Published in final edited form as:

Methods Mol Biol. 2015 ; 1230: 79–86. doi:10.1007/978-1-4939-1708-2_6.

Real-time imaging of Mu opioid receptors by total internal reflection fluorescence microscopy

Cristina Roman-Vendrell^{1,2,3} and Guillermo Ariel Yudowski^{1,2,*}

¹Department of Anatomy & Neurobiology, School of Medicine, University of Puerto Rico, Puerto Rico

²Institute of Neurobiology, University of Puerto Rico, San Juan, Puerto Rico

³Department of Physiology, School of Medicine, University of Puerto Rico, Puerto Rico

Abstract

Receptor trafficking and signaling are intimately linked, especially in the Mu opioid receptor (MOR) where ligand dependent endocytosis and recycling have been associated to opioid tolerance and dependence. Ligands of the Mu opioid receptor (MOR) can induce receptor endocytosis and recycling within minutes of exposure in heterologous systems and cultured neurons. Endocytosis removes desensitized receptors after their activation from the plasma membrane, while recycling promotes resensitization by delivering functional receptors to the cell surface. These rapid mechanisms can escape traditional analytical methods where only snapshots are obtained from highly dynamic events.

Total internal reflection fluorescence (TIRF) microscopy is a powerful tool that can be used to investigate, in real-time, surface trafficking events at the single molecule level. The restricted excitation of fluorophores located at or near the plasma membrane in combination with high sensitivity quantitative cameras, makes it possible to record and analyze individual endocytic and recycling event in real time. In this chapter, we describe a TIRF microscopy protocol to investigate in real time, the ligand dependent MOR trafficking in Human Embryonic Kidney 293 cells and dissociated striatal neuronal cultures. This approach can provide unique spatio-temporal resolution to understand the fundamental events controlling MOR trafficking at the plasma membrane.

Keywords

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

1. Introduction

Ligand-induced receptor endocytosis and recycling have been proposed to have important roles on physiological events such as the development of tolerance and resensitization (1, 2). Studies carried out in different neuronal and non-neuronal cell models have demonstrated

Corresponding author: Guillermo.yudowski@upr.edu.

that endocytosis of the Mu opioid receptor (MOR) is cell type and ligand specific (3–6). For instance, morphine fails to rapidly internalize receptors in most cells, whereas [D-Ala2, N-MePhe4, Gly-ol]-enkephalin (DAMGO) has been shown to induce receptor internalization and recycling (2). Moreover, several studies correlate the low endocytic efficacy of morphine with the development of tolerance in vivo (7, 8). These trafficking mechanisms have been mostly studied by biochemical and time-lapse imaging approaches. However, endocytosis and recycling occur within minutes of receptor activation and single and rapid events can escape traditional techniques. Furthermore, we are only beginning to grasp the complexity of the mechanisms and kinetics of individual trafficking events.

New technologies like quantitative live cell imaging can provide new insight into these complex mechanisms during receptor trafficking with high spatio-temporal resolution (9–11). In our laboratory, we use total internal reflection fluorescence (TIRF) microscopy to investigate MOR trafficking at the single molecule resolution (12). Relying on the selective illumination of a thin layer of the sample, TIRF allows visualization of the events occurring at plasma membrane with high signal-to-noise ratio. We and others have previously utilized TIRF to investigate the ligand dependent trafficking of the MOR in heterologous systems and neuronal cultures (12–15).

In these studies, we use Human Embryonic Kidney 293 cells (HEK293) and striatal neurons expressing a pH-sensitive GFP variant called superecliptic phluorin (SEP) fused to the amino terminal of MOR (16). SEP-MOR fluorescence is visible at the cell surface, where the extracellular pH is neutral. The fluorescence is reversibly quenched when the receptor enters the mildly acidic endocytic and recycling vesicles. This property of SEP-MOR facilitates the detection of discrete events mediating the removal and delivery of receptors at the cell surface (16, 17). Here, we describe the use of TIRF microscopy to investigate the kinetics and molecular mechanisms during MOR trafficking at the plasma membrane. This protocol describes the necessary steps, and important controls, to explore the ligand dependent endocytosis and recycling of MOR at the single vesicle level.

