Response to reviewers

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A high-content assay for monitoring AMPA receptor trafficking

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We thank the reviewers and editor for their helpful comments and suggestions to improve and strengthen this manuscript. To address concerns raised by the reviewers, we now include corrections and clarification in our revised version. These changes are summarized here and described in detail below (original reviewer comments in bold).

**Reviewers' comments:**  
  
**Reviewer #1:**  
**1. In the last part of the "2. Measuring AMPA Receptor Trafficking in Response to DHPG" section, (line 172) they described "2.7. Incubate the microplate at room temperature for 15 minutes". People would like to know whether the incubation was done in the CO2 incubator or not, since they used Neurobasal Medium whose pH is neutral at 5% CO2.**We have clarified this in the text (see below). Alterations in Neurobasal media pH in the absence of 5% CO2 could change overall neuron health. However, given the duration and requirement of multiple media changes for this specific trafficking assay, we opted to use Neurobasal media for the antibody incubation step in order to avoid the excessive buffer/media changes to the mature primary neurons, which could compromise their health more dramatically than the single room temperature incubation. Throughout the duration of this assay, we visually inspected neurons at every stage to ensure that they remained healthy. Note that if one is using this assay to measure surface receptors only, then exchanging the Neurobasal medium with DPBS, which maintains physiological pH at room temperature would be the preferred method.

Lines 175-176 edited: 2.7. Incubate the microplate at room temperature for 15 minutes. Incubation in 5% CO2 is preferred, but not necessary.

**2. In the "3. Immunolabelling" 3.6 section (line193) and 3.7 (line 196), the CO2 concentration and temperature should be described.**We have clarified this information in the text.

Lines 196-197 edited: 3.6. Remove existing media in wells. Add 100 µl/well of the 100 µM DHPG stock solution from Step 3.5. Incubate the microplate in the incubator at 37°C in 5% CO2 for 10 min.

Lines 199-200 edited: 3.7. Remove the DHPG solution from step 3.6 and add 100 µl/well Neuronal medium. Repeat this step one more time. Place the microplate in the incubator at 37°C in 5% CO2 for 5 min.

**3. In the 3.10 section, the composition of blocking buffer should be shown.**The blocking buffer is proprietary, however most likely contains fish gelatin. As per journal style, we have referred to this buffer in the Table of Materials section.

Lines 212-214 edited: 3.10. Remove DPBS from step 3.9. Add 150 µl/well blocking buffer (a ready to use formulation in TBS, refer to Table of Materials) and incubate at room temperature for 90 min. Alternatively, incubate overnight at 4oC.  
  
**Reviewer #2:**  
**1. In the introduction, there seems to be some confusion between a PDZ ligand and a PDZ domain. AMPAR subunits have PDZ ligands, which bind to PDZ domains in a number of proteins involved in scaffolding/trafficking. However, the introduction states AMPAR subunits have PDZ domains, which is incorrect. It is also worth noting that there are splice variants of each AMPAR subunit that have PDZ ligands - GluA1 has a class I PDZ ligand, while GluA2, 3 and 4 have class II PDZ ligands.**

We have corrected this statement below...

Lines 75-78 edited: GluA1 and GluA4 subunit-containing AMPA receptors are particularly apt to being trafficked to the cell surface during LTP, in part due to the presence of their PDZ ligands, ~90 amino acid sequences that promote membrane anchoring via interactions with various PDZ domain-containing proteins.  
  
**2. In section 1.4 of the protocol, it would be useful to state how much feeding media is added to the cells when they are initially fed - is removing 100ul of the conditioned media taking half off, for example, or all of it?**Lines 147-150 edited: 1.4. Feed neurons as previously described36 by removing half (100 µl) of the pre-existing media (referred to as conditioned media, which contains constituents that support neuron survival) from each well and replacing with 100 µl of 37oC prewarmed media prepared in step 1.3 every 3-4 days. Store the conditioned media from each feeding at 4˚C for step 2.3.

**Editorial comments:**  
**1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

We have thoroughly proofread the manuscript for spelling and grammatical errors.

**2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”**

We have re-used data in Figures 2 and 3 from our previous published manuscript. The link to the editorial policy that allows re-prints can be found on the following website (<https://www.elsevier.com/about/policies/copyright/permissions>). Note that this article is published as Open Access and is not protected by copyright. We have also uploaded this information as a .doc file.  
  
**4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Neurobasal, IRDye, Odyssey, Excel, etc.**

The word “NeurobasalTM” was replaced by “Neuronal” and are referenced, when appropriate in the Table of Materials and Reagents. The commercial language of IRDye®, Odyssey®, and Excel have been removed from manuscript.

**5. 1.3: Please provide composition of Neurobasal Media. If they are purchased, please cite the Table of Materials.**

Lines 143-145 edited: 1.3. Prepare media for feeding the neurons by adding 10 ml of 50 X B-27, 5 ml of 100X Glutamine, 242 µl of 10 mg/ml 5-Fluoro-2′-deoxyuridine (FUDR) and 10 µl of 10 mg/ml Gentamycin to 485 ml of Neuronal Media (refer to Table of Materials).

**6. 1.4: What is conditioned media?**

We have clarified the term “conditioned media”…

Lines 147-150 edited: 1.4. Feed neurons as previously described36 by removing half (100 µl) of the pre-existing media (referred to as conditioned media, which contains constituents that support neuron survival) from each well and replacing with 100 µl of 37oC prewarmed media prepared in step 1.3 every 3-4 days. Store the conditioned media from each feeding at 4˚C for step 2.3.

**7. 2.1: Please list an approximate volume to prepare TTX.**

Lines 154-155 edited: 2.1. At Day *In-Vitro* (DIV) 14, prepare a 500 µl stock solution of Tetrodotoxin (TTX) at a concentration of 2 mM using cell culture grade water.

**8. 2.4: Please specify the incubation temperature.**

Lines 166-167 edited: 2.4. Treat neurons with 100 µl/well of the 2 µM Solution of TTX from step 2.3. Place the microplate in a 5% CO2 incubator at 37°C for 4 hr.

**12. Please number the figures in the sequence in which you refer to them in the manuscript text.**

We have referenced Figure 1 in the Protocol section (line 133). Figures 2 and 3 are discussed in the Representative Results section.

**13. Please discuss all figures in the Representative Results. However for figures showing the experimental set-up, please reference them in the Protocol.**

As stated above, we have referenced Figure 1 is referenced in the Protocol section (line 133). Figures 2 and 3 are discussed in the Representative Results section.

**14. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.**

Lot numbers of antibodies have been added to the Table of Materials.

**15. References: Please do not abbreviate journal titles.**As requested, we have spelled out all journal titles in the reference section.