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Imaging GPCRs trafficking and signaling with total internal reflection fluorescence microscopy in cultured neurons

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Abstract

Total internal reflection fluorescence (TIRF) microscopy allows probing the cellular events occurring close and at the plasma membrane. Over the last decade we have seen a significant increase in the number of publications applying TIRF microscopy to unravel some of the fundamental biological questions regarding G protein-coupled receptors (GPCRs) function such as the mechanisms controlling receptor trafficking, quaternary structure and signaling among others. Most of the published work has been performed in heterologous systems such as HEK293 and CHO cells, where the imaging surface available is higher and smoother when compared with the narrow processes or the smaller cell bodies of neurons. However, some publications have expanded our understanding of these events to primary cell cultures, mostly rat hippocampal and striatal neuronal cultures. Results from these cells provide a bonafide model of the complex events controlling GPCR function in living cells. We believe more work needs to be performed in primary cultures and eventually in intact tissue, to complement the knowledge obtained from heterologous cell models. Here, we described a step-by-step protocol to investigate the surface trafficking and signaling from GPCRs in rat hippocampal and striatal primary cultures.

Keywords

G protein-coupled receptor; MOR; TIRF; Live cell imaging; Endocytosis; Recycling; Resensitization

The unique ability of TIRF microscopy to generate an evanescent field and excite fluorophores within a narrow optical section (~ 100nm) provides an ideal tool to investigate the multiple and dynamic events occurring close to and at the plasma membrane of living cells with reduced phototoxicity and bleaching [1–4]. This characteristic resulted in the application of TIRF microscopy to multiple biological areas ranging from single molecule analysis to cell migration. TIRF microscopy has been particularly used to investigate protein

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translocation and trafficking to the membrane, vesicular events and single molecule analysis among others [5–8]. Since astrocytes display a significant surface area, these cells have been used to visualize and analyze various events such as the molecular mechanisms controlling vesicular release [9]. However, the application of TIRF microscopy to investigate GPCRs in neurons is still very limited. Our laboratory was among the first to use TIRF microscopy to investigate GPCRs in neurons [10]. Our data showed that receptor recycling to the cell surface can be mediated by two different vesicular events, a rapid event without diffusion barriers and a slow event with a kinetic similar to kiss and run exocytosis [10,11]. These kiss and run events were not observed with the Mu opioid receptor and were dependent on the carboxy terminal sequence of the B2 Adrenergic receptor [7,12]. TIRF microscopy was also utilized to demonstrate that GPCR recycling after ligand induced internalization, is actively controlled by the activation of receptors still at the cell surface. Interestingly, identical results were observed in primary cell cultures and in heterologous systems supporting the idea that heterologous systems can provide valuable information [13–16]. Others have utilized TIRF in neuronal cultures to investigate the pathways by which receptors are recycled to the cell surface [17]. Our laboratory also utilized TIRF microscopy to investigate endogenous GPCR signaling in hippocampal neurons by loading neurons with calcium sensitive dyes and pharmacologically activating specific adrenergic receptors [18]. More recently, we have investigated how cannabinoid receptor 1 interacts with beta-arrestins at the cell surface of hippocampal neurons to regulate beta-arrestin signaling [19]. These are some of the applications of TIRF microscopy to investigate GPCRs. The application of TIRF and super resolution microscopy in combination with novel fluorescent tags and nanobodies should only expand the toolbox available to further probe the biology of these highly relevant receptors.

Important Considerations before Imaging

To visualize GPCRs at the cell surface, receptors must be tagged with fluorophores that are ideally resistant to quenching/photobleaching and with a significant quantum yield [20]. One of the fluorescent tags widely utilized in TIRF microscopy to investigate GPCRs is the pH sensitive eGFP variant super ecliptic phluorin (SEP) [21]. By attaching the SEP molecule to the extracellular domain of GPCRs, receptors are highly visible when they are located at the cell surface (neutral pH) and their fluorescence is rapidly quenched in intracellular compartments such as endosomes (pH acidic). The combination of TIRF microscopy with SEP-tagged GPCRs at their extracellular domain, it's an ideal approach to investigate events at the cell surface minimizing fluorescence from receptors in intracellular compartments while reducing phototoxicity. Other available tags such as antibodies labeled quantum dots or SNAP tags fusion proteins, which are smaller than GFPs and can be specifically and covalently label with many fluorophores, have been also utilized to investigate GPCRs surface trafficking [8,22–24]. However, their application to TIRF microscopy is not as frequent as conventional genetically coded fluorophores.