2. Materials

2.1 Cell Culture

1. Human embryonic kidney cells (HEK293; ATCC CRL-1573) (see Note 1).
2. Striatal primary cultures obtained from embryonic day 17–18 Sprague-Dawley rat embryos. Alternatively, brain tissue can be purchased from BrainBits LLC (Springfield, IL).
3. HEK293 culture media, Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies).
4. Neuron culture media: Neurobasal medium supplemented with B27 (according to manufacturer protocol) and 0.5mM glutaMAX™ (Life Technologies).

¹We do not recommend HEK293T, these cells achieve high expression levels preventing imaging of vesicular events.

5. Imaging media, Opti-MEM® reduced-serum medium supplemented with 20mM HEPES (Life Technologies) (see Note 2).
6. 30mm coverslips #1.5 thickness (Bioprotechs) acid wash treated and coated with poly D-Lysine (Sigma).
7. Transfection reagents, Lipofectamine 2000 (Life Technologies) or Effectene (Qiagen).
8. MOR-SEP cDNA (15) construct (see Note 3). Multiple fluorescently tagged proteins can be imaged by co-transfection.

2.2 Agonists

1. DAMGO, [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (Sigma)
2. Morphine sulfate salt (Sigma)

2.3 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 Note 4).
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 (Bioprotechs, Butler, PA)
4. Interchangeable Coverslip Dish (Bioprotechs) (<http://www.bioprotechs.com/Products/ICD/coverslipdish.html>)
5. Camera: iXonEM + DU897 back illuminated EMCCD camera (Andor, Belfast, UK).
6. Readout speed: 10Hz, exposure time: continuous 100msec. exposure for receptor recycling, EM gain 300, binning: 1×1, image: 512×512, BitDepth= 14 bits, camera temperature: -75°C and laser power: 10% for 488nm.
7. Software: NIS-Elements.

²HEPES is used to maintain the pH constant for up to 45–60 minutes outside a CO₂ incubator.

³High quality cDNA is required especially for neuronal transfection. To investigate mechanism involved in MOR trafficking, fluorescently tagged dominant negative versions of known recycling players can be co-transfected with SEP-MOR. These mutations produce an altered gene product that acts antagonistically to the wild-type allele (20). Fluorescently labeled siRNA can be used to selectively knock-down target proteins from individual cells while investigating SEP-MOR trafficking.

⁴Focal plane must be kept constant during imaging sessions.

3. Methods

3.1 Cell Culture

1. Acid clean coverslips by incubation in 1M HCl shaking overnight. Rinse the coverslips 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.
2. Coat acid clean coverslips with 100µg/mL poly-D-lysine for 2–4 h at 37°C. Wash 3 times with sterile water and air dry in sterile environment.
3. Passage HEK293 cells onto the prepared 35mm plates so that they reach 50–60% confluence on the day of transfection (see Note 5).
4. Plate 300,000 neurons per 35mm dish. Neurons are transfected at 4–5 DIV and imaged >15 DIV.
5. Transfect cells with DNA constructs using Lipofectamine 2000 or Effectene according to the manufacturer's instructions. We perform experiments 48–72 h after transfection to allow cell recovery from the transfection and achieve optimal expression levels (High expression levels will impair observation of individual events.)
6. The day of the imaging, carefully transfer the coverslip to the interchangeable coverslip dish and add 2mL of freshly prepared Opti-MEM with HEPES replacing the incubation media 15 to 30 minutes before imaging sessions (see Note 6).
7. Incubate the cells at 37°C for 10 min to allow acclimatization.

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.
2. Select TIRF objective, add a drop of immersion oil (Type LDF, RI: ~1.515) and carefully place the interchangeable coverslip dish on the stage (see Note 7).
3. Temperature of the imaging media must be controlled regularly and kept constant (37°C).
4. 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 Note 8) (Fig. 1).
5. Add agonist (DAMGO 10µM) diluted in warm imaging media by automated perfusion system or, manually outside the imaging area to minimize artifacts from media changes (see Note 9).

5It is very important that the cells grow in monolayer and are not more than 80–90% confluent the day of imaging.

6With forceps, take the coverslip from the 35mm dish and place on the dish base, tighten the threaded insert with O-ring and make sure that the media does not leak.

