibrated growth medium and culture for another 24 - 48 h.
      NOTE: Changing the equilibrated growth medium is useful because it removes dimethyl sulfoxide (DMSO) and protects against cell damage.

2. **Cell passage**
   1. After 24 - 48 h, when the cells have grown to near confluence, discard the equilibrated growth medium and wash the cells with 2 mL of phosphate-buffered saline (PBS). Swirl the dish gently.
   2. Discard the PBS and add 1 mL of Trypsin-EDTA (saline, 0.25% trypsin, and 0.02% EDTA). To completely digest the cells, swirl the dish carefully and aspirate off the Trypsin-EDTA solution. Put the dish in the 37 °C water-jacketed CO_2 incubator for 3 min.
      NOTE: During this period, prepare three new 10-cm cell culture dishes and add 10 mL of equilibrated growth medium to each.
   3. Using a light microscope, determine if the cells have lifted from the bottom of the dish. Add 2 mL of equilibrated growth medium to the dish. Mix by pipetting and immediately pipet 0.6 mL of the cell suspension to each of the new dishes. Gently mix by pipetting and allow the cells to incubate at 37 °C and 5% CO_2.

2. Cell Seeding

1. After 24 - 48 h, when the passaged cells have grown to near confluence, discard the equilibrated growth medium and wash the cells with 2 mL of PBS. Swirl the dish gently.
2. Discard the PBS and add 1 mL of Trypsin-EDTA. To completely digest the cells, swirl the dish carefully and aspirate off the Trypsin-EDTA. Put the dish in the 37 °C water-jacketed CO_2 incubator for 3 min.
   NOTE: During this period, prepare a 6-well plate by adding 2 mL of equilibrated growth medium to each well.
3. Add 1 mL of equilibrated growth medium to the dish, if, under microscopic observation, the cells to have lifted from the dish bottom. Mix by pipetting and immediately pipet 0.1 mL of cell suspension (the volume is adjusted according to the cell growth density) to each well of the prepared 6-well plate (cell-less 6-well plate with 2 mL of equilibrated growth medium). Gently mix by pipetting and allow the cells to incubate at 37 °C and 5% CO_2.

3. Cell Transfection

1. **Transfection method 1, with liposome formulations**
   NOTE: Please see the **Table of Materials** for transfection reagent 1.
   1. The following day, ensure that cells should be 85 - 95% confluent.
   2. Add 3 µg of plasmid DNA (500 ng/µL) and 100 µL of reduced serum media to a sterile microcentrifuge tube, and mix gently. Incubate for 5 min at room temperature.
   3. Prepare 9 µL of Reagent 1 and 100 µL of reduced serum media in another microcentrifuge tube, and mix gently. Incubate for 5 min at room temperature.
   4. Combine the diluted plasmid mixture with diluted Reagent 1 (total volume = 215 µL). Mix gently and incubate for 20 min at room temperature.
   5. Aspirate medium off cells-cultured plate, and add 1.5 mL fresh DMEM without FBS.
   6. Add 215 µL of DNA-transfection reagent mixture to each well dropwise, and mix gently by swirling the plate.
   7. Incubate at 37 °C and 5% CO_2 for 4 - 8 h. Replace with fresh DMEM with 10% FBS.
   8. Incubate cells at 37 °C and 5% CO_2 for another 24 - 48 h before running any experiments.

2. **Transfection method 2, with liposome formulations**
   NOTE: Please see the **Table of Materials** for transfection reagent 2.
   1. Add 2 µg of plasmid DNA (500 ng/µL), 4 µL of Reagent 2, and 100 µL reduced serum media into a sterile microcentrifuge tube. Mix gently and incubate for 15 min at room temperature.
   2. Add the transfection mixture to the cells in a dropwise manner.
   3. After transfection, incubate cells for 24 - 48 h at 37 °C and 5% CO_2, before running any experiments.

4. Cell Culture on Cover Slips

1. Remove the growth medium from the culture 6-well plate.
2. Wash the cell monolayer with 1 mL of PBS without calcium and magnesium.

