Video Article

# Imaging pHluorin-tagged Receptor Insertion to the Plasma Membrane in Primary Cultured Mouse Neurons

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## Abstract

A better understanding of the mechanisms governing receptor trafficking between the plasma membrane (PM) and intracellular compartments requires an experimental approach with excellent spatial and temporal resolutions. Moreover, such an approach must also have the ability to distinguish receptors localized on the PM from those in intracellular compartments. Most importantly, detecting receptors in a single vesicle requires outstanding detection sensitivity, since each vesicle carries only a small number of receptors. Standard approaches for examining receptor trafficking include surface biotinylation followed by biochemical detection, which lacks both the necessary spatial and temporal resolutions; and fluorescence microscopy examination of immunolabeled surface receptors, which requires chemical fixation of cells and therefore lacks sufficient temporal resolution[1-6] . To overcome these limitations, we and others have developed and employed a new strategy that enables visualization of the dynamic insertion of receptors into the PM with excellent spatial and temporal resolutions [7-17] . The approach includes tagging of a pH-sensitive GFP, the superecliptic pHluorin [18], to the N-terminal extracellular domain of the receptors. Superecliptic pHluorin has the unique property of being fluorescent at neutral pH and non-fluorescent at acidic pH (pH < 6.0). Therefore, the tagged receptors are non-fluorescent when within the acidic lumen of intracellular trafficking vesicles or endosomal compartments, and they become readily visualized only when exposed to the extracellular neutral pH environment, on the outer surface of the PM. Our strategy consequently allows us to distinguish PM surface receptors from those within intracellular trafficking vesicles. To attain sufficient spatial and temporal resolutions, as well as the sensitivity required to study dynamic trafficking of receptors, we employed total internal reflection fluorescent microscopy (TIRFM), which enabled us to achieve the optimal spatial resolution of optical imaging (~170 nm), the temporal resolution of video-rate microscopy (30 frames/sec), and the sensitivity to detect fluorescence of a single GFP molecule. By imaging pHluorin-tagged receptors under TIRFM, we were able to directly visualize individual receptor insertion events into the PM in cultured neurons. This imaging approach can potentially be applied to any membrane protein with an extracellular domain that could be labeled with superecliptic pHluorin, and will allow dissection of the key detailed mechanisms governing insertion of different membrane proteins (receptors, ion channels, transporters, etc.) to the PM.

## Video Link

The video component of this article can be found at http://www.jove.com/video/4450/

## Protocol

## 1. Preparing Mouse Glia Culture for Neuronal Culture Conditioning

1. T75 flasks are coated with collagen solution (1:3 dilution of Purecol in dd$H_2O$). The flasks are set upright and left to dry overnight in a tissue culture hood.
2. Dissection buffer (aCSF: 119 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 30 mM dextrose, 25 mM HEPES, pH 7.4, without calcium) and glia media (DMEM supplemented with 10% FBS, 10 units/ml penicillin, 10 µg/ml streptomycin, and Glutamax) are prepared and stored at 4 °C until ready for use.
3. On the day of glia culture, the glia media is warmed for at least 30 min in a 37 °C water bath before dissection of mouse brains. Embryonic or neonatal mice are euthanized by rapid decapitation. Cerebral cortexes are dissected out under a dissection microscope.
4. Dissected brain tissues are digested in papain solution (100 ml papain and 20 µl 1% DNase I in 2 ml dissection buffer) at 37 °C for 20 min.
5. Following papain digestion, brain tissues are rinsed with 10 ml pre-warmed glia media. Fresh glia media (2 ml) is then added to the digested brain tissues, and the tissues are triturated with a fire-polished glass Pasteur pipette.
6. Fresh glia media (8 ml) is added to the dissociated tissues. A 70 µm cell-strainer is used to filter the dissociated tissue suspension. Cells are then seeded into T75 flasks (usually 2-3 embryos can seed one T75 flask) and placed into a 37 °C cell culture incubator.
7. The next day, the media from the T75 flasks is aspirated. The flasks are rinsed with 10 ml PBS. Fresh glia media (10-15 ml) is then added to each flask.
8. Within 10-12 days, glia in the T75 flasks should be confluent. Glial cells are rinsed with PBS, and 5 ml of 0.05% trypsin is added to each T75 flask.