It is important to note that regardless of the florescent tag utilized, we strongly recommend tagging receptors at their extracellular domain and compare their function with wild type receptors before any further analysis. Only tagged receptors with identical pharmacological and functional properties to wild-type receptors must be used. In our experience, tags in

intracellular domains of GPCRs generally disrupt their signaling and or trafficking, rendering non-physiological behavior. Finally, TIRF microscopy is not exempt from the general rules and pitfalls from live cell imaging, image acquisition and analysis [25–28].

1. Materials

1.1 Cell Culture

1. Striatal primary cultures obtained from embryonic day 17–18 Sprague-Dawley rat embryos. Alternatively, brain tissue can be purchased from BrainBits LLC (Springfield, IL).
2. Neuron culture media: Neurobasal medium supplemented with B27 (according to manufacturer protocol) and 0.5mM glutaMAX™ (Life Technologies).
3. Imaging media such as Neurobasal nimus phenol red without serum and supplemented with 20mM HEPES (Life Technologies) (see Notes).
4. 30mm coverslips #1.5 thickness. Coverslips must be acid washed and coated with fresh poly D-Lysine (Sigma). Glass bottom dishes (MatTek) can be also utilized. They must be coated with PDL and the Glass Thickness must be 1.5
5. Transfection reagents, Lipofectamine 2000 or 3000 (Life Technologies).
6. Hippocampal cultures were >90% pure as calculated by MAP2 and GFAP staining as previously described [10,34].

2.2 TIRF Microscopy equipment and settings

1. Motorized Nikon (Melville, NY) Ti-E inverted microscope with a CFI-Apo×100 1.49 oil TIRF objective lens with color correction and a motorized stage with perfect focus (see Notes).
2. Light source: 488nm and 561nm Coherent sapphire lasers (Coherent Inc. Santa Clara, CA) 50 and 100 mW lasers respectively.
3. Temperature control is utilized to keep cells at 37°C with a Stable Z stage and objective warmer (Bioprotech, Butler, PA)
4. Interchangeable Coverslip Dish (Bioprotech) (<http://www.bioprotech.com/Products/ICD/coverslipdish.html>)
5. Camera: iXonEM + DU897 back illuminated EMCCD camera (Andor, Belfast, UK).
6. Readout speed: 10Hz, exposure time: continuous 50–100 milliseconds exposure for receptor recycling, EM gain 300, No binning, BitDepth= 14 bits, camera temperature set to minimum and laser power to 10% for 488nm 50 milliWatts.

3. Imaging

3.1 Cell Culture

1. Acid clean coverslips or glass bottom dishes by incubation in 1M HCl shaking overnight. Rinse two times with abundant ddH₂O and once with 70% ethanol. Leave in 95% ethanol until ready to use. Before coating, UV and dry in the culture hood no less than one hour.
2. Coat acid clean coverslips or glass bottom dishes with freshly prepared 100µg/mL poly-D-lysine for 2–4 h at 37°C. Wash 3 times with sterile water and air dry in sterile environment. It is important to prepare PDL fresh for every use. Old PDL solutions result in less than ideal cell attachment.
3. Plate 250,000–300,000 neurons per 35mm dish. Neurons are transfected at 4–5 DIV and imaged >15 DIV. Expression levels for receptor trafficking is ideal after >14 and <25 DIV.
4. Transfect cells with DNA constructs using Lipofectamine 2000–3000 or Effectene according to the manufacturer's instructions. We perform imaging after DIV 15. (High expression levels will impair observation of individual events. They can also result in trafficking artifacts such as reduced internalization). Lentivirus can be also used to infect targeted cells. In our experience, infected cells do not look as healthy as transfected cells.
5. The day of the imaging, change conditioned media to 2mL of freshly prepared Opti-MEM with HEPES 15 to 30 minutes before imaging sessions. Important: Do not to allow neurons to dry during this process. A small amount of media must be left at the dish covering all cells during the process.
6. Incubate cells at 37°C > 10 min to allow acclimatization to the new media.
7. Transfer neurons to the microscope.

3.2 Live cell imaging

1. At least 30 minutes before any acquisition, turn on the microscope and the temperature controllers. Turn on the laser key and let the laser warm up. Important: Objective and imaging chamber temperature must be controlled before and during experiments. Temperature must be 37°C at the glass.
2. Select TIRF objective, add a drop of immersion oil (Type LDF, RI: ~1.515) and carefully place cells on the stage (see Notes).
3. To reduce the effects of photobleaching, it is important to find and focus the cells using transmission light first. Then, find cells expressing tagged receptors using epifluorescence and then switch to TIRF illumination (see Notes).
4. Add agonist diluted in warm imaging media by automated perfusion system or, manually outside the imaging area to minimize artifacts (see Notes).