3. Add 0.5 mL Trypsin-EDTA. Swirl the dish carefully and aspirate off Trypsin-EDTA. Incubate the culture plate for 3 min at 37 °C and 5% CO_2. The actual incubation time varies with the cell line used.
   NOTE: During this period, prepare a new 12-well plate and add 15 mm diameter sterile cover slips to each well. Cover slips coated with lysine, laminin, or collagen may improve cell attachment for HEK293 cells that easily detach.
4. When cells are detached, add 1 mL pre-warmed complete growth medium to each well. Mix by pipetting and immediately transfer the required number of cells to the 12-well plate with cover slips. Add fresh complete growth medium to each well to adjust the total volume to 1 mL
5. Incubate the cells for 16 - 24 h at 37 °C and 5% CO_2.
   NOTE: After 30 - 60 min, the majority of cells will have adhered to the sterile cover slips.

5. Confocal Microscopy Detection

1. **Cellular localization**
   1. After 16 - 24 h, when the cells have grown to near confluence on the sterile cover slips in the 12-well plate, add the membrane probe, DiI, to each well, giving a final concentration of 5 μM. Allow the cells to incubate for 30 min at 37 °C and 5% CO_2.
   2. After 30 min, remove the equilibrated growth medium and wash the cells twice in cold PBS (2 - 8 °C).
   3. Fix the cells by adding 1 mL of a 4% paraformaldehyde solution (in PBS) to each well. Gently agitate for 15 min.
      Caution: Paraformaldehyde is moderately toxic by skin contact or inhalation, and designated as a probable human carcinogen. Chemicals fume hoods, vented balance enclosures or other protective measures should be used during the weighing and handling of paraformaldehyde.
   4. Wash the fixed cells twice in PBS. Add 200-300 μL of DAPI (4',6-diamidino-2-phenylindole) solution (0.5 μg/mL) to each well and incubate for 10 min at room temperature.
   5. Wash the cells twice with PBS. Carefully remove the cover slips from the plate and wick off excess PBS. Invert onto microscope slides loaded with one drop of mounting medium (50% v/v glycerol/water). Remove excess mounting medium from the slides.

2. **Cellular internalization**
   1. After 16 - 24 h, when the cells have grown to near confluence on the sterile cover slips in the 12-well plate, remove the equilibrated growth medium and add fresh, warmed DMEM medium (37 °C). Incubate the cells for 1 h at 37 °C and 5% CO_2.
   2. Treat the cells with different concentrations (0, 10^{-12}, 10^{-9}, and 10^{-6} M) of ligand for 30 min and treat them with 10^{-6} M ligand for different times (0, 10, 30, and 60 min).
   3. Wash the cells twice in cold PBS (2 - 8 °C). Fix the cells by adding 1 mL of a 4% paraformaldehyde solution (in PBS) to each well. Gently agitate for 15 min.
   4. Wash the cells twice in PBS. Carefully remove the cover slips from the plate and wick off the excess PBS. Invert onto microscope slides loaded with one drop of mounting medium (50% v/v glycerol/water). Remove excess mounting medium from the slides.

3. **Confocal microscopy**
   1. Turn on the hardware of confocal microscope, including the mercury lamp power, PC microscope, scanner, and laser. Login to operating system account, launch the software, check the configuration and select laser. Turn on the different units of confocal microscopy system in the order according to instructions.
   2. Place the prepared slides on the microscope stage. Position the sample over the objective lens using the stage controller. Add one drop of cedar oil onto coverslip when the oil immersion lens is selected. Place the cover slips on slides facing the lens.
   3. Find the cells of interest under the fluorescent microscopy.
   4. Switch to scan mode. Choose the laser power and the spectral range of the emission fluorophore. Capture the high quality confocal images by tuning laser intensity and other parameters.

Representative Results

Figure 1 shows an example of a confocal microscope system. **Figure 2** presents the expression of pEGFP-N1 in HEK293 cells. The GFP signal was detected in the cytoplasm and nucleus. **Figure 3** shows the subcellular localization of GnRHR from *S. japonica* in HEK293 cells, consistent with our previously published results[15]. The fusion protein with the EGFP tag at the C-terminus of *Sj*GnRHR (*Sj*GnRHR-EGFP) appeared green in this experiment when under CLSM. The nucleus stained with DAPI was revealed in blue. DiI staining highlighted the cell membrane in red. The yellow signal on the cell surface in the merged image indicated the coincidence of red and green, demonstrating the plasma membrane localization of *Sj*GnRHR.

