Point-by-point response to comments from the editor and reviewers

Editor's comments:

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

Thank you for your suggestion. The manuscript was thoroughly proofread and revised by native English-speaking editors.

2. Please revise lines 85-87 to avoid previously published text.

We are sorry that the previous manuscript included previously published text, even though we had used "iThenticate (plagiarism detecting software)" on the manuscript before the first submission. Thank you very much for pointing this out. This sentence is now revised as follows. "Specific spatio-temporal patterns of Ca^{2+} signals activate specific downstream enzymes." (line 59)

3. Figure 2: Please include a space between all numbers and their units (30 s, 80 °C).

A space was inserted between all numbers and their units, except for % in Figure 2 and the manuscript was also revised in this regard.

4. Figure 5: Please describe what the yellow circles represent in the figure legend.

We regret that we did not provide the description of the yellow circles. The description has now been added in the figure legend.

5. Videos: Please provide a title for each video and place them in the Figure Legend section. Please include a scale bar and define its scale in each video.

We have now included all these requirements.

6. Please define all abbreviations before use.

We have defined all abbreviations in the revised manuscript.

7. 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: CELLBANKER1, Neurobasal, OPTIMEM, Lipofectamine, X-tremegene, Hamamatsu Photonics, etc.

We have removed all commercial language from the manuscript and replaced them with the respective generic terms. In some procedures requiring the use of a specific commercial product, we provided the name of the commercial product in the Table of Materials.

8. Please add more details to your protocol steps. There should be enough detail in each step

to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Thank you very much for your instruction. To ensure that the procedures can be replicated, we have added more details to the protocols. In particular, the procedure in section **1.3 ("Preparation of hippocampal neuron-astrocyte mixed culture from rats or mice")** has been heavily revised to include elaborate details**.** We hope that these revisions greatly improve the manuscript, and we would be happy to provide further details if necessary.

9. 1.1.2: Please list an approximate volume of PEI solution to prepare.

The recommended volume of 0.04% PEI solution (12.5 ml/12-well plate) is now listed.

10. 1.1.4: Please specify the incubation temperature.

This has been specified in the revised manuscript.

11. 1.2.1: Please specify the cell type used and provide the composition of culture medium used as well as the culture conditions.

We have specified the cell type (HeLa). The composition of culture medium is provided in the Table of Materials and Reagents.

12. 1.2.3: What is PBS (-)?

PBS (-) is now fully defined as "Phosphate-buffered saline without Ca^{2+} and Mg^{2+}".

13. 1.2.4: Please specify the concentration of trypsin-EDTA solution and incubation temperature and time.

The concentration of trypsin-EDTA (0.5%) and the approximate incubation time (90 s) are now provided.

14. 1.3.2: Please describe how the rat or mouse is anesthetized and how to extract the uterus.
These details are now provided (steps 1.3.6 – 1.3.9).

15. 1.3.3-1.3.5: Please describe how these are done. Specify all surgical instruments used. Please provide the composition of the dissection medium.

All surgical instruments are now described in the dissection steps and the Table of Materials.

16. 2.1.5: Please specify the incubation temperature.

It has now been specified.

17. 3.2.3, 3.3.2: Please specify the filter set and light source that are chosen in this step.

We agree that the excitation light information should be provided in this step. Now, the corresponding test (3.2.3, 3.2.4, and 3.3.2) includes the excitation wavelength.

18. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We have made this modification.

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

We have made this modification.

20. 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.

In addition to making the changes suggested by the editor and the reviewer, we have further highlighted the important step in the protocol for the video.

21. 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.

We have now highlighted the complete sentences. We have not highlight the step describing anesthetization and euthanasia.

22. 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.

We hope that you will agree that the current manuscript includes all the required steps for filming.

23. References: Please do not abbreviate journal titles.

We have made this modification.

24. Table of Materials: Please use SI abbreviations for all units (L, mL, µL) and include a space between all numerical values and their corresponding units (15 mL, 37 °C, etc.). Please sort the items in alphabetical order according to the Name of Material/Equipment.

The Table of Materials was revised to use SI abbreviations for all units, and to include space between all numerical values and corresponding units (except for %). The table is now sorted in alphabetical order according to the Name of Material/Equipment.

Reviewers' comments:

Reviewer #1:
In this manuscript, Bannai and coworkers provided a protocol for two-coloring Ca2+ imaging in live cells using membrane-targeted genetically encoded Ca2+ indicators (GECIs). The protocol focuses on preparation of cell culture, transfection, and Ca2+ imaging in two

fluorescence channels. Overall, the protocol is widely applicable and provides details for reader to carry out described experiments.

The authors sincerely thank Reviewer #1 for thoroughly reading the manuscript, and for constructive comments that have helped improve this manuscript.

One major concern is the imaging part of the protocol, being unique to this protocol, is however relatively lacking. Although the authors mentioned: "The condition of recording, i.e. length of excitation, recording frequency, the excitation light intensity, and the duration of recording, should be optimized according to the purpose of the experiment…" The optimization process and some example settings are of great importance, thus should be included to further strengthen the manuscript.

We sincerely thank Reviewer #1 for this constructive suggestion. We have described the optimization process in the "Note" of section 3.1 as follows.

