Single-Molecule Imaging of GPCR with TIRF Microscopy

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Abstract

GPCR is the largest family of membrane receptors and relays signals in various physiological contexts. It is the target of approximately 50% of marketed drugs, and further characterization of GPCR’s molecular details is crucial in drug improvements and discovery of novel therapeutic methods. Hence, GPCR’s activity has been studied extensively; however, these experiments often employ biochemical or bulk biophysical assays. Though these methods uncover protein-protein interactions and receptors’ physical states, they lack in spatial and temporal resolution and do not yield conclusive data on its activity throughout its lifetime. Hence, we observed GPCR at single-molecule level using TIRFM imaging and computational programs in deriving the activity of individual GPCR molecules. This novel method allows for the illustration of single molecule activity, which may contrast significantly from that observed in ensemble level studies.
Introduction

G-protein-coupled receptors (GPCRs) are a group of membrane receptors involved in various cellular signaling pathways. Consisting of approximately 1000 different types in humans alone, GPCRs bind many types of molecules, such as peptides, nucleotides, ions, and amino acids, and relay signals in various contexts, from photon sensing in the retina of the eye to hormone signaling in vasoregulation. Hence, GPCRs have been exploited as targets of approximately fifty-percent of marketed drugs, being a major focus of modern scientific research.

GPCR consist of seven conserved transmembrane α-helices along with extracellular and intracellular elements that vary among different types of receptors. The inactive form of GPCR is thought to be bound to a heterotrimeric G-protein, consisting of α, β and γ subunits. Agonist binding activates the receptor by inducing the exchange of GDP molecule, already present on the Gα subunit, with GTP. The Gα and Gβγ subunits are subsequently released from GPCR, proceeding to act on secondary messenger molecules on the membrane.

In response to prolonged exposure to ligands, GPCR must be desensitized, or downregulated. One prominent mechanism for desensitization begins with the phosphorylation of active GPCRs by G-protein-coupled receptor kinases (GRKs). Phosphorylated GPCRs are bound by arrestin, which are then translocated into the cell, where it is either degraded within lysosomes or recycled and brought up to the membrane for further signaling activity.

Various studies have shown that GPCRs form and act as dimers and/or oligomers. For instance, Beta2-adrenergic receptors and Rhodopsin receptors have been shown to form homodimers, while GABA receptors form heterodimers with multiple types of GPCRs. Many methods have been utilized in exploring GPCR’s activity and interaction with other molecules; however, many have relied on biochemical techniques, such as western blotting and SDS-PAGE, and bulk biophysical techniques, including FRET. The issue with such procedures is that the former is conducted in vitro, lacking in spatial and temporal resolution, while the latter is insufficient in concluding that GPCR interacts with other molecules, as to simply existing in close proximity. Hence, many alternative methods have been proposed in recent years to further investigate GPCRs’ activity throughout its lifetime.

Objective

GPCR activity, including oligomerization state and protein-protein interaction, can be represented through the motion of GPCR. In other words, GPCRs interaction with other GPCRs and interactive molecules must alter its kinetics. Hence, we conducted live-cell imaging of human embryonic kidney cells (HEK cells) using confocal and single molecule total internal reflection fluorescence microscopy (TIRFM) to characterize GPCR activity in cellulo.
Materials and Methods

Plasmid construction: Plasmids containing various types of GPCR DNA had been previously constructed in the Ozawa lab. GPCRs were inserted along SNAP tags to allow for labelling and were prepared in pcDNA3.1 containing V5 and HisB tag sequences. These constructs were amplified through bacterial transformation, followed by maxiprep purification. The prepared plasmids were stored at -30 °C until use.

Cell culture and Transfection: HEK cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% Penicillin Streptomycin at 37 °C. These cells were plated onto 35 mm dishes at approximately 3 x 10^5 cells per dish in 1 ml medium. Cells were incubated overnight for 2 days to grow until ready for transfection. Transfection solution was prepared from 1 µl of plasmid, 3 µl of TransIT-LT transfection reagent, and 100 µl of OPTI-MEM medium. After 15 min incubation at room temperature, the solution was added to the cell cultures. The dishes were incubated at 37 °C for approximately 5 hours for single molecule imaging, over night for confocal imaging, and 2 days for western blotting.

Western Blotting: Cells were scraped in 500 µl phosphate-buffered saline (PBS). After centrifugation, the samples were lysed in 50 µl lysis buffer (4% complete protease inhibitor) and incubated with sodium dodecyl sulfate (SDS) solution. The samples were run on acrylamide gels at 110 V for around 2 hours. The samples on the gel were then transferred onto a membrane, which was washed with tris-buffered saline with tween (TBST), and incubated overnight with V5 tag antibody (mouse monoclonal). The following day, the membrane was washed, incubated with anti-mouse secondary antibody, and imaged.

Confocal imaging: Imaging was conducted using Olympus FV1000 confocal microscope. After an overnight transfection, the cell media were replaced with that containing Alexa647 (SNAP tag) and Tetramethylrhodamine (TMR, HALO tag) at 1:1000 dilution. Prior to observation, GPCR ligands were added to the dishes setup on the microscope. Following ligand application, a video was taken at 1 frame per 30 seconds for 30 minutes. Lasers with wavelengths 488, 559, and 635 were used for observation of different proteins, and the movies were saved as TIF files.