Figures 4 and **5** display the results of the *Sj*GnRHR internalization assays in HEK293 cells. To visualize the internalization of *Sj*GnRHR, cells were treated with gonadotropin-releasing hormone (*Sj*GnRH)[15] at different concentrations for 30 min (**Figure 4**), or they were treated with a single concentration (10^{-6} M) for different durations(**Figure 5**). In the absence of *Sj*GnRH, the control (CTL) receptor fusion had plasma membrane localization. As shown in **Figure 4**, at different ligand concentrations, the internalization of the *Sj*GnRHR proceeded in a dose-dependent manner, and the receptor was completely internalized from the cell surface with the 10^{-6} M ligand. As shown in **Figure 5**, the results of the time course analysis of receptor internalization with 10^{-6} M ligand demonstrated that internalized *Sj*GnRHR was detectable 10 min after agonist stimulation, and extreme internalization occurred by 60 min. Observation with confocal microscopy from these two figures revealed that the fluorescent *Sj*GnRHR-EGFP fusion protein was primarily localized in the plasma membrane and was dramatically and rapidly internalized into the cell in response to the *Sj*GnRH peptide in HEK293 cells.

Figure 1. An Example of a Confocal Microscope System. Please click here to view a larger version of this figure.

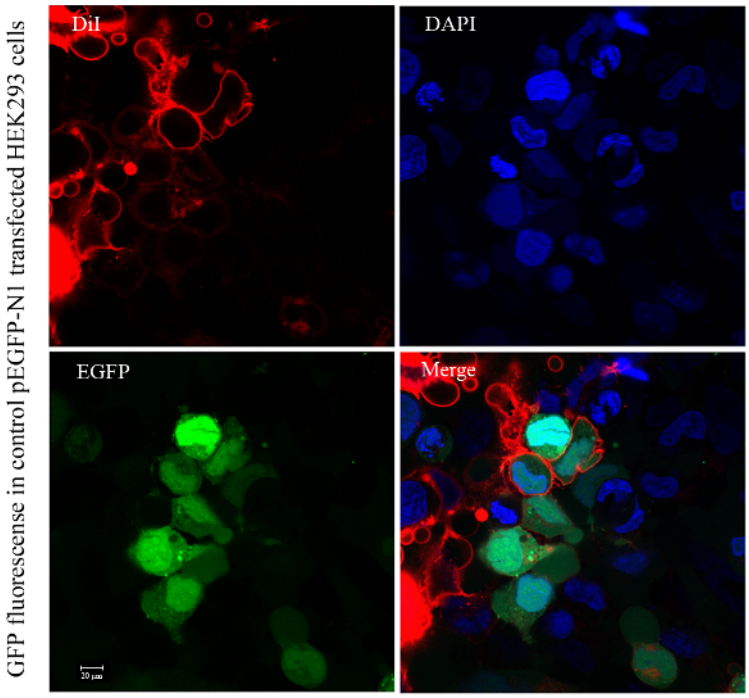

Figure 2. Confocal Microscopy of GFP Protein in Control pEGFP-N1-transfected HEK293 Cells. Please click here to view a larger version of this figure.