7It is very important that the bottom of the imaging dish is completely dry and clean. Any liquid or dirt will interfere during TIRF imaging.

8The 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.

6. Acquisition settings for endocytosis: 100–300msec exposures every 2–3 seconds. Total time: 10–30 minutes.
7. Acquisition settings for recycling: Continuous illumination and acquisition at 100msec exposures for 1–2 minutes (see Note 10).
8. Imaging sessions will generate large amounts of data. Careful data management must be implemented in advance. Standardized electronic notebooks or spreadsheets are recommended.

3.3 Analysis

1. To obtain single molecule information, single EGFP analysis (See Note 11) is performed regularly to help compensate for day-to-day variability. Mean fluorescent intensity of single EGFPs is obtained by combining all single measurements from multiple experiments (Fig. 2). This information is used to correlate fluorescence intensity with the number of SEP-MOR receptors per recycling vesicle.
2. Endocytic events are analyzed by double blind analysis, multiple times manually and using the particle tracking algorithm 2D spot tracker (18). Individual event location, time and fluorescence profile are logged and recorded electronically. Endocytic events are identified and scored following previously described behaviors, briefly: (i) events must appear and disappear within the time series; (ii) events must display limited movement (no more than 4 by 4 pixels through their lifetime) (iii) events must not fuse or collide with each other (19).
3. Recycling events are analyzed using the open source program, ImageJ (NIH). Recycling receptors are observed as abrupt increases of surface fluorescence in diffraction-limited spots. Maximum intensity projections of each treatment can be compared for changes in recycling frequency (Fig. 3).

4. Notes

¹We do not recommend HEK293T, these cells achieve high expression levels preventing imaging of vesicular events.

²HEPES is used to maintain the pH constant for up to 45–60 minutes outside a CO₂ incubator.

³High quality cDNA is required especially for neuronal transfection. To investigate mechanism involved in MOR trafficking, fluorescently tagged dominant negative versions of known recycling players can be co-transfected with SEP-MOR. These mutations produce an altered gene product that acts antagonistically to the wild-type allele (20). Fluorescently

⁹DAMGO is dissolved in DMSO, a highly viscous solvent, and must be mixed well with imaging media before adding to the cells. If adding manually, be very careful not to disturb the cells within the imaging area. Controls should be performed to test the effects of DMSO on surface fluorescence and basal cell activity.

¹⁰Agonist-induced MOR recycling can be observed 2–3 minutes after agonist exposure. A constant rate of vesicular fusion is generally observed at ~10 minutes.

¹¹Utilizing the linear range of our EMCCD camera, we correlated the number of single EGFPs to the number of SEP-MORs observed during our imaging.

labeled siRNA can be used to selectively knock-down target proteins from individual cells while investigating SEP-MOR trafficking.

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 With forceps, take the coverslip from the 35mm dish and place on the dish base, tighten the threaded insert with O-ring and make sure that the media does not leak.

7 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.

8 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.

9 DAMGO is dissolved in DMSO, a highly viscous solvent, and must be mixed well with imaging media before adding to the cells. If adding manually, be very careful not to disturb the cells within the imaging area. Controls should be performed to test the effects of DMSO on surface fluorescence and basal cell activity.

10 Agonist-induced MOR recycling can be observed 2–3 minutes after agonist exposure. A constant rate of vesicular fusion is generally observed at ~10 minutes.

11 Utilizing the linear range of our EMCCD camera, we correlated the number of single EGFPs to the number of SEP-MORs observed during our imaging.

Acknowledgments

This work was supported by research grants from NIH DA023444, Puerto Rico Science Trust, NIMHD 8G12-MD007600 (RCMI) and Stephanie Palacio.