 

9. After trypsin incubation at 37 °C for 5 min, glial cells are dissociated and glial cells from each T75 flask are split into two different collagen-coated T75 flasks.
10. Culture inserts (Millipore PICM03050) are prepared by placing them into 6-well plates, coating them with collagen, and air-drying overnight. Glia media (2 ml each) is placed underneath each culture insert.
11. Glia from confluent T75 flasks are trypsinized and seeded onto the culture inserts (one T75 flask per 6-well plate, 2 ml each insert). These glia culture inserts will be used to condition the neuronal cultures.

## 2. Neuronal Culture

1. The glass coverslips for neuronal culture are cleaned by soaking in 70% $HNO_3$ at least overnight. Coverslips are then washed with $ddH_2O$ at least 3 times, and dried in an Isotemp Oven (>200 °C) overnight.
2. Cleaned coverslips are placed in a 6-well plate, one coverslip per well. Coverslips are coated with poly-L-Lysine solution (2 ml, 0.1% in 100 mM boric acid, pH = 8.5) overnight in a 37 °C incubator.
3. The poly-L-Lysine solution is removed from the coverslips and coverslips are washed 3 times with autoclaved $ddH_2O$. Coverslips are then incubated in a 37 °C incubator overnight in plating media (Neurobasal supplemented with 5% heat-inactivated horse serum, 10 units/ml penicillin, 10 µg/ml streptomycin, Glutamax; 2% B27 supplement is added to the plating medium on the day it is used).
4. On the following day, the plating media is removed and 2 ml of fresh plating media with B-27 is added to each well that has a glass coverslip.
5. Pregnant mice (E15-18) are euthanized with $CO_2$, and embryonic mice are euthanized by decapitation. The hippocampus or striatum is dissected from the brain tissue and placed into a 15 ml conical tube with ice-cold dissection buffer, for culture of hippocampal neurons or striatal medium spiny neurons, respectively.
6. Dissected brain tissues are dissociated with papain as described in steps 1.4) and 1.5).
7. Following dissociation, the number of cells in each neuronal suspension is counted with a hemocytometer. Neurons are then seeded on each coverslip at a density of $0.4 \times 10^6$ cells per well of a 6-well plate, and cultured in a 37 °C incubator overnight (neurons are DIV 0).
8. To prepare glia culture inserts for conditioning the neuronal cultures, the glia media is removed from the glia culture inserts and fresh plating medium is added (2 ml in the insert and 2 ml underneath the insert).
9. The next day (DIV 1), coverslips with neurons are placed underneath the glia culture inserts, one coverslip in each well.
10. Neurons are fed with half the volume of fresh pre-warmed feeding media (with B-27) every 3-4 days until they are ready for transfection and imaging experiments.

## 3. Transfection

1. Neuronal transfection is performed using Lipofectamine 2000. For each neuronal culture (one culture per coverslip), 2 µl Lipofectamine 2000 and 1 µg of DNA are used. Fresh Neurobasal medium with no supplement is used to dilute Lipofectamine and DNA.
2. DNA and Lipofectamine are incubated for 20 min at room temperature after mixing.
3. Save 1 ml of the media from underneath each glia culture insert, and 1 ml from inside each insert, and transfer the media to a conical tube. Add the same volume of feeding media + B27 to the saved medium and incubate at 37 °C. This medium is used for neuronal culture following transfection.
4. Following the 20-min Lipofectamine/DNA incubation, glia culture inserts are removed momentarily from the 6-well plates. The 200 µl Lipofectamine/DNA mixture is added to each coverslip with neurons. The glia culture inserts are then returned to each well, above the neurons. Generation of bubbles under the membrane of the culture inserts should be avoided. Neurons are incubated with the Lipofectamine/DNA mixture at 37 °C for 2-4 hr.
5. After the 2-4 hr incubation period, glia inserts are removed again. The culture media with the Lipofectamine/DNA mixture is removed from each well. The media saved in step 3.3) is added to neurons (2 ml per well). Glia culture inserts are returned to each well, and the glia media from each insert is removed, and 2 ml of the media from step 3.3) is added into each insert.
6. Neurons are cultured for an additional 24-72 hr to allow expression of transfected cDNA before TIRF imaging is performed on these neurons.