5. Acquisition settings for endocytosis: 100–300msec exposures every 2–3 seconds. Total time: 10–30 minutes.
6. Acquisition settings for recycling: Continuous illumination and acquisition at 50–100msec exposures for 1–2 minutes (see Notes).
7. Depolarization with 25mM KCL is performed to test viability at the end of experiments with Fluo-4.
8. Imaging sessions will generate large amounts of data. Careful data management must be implemented in advance. Standardized electronic notebooks or spreadsheets are recommended.

Note

1. Healthy primary cultures are essential to obtain reliable and valid results. Neurons must conserve their integrity, without blebbing or detachments.
2. HEPES is used to maintain the pH constant for up to 45–60 minutes outside a CO₂ incubator.
3. High quality cDNA is highly desired for neuronal transfection. Multiple transfection agents are commercially available. We utilize lipofectamine 2000 on DIV 4–5 and perform imaging on div>15. This delay results in optimal expression levels for TIRF imaging.
4. Focal plane must be kept constant during imaging sessions.
5. It is very important that the cells grow in monolayer and are not more than 80–90% confluent the day of imaging.
6. It is very important that the bottom of the imaging dish is completely dry and clean. Any liquid or dirt will interfere during TIRF imaging.
7. The most critical step is to find the exact angle for TIRF. To align the laser properly, focus on the plasma membrane. You can find the cell sharp edges and use them as reference.
8. If ligands are added manually, extreme care is needed to not disturb the cells within the imaging area. Controls should be performed to test the effects of DMSO and other solvents on surface fluorescence and basal cell activity.
9. Endocytosis should be visible within 1 or 2 minutes of agonist addition. Agonist-induced recycling can be observed 2–3 minutes after initial exposure. A constant rate of vesicular fusion is generally observed at ~10 minutes.

Image Analysis

Exocytotic events are easily identified by direct visualization the abrupt increase (~1 second) in intensity at discrete points in the cell surface. Image sequences can be analyzed using the acquisition software available from the microscope or by using the public domain NIH Image program ImageJ/FIJI software, which is freely available at <http://fiji.sc/Fiji>.

Orthogonal views (Kymographs) can be used to distinguish these events from other vesicular event due to their characteristic kinetics and to quantify their frequency, decay kinetics and location. Maximum intensity fluorescence can be extracted and plotted to demonstrate lateral diffusion and number of exocytic molecules among others. More recently, software specifically designed to identify exocytic events has been developed such as the exocytosis detection recipe from SCell (<https://www.svcell.com/recipes/exocytosis-detection>). Further development of this and other open source codes should provide better tools to extract and analyze exocytic events. We recommend performing all the analysis blindly to reduce bias in the quantification.

Endocytic events are intrinsically more challenging to analyze due to their lower signal to noise ratio. Manual detection was initially utilized following specific predetermined clathrin coated pits behaviors including: (1) events must appear and disappear within the time series (<250 sec); (2) events must display limited movement (no more than 4 by 4 pixels through their lifetime) (3) events must not fuse or collide with each other [29]. More recently particle tracking algorithms have been developed to follow these events such as the imageJ 2D spot tracker plugin [30] or the matlab code developed by the groups of Danuser and Schmid [31,32]. These codes were developed to analyze movies with ideal signal to noise ratio such as those obtained by imaging fluorescently tagged clathrin and dynamin. Analysis of GPCR endocytosis utilizing these tools has been more challenging with limited success. Nevertheless, these are fundamental steps towards a more rigorous and unbiased tool to investigate endocytosis. Finally, to achieve statistical data, we define individual experiments by the number of cells analyzed from the same transfection (n=1 experiment, × number of cells). To achieve statistical significance we analyze no less than 10 different cells from more than 4 different experiments (different transfection). A minimum of 20 endocytic events are expected from every cell to secure results from healthy cells.

Final comments

The availability of turn-key TIRF microscopes and the development of multiple fluorophores have allowed the use of TIRF microscopy to peer into the complex lives of GPCRs at the cell surface. We have learned that their surface trafficking, signaling and general function is more complex and exquisite than previously thought. Novel layers of regulatory mechanisms and trafficking pathways have been discovered along with complex associations between receptors and other proteins. This is only the beginning of a new era of GPCR research in which novel imaging tools such as TIRF and super-resolution microscopy are key to unravel some of the fundamental questions in GPCRs biology.

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