**Rebuttal Document**

**Editorial comments:**  
Changes to be made by the author(s) regarding the manuscript:

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

2. Please note that Open Access is checked in the uploaded ALA, while in the Questionnaire Responses Standard Access is selected. Please be consistent.

We wish to choose standard access. We were not able to correct this when uploading.

3. Please revise the title to avoid the use of colon.

Revised to ‘Real time live cell flow cytometry to investigate calcium influx, pore formation and phagocytic function of P2X7 receptors in adult neural progenitor cells’

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: NeuroCult, TrypLE, Fluo-8, Cytek Biosciences, Microsoft Excel, etc.

All commercial language has been removed. However, the authors would like to confirm if ‘TrypLE’ should be removed as commercial language. There are many different examples of dissociation reagents and we would prefer to be specific as the reagent used can have significant impacts on the resulting cultures. We are able to find numerous examples in JoVE where TrypLE is described, as well as other common dissociation reagents such as Accutase and Dispase. The brand names of TrypLE (Gibco or Thermofisher) have obviously been excluded.

Please advise.

5. Please spell out each abbreviation the first time it is used.

Abbreviations have been defined.

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Steps have been significantly revised to read in the imperative tense. The phrases ‘should be’ etc have been removed. Notes have been revised and either moved to discussion or included as their own method step. Safety information has been added where necessary.

7. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Notes have been revised and either moved to discussion or included as their own method step.

8. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Steps revised significantly

9. 1.2: Please specify the age, gender and strain of mice.

This information has been added

10. 1.3.8: Please specify what medium is added.

Culture medium has been specified

11. Please include single-line spaces between all paragraphs, headings, steps, etc.

Spaces added everywhere.

12. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

13. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

14. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Steps for video have been highlighted.

15. Figures 2-4: Please indicate the time unit for the x-axis.

This has been corrected

16. Discussion: Please move lines 357-369 to the Discussion section. Please also discuss critical steps within the protocol.

Done. Some crucial steps have been added – though these were mainly the Notes that have been moved from the method to the discussion.

17. References: Please do not abbreviate journal titles.

This has been corrected

18. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Done

**Reviewers' comments:**  
  
**Reviewer #1:**  
  
Manuscript Summary:  
The authors present a video script demonstrating time-resolved FACS measurements of P2X7 receptor-dependent changes of the intracellular Ca2+ concentration ([Ca2+]i), ethidium fluorescence and latex bead bead phagocytosis. It is potentially an interesting project but several questions remain.  
  
Major Concerns:  
I am not aware if there are already videos published showing time-resolved FACS measurements. In life science it is a rather standard method, especially the measurement of [Ca2+]i and the uptake of fluorescent dyes.

To the best of our knowledge, no video has been published showing time-resolved FACS measurements. Our manuscript with detailed protocol and video would contribute to this gap in the literature. In addition, there are very few reports detailing the use of adult neural progenitor cells for functional P2X7 analysis. This manuscript details their culture and use for P2X7 research, and hopefully will lead to further research being conducted in this specific field.

The kinetics of [Ca2+]i does not reflect the kinetics of receptor activation. The latter is much faster (within about 10 ms). [Ca2+]i change rather reflects the integral of the Ca2+ influx and is additionally dependent on membrane potential, buffering and other Ca2+ transport processes. A [Ca2+]i increase can even be absent although P2X7 receptors have been activated (see Loehn et al Cell calcium 29, Pippel et al. Cell Calcium 57).

One paragraph has been added in the Discussion to address this concern.

Furthermore, in several cells (as mentioned by the authors) P2Y receptors which release Ca from intracellular stores may become activated by ATP too. It should be mentioned how P2X7- and P2Y-dependent Ca2+ signals can be differentiated.

One paragraph has been added in the Discussion.

Is it verified that the introduced phagocytosis assay really reflects phagocytosis and not binding of the beads to the cells? The very fast increase of bead fluorescence under control conditions after addition of the beads is suspicious.