## 4. TIRF Imaging

1. Artificial Cerebrospinal Fluid (aCSF: 119 mM NaCl, 2.5 mM KCl, 30 mM glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM HEPES, pH = 7.4) will be used for live-imaging experiments. Our TIRF imaging system is custom set-up with a 100-mW Cyan laser for excitation, and an Electron Multiplying Charge Coupled Device (EMCCD) camera for a detector.
2. Prior to imaging experiments, the aCSF is warned in a 37 °C water bath for at least 30 min. The heating insert for live imaging is placed on the microscope stage, and the heating insert, laser, and camera are warmed for at least 30 min before the start of the imaging experiments.
3. A coverslip with transfected neurons is assembled into the live-imaging chamber. The pre-warmed aCSF is placed in the chamber after the chamber is assembled; this step should be completed as quickly as possible.
4. Once the chamber is placed on the heating insert on the microscope stage, transfected neurons are identified visually under epi-fluorescent imaging mode.
5. After healthy transfected neurons are identified (refer to **Figures 1 and 2**), we typically perform 1 min of photo-bleach to eliminate pre-existing pHluorin fluorescence on the plasma membrane, as pHluorin-receptors on the plasma membrane have very high fluorescence levels when TIRF imaging is first started, which interferes with visualization of individual insertion events.
6. Following photobleach, the gain setting of the electron multiplier on the EMCCD camera is set to maximum, and recording is performed for 1 min at 10 Hz.
7. Each recording will contain 600 images. Individual insertion events are identified by playing the recording back using ImageJ. Individual insertion events are analyzed manually using ImageJ.

## 5. Representative Results

Consistent neuronal culture is the key to successful live-imaging experiments. **Figure 1** shows mouse hippocampal neurons cultured using our protocol from DIV 11 to DIV 18. It is clear from the images that the neurons have developed extensive processes and that the dendritic processes are covered densely with dendritic spines, indications that these neurons are healthy in culture. Our culture protocol could also be used for other types of neurons in the brain. For example, we recently used this protocol to culture striatal medium spiny neurons in a study to examine dopamine D2 receptor (DRD2) insertion in cultured mouse striatal medium spiny neurons [9]. **Figure 2** shows mouse striatal medium spiny neurons cultured using our protocol at DIV 8. We have also successfully performed TIRF imaging of superecliptic pHluorin-tagged DRD2s in this type of neuron. **Figure 3** shows expression of pHluorin-tagged DRD2 (pH-DRD2) in a medium spiny neuron and visualization of pH-DRD2 insertion in this neuron.

