Journal of Visualized Experiments

An antibody feeding approach to study glutamate receptor trafficking in dissociated primary hippocampal cultures

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59982R1
Full Title:	An antibody feeding approach to study glutamate receptor trafficking in dissociated primary hippocampal cultures
Keywords:	Receptor Trafficking; Glutamate Receptors; Antibody Feeding; Dissociated Primary Cultures; NMDA Receptors; AMPA Receptors; Internalization; Recycling
Corresponding Author:	Antonio Sanz-Clemente, Ph.D Northwestern University Chicago, IL UNITED STATES
Corresponding Author's Institution:	Northwestern University
Corresponding Author E-Mail:	antonio.sanz-clemente@northwestern.edu
Order of Authors:	Andrew M Chiu
	Levi Barse
	Pavla Hubalkova
	Antonio Sanz-Clemente, Ph.D
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Chicago, Illinois, USA

Northwestern University
Feinberg School of Medicine

DEPARTMENT OF PHARMACOLOGY
Searle Medical Research Building
Room 8-510
320 East Superior Avenue
Chicago, Illinois 60611
Tel: 312-503-4892

NORTHWESTERN
UNIVERSITY

Antonio Sanz-Clemente Ph.D
Assistant Professor of Pharmacology
Feinberg School of Medicine
Northwestern University. Chicago, IL
antonio.sanz-clemente@northwestern.edu
http://labs.feinberg.northwestern.edu/sanz-clemente/

Phillip Steindel, Ph.D.
Review Editor,
Journal of Visualized Experiments (JoVE)
1 Alewife Center, Suite 200
Cambridge, MA 02140
USA

Re: Manuscript Resubmission to *JoVE*

Dear Dr. Steindel,

We are pleased to resubmit our Methods Article entitled ***"An antibody feeding approach to study glutamate receptor trafficking in dissociated primary hippocampal cultures"*** to be considered for publication in JoVE under the "Neuroscience" category. We would like to thank the editors and the reviewers for their positive and constructive comments on our original submission. We have spent the past few weeks addressing the concerns raised by each reviewer, and, as a result, we now include revised Figures 2, 3 and 4. As suggested, we have performed additional controls to reinforce the reliability of our protocol and modified the text to include detailed instructions for image analysis. Importantly, we have written a macro which can be opened in FIJI to automate the analysis routine. This macro can easily be altered to provide more individualized workflows that are pertinent to an individual experimenter's data. These modifications are outlined in our point-by-point "response to the reviewers" document.

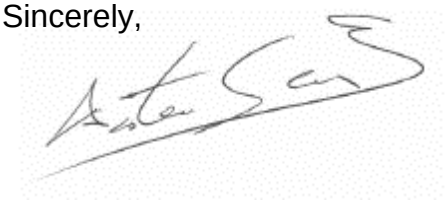
In this article, we present a method to study glutamate receptor (GluR) trafficking in dissociated primary hippocampal cultures. Specifically, we use an antibody-feeding approach to evaluate the surface expression, internalization, and recycling of GluRs. This is a very versatile protocol that can be used in combination with pharmacological approaches or overexpression of altered receptors to gain valuable information about stimuli and molecular mechanisms affecting GluR trafficking. Similarly, it can be easily adapted to study other receptors or surface expressed proteins.

To exemplify the utility of this protocol, we have analyzed the increase in the surface expression of the endogenous GluA1 subunit of AMPAR after inducing chemical LTP (cLTP). Similarly, we have overexpressed a phospho-mimetic mutant of the GluN2B subunit of NMDARs (GluN2B S1480E) and quantified its internalization and recycling ratio in comparison to GluN2B wild-type (WT). This is an example of the utility of this protocol to investigate molecular mechanisms affecting GluR trafficking and surface expression.

Although other protocols explaining antibody-feeding approaches have been published elsewhere, here we provide some technical advice that we expect to be useful for the neuroscience community.

Thanks again for the opportunity.

Sincerely,



Antonio Sanz-Clemente. Ph.D.
Assistant Professor of Pharmacology
Feinberg School of Medicine
Northwestern University

TITLE:

An Antibody Feeding Approach to Study Glutamate Receptor Trafficking in Dissociated Primary Hippocampal Cultures

AUTHORS AND AFFILIATIONS:

Andrew M. Chiu[1], Levi Barse[1], Pavla Hubalkova[1,2], Antonio Sanz-Clemente[1]

[1]Department of Pharmacology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

[2]Department of Cellular Neurophysiology, Institute of Physiology CAS, Prague, Czech Republic

Corresponding Author:

Antonio Sanz-Clemente (antonio.sanz-clemente@northwestern.edu)

Email Addresses of Co-Authors:

Andrew M. Chiu (andrew.chiu@northwestern.edu)

Levi Barse (levi.barse@northwestern.edu)

Pavla Hubalkova (pavla.hubalkova@northwestern.edu)

KEYWORDS:

glutamate receptor, surface expression, receptor internalization, receptor recycling, antibody feeding, transfection, neurons

SUMMARY:

This article presents a method to study glutamate receptor (GluR) trafficking in dissociated primary hippocampal cultures. Using an antibody-feeding approach to label endogenous or overexpressed receptors in combination with pharmacological approaches, this method allows for the identification of molecular mechanisms regulating GluR surface expression by modulating internalization or recycling processes.

ABSTRACT:

Cellular responses to external stimuli heavily rely on the set of receptors expressed at the cell surface at a given moment. Accordingly, the population of surface-expressed receptors is constantly adapting and subject to strict mechanisms of regulation. The paradigmatic example and one of the most studied trafficking events in biology is the regulated control of the synaptic expression of glutamate receptors (GluRs). GluRs mediate the vast majority of excitatory neurotransmission in the central nervous system and control physiological activity-dependent functional and structural changes at the synaptic and neuronal levels (e.g., synaptic plasticity). Modifications in the number, location, and subunit composition of surface expressed GluRs deeply affect neuronal function and, in fact, alterations in these factors are associated with different neuropathies. Presented here is a method to study GluR trafficking in dissociated hippocampal primary neurons. An “antibody-feeding” approach is used to differentially visualize GluR populations expressed at the surface and internal membranes. By labeling surface receptors on live cells and fixing them at different times to allow for receptors

endocytosis and/or recycling, these trafficking processes can be evaluated and selectively studied. This is a versatile protocol that can be used in combination with pharmacological approaches or overexpression of altered receptors to gain valuable information about stimuli and molecular mechanisms affecting GluR trafficking. Similarly, it can be easily adapted to study other receptors or surface expressed proteins.

INTRODUCTION:

Cells utilize the active process of trafficking to mobilize proteins to specific subcellular localizations and exert strict spatiotemporal regulation over their function[1]. This process is especially important for transmembrane receptors, as cellular responses to different environmental stimuli rely on intracellular cascades triggered by receptor activation. Cells are able to modify these responses by altering the density, localization, and subunit composition of receptors expressed at the cell surface via receptor subcellular trafficking regulation[2]. Insertion of newly synthetized receptors into the plasma membrane, along with endocytosis and recycling of existing receptors are examples of trafficking processes that determine the net pool of surface-expressed receptors[2]. Many molecular mechanisms cooperate to regulate protein trafficking, including protein-protein interactions and posttranslational modifications such as phosphorylation, ubiquitination, or palmitoylation[2].

Regulation of receptor trafficking is particularly required in strongly polarized cells with highly specialized structures. The paradigmatic example is the control of neuronal function by regulated trafficking of glutamate receptors (GluRs)[3,4]. Glutamate, the main excitatory neurotransmitter, binds and activates surface-expressed GluRs to control fundamental physiological neuronal functions such as synaptic neurotransmission and synaptic plasticity. The fact that altered GluR trafficking has been observed in a broad spectrum of neuropathies, ranging from neurodevelopmental disorders to neurodegenerative diseases, highlights the importance of this process[5]. Thus, understanding the molecular events that control GluR trafficking is of interest in many areas of research.

In this protocol, an antibody-feeding based method is used to quantify the level of surface-expressed GluRs in primary hippocampal neurons as well as evaluate how changes in internalization and recycling result in the observed net surface expression. The use of pharmacology and/or overexpression of exogenous receptors harboring specific mutations makes this protocol a particularly powerful approach for studying molecular mechanisms underlying neuronal adaptation to different environmental stimuli. A final example of the utility of this protocol is studying how multifactorial changes in the environment (such as in a disease models) affects GluR trafficking through the examination of surface expression in such models.

Using specific examples, it is initially demonstrated how a pharmacologic manipulation mimicking physiological synaptic stimulation [chemical LTP (cLTP)] increases the surface expression of the endogenous GluA1 subunit of the AMPA-type of GluRs (AMPARs)[6]. The trafficking of an overexpressed phospho-mimetic form of the GluN2B subunit of NMDA-type of GluRs (NMDARs) is also analyzed to exemplify how this protocol can be used to study the

regulation of GluR trafficking by specific posttranslational modifications. Though these specific examples are used, this protocol can easily be applied to other GluRs and other receptors and proteins that possess antigenic extracellular domains. In the case that there are no antibodies available for extracellular domains, overexpression of extracellular epitope-tagged (e.g., Flag-, Myc-, GFP-tagged, etc.) proteins can assist in protein labeling.

The current protocol provides instructions for quantifying specific GluR subtype density and trafficking using specific antibodies. This protocol can be utilized to study 1) total GluR surface expression, 2) GluR internalization, and 3) GluR recycling. To study each process individually, it is advised to begin with sections 1 and 2 and continue with either section 3, 4, or 5. In all cases, finish with sections 6 and 8 (**Figure 1**).

PROTOCOL:

Work pertaining to hippocampal primary culture preparation was reviewed and approved by the Northwestern University Animal Care and Use Committee (protocol #IS00001151).

1. Preparation before labeling

1.1. Preparation and maintenance of primary hippocampal cultures

1.1.1. Prepare primary hippocampal cultures at a density of 150,000 cells plated on poly-D-lysine-coated (0.1 mg/mL) 18 mm cover glasses. Excellent guides for dissociated neuronal culture preparation are available[7,8].

NOTE: If required, the cultures may be treated with cytosine arabinoside (Ara-C, 10 μM from DIV1) to avoid glial proliferation in the preparation.

NOTE: Alternative coating reagents such as fibronectin (1 mg/mL) or laminin (5 μg/mL) may be used instead of poly-D-lysine.

1.1.2. Maintain cultures in a cell incubator at 37 °C and 5% CO_2 in 2 mL/well of neurobasal media supplemented with B27 and 2 mM L-glutamine.

NOTE: Substitutes for L-glutamine (e.g., Glutamax) can be used, if desired.

1.1.3. On weekly-basis, remove half the volume of media and replace with the same volume of supplemented neurobasal media.

1.2. Optional: Transfection of mutated and/or epitope-tagged receptors

NOTE: Neurons should be transfected at least 3–4 days prior to the analysis time point to allow for receptor expression. The use of young neurons [Days in vitro 6–9 (DIV6–9)] results in better transfection efficiency than older (DIV15–20) neurons, but a sufficient number of transfected

cells (>20) can be achieved regardless of the DIV employed.

1.2.1. For each well of a 12-well plate, dilute 1.5 µg of plasmid containing the construct of interest in 100 µL of fresh neurobasal media without B27 or glutamine supplementation in a microcentrifuge tube and mix by vortexing quickly.

NOTE: For successful transfection, it is critical that the neurobasal media used is as fresh as possible, ideally less than 1 week after bottle opening.

1.2.2. In a second microcentrifuge tube, mix 1 µL of an appropriate lipofection reagent in 100 µL of fresh neurobasal media and mix gently.

NOTE: Do not vortex the lipofection reagent mixture. Use of fresh lipofection reagents can improve transfection efficiency.

1.2.3. Incubate the tubes for 5 min at room temperature (RT).

1.2.4. Add the lipofection reagent mixture dropwise to the DNA mixture, mix gently, and incubate for 20 min at RT.

1.2.5. Adjust the volume of media in each well to 1 mL of conditioned media.

1.2.6. Add the lipofection reagent -DNA mixture dropwise to the well.

1.2.7. Return cells to the incubator and allow at least 3–4 days for protein expression.

NOTE: For the purposes of the internalization and recycling protocols outlined below, hippocampal neurons were transfected at DIV11–12 with constructs expressing GluN2B tagged with GFP in the extracellular domain (GFP-GluN2B) and imaged at DIV15–16.