The nature of this real-time bead uptake method as a measurement of phagocytosis has been extensively studied. We found that bead uptake was completely abolished at low temperature (4oC), at which phagocytosis is completely blocked. This indicates there is little adhesion of beads to cells in such constantly stirred condition. We have also found that 5-10% serum is able to completely block bead uptake at 37oC in both a stirred condition and a static condition under microscopy (Gu et al, Blood, 2010; J Biol Chem, 2012). Thus, it is unlikely the rapidly increased cellular fluorescence intensity is due to nonspecific binding of beads to the cell. In addition, inhibitors for phagocytosis, e.g. cytochalasin D and Latrunculin A, are used routinely to assess nonspecific binding. This is the method we have been applied for many years and we are very happy to share our knowledge with other researchers.

Minor Concerns:  
What is the idea behind the complicated preparation of the P2X7 expressing neural progenitor cells? Why haven't macrophages been used? They are much easier to be prepared.

This manuscript aims to provide researchers the means to continue P2X7 research in adult neural progenitor cells, which have very different applications and clinical implications to macrophages. Macrophages may be substituted if the sole purpose of the research is to examine P2X7 receptor activity, though in macrophages this has already been established. The field is shifting to explore the function of these receptors in other cell types, and subsequently their potential as a therapeutic target.

In Figures 2 to 4, the time unit is not shown. Which extracellular solutions were used?

Unit has been added. The extracellular solutions are detailed in the method. Na2+ medium is used for the phagocytosis assays, while K+ medium is used for calcium and pore formation assays.

How is the application of definite concentrations of agonists or antagonists managed? The substances always become diluted by the amount of water in the cell suspension to which they are added.

The volume in the FACS tube is known, and the exact volume of a stock to add to a known volume to end up with the desired final concentration was calculated (C1V1 formula).

All abbreviations must be explained.

Addressed

Figure 3: Ethidium uptake takes place at 300 µM ATP, and not only at 500 and 1000 µM. 3B demonstrates concentration dependence and not does dependence.

The ethidium uptake occurring at 300 µM is not statistically significant. As JoVE required that representative results only are shown, repeats and data analysis calculations have not been included.

Fig. 4: Why oxATP was used as blocker (which may be harmful to cells) and not the more "modern" blockers like AZ10606120?

OxATP is the only inhibitor of P2X7 receptor which not only irreversibly blocks P2X7 channel/pore function but also inhibits its phagocytic function.

P6 L290: To determine significance between treatments, take an average of the fluorescence intensities over the final 10-20 seconds". This is an unclear statement.

Statement has been reworded

P1 L87: "Plate reader methods, which are rapid and allow for up-scale, do not allow for the observation of kinetics." Why not?

Most of plate readers do not have the adapter for agonist injection, constantly stirring and temperature control which are essential for real-time measurement. Additionally, the high background of each well can make it difficult to measure small changes.

**Reviewer #2:**  
  
Manuscript Summary:  
  
The technique proposed by the authors is quite simple, with an excellent cost benefit, although other works have even used flow cytometry to show the functionality of P2X7 receptor, especially in ethidium bromide incorporation or calcium signaling assays. However, none used in a combined way to show the three properties of the P2X7 receptor live., becoming a good tool for the study of this receptor in different cellular models.  
  
The methodology of both neurospheres culture and preparation for flow cytometry is well detailed, but some informations are missing as stated below.  
  
Minor Concerns:  
  
1) During calcium influx assay, the authors used 1 mM of ATP or 300 μM BzATP which are known to be a high concentration of agonists and can induce pore formation. How could the authors guarantee that calcium influx is being mediated by P2X7 channel opening but not by pore formation? What would happen in calcium influx if lower concentrations of ATP and/or BzATP was used?

The P2X7 channel dilates in 20-30 sec in the presence of its agonist ATP or BzATP. Calcium influx can go through both the channel and pore formed by P2X7 receptors. Currently, there is no method which can separate the two processes. In practice, we only take consideration of the first 20 sec of calcium influx curve following the addition of ATP as an estimate of P2X7 channel function.