References

1. Koch T, Widera A, Bartsch K, et al. Receptor endocytosis counteracts the development of opioid tolerance. *Mol Pharmacol*. 2005; 67:280–287. [PubMed: 15475572]
2. Koch T, Höllt V. Role of receptor internalization in opioid tolerance and dependence. *Pharmacol Ther*. 2008; 117:199–206. [PubMed: 18076994]
3. Whistler JL, Chuang HH, Chu P, et al. Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron*. 1999; 23:737–746. [PubMed: 10482240]
4. Bushell T, Endoh T, Simen AA, et al. Molecular components of tolerance to opiates in single hippocampal neurons. *Mol Pharmacol*. 2002; 61:55–64. [PubMed: 11752206]
5. Bailey CP, Couch D, Johnson E, et al. Mu-opioid receptor desensitization in mature rat neurons: lack of interaction between DAMGO and morphine. *J Neurosci*. 2003; 23:10515–10520. [PubMed: 14627635]
6. Habersack-Debic H, Kim K-A, Yu YJ, Von Zastrow M. Morphine promotes rapid, arrestin-dependent endocytosis of mu-opioid receptors in striatal neurons. *J Neurosci*. 2005; 25:7847–7857. [PubMed: 16120787]
7. Grecksch G, Bartsch K, Widera A, et al. Development of tolerance and sensitization to different opioid agonists in rats. *Psychopharmacology (Berl)*. 2006; 186:177–184. [PubMed: 16572262]
8. Enquist J, Kim J, Bartlett S. A novel knock-in mouse reveals mechanistically distinct forms of morphine tolerance. *J Pharmacol*. 2011; 338:633–640.
9. Schmoranz J, Goulian M, Axelrod D, Simon SM. Imaging constitutive exocytosis with total internal reflection fluorescence microscopy. *J Cell Biol*. 2000; 149:23–32. [PubMed: 10747084]
10. Steyer JA, Almers W. A real-time view of life within 100 nm of the plasma membrane. *Nat Rev Mol Cell Biol*. 2001; 2:268–75. [PubMed: 11283724]

11. Wennmalm S, Simon SM. Studying individual events in biology. *Annu Rev Biochem.* 2007; 76:419–446. [PubMed: 17378765]
12. Roman-Vendrell C, Yu YJ, Yudowski GA. Fast modulation of μ -Opioid receptor (MOR) recycling is mediated by receptor agonists. *J Biol Chem.* 2012; 287:14782–14791. [PubMed: 22378794]
13. Yu YJ, Dhavan R, Chevalier MW, et al. Rapid delivery of internalized signaling receptors to the somatodendritic surface by sequence-specific local insertion. *J Neurosci.* 2010; 30:11703–11714. [PubMed: 20810891]
14. Soohoo AL, Puthenveedu MA. Divergent modes for cargo-mediated control of clathrin-coated pit dynamics. *Mol Biol Cell.* 2013; 24:1725–1734. [PubMed: 23536704]
15. Henry AG, Hislop JN, Grove J, et al. Regulation of Endocytic Clathrin Dynamics by Cargo Ubiquitination. *Dev Cell.* 2012; 23:519–532. [PubMed: 22940114]
16. Miesenbock G, De Angelis DA, Rothman JE. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature.* 1998; 394:192–195. [PubMed: 9671304]
17. Sankaranarayanan S, De Angelis D, Rothman JE, Ryan TA. The use of pHluorins for optical measurements of presynaptic activity. *Biophys J.* 2000; 79:2199–2208. [PubMed: 11023924]
18. Sage D, Neumann FR, Hediger F, et al. Automatic tracking of individual fluorescence particles: application to the study of chromosome dynamics. *IEEE Trans Image Process.* 2005; 14:1372–1383. [PubMed: 16190472]
19. Saffarian S, Cocucci E, Kirchhausen T. Distinct Dynamics of Endocytic Clathrin-Coated Pits and Coated Plaques. *PLoS Biol.* 2009; 7:e1000191. [PubMed: 19809571]
20. Herskowitz I. Functional inactivation of genes by dominant negative mutations. *Nature.* 1987; 329:219–222. [PubMed: 2442619]

Figure 1.
Example of a neuron imaged using epifluorescence illumination and TIFR microscopy.

Figure 2. Single molecule quantification of EGFPs. *A*, representative fluorescence intensity measurement of a single EGFP. *B*, histogram depicting single GFP intensity distribution.

Figure 3. Example of SEP-MOR expressing HEK293 cell. *A*, Vesicles at the cell surface and recycling events can be visualized by maximum intensity projection for a HEK293 cell. The image represents 60 s acquired at 10 Hz. Each fluorescence spot surrounded by the circle represents a recycling event. *B*, Kymograph of the representative cell, with increasing time from left to right. An example of recycling is indicated by the arrow.