1.3. Optional: Incubation of cells with drugs (chronically or acutely) in the conditioned media until fixation.

NOTE: For acute treatment, begin treating cells before labeling. Depending on the drug treatment protocol used, cells can be maintained in drug-containing media during section 2. In our example, DIV21 cells were subject to a cLTP protocol to increase surface-expressed AMPAR[9].

1.3.1. Exchange conditioned media for extracellular solution (ECS).

1.3.2. Treat cells with 300 µM glycine in ECS for 3 min at RT. As a control, treat a sister coverslip with ECS (without glycine).

1.3.3. Wash cells 3x with 37 °C ECS and return the cells in ECS (without glycine) to the cell

incubator for 20 min prior to continuing with section 2.

NOTE: ECS (in mM): 150 NaCl, 2 $CaCl_2$, 5 KCl, 10 HEPES, 30 Glucose, 0.001 TTX, 0.01 strychnine, and 0.03 picrotoxin at pH 7.4.

2. Live labeling of surface-expressed receptors

2.1. Prepare coverslips for labeling

2.1.1. To save reagents and facilitate manipulation, transfer coverslips cell side up to a paraffin film-covered tray.

NOTE: It is critical to never let the samples dry out.

2.1.2. Save and maintain conditioned media at 37 °C for incubation and washing steps.

NOTE: For an 18 mm coverslip, incubation with 75–100 µL of media for antibody labeling and 120–150 µL for internalization/recycling are recommended.

2.2. Labeling of surface receptors with primary antibody

2.2.1. Incubate cells with primary antibody diluted in conditioned media for 15 min at RT.

NOTE: For GFP-tagged receptors, rabbit anti-GFP antibody at a dilution of 1:1000 was used. For endogenous GluA1, mouse anti-GluA1 at a 1:200 dilution was used.

2.2.2. Carefully aspirate off the antibody-containing media using a vacuum pipette and wash cells three times with conditioned media.

NOTE: If conditioned media is unavailable, all washing steps may be performed using PBS+ [phosphate buffered saline (PBS) containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$]. Manual aspiration using a micropipette may be performed if gentle vacuum aspiration is not available.

3. Surface expression (Figure 2)

3.1. Secondary antibody labeling of surface-expressed receptors

3.1.1. Wash once with PBS+.

3.1.2. Fix cells by incubating with 4% paraformaldehyde (PFA) and 4% sucrose in PBS for 7–8 min.

NOTE: Unlike other fixation methods such as methanol incubation, PFA does not permeabilize the plasma membrane and is therefore suitable for surface-expression analysis. For optimal

results, use freshly prepared PFA. Short-term storage of PFA at 4 °C or long-term (up 30 days) storage at -20 °C is permissive for adequate fixation.

CAUTION: PFA is a known carcinogen. Use proper personal protective equipment and a safety hood when handling.

3.1.3. Wash cells three times with regular PBS.

NOTE: Alternatively, 0.1 M glycine can be used for washing PFA instead of PBS, as glycine will quench any remaining fixative that may increase the background in the preparation.

3.1.4. Block nonspecific binding sites by incubating with 10% normal goat serum (NGS) in PBS for 30 min at RT.

NOTE: Blocking time can be extended without adverse effects on labeling.

3.1.5. Incubate with fluorescently-tagged secondary antibody diluted in 3% NGS in PBS for 1 h at RT to label primary antibody-labeled receptors (i.e., surface-expressed).

NOTE: In these examples, a 1:500 dilution of Alexa 555-conjugated secondary antibodies:goat anti-rabbit for GFP-labeled receptors and goat anti-mouse for GluA1 was used.

3.1.6. Wash cells with PBS 3x.

3.2. Labeling of intracellular receptors

3.2.1. Permeabilize cells with 0.25% Triton X-100 in PBS for 5–10 min at RT.

NOTE: To check that the initial round of antibody labeling occupies all surface epitopes, this permeabilization step can be skipped in a sister culture. In this case, no signal for intracellular receptors should be obtained. Additionally, to check that no internal receptors have been labeled in the previous section 2 (i.e., showing the integrity of the plasma membranes in culture), the permeabilization step can be skipped in a sister culture, and a primary antibody against an intracellular protein (e.g., PSD-95 or MAP2) can be utilized in step 2.2.3. No signal should be obtained from this primary under these conditions. In this case, a rabbit anti-PSD-95 antibody (1:500) was used.

3.2.2. Block with 10% NGS in PBS for 30 min at RT.

3.2.3. Label intracellular receptors by incubating permeabilized cells with the same primary antibody used in section 2.2 diluted in 3% NGS in PBS for 1 h at RT.

NOTE: The antibody dilution for labeling intracellular receptors may be different than that required for labeling surface-expressed receptors. In the example of GluA1, the same antibody

dilution (1:200) was used.

3.2.4. Wash cells 3x with PBS.

3.2.5. Label with second fluorescently-tagged secondary antibody diluted in 3% NGS in PBS for 1 h at RT.

NOTE: In these examples, a 1:500 dilution of Alexa 647-conjugated secondary antibodies:goat anti-rabbit for GFP-labeled receptors and goat anti-mouse for GluA1 was used.

3.2.6. Wash cells 3x with PBS.

4. Internalization (Figure 3)

4.1. Internalization of antibody-labeled surface receptors

4.1.1. After labeling of surface-expressed receptors and antibody washing (section 2.2), maintain cells in conditioned media without antibody and return them to the incubator (37 °C) to allow for internalization.

NOTE: For NMDA receptors, 30 min for internalization is recommended. As a control, a sister culture may be maintained with conditioned media at 4 °C during the internalization process. Minimal receptor internalization should occur under these conditions.

4.2. Labeling of surface receptors

4.2.1. Wash cells once with PBS+.

4.2.2. Fix cells with 4% PFA and 4% sucrose in PBS for 7–8 min.

CAUTION: Use proper personal protective equipment and a safety hood when handling PFA.

4.2.3. Wash cells 3x with regular PBS.

4.2.4. Block with 10% NGS in PBS for 30 min at RT to prevent nonspecific binding.

4.2.5. Incubate samples with fluorescently-tagged secondary antibody diluted in 3% NGS in PBS for 1 h at RT to label primary antibody-labeled receptors (i.e., surface-expressed receptors which were not internalized).

NOTE: For this example, Alexa 555-conjugated goat anti-rabbit secondary antibody (1:500) was used for labeling.

4.2.6. Wash cells 3x with PBS.

4.3. Labeling of internalized receptors

4.3.1. Permeabilize cells with 0.25% Triton X-100 in PBS for 5–10 min.

4.3.2. Block nonspecific binding by incubation with 10% NGS in PBS for 30 min at RT.

4.3.3. Incubate samples with fluorescently tagged secondary antibody diluted in 3% NGS in PBS for 1 h at RT to label internalized antibody-labeled receptors.

NOTE: For this example, Alexa 647-conjugated goat anti-rabbit secondary antibody (1:500) is used for labeling.

4.3.4. Wash cells 3x with PBS.

5. Recycling (Figure 4)

5.1. Internalization of antibody-labeled surface receptors

5.1.1. After labeling of surface-expressed receptors and antibody washing (section 2.2), maintain cells in conditioned media without antibody and return them to the incubator (37 °C) to allow for internalization.

NOTE: For NMDA receptors, 30 min for internalization is recommended.

5.2. Blocking of stable surface expressed receptors

5.2.1. To block the epitopes on the primary antibody attached to surface-expressed receptors that have not been internalized, incubate cells with unconjugated Fab anti-IgG (H+L) antibody fragments (against the primary used in section 2.2) diluted in conditioned media (20 µg/mL) for 20 min at RT. This treatment prevents future interaction with secondary antibodies.

NOTE: For this example, Goat anti-rabbit Fab fragments were used.

NOTE: Control experiment: to ensure that complete blocking of surface-expressed receptors has occurred, sister coverslips can be incubated with and without Fab. Cultures should be fixed immediately after Fab treatment, and both cultures are incubated with Alexa 555-conjugated secondary antibody. No Alexa 555 signal in the Fab-incubated cells indicates proper antibody blocking.

5.2.2. Wash cells 3x with conditioned media.

5.2.3. Incubate cells with conditioned media containing 80 µM dynasore to prevent further internalization and return cells to the incubator (37 °C) to allow for recycling of internalized

receptors. Dynasore is a GTPase inhibitor that inhibits dynamin and therefore prevents internalization.

NOTE: 45 min for NMDAR recycling is recommended. Note that Dynasore exclusively blocks the dynamin-dependent internalization process (e.g., NMDARs internalization). However, internalization of other synaptic protein (dynamin-independent) can still occur in the presence of Dynasore.

5.3. Labeling of recycled receptors

5.3.1. Wash cells once with PBS+.

5.3.2. Fix cells with 4% PFA and 4% sucrose in PBS for 7–8 min.

CAUTION: Use proper personal protective equipment and a safety hood when handling PFA.

5.3.3. Wash cells 3x with PBS.

5.3.4. Block with 10% NGS in PBS for 30 min at RT to prevent nonspecific binding.

5.3.5. Label cells with first fluorescently-tagged secondary antibody diluted in 3% NGS in PBS for 1 h at RT.

NOTE: For this example, Alexa 555-conjugated goat anti-rabbit antibody (1:500) was used for labeling.

5.3.6. Wash cells 3x with PBS.

NOTE: Longer washes with PBS (5–10 min) may help to reduce background in the preparation.

5.4. Labeling of internalized receptors

5.4.1. Permeabilize cells with 0.25% Triton X-100 in PBS for 5–10 min.

5.4.2. Block with 10% NGS in PBS for 30 min at RT.

5.4.3. Label with second fluorescently-tagged secondary antibody diluted in 3% NGS in PBS for 1 h at RT.

NOTE: For this example, Alexa 647-conjugated goat anti-rabbit antibody (1:500) was used for labeling.

5.4.4. Wash cells 3x with PBS.

6. Mounting and imaging of samples

6.1. Mount cells by gently placing the coverslips cell side down on 12–15 µL of the appropriate mounting media.

NOTE: Aspiration of excess mounting media will improve the quality of images.

6.2. Image cells on an appropriate confocal microscope.

NOTE: It is recommended to image a z-stack at 60x magnification with 0.35 µm steps, encompassing the entire thickness of the neuron.

7. Time considerations

7.1. This is a long protocol that can be stopped at several points. If desired, perform blocking and primary antibody incubation steps overnight at 4 °C in a humid chamber.

7.2. Alternatively, if desired, use a microwave tissue processor to vastly speed up post-fixation incubation times. For all steps, use 150 W at 30 °C for "On" settings.

7.2.1. To block, run the processor at 2 min "On," 1 min "Off," and 2 min "On."

7.2.2. For primary and secondary antibody incubation steps, run the processor at 3 min "On," 2 min "Off," and 3 min "On."

NOTE: We observe no difference in quality by making the above alterations to the protocol.

8. Image analysis

8.1. It is recommended to use FIJI <https://fiji.sc/> to conduct image analysis, as it is compatible with multiple file formats. For our data, images in the Nikon ND2 file format were acquired.

8.2. A macro script is provided for easy batch quantification of different parameters pre-selected by FIJI. The following steps are included in the macro:

NOTE: For these examples, "integrated intensity" was measured.

8.3. Open the image files in FIJI and separate channels.

8.4. Z-project each channel stack as a maximum intensity projection.

8.5. Set a lower threshold for each channel.

NOTE: Thresholds should be empirically determined for each experimental data set. While each

channel can have a separate lower threshold value, it crucial that channel threshold values are consistently maintained for all images of the same data set.

8.6. Select three to five secondary or tertiary dendrites and save them as regions of interest (ROIs).

8.7. Measure the integrated density of each ROI in surface and intracellular channels.

8.8. Normalize the signal for each ROI by dividing the integrated density value of the surface channel by the intracellular channel.

8.9. Repeat the measurements for all control and experimental images and normalize experimental values to control values (e.g., GluN2B WT or no-glycine conditions).