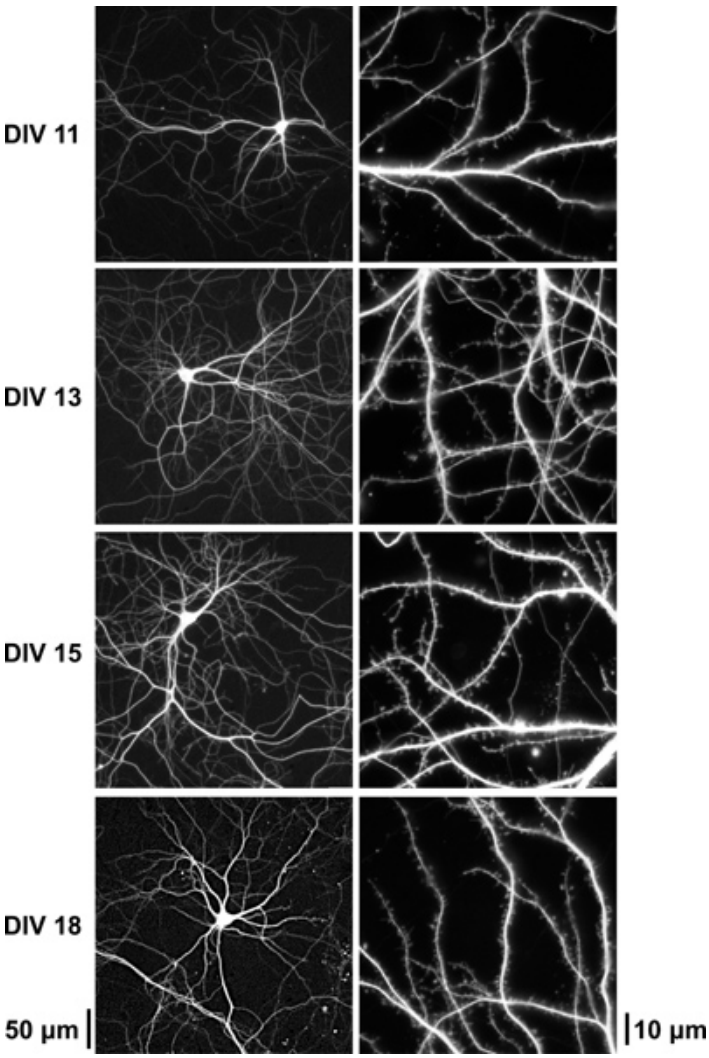


**Figure 1.** Mouse hippocampal culture using the interface culture method. DIV: days *in vitro*, the day of culture is considered as DIV 0. From the images, it is evident that hippocampal neurons mature in this type of culture between DIV 11 and DIV 15. At DIV 11 and 13, neuronal dendrites are covered with filipodia and immature spines. At DIV 15 and 18, neuronal dendrites are covered with large spines, an indication of more mature neurons. Left panels: low-magnification images of mouse hippocampal neurons at different ages. Right panels: high-magnification images (zoom in on neuronal dendrites of the left panel) of neuronal dendritic processes with dendritic spines at different ages.