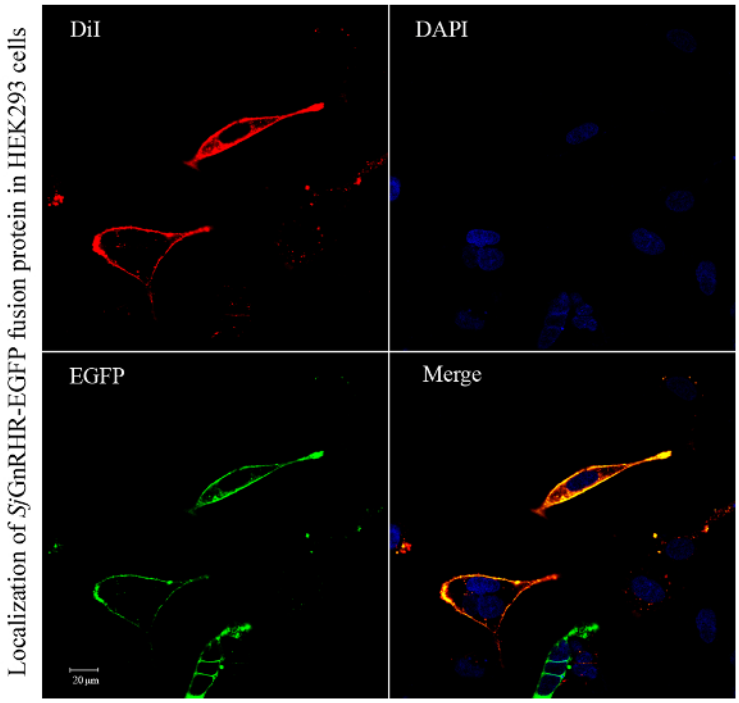

Figure 3. Localization of *Sj*GnRHR-EGFP Fusion Protein in HEK293 Cells by Confocal Microscopy. The nucleus stained with DAPI is shown in blue. DiI staining, shown in red, highlights the cell membrane. *Sj*GnRHR-EGFP fusion protein is shown in green. In the merged image, yellow indicates the coincidence of the red and green signals. All images represent at least three independent experiments. Please click here to view a larger version of this figure.

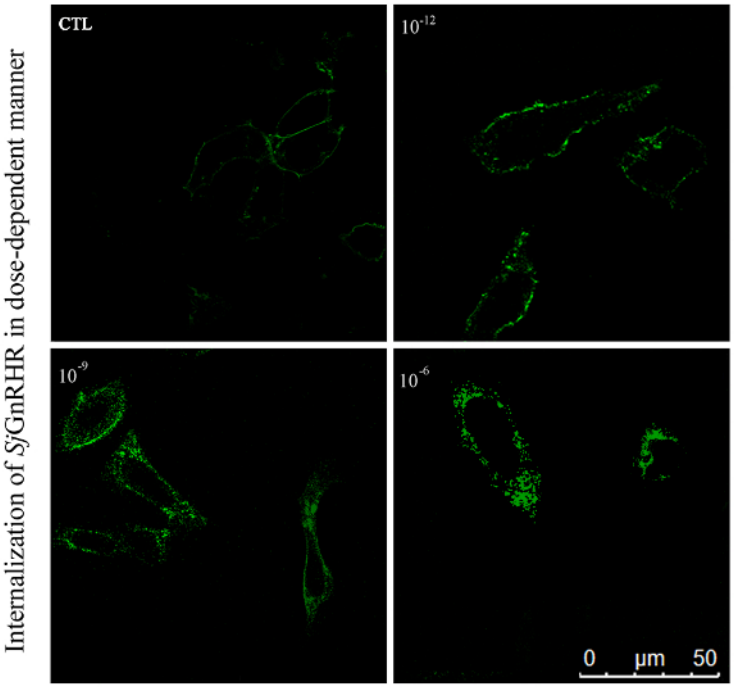

Figure 4. Internalization of *Sj*GnRHR-EGFP Fusion Protein in HEK293 Cells in a Time Course by Confocal Microscopy. HEK293 cells transfected with *Sj*GnRHR-EGFP plasmid were activated by treatment with different concentrations of GnRH for 30 min. The control case was without GnRH stimulation (CTL). All images represent at least three independent experiments. Please click here to view a larger version of this figure.