REPRESENTATIVE RESULTS:

This protocol to study glutamate receptor trafficking is based on differential labeling of receptors expressed at the cell surface and those expressed in internal membranes. Segregation is achieved by the labeling the receptors before and after membrane permeabilization, using the same primary antibody but a secondary antibody conjugated to a different fluorophore. As outlined by the optional steps included the protocol, this is a very versatile method for interrogating different receptor trafficking processes, such as internalization and recycling, and can be easily adapted to the investigator's needs (**Figure 1**).

First, a method is provided for the quantification of receptors expressed at the neuronal surface. This protocol allows for quantification of basal surface expression as well as studying the molecular mechanisms by which different drugs or mutations alter basal levels of surface-expressed receptors. Surface-expressed receptors were first labeled by incubating with both primary and secondary antibody prior to permeabilization. After permeabilization with 0.25% Triton X-100, the intracellular pool of receptors is accessible for antibody labeling. To illustrate this protocol, surface vs. intracellular staining of AMPA receptors (AMPARs) in control cultures and after induction of cLTP was conducted. Specifically, live cells were labeled using an antibody against an extracellular epitope in the GluA1 subunit of the AMPAR, and cells were fixed then labeled with Alexa 555-conjugated secondary. The cells were then permeabilized and labeled with same anti-AMPAR antibody and incubated with a secondary antibody conjugated to Alexa 647. This dual labeling allows clear visualization of the two GluA1 populations.

After acquiring confocal images, the fluorescent signal can be easily quantified to show an increase in surface expression, relative to the intracellular population, following cLTP (**Figure 2A,B**). As a control, the permeabilization step was skipped (incubation with 0.25% Triton X-100) in a sister coverslip and a primary antibody was used against PSD-95, a standard intracellular marker for excitatory synapses. As shown in **Figure 2C**, no signal for PSD-95 can be obtained in non-permeabilized cells, demonstrating the integrity of the plasma membrane. This indicates that the signal obtained for "surface GluA1" indeed corresponds to surface-expressed receptors

(i.e., signal for surface-expressed GluA1 does not include intracellular receptors). Importantly, a minimal signal for "internalized GluA1" can be observed under non-permeabilization conditions, showing that all surface epitopes are occupied by the initial round of antibody labeling (i.e., signal for intracellular GluA1 does not included surface-expressed receptors).

A second process that can be examined utilizing this protocol is the internalization of surface-expressed receptors. Specifically, surface receptors are labeled on a live cell, and neurons are returned to the incubator for a given time to undergo receptor internalization by clathrin-mediated endocytosis. Following this step, cells are fixed to preserve the spatial expression of primary antibody-labeled receptors (i.e., surface-expressed and internalized receptors). Then, surface receptors (i.e., those which have not been internalized in the time period of interest), are labeled with a secondary antibody prior to permeabilization. Following permeabilization, receptors that have been internalized are labeled by a secondary antibody with a different fluorophore.

In this protocol, it was examined how a particular phosphorylation within the PDZ ligand of the GluN2B subunit of NMDARs (at S1480) induces NMDAR internalization. To do so, primary cultures were transfected with a phospho-mimetic receptor, in which serine (S1480) had been substituted by glutamate (E). The resultant mutant, GluN2B S1480E, acts as a "constitutively-phosphorylated" form of GluN2B. To ease labeling of GluN2B and identify the phospho-mimetic mutant, GFP was used as an epitope tag on the extracellular side of GluN2B (GFP-GluN2B S1480E). Surface receptors on live cells were labeled with an anti-GFP antibody for 15 min at RT. Next, the excess antibody was washed with conditioned media and returned the cells to 37 °C for 30 min to allow for endocytosis. Then, cells were fixed to freeze receptor movement. Receptors that remained on the surface were then labeled with Alexa 555-conjugated secondary antibody prior to permeabilization.

To identify receptors that had been internalized during the 30 min incubation period, cells were permeabilized, and the internalized receptors (already labeled with a primary antibody) were then labeled with Alexa 647-conjugated antibody. Again, this dual-labeling strategy allows quantification of the proportion of internalized receptors. This example highlights that GluN2B phosphorylation at S1480 promotes receptor internalization, as the phospho-mimetic mutant S1480E displayed a much higher internalization ratio compared to WT receptors (**Figure 3A,B**). As a control, a sister culture at 4 °C was maintained in conditioned media during internalization to strongly slow the process. As expected, no signal was obtained for "internalized" receptors under these conditions (**Figure 3C**).

Lastly, this protocol can be utilized to examine the recycling of previously internalized receptors. This protocol variation is a continuation of the internalization protocol, by following these receptors back to the cell surface. There are two crucial components to this variation. Firstly, receptors which remained stably expressed at the surface during the entire protocol (i.e., receptors that were not internalized or recycled) must be "blocked" so that they are not mistaken for recycled receptors. To do so, before performing the recycling step, live neurons are incubated with high concentrations of Fab that interact with primary antibody-labeled

receptors expressed at the surface to prevent further binding of the fluorophore-conjugated secondary antibody. Second, internalization should be prevented during the recycling phase, so that recycled receptors are not repeat internalized. This was done by adding dynasore to the media during recycling as this drug blocks processes reliant on dynamin such as clathrin-mediated endocytosis, such as NMDAR internalization. In this protocol, studying the trafficking of the phospho-mimetic mutant GluN2B S1480E was performed as well as surface labeling of GFP-GluN2B on live cells, which allowed for internalization during a 30 min period as explained before.

Following this, surface receptors that were not internalized during this period were blocked by incubating the cells with Fab for 20 min at RT. Next, cells were again incubated at 37 °C for 45 min to allow for previously internalized GluN2B to be recycled back to the cell surface. Dynasore was present in the media during the recycling step. Fixation of the cells following this step allows for the identification of recycled receptors (i.e., those that are unblocked and expressed on the cell surface) and internalized receptors (i.e., those that are primary antibody labeled and intracellular). By 1) labeling recycled receptors with an Alexa 555-conjugated secondary antibody prior to permeabilization and 2) labeling internalized, but not recycled, receptors with Alexa 647-conjugated secondary antibody, a recycling ratio was generated to show that GluN2B S1480E does not have any effect on NMDAR recycling (**Figure 4A,B**). As a control, it was ensured that complete blocking of surface-expressed receptors occurred by incubating sister coverslips in the presence or absence of Fab, followed with PFA fixation. As shown in **Figure 4C**, a strong signal can be observed for surface-expressed GluN2B in the absence of Fab blocking. This signal disappears in Fab-treated cultures, demonstrating that the blocking protocol is sufficient to completely block the surface-expressed epitopes and that the surface signals observed after recycling indeed correspond to receptors trafficked back to the plasma membrane.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of protocol variations to study receptor surface expression, receptor internalization (endocytosis), and receptor recycling.

Figure 2: cLTP increases surface expression of GluA1. Primary hippocampal neurons at DIV21 were subjected to chemical LTP (cLTP) by incubating with glycine-containing ECS. Distinct labeling of surface-expressed (red) vs. intracellular (blue) GluA1 populations reveals the expected increase in surface expression of AMPAR. (**A)** Single plane and (**B)** Z-stacked (maximum intensity projection) confocal pictures. Scale bars = 50 µm (whole cell) or 5 µm (dendrite). Graph shows the increased surface expression of GluA1 after cLTP protocol. Surface expression index: surface/intracellular receptors (n = 3; number of cells: con = 7; cLTP = 7; values represent mean ± SEM; ****$p < 0.0001$ using Mann-Whitney U test). (**C)** Control experiment in which the permeabilization step was skipped. In addition to surface and internal GluA1, the intracellular excitatory synaptic marker PSD-95 was evaluated. Scale bars = 50 µm (whole cell) or 5 µm (dendrite).

Figure 3: Phosphorylation of GluN2B at S1480 promotes NMDAR internalization. Primary hippocampal neurons were transfected with either GFP-GluN2B WT or the phospho-mimetic mutant GFP-GluN2B S1480E on DIV11-12. Following 3–4 days of protein expression, surface GFP was labeled on live cells with a rabbit anti-GFP antibody and cells were then returned to 37 °C to allow for receptor internalization by endocytosis. Surface-expressed exogenous receptors were visualized with Alexa 555-conjugated secondary antibody, and the internalized population identified after permeabilization using Alexa 647-conjugated antibody. For clarity, surface GFP-GluN2B is pseudocolored in green and internalized GFP-GluN2B is pseudocolored in white. (**A)** Single plane and (**B)** Z-stacked (maximum intensity projection) confocal pictures. Scale bars = 50 µm (whole cell) or 5 µm (dendrite). Graph shows the elevated internalization displayed by the phospho-mimetic mutant GluN2B S1480E. Internalization index: internalized receptors/surface-expressed receptors (n = 6; number of cells: WT = 34; S1480E = 28; values represent mean ± SEM; ***p < 0.001 using Mann-Whitney U test). (**C)** Control experiment in which the internalization step (Intenaliz.) was performed at 4 °C. Scale bars = 50 µm (whole cell) or 5 µm (dendrite).

Figure 4: Phosphorylation of GluN2B at S1480 does not modify NMDAR recycling. Primary hippocampal neurons were transfected with either GFP-GluN2B WT or the phospho-mimetic mutant GFP-GluN2B S1480E on DIV11-12 as shown in **Figure 3**. Following 3–4 days of protein expression, surface GFP was labeled on live cells with a rabbit anti-GFP antibody and cells were then returned to 37 °C to allow for receptor internalization by endocytosis. Remaining surface-expressed receptors were blocked by Fab incubation and recycling was allowed for 45 min. Available surface expressed exogenous receptors (recycled) were visualized with Alexa 555-conjugated secondary antibody, and the internalized population identified after permeabilization using Alexa 647-conjugated antibody. For clarity, surface GFP-GluN2B is pseudocolored in white and internalized GFP-GluN2B is pseudocolored in green.
(A) Single plane and (**B)** Z-stacked (maximum intensity projection) confocal pictures. Scale bars = 50 µm (whole cell) or 5 µm (dendrite). Graph shows the lack of effect the GluN2B S1480 phosphorylation has on recycling. Recycling index: recycled receptors/internalized receptors (n = 5; number of cells: WT = 27; S1480E = 24; values represent mean ± SEM; n.s. = non-significant using Mann-Whitney U test). **(C)** Control experiment in which the Fab incubation step to block surface-expressed epitopes was skipped. Scale bars = 50 µm (whole cell) or 5 µm (dendrite).

DISCUSSION:

The interaction between a cell and its environment (e.g., communication with other cells, response to different stimuli, etc.), heavily relies on the correct expression of receptors at the cell surface. The rapid and fine-tuned regulation in surface-expressed receptor content enables proper cellular response to a constantly changing environment. In the particular case of neurons, alterations in the number, localization, and subunit composition of synaptically expressed receptors heavily influences synaptic communication, synaptic plasticity, synaptogenesis, and synaptic pruning[3,5,10]. Therefore, accurate analysis of receptor surface expression and the mechanism underlying its regulation is an important topic of research.

A number of modalities exist by which receptor surface expression can be studied. In general, these fall under three main categories: (i) biochemical isolation of the surface expressed receptor population, (ii) imaging techniques to visualize receptors at the surface, and (iii) functional techniques to monitor the consequences of receptor activation. These approaches are complementary and can be utilized in conjunction to answer specific questions about surface expression of receptors.

Examples of biochemical isolation of surface expressed receptors include biotinylation protocols in cultured cells or brain slices and subcellular fractionation of cultured cells or tissue[11]. Surface biotinylation is based on the labeling of surface-expressed receptors with biotin by incubating the cultures with ester-activated biotin. The ester group reacts with primary amino groups ($-NH_2$) to form stable amide bonds. Because this reagent is cell-impermeable, only surface-expressed proteins are labeled with biotin. After cellular lysis using detergents, labeled receptor can be recovered by incubating the cell lysate with Agarose beads conjugated to avidin or its variants which have a strong affinity for biotin, then evaluated by immunoblotting. Subcellular fractionation is a biochemical protocol that uses several centrifugation steps to isolate different cellular membranous compartments based on their different densities[12]. Both techniques are useful to quantify changes in surface expression of endogenous proteins and can be used in conjunction with pharmacological approaches (both *in vivo* and in culture) to determine how molecular manipulations affect surface expression of receptors. They are particularly useful because changes in multiple endogenous receptors, relative to a standard, can be quantified simultaneously. However, unlike imaging modalities, such as that described in this protocol, spatial resolution is lost through biochemical processing of samples.