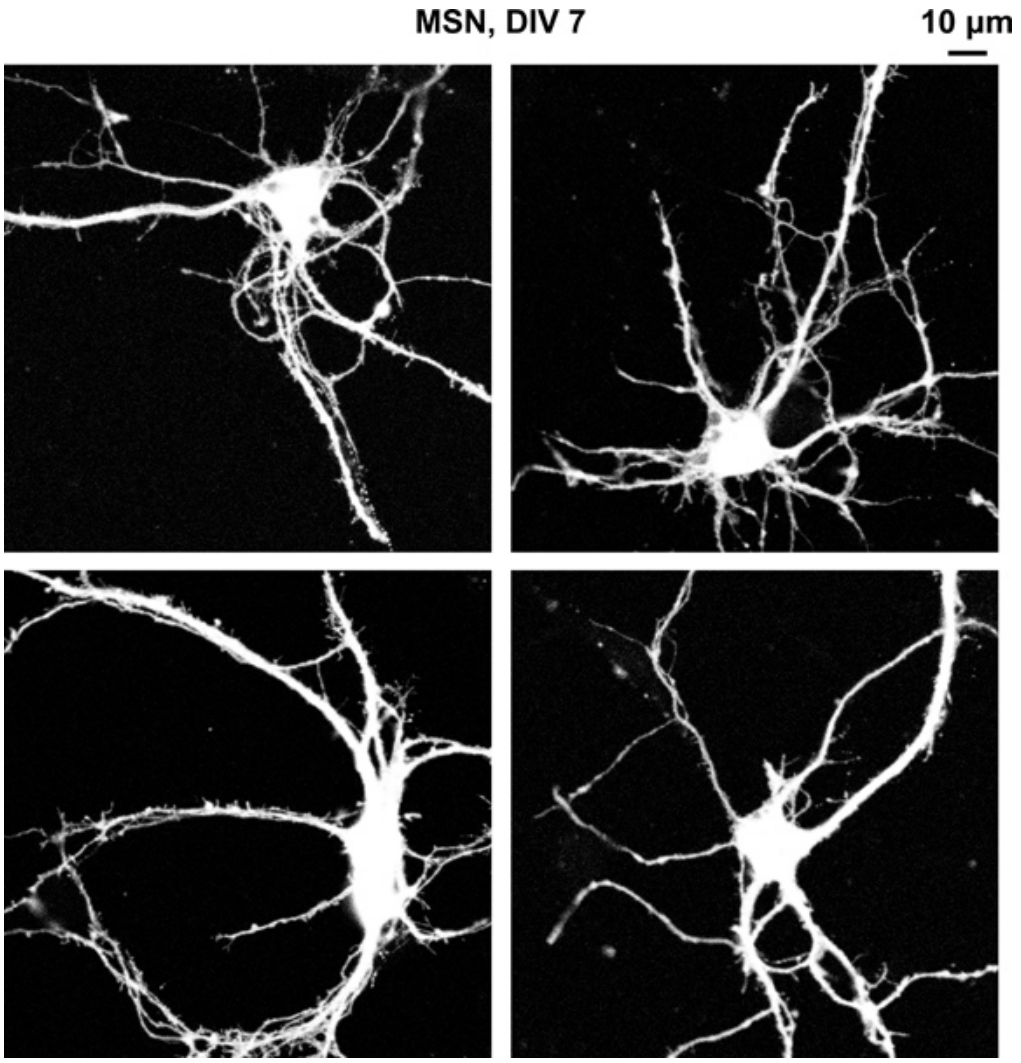


**Figure 2.** Mouse striatal medium spiny neuron culture using the interface culture method. Neurons in the images are at DIV 8.

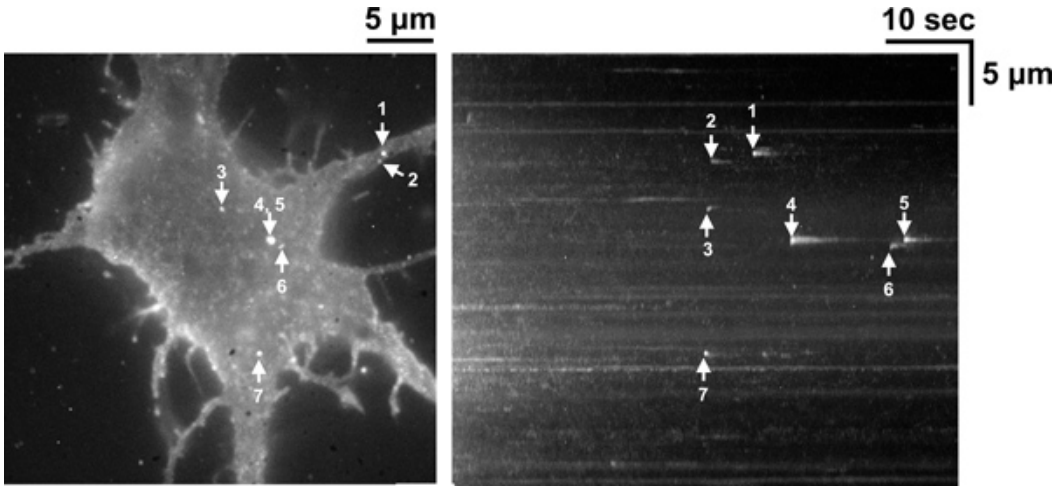


**Figure 3.** A mouse striatal medium spiny neuron transfected with super ecliptic pHluorin-tagged dopamine D2 receptor (pH-DRD2). Image on the left is maximum intensity projection of a time-lapse recording (600 images, 100 msec per image). White arrowheads indicate individual vesicular insertion of pH-DRD2. This image retains insertion information in the x-y dimension but loses the time information. Image on the right shows y-

t maximum intensity projection, in which the x-y-t stack is rotated 90 degrees around the y-axis, and the maximum pixel on each x-axis line is projected onto a single y-axis pixel. This image retains insertion information along the y-t axis but loses the x-axis information.