We have additionally observed calcium influx by live cell microscopy following applications of ATP as low as 0.1 µM, though as this additional method is not included here and is beyond the scope of this manuscript.

2) Please describe better YG latex beads: what is best particle size? Is there any specification for the beads? Are they diluted as manufacturer's protocol?

This information is provided in the protocol (1 µm beads are used). Optimal size of the latex bead was previously established and this reference is provided. Specifications (brand name) is provided in the table of materials.

3) I did not understand the NOTE in item 5.5: which is phagocyte:target ratio? The protocol said that was 1 x 10⁶ cells/mL + 5 μL of 1 μm YG latex beads, isn't it?

This comment has been revised. For adult neural progenitor cells, 5 µL of YG beads is adequate, though this may need to be adjusted in other cell types.

4) Please, include the parameters of FACSCalibur Configuration and Settings such as filters, lasers and channels. Was done any compensation?

As JoVE is not able to publish brand names, the term ‘FACS Calibur’ has been removed, and discussion of the specifics of the flow machine is not applicable.

For the reviewers information, FACSCalibur comes with two lasers: 488nm blue laser and 635nm red diode laser. The voltage settings in different machine may differ. Our three methods only describe single colour usage therefore no compensation is needed. Ca2+ influx and YG bead uptake signals are collected in the FL1 (488) channel and ethidium uptake is collected in the FL2 channel, all in linear mode.

For Ca2+ influx: The FL3 voltage is adjusted to give a linear mean channel fluorescence intensity of ~700 for the gated population. No compensation is required between FL1 and FL3.

For ethidium uptake: The FL2 voltage is set at around 595V with a gain of 5.0, at which the linear mean channel fluorescence intensity for Quantum PE standard beads with MESF 300747 is 48±1 (256 linear scale) and the peak channel for right reference standard PE high level beads (MESF ~560,000) is 100±1 (256 linear scale). The compensation of FL1-FL2 and FL2-FL1 is 7% and 8% respectively.

For YG beads uptake: Linear MFI of YG fluorescence is collected in FL1 (voltage: 380-420, gain: 2.0).

5) Please specify more about the use of Time Zero Module (Cytek Biosciences).

As JoVE is not able to publish brand names, the term ‘Time Zero Module’ has been removed, and discussion of the specifics of is not applicable. A general description has been added to the introduction.

For the reviewers information:

Time Zero Module (Cytek, <https://www.cytebio.com>) and a circulating water bath device allows precise temperature control and stirring of cells suspension to which a stimulus is delivered within one second, allowing uninterrupted measurement of cellular response. It consists of two modules, the Time Zero module with water-jacket tube holder and the Air Supply module. An additional circulating water bath is also needed if temperature control is required. To install this device, the Air Supply module has to be connected to the air pressure system of the flow cytometer via a three-way valve, the sample nozzle has to be connected with a soft tubing and the short tubes (2.5 mL) have to be used instead of regular 5 mL FACS tubes. These changes may take 10-15 min to setup and another 10-15 min to clean up after each run. Since most flow cytometers are shared core facilities, other users may be affected by these changes. If subsecond cell response is not crucial for the study, an alternative way is to unscrew the sample platform (takes about 10 seconds) and fit the water-jacket tube holder on the sample bar of a BD flow cytometer (Fig. 1). The Air Supply module is therefore not needed, and regular 5 mL tubes can be used. However, the tube has to be physically removed and replaced after the addition of stimulus, which incurs a delay of 2-5 seconds before recording. In either case, a tiny stir bar (1x3 mm) has to be placed in the bottom of tube in order to mix cells. A major advantage of the Time Zero system is the device for magnetic stirring of the reaction cuvette, which maximizes the number of cell-cell interactions as well as rapidly mixing agonist or probe into the suspension. It is also a good idea to leave a small amount of water inside the water-jacket tube holder to ensure good thermal conductivity to the tube.