The protocol described here belongs to the imaging techniques category. This group of approaches (such as surface labeling and live-cell imaging of fluorescently tagged overexpressed proteins) are useful to give spatiotemporal resolution to surface expression of receptors. For instance, by examining surface vs. intracellular expression of AMPAR, as exemplified previously, one can examine how changes in expression are pronounced in the dendrites (the biological compartment of interest for this particular application). Like biochemical fractionation, drugs can be used with imaging techniques to determine the effects of a drug on surface expression. In addition, transfection of neurons with receptors containing modifications (mutations or tags) further elaborates the possiblities for imaging. Transfection with mutant receptors can delineate the effects of a particular mutation on surface expression.

Another useful approach to visualize receptor trafficking is live imaging microscopy after transfection of primary cultures with fluorescently tagged constructs, such as Superecliptic pHluorin (SEP)- or other fluorophore-tagged constructs[13]. SEP-tagged receptors can be particularly useful for studying surface expressed receptors, as this fluorophore will only fluoresce when exposed to the extracellular medium. Like previous examples, pharmacological approaches can be used, or mutations made on the receptor, to determine if these change the surface expression characteristics of the receptor. In the case of drugs, live-cell imaging can be used to monitor the modifications in surface expression in real-time. A limitation to live imaging

is the lack of mechanistic insight into changes in surface expression. For example, a decreased surface expression might be a result of either increased receptor internalization, reduced receptor recycling, or both.

To answer this question, the protocol described above may be used. Another important trafficking event that can be monitoring using live imaging approaches is the lateral diffusion of receptors between different compartments in the plasma membrane (e.g., between synaptic and extrasynaptic sites). In this case, the technique of choice is the use of single-particle tracking approaches in which a fluorescence semiconductor nanocrystal [i.e., Quantum Dot (QD)] is attached to an antibody against an extracellular epitope on the protein of interest. QDs are characterized by remarkable stability (allowing much less photobleaching than traditional fluorescent proteins), strong brightness, and the alternation between on/off status ("blinking"). By using the appropriate microscope settings, it is possible to track the movement of a single QD (and, therefore, a single protein of interest) through the cellular plasma membrane[14].

The current protocol provides an examination of receptors in the surface population, internalized population, and recycled population, allowing for the calculation of ratios to determine how these processes control total surface expression. Furthermore, stopping the internalization or recycling processes at different timepoints adds temporal resolution. This method, therefore, allows for spatiotemporal surface expression studies. Unlike the other imaging techniques, levels of endogenous surface expression can also be studied (e.g., AMPAR), provided there exists an antibody against the extracellular portion of the receptor. However, because this method requires fixation of cells, it is not compatible with live-cell imaging and thus cannot provide real-time information.

Finally, functional approaches like electrophysiology[15] or calcium imaging[16] are powerful, though often indirect, manners to study the surface expression of receptors. These methods are based on the quantification of functional changes in the cell after receptor activation (e.g., change in membrane potential or calcium concentration). Using pharmacology to isolate the response of a given receptor, these methods allow for estimation of the receptors expressed at the cell surface in a precise, spatiotemporal manner. These methods are versatile and allow for the study of cells in culture and in more physiological conditions *ex vivo* (e.g., acute brain slices) and *in vivo*. However, as in the case of the live imaging approaches, mechanistic information is often missed when using functional approaches.

In summary, a combination of techniques to study receptor surface expression can be employed to fully understand how surface expression is controlled. The protocol presented here is particularly powerful as it provides a mechanistic understanding of the spatiotemporal surface expression of receptors in dissociated primary cultures.

ACKNOWLEDGMENTS:
We thank the Northwestern Center for Advanced Microscopy for the use of the Nikon A1 Confocal microscope and their assistance in planning and analyzing the experiments. This research was supported by NIGMS (T32GM008061) (A. M. C.), and NIA (R00AG041225) and a

NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation (#24133) (A. S. -C.).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Enns, C. Overview of protein trafficking in the secretory and endocytic pathways. *Current Protocols in Cell Biology.* **Chapter 15,** Unit 15 11, doi:10.1002/0471143030.cb1501s03 (2001).

2 Bedford, F. K. *Encyclopedia of Neuroscience* (eds, Marc D. Binder, Nobutaka Hirokawa, & Uwe Windhorst). 3385-3389 (Springer Berlin Heidelberg, 2009).

3 Diering, G. H., Huganir, R. L. The AMPA Receptor Code of Synaptic Plasticity. *Neuron.* **100** (2), 314-329, doi:10.1016/j.neuron.2018.10.018 (2018).

4 Lussier, M. P., Sanz-Clemente, A., Roche, K. W. Dynamic Regulation of N-Methyl-d-aspartate (NMDA) and alpha-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptors by Posttranslational Modifications. *The Journal of Biological Chemistry.* **290** (48), 28596-28603, doi:10.1074/jbc.R115.652750 (2015).

5 Traynelis, S. F. *et al.* Glutamate receptor ion channels: structure, regulation, and function. *Pharmacological Reviews.* **62** (3), 405-496, doi:10.1124/pr.109.002451 (2010).

6 Molnar, E. Long-term potentiation in cultured hippocampal neurons. *Seminars in Cell & Developmental Biology.* **22** (5), 506-513, doi:10.1016/j.semcdb.2011.07.017 (2011).

7 Seibenhener, M. L., Wooten, M. W. Isolation and culture of hippocampal neurons from prenatal mice. *Journal of Visualized Experiments.* (65), doi:10.3791/3634 (2012).

8 Nunez, J. Primary Culture of Hippocampal Neurons from P0 Newborn Rats. *Journal of Visualized Experiments.* (19), doi:10.3791/895 (2008).

9 Lu, W. *et al.* Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. *Neuron.* **29** (1), 243-254 (2001).

10 Sanz-Clemente, A., Nicoll, R. A., Roche, K. W. Diversity in NMDA Receptor Composition: Many Regulators, Many Consequences. *The Neuroscientist: A Review Journal Bringing Neurobiology, Neurology and Psychiatry.* **19** (1), 62-75, doi:10.1177/1073858411435129 (2013).

11 Tham, D. K. L., Moukhles, H. Determining Cell-surface Expression and Endocytic Rate of Proteins in Primary Astrocyte Cultures Using Biotinylation. *Journal of Visualized Experiments.* (125), e55974, doi:doi:10.3791/55974 (2017).

12 Bermejo, M. K., Milenkovic, M., Salahpour, A., Ramsey, A. J. Preparation of Synaptic Plasma Membrane and Postsynaptic Density Proteins Using a Discontinuous Sucrose Gradient. *Journal of Visualized Experiments.* (91), e51896, doi:doi:10.3791/51896 (2014).

13 Makino, H., Malinow, R. AMPA receptor incorporation into synapses during LTP: the role of lateral movement and exocytosis. *Neuron.* **64** (3), 381-390, doi:10.1016/j.neuron.2009.08.035 (2009).

14 Bailey, D. M., Kovtun, O., Rosenthal, S. J. Antibody-Conjugated Single Quantum Dot

Tracking of Membrane Neurotransmitter Transporters in Primary Neuronal Cultures. *Methods in Molecular Biology.* **1570,** 165-177, doi:10.1007/978-1-4939-6840-4_11 (2017).

15 Trussell, L. Recording and analyzing synaptic currents and synaptic potentials. *Current Protocols in Neuroscience.* **Chapter 6,** Unit 6 10, doi:10.1002/0471142301.ns0610s07 (2001).

16 Barreto-Chang, O. L., Dolmetsch, R. E. Calcium Imaging of Cortical Neurons using Fura-2 AM. *Journal of Visualized Experiments.* (23), e1067, doi:doi:10.3791/1067 (2009).