Single molecule imaging: Imaging was conducted using Olympus microscope equipped with 100x oil immersion TIRFM objective lens. After a 5-hour transfection, the cell media were replaced with that containing SeTau647 (SNAP tag) at 1:1000 dilution. Prior to observation, GPCR ligands were added to the dishes setup on the microscope. Following ligand application, a video was taken at 30 frames per second for around 5 minutes. Laser with wavelengths 635 was used for observation of GPCR, and the movies were saved as TIF files.

Analysis: Fluorescence detection and single molecule tracking was conducted through the TrackMate plugin of ImageJ. Membrane and cytosolic fluorescence distribution from confocal imaging data was quantified manually on ImageJ. Fluorescence data for TIRFM imaging were passed through computational programs previously written in the Ozawa lab. Mean squared displacement was derived from individual tracks and was used in the diffusion equation to derive the diffusion coefficient. Intensity and displacement were additionally calculated.
Confocal Imaging Results

Ligand activation of GPCR induced endocytosis and recruitment of β-arrestin and Gai-protein to membrane: Confocal imaging data showed that ligand application induces an increase in the membrane distribution of β-arrestin and Gai-protein and the cytosolic distribution of GPCR (Figure 1).

Figure 1: Quantification of average membrane to whole cell fluorescence intensity of β2-AR GPCR, β-arrestin, and Gai-protein. Proteins were observed using SNAPf-Alexa647, EGFP, and HALO-TMR respectively. The quantification is based on videos recorded over 30 minutes with 1 frame per 30 seconds. (mean +/- SD, n=3)

This data is consistent with the previous understanding of GPCRs mechanism, in which arrestin is recruited to the membrane after GPCR activation, followed by its internalization through endocytosis. Though the change in G-protein was small, activation of GPCR may be reinforcing its recruitment to the membrane where it binds GPCR and relays signals.
TIRFM Imaging Results

Multiple Diffusion patterns exist among GPCR observed at single-molecule level: GPCR observed with TIRFM showed multiple types of diffusion patterns. In the first notable pattern, GPCR (μ-OR) existing in a rapid motion state suddenly transitioned into long-term restricted motion state (Figure 2A). Interestingly, fluorescence intensity of GPCR rapidly declines simultaneously (Figure 2B). The rapid transition may be an indication of GPCR’s interaction with other molecules, though, the data is inconclusive in determining the physical state of the protein (n=3).

Figure 2: Diffusion coefficient and intensity of a single-molecule track illustrating diffusion pattern 1. μ-OR GPCR labelled with SNAPf-SeTau647 was observed for 5 minutes using TIRFM. Diffusion coefficient shows a rapid decrease in long-term activity (A). Intensity quantification shows a similar decrease slightly after the decrease in activity occurs (B).
A second pattern showed a GPCR in rapid motion state transitioning into short stoppage and returning back to rapid motion state within 10 seconds (Figure 3A). There also seems to be a correlated increase in fluorescence intensity at the same time (Figure 3B). This opposition in intensity change indicates a different type of diffusion pattern from the first, though, data is similarly inconclusive in determining the specific physical state of the protein (n=3).

**Figure 3:** Diffusion coefficient and intensity of a single-molecule track illustrating diffusion pattern 2. μ-OR GPCR labelled with SNAPF-SeTau647 was observed for 5 minutes using TIRFM. Diffusion coefficient shows sudden stoppage and return to rapid motion (A). Intensity quantification shows an opposite increase (B).
No significant change occurs when single-molecule tracks are combined in ensemble analysis:
Interestingly, when the single-molecule tracks were analyzed at the ensemble level, no
temporal trend emerged (Figure 4). Given the existence of multiple single molecule tracks with
different diffusion patterns, this data indicates that average trend in activity is not necessarily
representative of individual molecular activity. This further underscores the importance of
single-molecule imaging studies of GPCR in studying its kinetics.

Figure 4: Diffusion coefficient of multiple tracks represented as line and dot. There is a clear gap
between molecules with relatively high and low diffusion coefficient. Data is of μ-OR GPCR with
180 seconds total imaging time (A, B) and RHO GPCR with 200 seconds total imaging time (C,
D).
**Discussion and Future Directions**

In conclusion, data from confocal imaging showed that Membrane localized GPCR visibly starts to internalize about 15 minutes after ligand application, while ubiquitous β-arrestin seems to begin membrane localization about 10 minutes after ligand application. Both of these reinforce the currently understood mechanism behind GPCR activity. Furthermore, TIRFM data showed that GPCR undergoes multiple single-molecule diffusion patterns that may be related to its interaction with other molecules, while this single-molecule pattern is not seen at ensemble level within 3 minutes after ligand application. It is important to note here that the contrast between the single-molecule and ensemble observations indicate that individual GPCRs are acting according to various diffusion patterns and that this is collectively not apparent. Hence, this study serves as an indication of the importance of single-molecule observation of GPCR in elucidating its kinetics and could potentially become an effective way to uncover its activity throughout its lifetime.

However, for this method to become effective, many issues must be resolved. One of the primary problems with this project was the tracking of individual GPCR using TIRFM. Bleaching of fluorophores, z-axis fluctuation of individual molecules, overlap of multiple tracks, and multitudes of other factors impacted tracking of GPCR fluorescence. Hence, optimization of imaging and tracking techniques is necessary in order to effectively track individual molecules for a long duration. Once this is established, we can derive diffusion patterns with higher accuracy and relevance to its kinetics. Furthermore, multi-channel TIRFM imaging of GPCR with other associating molecules, such as β-arrestin and G-protein, may illuminate the effect they have on GPCR’s motion. Lastly, characterization and comparison of multiple types of GPCR could uncover the kinetic similarities and differences within the GPCR family.
References