## Discussion

For unknown reasons, mouse neurons are always more difficult to culture than rat neurons. In our experience, a mixed culture of neurons and glia works well for primary cultured mouse neurons. However, such a mixed culture is not suitable for TIRF imaging experiments, as in this type of culture neurons and their processes tend to grow on top of glia cells, situating the neuronal somata and dendritic processes beyond the reach of TIRF microscopy. Therefore, a lower-density neuronal culture with few glia on the coverslip is ideal for TIRF imaging. We first tested the Banker method [19;20], in which the bottom of a culture dish is seeded with glia, and neurons are seeded on a coverslip and placed upside-down in the culture dish. The coverslip and the glia layer are separated by three small drops of wax on the coverslip. This method works well for smaller-size coverslips (such as 15-mm or 18-mm coverslips), but when we tested the use of 25-mm coverslips, neurons near the center of the coverslips were not as healthy as those near the coverslip edges.

While the cause of this difference in neuron health was not clear to us, we reasoned that it may be due to limited diffusion of nutrients to the center of the coverslip area. We therefore turned to the interface culture method described in this manuscript, as the membrane of the culture insert allows free diffusion of nutrients to neurons on the coverslips. We seeded neurons on 25-mm coverslips, with the neurons facing upward. We seeded glia on a culture insert, and placed the glia feeder insertion on top of the coverslip. We determined that the interface culture method is superior to other methods we have tested when using 25-mm coverslips for low-density mouse neuronal culture, and yields consistent results for TIRF imaging. To quantify the properties of receptor insertion (frequency, amplitude), a control group with neurons transfected with wildtype pHluorin-tagged receptors should always be included. This control group also serves as a quality check for neuronal culture.

Our TIRFM system is constructed based on a manual Zeiss AxioObserver microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). The excitation laser is a Newport 488nm-100mW Cyan Laser System (Newport Corporation, Irvine, CA). The laser is coupled to a Zeiss TIRF slider *via* a KineFLEX-P-2-S-488-640-0.7-FCP-P2 optical fiber (Point Source, Mitchell Point, Hamble, UK). A Z488RDC dichroic mirror (Chroma Technology Corporation, Bellows Falls, VT) was used to reflect the incoming laser onto a Zeiss α-plan 100X objective lens (N.A. = 1.46, Carl Zeiss). An ET525/50 emission filter (Chroma Technology Corporation) was used for GFP fluorescence detection. An Evolve EMCCD camera (Photometrics, Tucson, AZ) was used as the detector. A 2.5X relay lens was inserted between the microscope camera port and the camera in order to achieve optimal spatial resolution (0.064 μm per pixel when using the 100X N.A. = 1.46 objective). The camera was maintained at -80 °C during imaging experiments. A Uniblitz LS6 shutter controlled by a VMM-D3 controller (Vincent Associates, Rochester, NY) was integrated between the laser head and fiber launcher. Data were acquired using μManager software (http://www.micro manager.org/).

All imaging experiments were performed at 37 °C in aCSF solution containing 2 mM $CaCl_2$. This aCSF solution is adjusted to pH=7.4 at room temperature. When warmed to 37 °C, the pH of this aCSF becomes 7.2, which is optimal for neurons in culture. During acquisition, laser power is set at maximum, both for photo-bleach and for data acquisition. Camera exposure was set at 100 msec, and acquisition rate was 10 images per second (10 Hz). EMCCD gain was set at maximum. Recordings were analyzed using ImageJ software (Rasband, W.S., NIH, http://rsb.info.nih.gov/ij/, 1997-2012), and insertion events were registered and analyzed manually. Total events per minute per unit surface area were taken as the frequency of insertion, and were normalized to the control group as 100%. Y-t rendered images were generated in ImageJ by rotating the original x-y-t stack 90° along the y-axis, and the maximum intensity of each x line was projected onto a single pixel of the y axis using the maximum intensity projection algorithm in ImageJ.