Figure 1

Click here to access/download;Figure;Antonio Sanz-Clemente - Figure 1.pdf

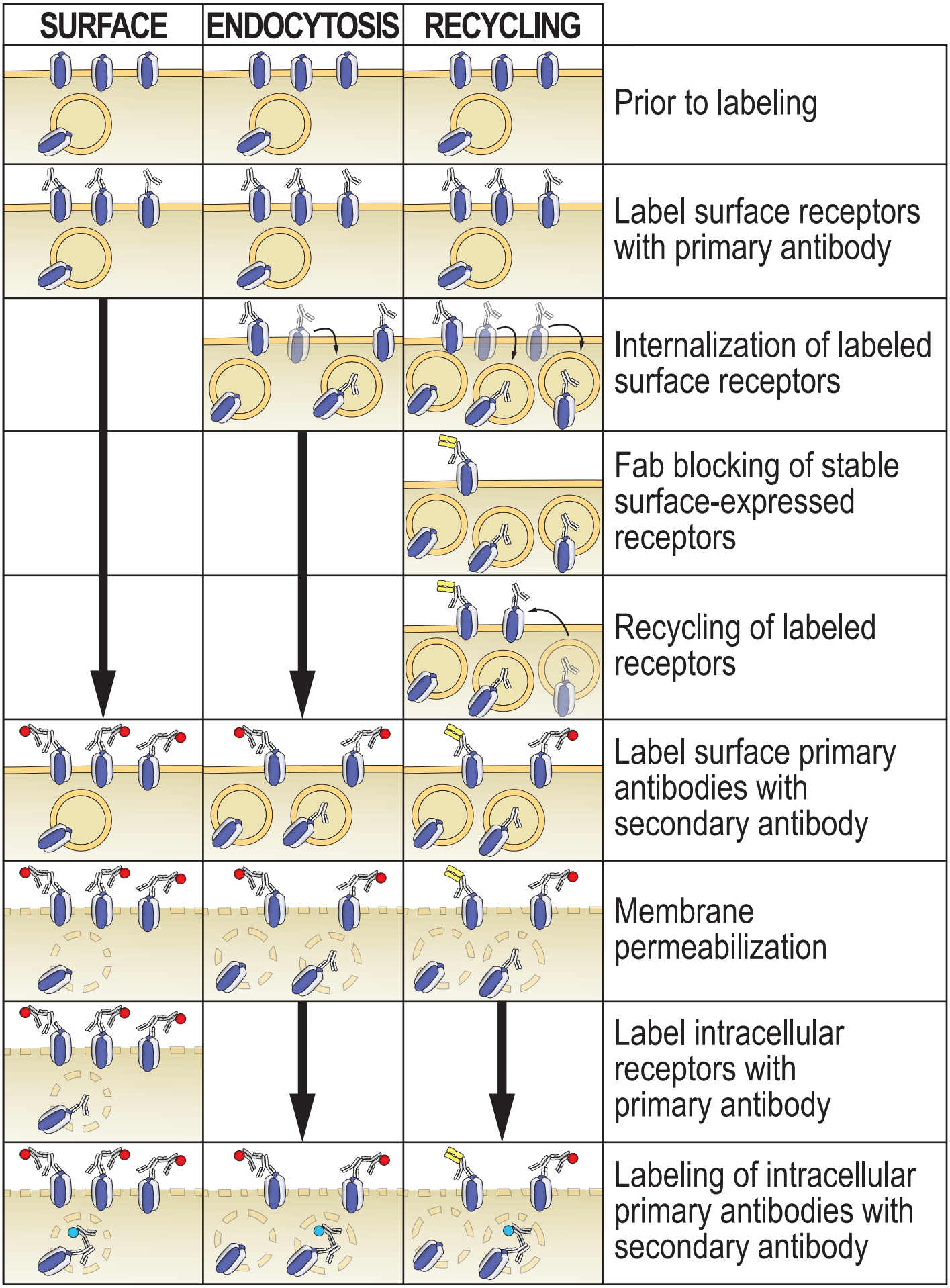
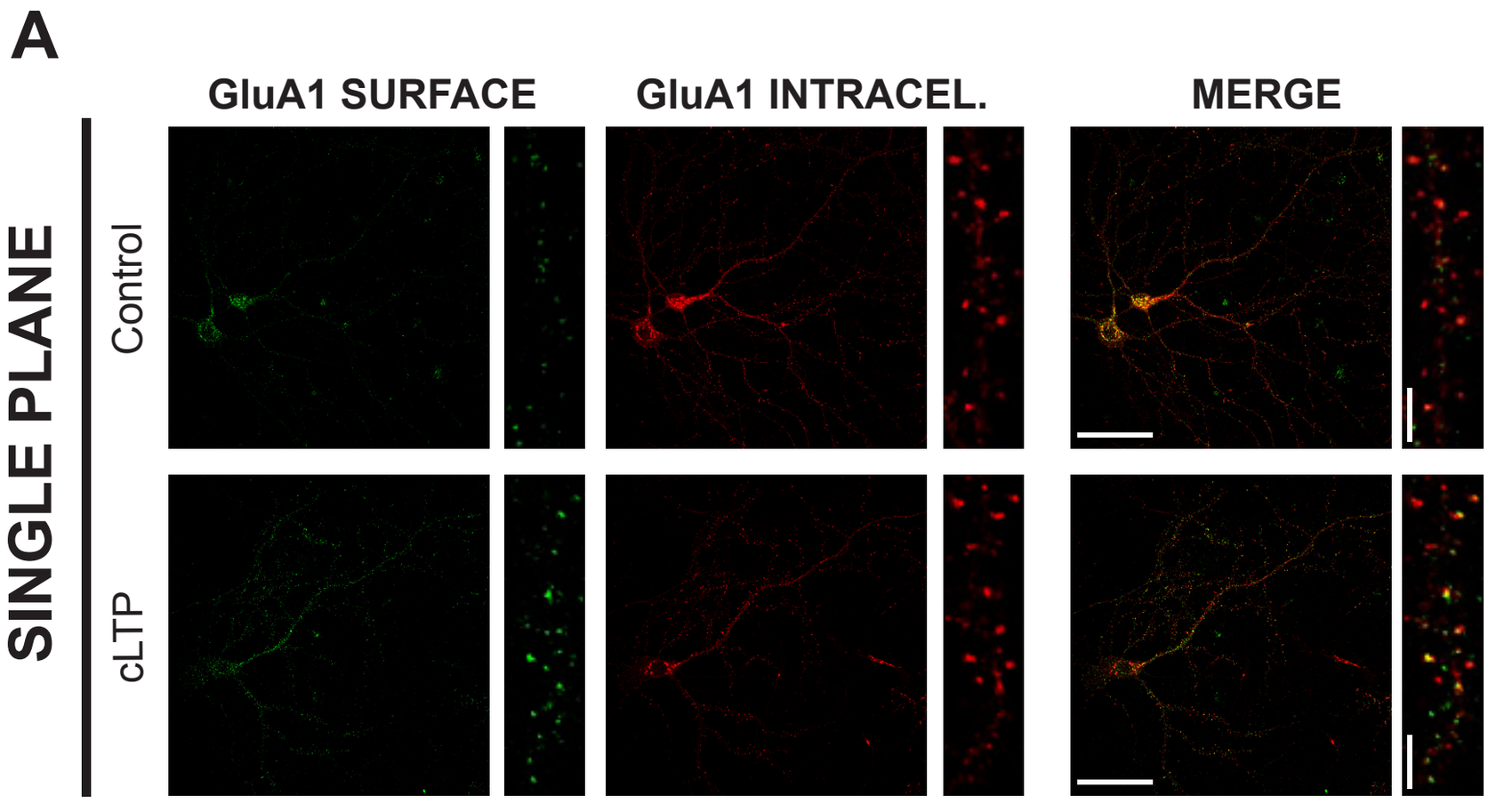
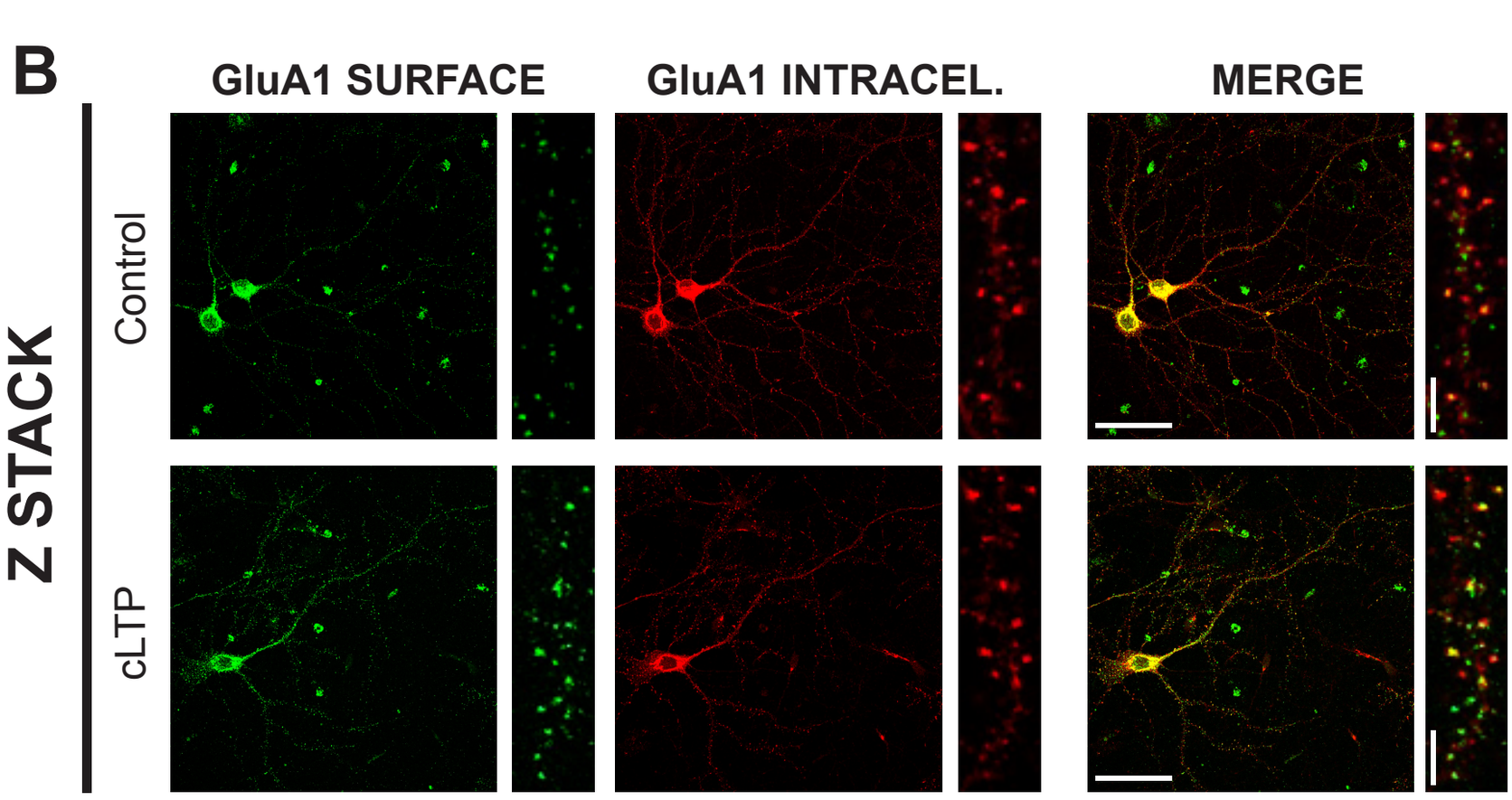
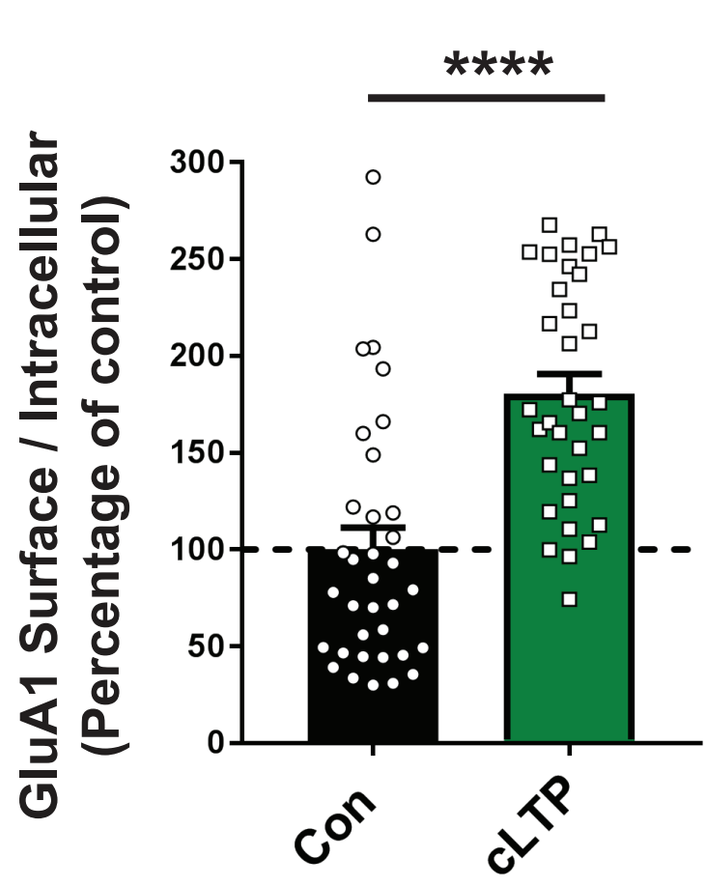