"We recommend reducing the exposure time and the excitation light intensity as low as possible to avoid photobleaching and photo-toxicity to the cell. The recording frequency and the duration of recording should be sufficient to cover the Ca^{2+} elevation events of interest, but should be kept as low as possible to also avoid photobleaching and photo-toxicity. We recommend determining the recording frequency and the duration first and optimizing the light intensity and the exposure time so that the photobleaching of the GECIs is minimized."

We have also provided the example in recording frequency in 3.1.4.

Following are some other concerns/suggestions regarding this manuscript:

1. Title of 1.2 should be "Plating cell lines" as transfection is not mentioned in this section.

Thank you very much for pointing this out. The title of 1.2 has been revised to "Plating cell lines".

2. In 1.3.2 and 1.4.1, it would be better accessible to the readers if it were rephrased as 'Extract E18-19 embryos from the uterus of an anesthetized female rat or mouse'.

We thank the reviewer for this suggestion. We have made this modification proposed by Reviewer #1 in all applicable instances (currently sections 1.3.9 and 1.4.3).

3. In 1.3.7 and 1.4.6, It would be preferred if 'thrice' could be replaced by more commonly-used 'three times'.

We have made this modification.

4. In Figure 2, it would be better to label the corresponding step number on the flowchart.

We have made this modification.

5. In the results section, the authors stated:" … RCaMP2 remained at a higher level compared to that shown by OER-GCaMP6f, suggesting that Ca2+ dynamics at the plasma membrane shows a different temporal pattern than that in the vicinity of ER, i.e. the source

of this Ca2+ signal…" It is unclear to me how a higher level of RCaMP2 signal would suggest a different temporal pattern.

We regret that the previous description on the time-course of Lck-RCaMP2 and OER-GCaMP6f was unclear to readers. To describe it more clearly, the sentence was replaced with the following sentence:

"The results indicate that the Ca^{2+} elevation is prolonged in the vicinity of the plasma membrane, while it is terminated earlier around the ER, which is the source of this Ca^{2+} signal induced by His stimulation."

6. A brief explanation on baselines drifting would be helpful for Figure 4 and Figure 5A.

Thank you very much for this suggestion. The baseline drift suggests the changes in the global Ca^{2+} level in the cell. Now the baseline drift is briefly explained in the Figure Legend for Figure 4 and Figure 5A.

7. The 'dissection medium' mentioned in 1.3.4, 1.3.5, 1.4.3 and 1.4.4 was not described in Table of Materials.

We regret that the "dissection medium" was not mentioned. It is now mentioned in section 1.3.2 and fully described in the Table of Materials and Reagents.

8. Since the protocol is emphasizing the simultaneous/ sequential recording from both GECIs, it would be highly desirable if the methods of co-transfection/co-infection could be discussed in part 2.

Thank you very much for this suggestion. The information required to perform co-transfection and co-infection is provided in part 2 (2.1.1, 2.1.2; 2.2.3, 2.2.4; 2.2.9)

9. In Table of Materials, what does '(-)' mean in 'PBS (-)'?

We regret that we did not describe PBS (-). PBS (-) denotes "Phosphate-buffered saline without Ca^{2+} and Mg^{2+}". The absence of Ca^{2+} and Mg^{2+} is critical in this protocol to prevent inhibit the activity of trypsin. In the present Table of Materials, PBS (-) is fully described. In the manuscript, we also fully define PBS (-) as "Phosphate-Buffered Saline without Ca^{2+} and Mg^{2+}" (1.2.3).

10. In Table of Materials, the Penicillin-Streptomycin concentration is used at only 1/20 of the that recommended by the manufacturer. Please briefly explain the considerations for using a reduced antibiotics concentration?

Thank you very much for pointing out this important point. Indeed, neuronal survival is severely decreased when Penicillin-Streptomycin (PS) is used at the recommended concentration. Therefore, using a concentration of PS that is just 1/20th of that recommended in the conventional protocol is critical here. Now, we have also included the following sentence in the Table of Materials.

"This concentration of penicillin-streptomycin, which is 1/20 of the concentration recommended by the manufacturer, is critical for neuronal survival."

11. In Table of Materials, there exist quite a few typos, such as 'Altanative', 'Pfiser', 'concentation', 'microsocope', 'Appropreate'. A careful and exhaustive spell check is strongly recommended before publishing.

We apologize for the typographical errors in the Table of Materials. The editor (from the language-editing service) as well as the authors have now performed a careful spell check and proofread the manuscript.

12. Paper describing RCaMP2 should be properly referenced.

The authors sincerely thank Reviewer #1 for finding this error in our manuscript. We have now provided a reference for RCaMP2 (Inoue et al. 2015 Nature Methods).

Reviewer #2:
Manuscript Summary:
This is an excellent manuscript which will be of great interest to the calcium-research community. The authors may want to consider some English editing. I do not have additional comments and recommend publishing of the MS in JOVE.

The authors thank Reviewer #2 for stating that our manuscript is of great interest to the calcium-research community. We deeply thank the reviewer for the careful reading of the manuscript. The manuscript has now been edited by a native English-speaking editor.