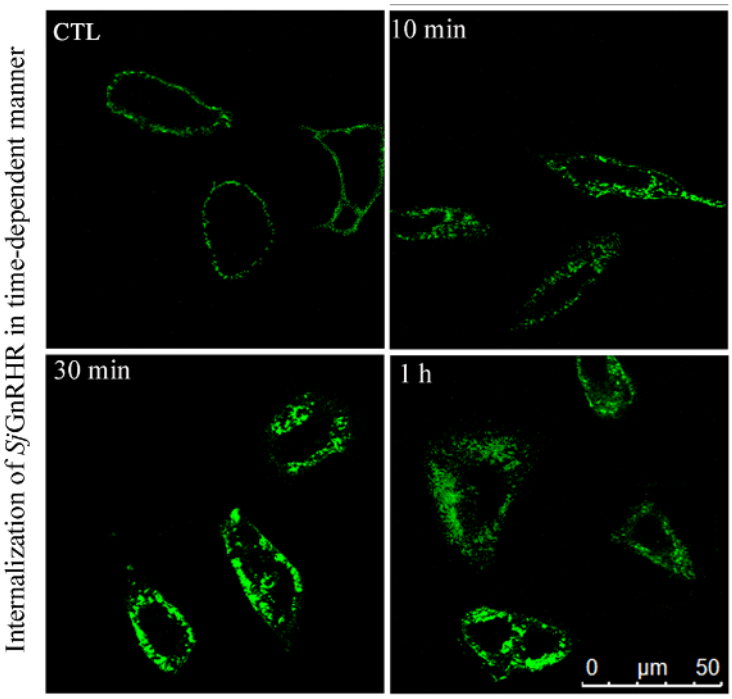

Figure 5. Internalization of *Sj*GnRHR-EGFP Fusion Protein in HEK293 Cells in a Dose Dependent Manner by Confocal Microscopy. HEK293 cells transfected with *Sj*GnRHR-EGFP plasmid were activated by treatment with 10^{-6} M ligand for different durations. The control case was without GnRH stimulation (CTL). All images represent at least three independent experiments. Please click here to view a larger version of this figure.

Discussion

The protocol presented here provides an efficient and easily interpretable method to detect the subcellular localization and internalization of expressed GPCR fusion protein in HEK293 cells. The technique can be easily adapted for many different genes and cell types, such as for the cellular localization and internalization of the corazonin receptor from *Bombyx mori* in HEK293 and BmN cells[16].

The successful application of this powerful assay relies on a few key factors. Foremost is the good health of the cells, which is essential for efficient transfection and data quality. The transfection incubation time, the interval between transfection and other experiments, and the passage of transfected cells to cover slips before imaging, are all steps where damage to the cultured cells is possible. Therefore, maintaining the good health of the cells is an important prerequisite for all steps in this experiment. Moreover, avoiding tightly compressed cells in dense cultures is vital in order to acquire high-quality images with confocal microscopy. To minimize the influence of the fluorescent tag on the localization of the GPCRs, we generated receptors with EGFP fused to the intracellular C-terminus. The successful and efficient transfection of the gene into the cells is also important. Transfection efficiency may vary greatly in different cell lines. Therefore, the optimization of certain steps in the protocol may be necessary, depending on the particular cell type used. For example, in transfection method 1, the incubation duration of transfected cells in DMEM without FBS could be optimized according to the cell condition. In addition to the dosage of the DNA plasmid, the dosage of the liposome reagent and reduced serum media should be adjusted according to specific cell lines. Otherwise, the electrotransfection technique could be conducted instead of liposome reagent mediated transfection for acceptable transfection efficiency in a specific cell line. However, additional hardware is required, and cells in suspension and in a small volume are difficult to transfect by electrotransfection. The true strength of the current assay, however, lies in the comparison between changes in the same cells under identical culture conditions after acute manipulations, such as the internalization of *Sj*GnRHR in response to the *Sj*GnRH ligand.

Internalization is one of the predominant mechanisms controlling GPCR signaling to ensure the appropriate cellular responses to stimuli[11]. We can easily observe changes in internalization through imaging with confocal microscopy. The internalization of *Sj*GnRHR in a dose- and time-dependent manner is clearly revealed in **Figure 4**.

The protocol we have described can be easily adapted to the subcellular tracing of other eukaryotic proteins, such as the nuclear estrogen receptor[17]. However, the optimization of certain steps in the protocol will be necessary, depending on the particular cell types used, which will require further exploration and practice. Nevertheless, considering that the subcellular location of proteins has a great influence on the identification of genes, protein modifications, and signaling pathways, this protocol, based on the published research, can provide reliable data and information.

Disclosures

The authors declare no conflicts of interest.

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