Figure 2







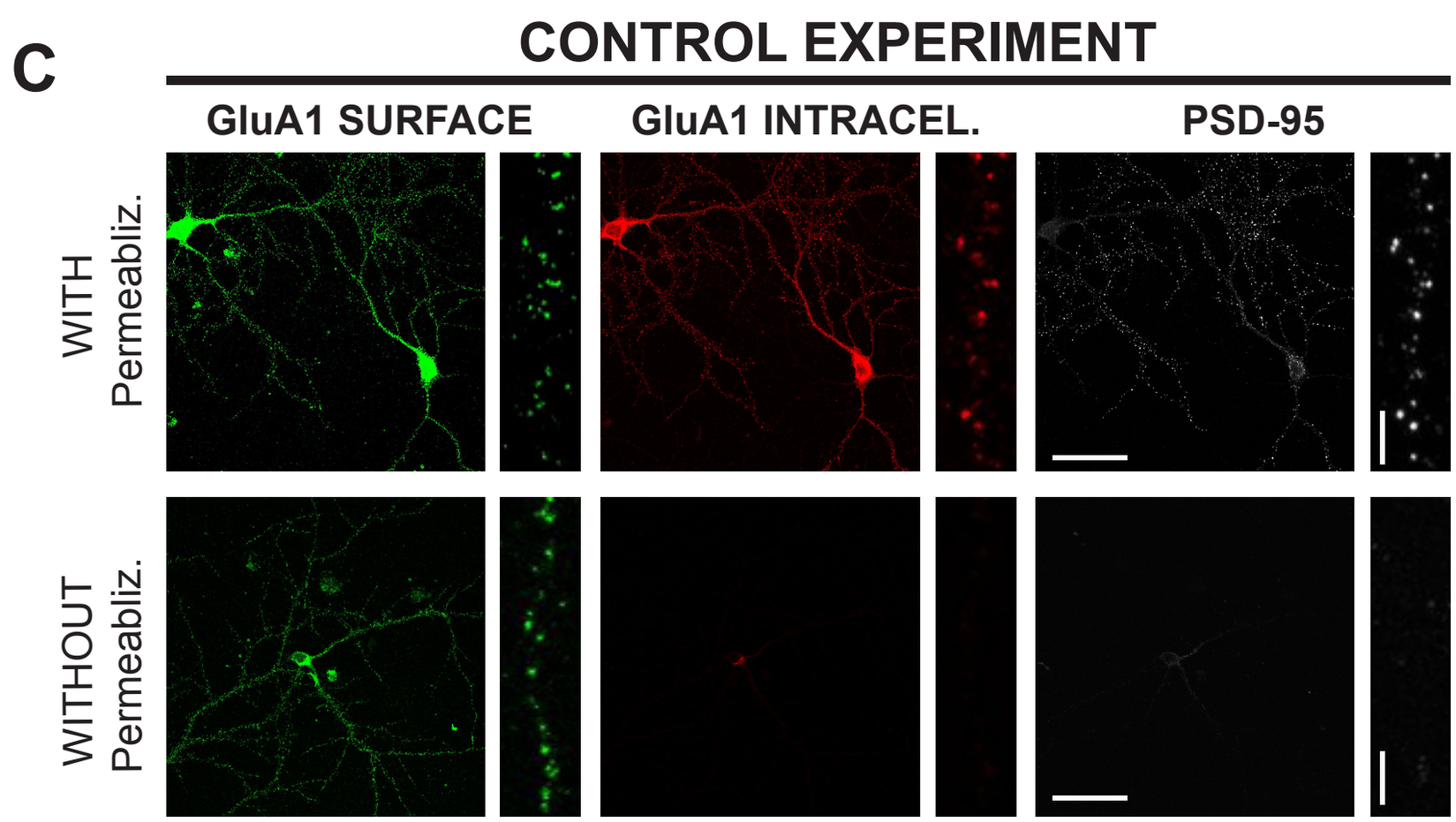
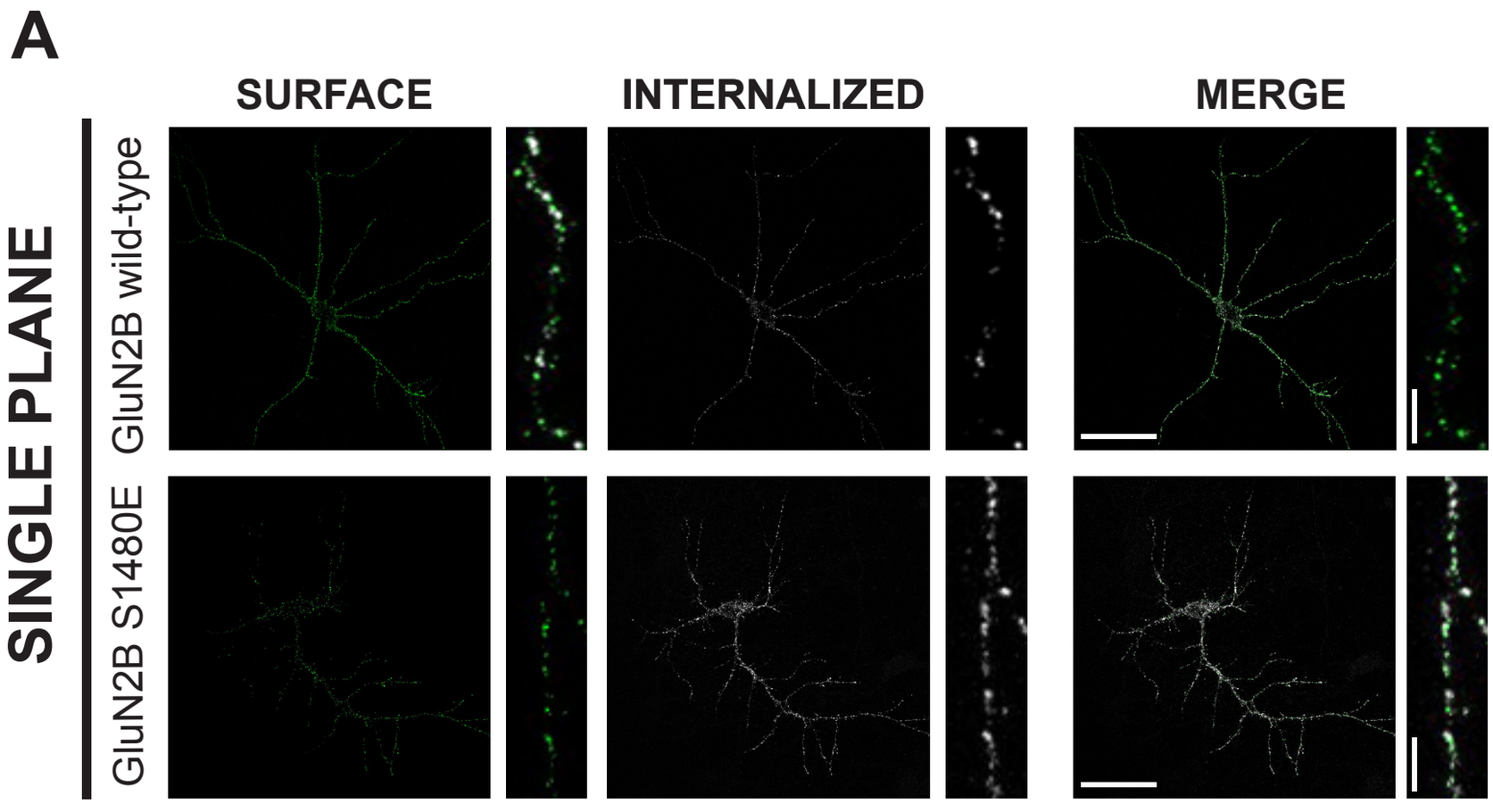