Insertion events are typically observed as the sudden appearance of fluorescent punta (fast rising phase), followed by a decaying phase that represents receptor diffusion on the plasma membrane [7;9]. The appearance of fluorescent punta with a slow rising phase or those without an apparent decay phase (sudden disappearance) are excluded from data analysis, as these events likely represent trafficking of intracellular vesicles that have a less acidic lumen (such as those from the endoplasmic reticulum). Photobleach of pre-existing surface receptor populations will also minimize the contribution of pre-existing surface receptor clustering on the plasma membrane. To date, all our data are analyzed manually using ImageJ. Future development of computer algorithms for automatic event detection and data analysis will be important for facilitating adaptation and application of this imaging method for examination of receptor insertion to the plasma membrane.

In order to visualize individual vesicular insertion events of pHluorin-labeled receptors, several important parameters need to be taken into consideration. First, the laser excitation needs to be strong enough to enable detection of fluorescence from single vesicles. In our custom-designed system, we used a 100-mW 488-nm laser as our excitation source. Second, the detector of the imaging system needs to have both the sensitivity and the speed to acquire data from real-time, dynamic receptor insertion. For these reasons, we use an EMCCD in our system. The combination of a strong excitation laser and an EMCCD detector offers single GFP molecule detection capability in our imaging system. Our choice of a custom-designed TIRF system is based on achieving maximum performance from limited available resources. It is worth noting that any commercially available TIRF system with sufficient laser excitation power (a 100-mW 488-nm laser is sufficient for our purposes, and is readily available on most commercial platforms) and an EMCCD camera should also be suitable for such imaging studies. Therefore, adaptation of such imaging studies for neurons is not limited by performance of the TIRFM system, but by the quality of neuronal culture and consistency of neuronal cultures.

Third, due to the initial fluorescence from existing pHluorin-tagged plasma membrane receptors, photobleach under the TIRF mode is typically necessary for detection of fluorescence from a single vesicle. We normally perform 1-minute photobleach using the TIRF imaging mode at maximum laser power once a neuron is visually identified, which results in elimination of the majority of pre-existing surface pHluorin fluorescence. It is also important to keep in mind that high laser excitation power also increases the possibility of laser damage and phototoxicity. In our experience, using a 100-mW 488-mW laser as the excitation source does not appear to introduce noticeable damage to neurons within the data acquisition period, while providing sufficient laser power for visualizing receptors in single vesicles. An additional important consideration is that differences in the types and numbers of receptors in each vesicle will also affect the laser excitation requirement. For example, we previously were able to examine regulation of glutamate receptor GluA1 subunit insertion to the plasma membrane using a 50-mW excitation laser [7]. We estimate that each vesicle examined in that study contained 56 ± 6 GluA1 molecules, similar to the estimate made by another group using similar methods [12]. In our recent study of DRD2, we estimated that each vesicle contained 30 ± 1 molecules, and a 100-mW laser was

sufficient for that study. However, because of the high level of background noise in live cells, examination of a vesicle containing only several receptor molecules (for example, 5-10) may require a laser power higher than 100 mW in order to achieve a sufficient signal-to-noise ratio for visualization of receptor insertion to the plasma membrane in neurons. Finding the right balance of sensitivity and excitation power is important for planning a successful study.

The use of TIRFM to image superecliptic pHluorin-tagged receptors has been applied to several different types of neuronal receptors [7-17]. However, it is very important to keep in mind that our current method relies on tagging receptors with a GFP molecule and overexpression of tagged receptors. Attaching a GFP molecule to the extracellular domain of a membrane protein may interfere with the protein's function, and it is therefore critical to verify that this tagging strategy does significantly interfere with the function of the protein under investigation [9]. Moreover, an overexpressed receptor may or may not be subject to the regulatory mechanisms that govern the trafficking of endogenous receptors. Independent methods are therefore needed to validate results obtained from imaging overexpressed pHluorin-tagged receptors. For example, in our studies of GluA1 insertion [7], surface expression and synaptic trafficking of endogenous GluA1 receptors were validated using standard immunostaining/biochemical methods or electrophysiological approaches. In sum, our imaging approach, in combination with other standard methods for membrane protein trafficking, is likely to be instrumental in dissecting detailed molecular and cellular mechanisms governing plasma membrane insertion of other membrane proteins in neurons.

## Disclosures

No conflicts of interest declared.

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