Figure 3



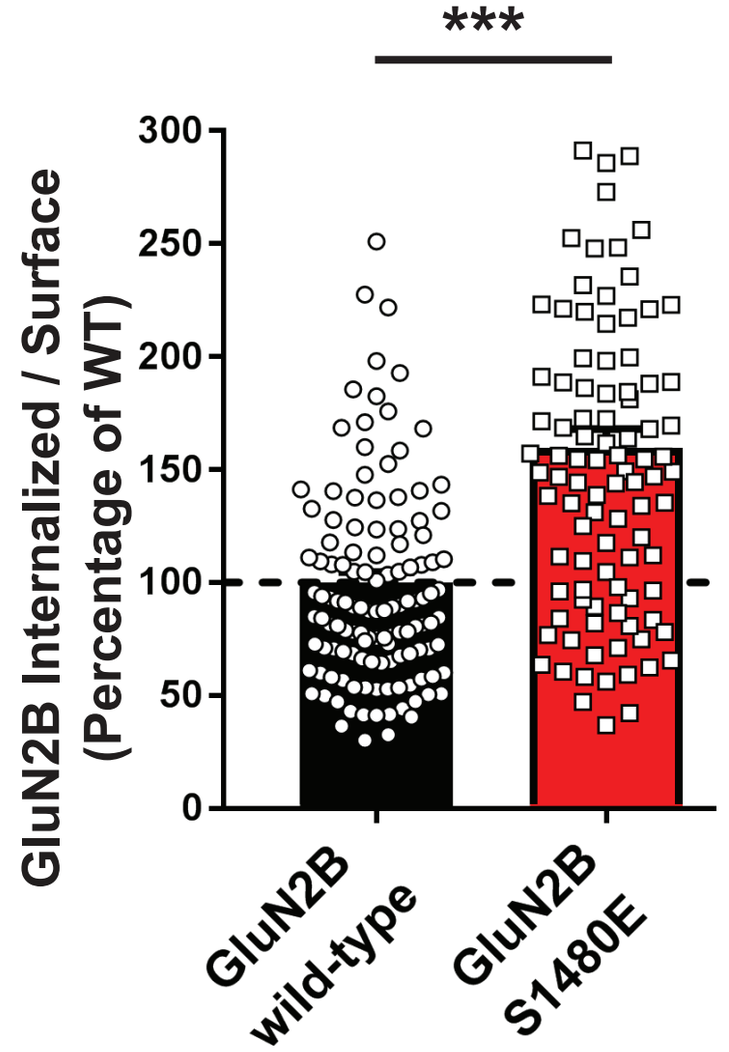
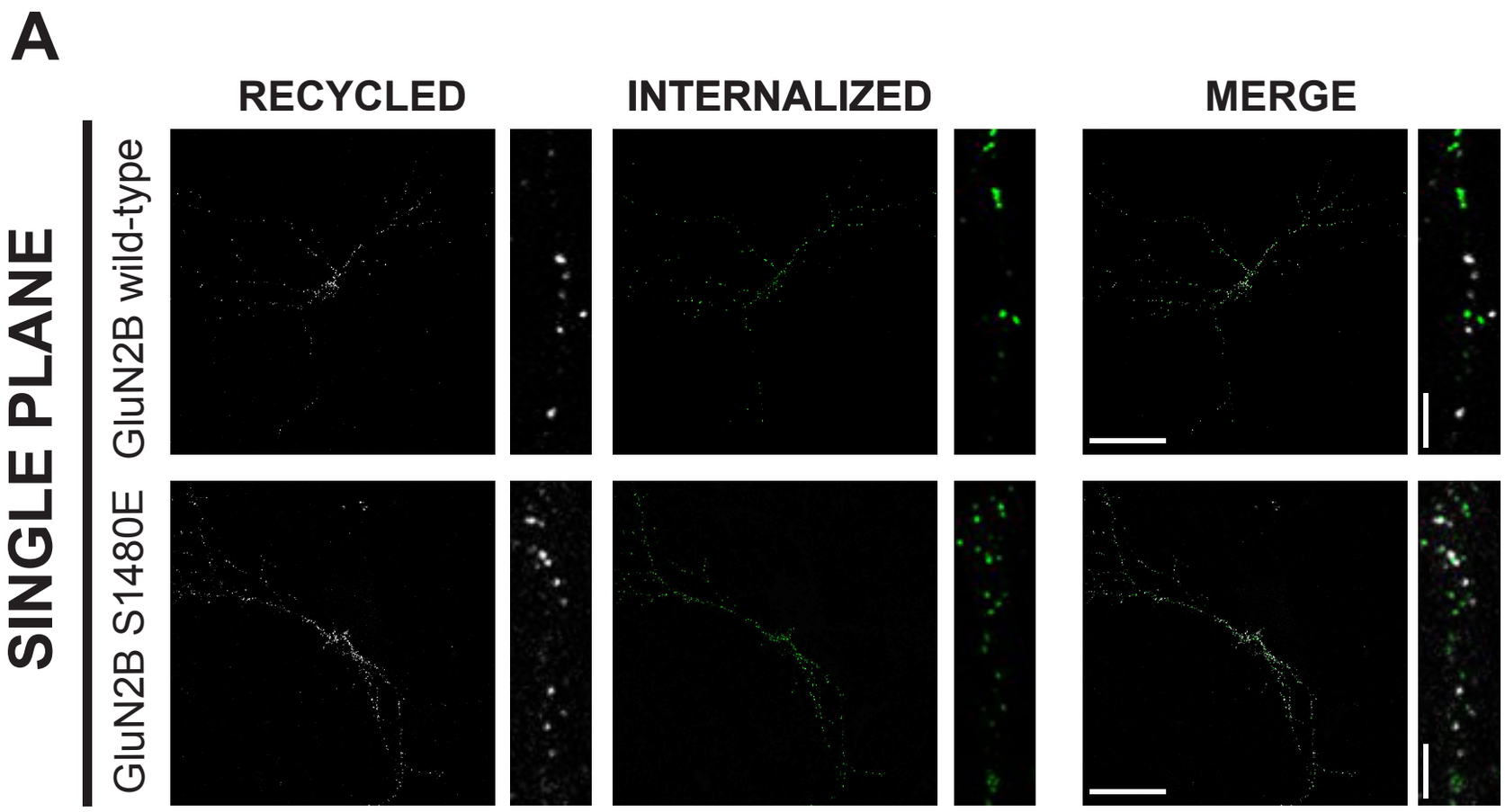
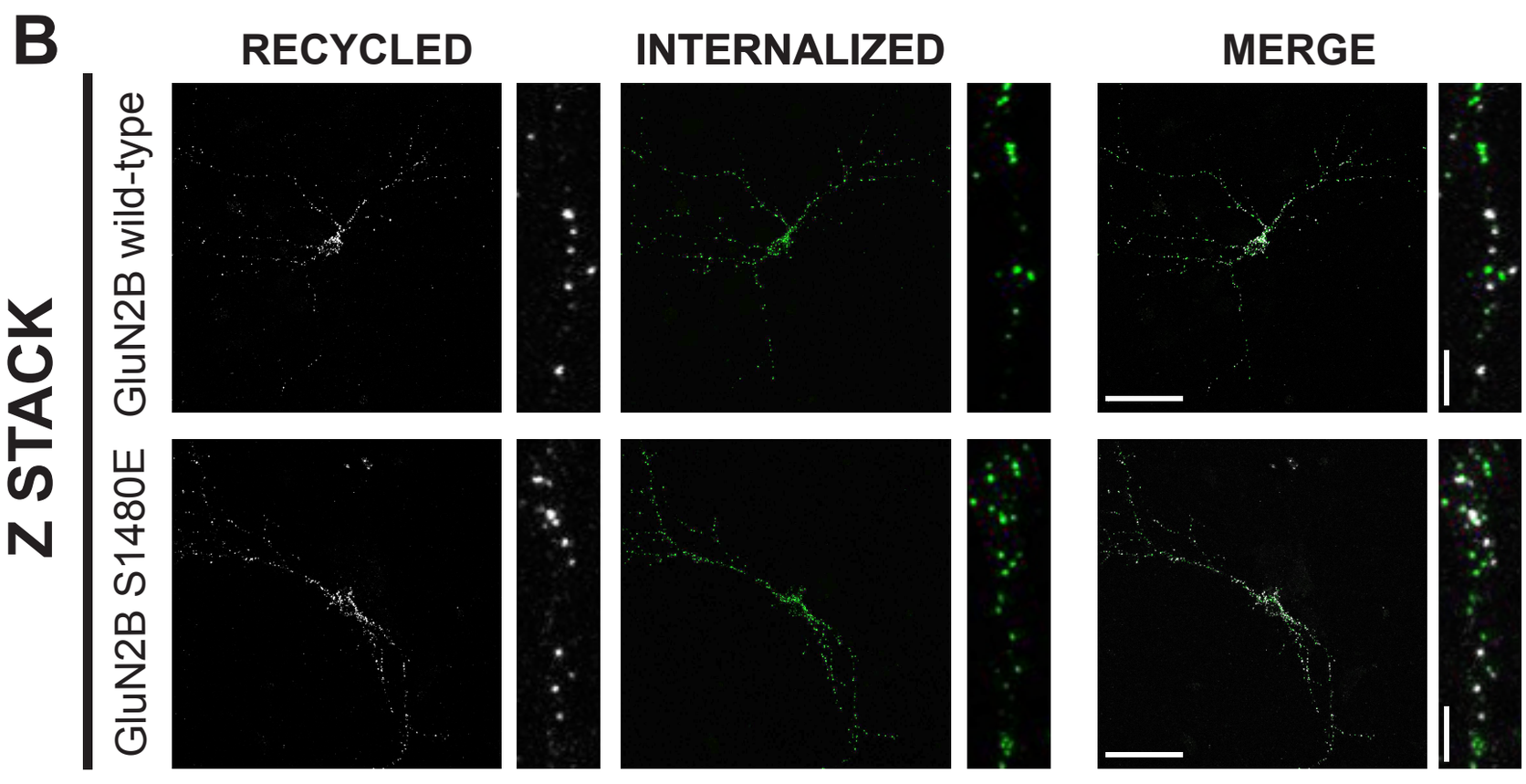
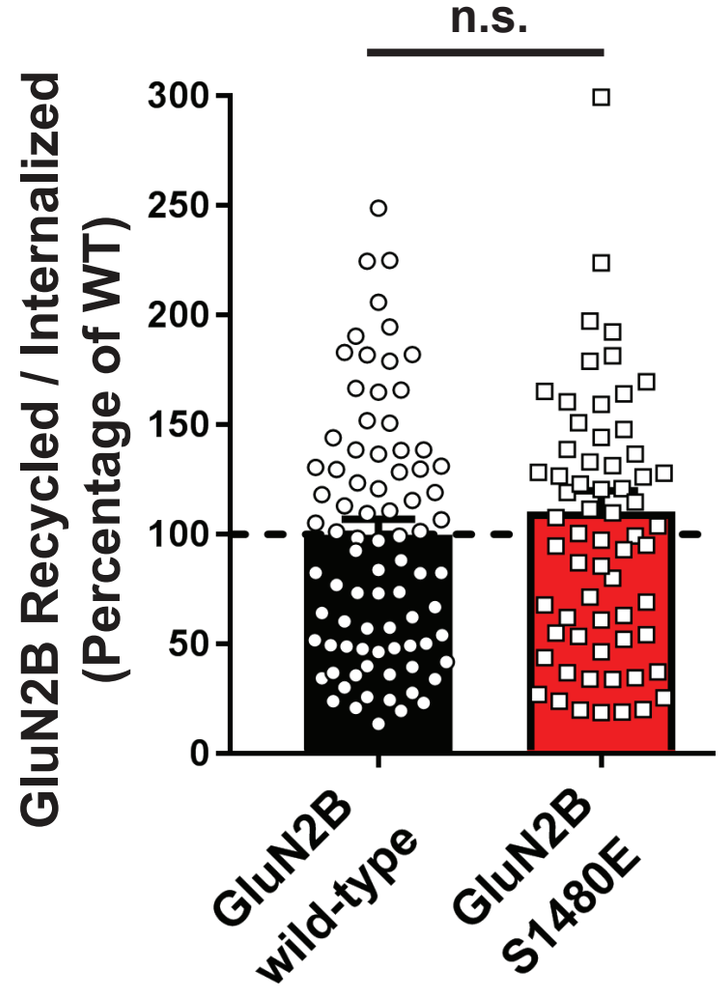
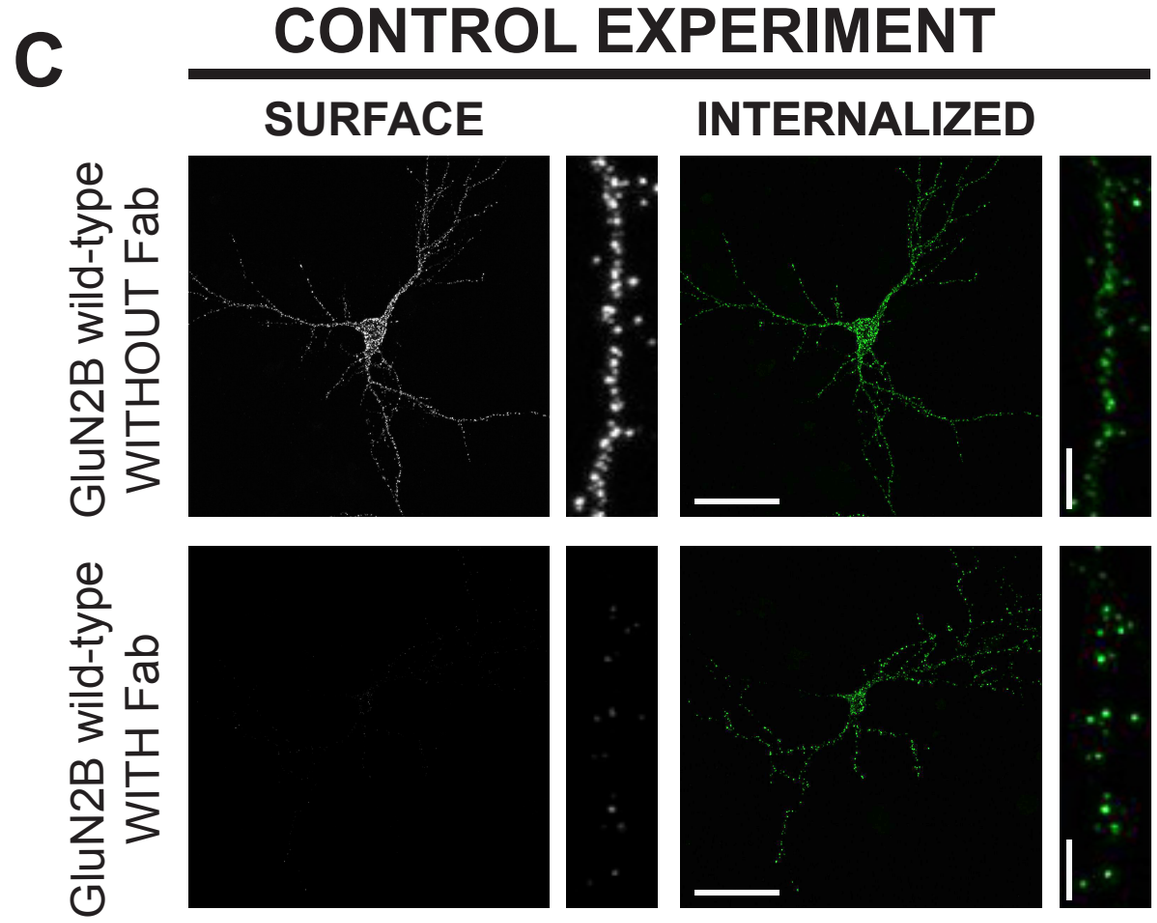


Figure 4









Name of Material/ Equipment	Company	Catalog Number
18 mm dia. #1.5 thick coverglasses	Neuvitro	GG181.5
Alexa 555-conjugated goat anti-mouse secondary	Life Technologies	A21424
Alexa 555-conjugated goat anti-rabbit secondary	Life Technologies	A21429
Alexa 647-conjugated goat anti-mouse secondary	Life Technologies	A21236
Alexa 647-conjugated goat anti-rabbit secondary	Life Technologies	A21245
B27	Gibco	17504044
CaCl2	Sigma	C7902
Corning Costar Flat Bottom Cell Culture Plates	Corning	3513
Dynasore	Tocris	2897
Glucose	Sigma	G8270
Glycine	Tocris	0219
Goat anti-rabbit Fab fragments	Sigma	SAB3700970
HEPES	Sigma	H7006
KCl	Sigma	P9541
L-Glutamine	Sigma	G7513

Lipofectamine 2000	Invitrogen	11668019
Mouse anti-GluA1 antibody	Millipore	MAB2263
NaCl	Sigma	S6546
Neurobasal Media	Gibco	21103049
NGS	Abcam	Ab7481
Parafilm	Bemis	PM999
PBS	Gibco	10010023
Pelco BioWave	Ted Pella	36500
PFA	Alfa Aesar	43368
Picrotoxin	Tocris	1128
Poly-D-lysine hydrobromide	Sigma	P7280
ProLong Gold Antifade Mountant	Life Technologies	P36934
Rabbit anti-GFP antibody	Invitrogen	A11122
Rabbit anti-PSD-95 antibody	Cell Signaling	2507
Strychnine	Tocris	2785
Sucrose	Sigma	S0389
Superfrost plus microscope slides	Fisher	12-550-15
Triton X-100	Sigma	X100
TTX	Tocris	1078

Comments/Description

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: An Antibody feeding approach to study Glutamate Receptor Trafficking in Primary Hippocampal Cultures

Author(s): A.M. Chiu, [redacted] Barse, P. Huballoda, A. Sanz-Clemente

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

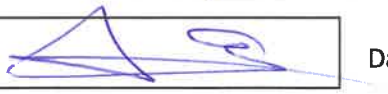
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Antonio Sanz-Clemente
Department:	Pharmacology
Institution:	Feinberg School of Medicine. Northwestern University
Title:	Assistant Professor
Signature:	
Date:	8.10.19

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

We would like to thank all the reviewers for their comprehensive review of our manuscript and their overall positive comments. We believe their thorough feedback has greatly strengthened the quality of our protocol. All the three reviewers have identified two major concerns in our original manuscript: 1) the need of explaining how our image analysis has been conducted and 2) the need of performing control experiments to help to identify successfully completed protocols, rather than just mention them in the protocol. We will address these common concerns first, and we will follow with a point-by-point response to individual concerns.

Common Concerns:

1. Need of explaining image analysis details

We recognize that the analysis of the acquired confocal images is an important component of this protocol and we apologize for not having included it in our original manuscript. We have now added a completely new section within the protocol entitled "Image Analysis" which outlines the general steps that we used for analysis. Briefly, we have quantified "integrated intensity" in selected ROIs within secondary/tertiary dendrites after z-projecting the image stack as maximum intensity projections. Importantly, we have written a macro which can be opened in FIJI to automate the analysis routine. This macro can easily be altered to provide more individualized workflows that are pertinent to an individual experimenter's data.

2. Performing control experiments, rather than merely mentioned them in the text

We agree that control experiments are critical to have confidence in the data obtained and that was our reason for mentioning them in our original submission. We understand that actually performing those controls and showing representative results will strengthen our manuscript and therefore, we have conducted a series of control experiments that can be found in the new figures 2C, 3C, and 4C.

Specifically, we have first skipped the permeabilization step in our "Surface vs. Intracellular" protocol and, additionally, we have used a primary antibody against PSD-95, an intracellular protein. As shown in **Fig 2C**, no signal for PSD-95 or "intracellular GluA1" is obtained in non-permeabilized cells demonstrating i) the integrity of neuronal membranes (i.e., signal for surface-expressed GluA1 does not include intracellular receptors) and ii) all the extracellular epitopes are saturated with the first round of labeling (i.e., signal for intracellular GluA1 does not include surface-expressed receptors).

Then, we performed "internalization" at 4 ºC in a sister culture. Because this trafficking process is abolished (or greatly reduced) at 4 ºC, no signal for internal GluN2B can be obtained under these conditions **(Fig 3C).**

Finally, we have controlled for the complete blockade of the surface-expressed receptors before recycling, by incubating cultures ± Fab. As shown in **Fig 4C,** incubation with Fab eliminated the signal for surface-expressed GluN2B, demonstrating that the "recycling" signal corresponds to actual recycled receptors and not to receptors that have been always present at the cell surface.

Reviewer #1:

Manuscript Summary:
The summary seems OK

Major Concerns:

The authors suggest critical control experiments "to verify that the labeled populations are indeed correct". The authors should present data from these controls to demonstrate that their labelling procedures are indeed appropriate. Since these control experiments are so important, I suggest that they are included in the main protocols, rather than added on to the end.

We have included the controls and representative figures in our protocol. As this is a concern shared by the other reviewers, we have responded in the "Common Concerns" section above.

In protocol II, the authors state, "NOTE: For recycling applications, it may be beneficial to use a lower antibody dilution (in our example, we used anti-GFP 1:500)." The very fact that the authors recommend increasing the antibody concentration for recycling applications suggests that the recommended dilutions are not saturating, and not all the surface antigens are labelled.

The reviewer is absolutely right, and we apologize for the recommendation of increasing the antibody concentration. We have, in fact, repeated this experiment using our "regular" GFP and secondary antibodies dilution (1:1,000 and 1:500, respectively) and observed no difference to neurons incubated with higher concentration. This was expected because, as stated by the reviewer, the "regular" concentrations should saturate all the epitopes available.

The representative images are not convincing. In Figure 2, the surface staining for the control condition shows very strong signal in the cell body. This looks very much like intracellular staining, and appears to precisely overlap with the cell body staining for the intracellular pool. In figure 3, the surface and internalised pools seem to overlap almost entirely for the WT construct. Even at the resolution of the light microscope, receptors in endosomal structures should be clearly distinct from synaptic puncta. Unfortunately, this fuels my concern that the labelling methods are not specific for surface vs internalised pools of receptors.

We apologize for the confusing pictures as we think that the apparent lack of specificity of our staining is a consequence of showing our images as Z-stacks. We Z-stacked our pictures before quantifying the intensity in the different channels (see new section "Image Analysis"), and this projection may give the impression that the "surface signal" is present in the cell body. To clarify this issue, we have now included single plane examples in our Figures (new Fig 2A, 3A, and 4A) to show clearly that the surface and internalized pools are indeed discrete populations.

In addition, our control experiment showed in the new Fig 2C, clearly demonstrates that our manipulations selectively label surface-expressed or intracellular receptors.

I do not agree that compressed z-stacks are the right way to image these cells. The separate pools of surface and internalised receptors are more easily distinguished in a single confocal plane.

Indeed, we acquired our images as single-planes using 0.35 um steps (as previously mentioned, one of these single planes have been added to our figures as they allow for clear identification of the two populations, as stated by the reviewer). However, as we are interested in quantifying both pools within a single dendrite, z-stacks allow for the summation of these populations so as not to skew that data towards one population vs the other, which can occur with single plane analysis.

There is no mention of how the images are analysed; how are cell outlines/regions of interest defined? Is the whole cell analysed or just dendrites? If just dendrites, what length of dendrite?

As stated in our "Common Concerns" section above, we have updated the analysis information. We try to capture the full length of a secondary or tertiary dendrite.

The use of Dynasore will obviously only block dynamin-dependent endocytosis. It has been demonstrated that AMPARs can also be internalised by dynamin-independent mechanisms (Glebov et al., J.Neurosci 35:4830-6 (2015); Zheng et al., eLife 4:e06878 (2015)).

The reviewer is correct as Dynasore exclusively blocks dynamin-dependent internalization processes. We have added a note in the text to clarify this fact, although please note that we have used Dynasore to prevent the internalization of GluN2B subunit of NMDARs, a process that, as we and others have demonstrated, is dynamin-dependent: interaction between the clathrin adaptor AP-2 and the YEKL endocytic motif present at the end of the C-terminal of GluN2B.

Minor Concerns:

I'm surprised the authors do not include a step to quench residual PFA reactivity, with ammonium chloride or glycine solution. The absence of this step might lead to non-specific antibody binding.

The reviewer is correct, and we have added this option as note within the text. In our particular experiment, we observe minimal non-specific binding even without washing with glycine but, of course, this may help with troubleshooting for other antibodies and we appreciate reviewer's suggestion.

For figures 2-4, the two channels should also be shown in a merged image (as well as the separate images as are currently shown).

Merged images have been added to Figures 2, 3 and 4.

Reviewer #2:

Manuscript Summary:
This methods paper by Sanz-Clemente and colleagues is a nice description of imaging techniques to quantify surface and intracellular receptors and further show internalization and recycling of membrane receptors. This approach strengthens existing approaches by allowing for measurement of signal intensity for receptors that have internalized or recycled. Overall this is well written and easy to read and the protocol is very well described and clear.

We thank review #2 for his/her feedback and are pleased that he/she find our manuscript "well written and easy to read" and that "the protocol is very well described and clear."

Major Concerns:

I believe The addition of some of the controls that they discuss in the manuscript would strengthen the manuscript. Specifically, perform experimental controls and show the control data for point 2 and point 3 under controls. This will show that when permeabilization does not occur there is no labeling of intracellular receptors and that the Fab can block all unlabeled extracellular receptors.

This is a shared concern between the three reviewers and we have now shown these controls in our revised figures. Please see "Common Concerns" section at the beginning of this document for details.

Additional details on how the quantitation was performed. Is this total intensity? If so per what length? Is it number of puncta? More information on the data analysis would help with using this paradigm.

Again, this concern has been raised by the other reviewers. Quantification details have been now added to the revised manuscript ("Image Analysis" section). A more detailed response to this concern can be found in the "Common Concerns" section above but, briefly, we quantify total intensity in secondary/tertiary dendrites. As we present our data as a ratio between surface/internal receptors, we do not need to control for length or area of the ROI.

Reviewer #3:

Manuscript Summary:
The authors describe a protocol describing the immunofluorescence analysis of GluRs trafficking, with potential use for the study of other membrane-assocaited proteins. This is a very interesting protocol that allows to address a critical aspect of synaptic plasticity mechanisms, namely the dynamics of neurotransmitter receptors underlying synaptic plasticity processes. While some reports describe the use of this protocol, to my knowledge this protocol is not visually available. It is a very well described protocol with clear illustrative exemples, that will facilitate the incorporation of this technique to many different laboratories. Indeed, compared to live imaging methods, the protocol presented here can be generally implemented in cell biology laboratories and complement electrophysiological and biochemical studies. The protocol is very well described, and only a major concern (the need to describe image analysis, for s quantitative measurement) and minor concerns are stated, as follows.

We would like to thank reviewer #3 for his/her comprehensive evaluation of our manuscript. We are glad that the reviewer finds our manuscript to be a "very well described protocol with clear illustrative examples" and that the reviewer believes that this manuscript "will facilitate the incorporation of this technique to many different laboratories," as this is our goal in publishing this protocol.

Major Concerns:

The authors should include Ia description of the image analysis / software used, to provide a complete protocol for the quantification of GluR density in the different subcellular compartments. Please include a description of clusters / particles analysis, indicating the software used (ImageJ or alternative software), the need to setup and fix the confocal parameters for each set of experiments. Since this technique is highly-sensitive and depends on neuronal density (experiment-dependent), please describe the need to use internal controls (normalisation with wt conditions, etc).

The need for an explanation of our analysis protocol and the addition of controls are the two concerns shared by all the reviewers. We have responded to these thoughtful suggestions at the beginning of this document ("Common Concern" section). As stated there, we have added details on quantification in our revised manuscript and provided a macro to help others analyze their images. As mentioned, we have emphasized the importance of setting parameters for each experimental data set and the need for normalization to controls.

Minor Concerns:

Please find below a list of minor concerns that might help to clarify some technical tips and to define more clearly the different aspects treated along this interesting protocol.

Thank you for identifying these areas that improve the quality of our manuscript.

Line95: Please specify that this protocol allows the labelling of "specific GluR subtypes density and trafficking", upon antibodies availability and specificity.

We have added this clarification

Line 104. Please refer about the potential use of other coating reagents.

This clarification has been added.

Line108. Should Glutamax be used, in spite of L-Glutamine? Different groups apply Ara-C to the medium, to avoid glial proliferation. Please clarify if Ara-C is required or is optional.

We have added this information to the protocol.

Line141. Please reformulate: GluA1 subunit surface recruitment is used as a cellular readout of AMPARs recruitment. Obviously, this phenomenon is associated with an increased synaptic efficacy, but being strict, IF analysis reflects the surface incorporation of AMPARs. Gly-chemLTP protocol has been particularly originally described by Lu et al., 2001 (Neuron, Vol. 29, 243-254, January, 20019). This important paper describes the rationale of the drugs used along Gly-chemLTP protocol, and so the reference should be included.

The reviewer is correct and we have eliminated our sentence about "synaptic potentiation". Similarly, we have included the mentioned reference in our revised manuscript, and we thank for the thoughtful indications.

Line 143. Please define the control conditions (ECS without glycine) that must be run in parallel.

The text has been clarified.

Line166. Shall manual aspiration (using a micropipette) be more suitable, to avoid cellular stretch that might affect GluRs surface mobility?

We have added a note on aspiration to the text.

Line182. Please add a note on the potential use of frozen PFA.

A note has been added to the text.

III Surface expression

1.2 Please add a note on the potential use of frozen PFA

See above.

1.3 and 1.6, please define the washing time (5-10 min each wash?)

Although most of the protocols available for ICC suggest 5-10 min for washing time, we have found no differences using 3 quick washes with PBS (similar background, signal intensity, etc.) Nevertheless, we have added a note to our protocol stating the possibility of performing longer washes if needed.

2.3 For the comparative analysis of surface to total GluRs expression, are the authors using a different dilution of primary antibodies (agains GFP)?

We used the same antibody dilution for surface and intracellular. This has been clarified.

VI. Mounting and imaging of samples
1. Please state that the cover slip should be smoothly left upside down, on a given volume of mounting medium (10ul for a 12mm coverslip), to prevent damaging the fine dendritic processes.

We have added this helpful information.

2. The recruitment of AMPARs is dependent on the dendritic process analized. Indeed, for Gly-chemLTP many different groups focus on the analysis of secondary and tertiary dendritic processes, that provide a higher dynamic range to detect GluA1 surface insertion. Please clarify this issue.

Thank you for pointing this out. We have emphasized that we examine secondary and tertiary dendrites in our new “Image Analysis” section.

Line 343: please use a consistent terminology for the amino acids (Ser vs. Glutamate) and the term "phosphomimetic" (sometimes an hyphen is used throughout the text)

Thank you for identifying this issue. We have made appropriate alterations to the text.

Line 399: the authors mentioned that the experiments were conducted at DIV15-16 (line 133), but it is not clear whether this developmental stage was used for the different applications. Please clarify the optimal time-points for the different applications.

This developmental information has been added to the manuscript. We used DIV21 neurons for cTLP and DIV15/16 for internalization/recycling of overexpressed GluN2B.

Discussion. Live imaging methods: Quantum dots allow the monitoring of GluRs surface mobility in living cells / tissue, and its use provides interesting insights in the field. Although this technique requires a complex technical setup and expertise (in this sense, the use of the antibody-feeding technique has a much broader use), it is noteworthy to mention in the discussion section.

Thank you for suggesting this. We have added this to our section on microscopy techniques.

Figures: The figures are very illustrative of the different protocol modalities. Regarding the graphs, the authors should consider whether a Scatter dot representation could provide a more visual representation of data distribution.

We have added scatter plots to our graphs to help to visualize the distribution of the data.

Click here to access/download

Supplemental Coding Files

Sanz-Clemente.ijm