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Cover Letter

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Dear Dr. Vineeta Bajaj

Thank you very much for this opportunity for revising this manuscript. Indeed, we are grateful to the editor and two reviewers for their comments that will help us to improve this manuscript for publication in ***JoVE***. The authors have fully revised the manuscript according to advices.

Briefly, the authors made following revisions:

1) **Detailed procedures that the editor and the reviewer required were now included in the manuscript. Abbreviation is defined before use.**

2) **The fatal error (the published sentence and inappropriate citation) is completely revised in this version.**

3) **Titles and the legends for all videos are now provided in the Figure Legend section.**

4) **The manuscript was edited to fully match the requirement of *JoVE*.**

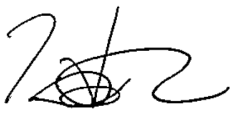
5) **The manuscript and the Table of Materials were thoroughly proofread and revised by native English-speaking editors to amend typos, grammatical errors.**

I believe that these revisions have improved the manuscript, and I hope that the reviewers and you will agree. Point by point responses to the comments and concerns of the editor and the reviewers (each comments are in ***blue italics***) are detailed below.

Thank you for your consideration. I look forward to hearing from you.

With Best Wishes,

Hiroko Bannai



TITLE:

Dissection of Local Ca^{2+} Signals in Cultured Cells by Membrane-targeted Ca^{2+} Indicators

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KEYWORDS:

Ca^{2+} imaging, local Ca^{2+}, GCaMP6f, RCaMP2, Ca^{2+} influx, Ca^{2+} release, plasma membrane, endoplasmic reticulum, cell line, neuron, astrocyte, dissociated culture

SUMMARY:

Here we present a protocol for Ca^{2+} imaging in neurons and glial cells, which enables the dissection of Ca^{2+} signals at subcellular resolution. This process is applicable to all cell types that allow the expression of genetically encoded Ca^{2+} indicators.

ABSTRACT:

Calcium ion (Ca^{2+}) is a universal intracellular messenger molecule that drives multiple signaling pathways leading to diverse biological outputs. The coordination of two Ca^{2+} signal sources, Ca^{2+} influx from outside the cell and Ca^{2+} release from the intracellular Ca^{2+} store endoplasmic reticulum (ER), is considered to underlie the diverse spatiotemporal patterns of Ca^{2+} signals that cause multiple biological functions in cells. The purpose of this protocol is to describe a new Ca^{2+} imaging method that enables the monitoring of the very moment of Ca^{2+} influx and Ca^{2+} release. OER-GCaMP6f is a genetically encoded Ca^{2+} indicator (GECI) comprising GCaMP6f, which is targeted to the ER outer membrane. OER-GCaMP6f can monitor Ca^{2+} release at a higher temporal resolution than conventional GCaMP6f. Combined with plasma membrane-targeted GECIs, the spatiotemporal Ca^{2+} signal pattern can be described at a subcellular resolution. The subcellular-targeted Ca^{2+} indicators described here are, in principle, available for all cell types, even for the in vivo imaging of *Caenorhabditis elegans* neurons. In this protocol, we introduce Ca^{2+} imaging in cells from cell lines, neurons, and glial cells in dissociated primary cultures and describe the

preparation of frozen stock of rat cortical neurons.

INTRODUCTION:

Ca^{2+} signals represent the elevation of the intracellular Ca^{2+} concentration. Ca^{2+} is the universal second messenger for eukaryotic cells. Using Ca^{2+}, cells function via diverse intracellular signaling pathways and induce various biological outputs. For example, in neurons, synaptic vesicle release at the presynaptic terminal, gene expression in the nucleus, and induction of synaptic plasticity at the postsynapse are regulated by distinct Ca^{2+} signals that precisely activate the appropriate downstream enzymes at the right sites and with precise timing[1].

Specific spatiotemporal patterns of Ca^{2+} signals activate the specific downstream enzymes. Ca^{2+} signals are generated by the coordination between two different Ca^{2+} sources: Ca^{2+} influx from the extracellular space and Ca^{2+} release from the endoplasmic reticulum (ER), which serves as an intracellular Ca^{2+} store. The meaningful spatiotemporal Ca^{2+} signaling pattern to induce a specific cell function is also supported by nanodomains of 10–100 μM Ca^{2+} generated in the vicinity of Ca^{2+} channels on the plasma membrane or ER membrane[2]. Importantly, the source of Ca^{2+} signals is one of the most critical factors determining the downstream biological output. In neurons, Ca^{2+} influx and Ca^{2+} release have opposite effects on the clustering of gamma-aminobutyric acid $(GABA)_A$ receptors ($GABA_AR$) at the GABAergic synapses, which is responsible for the inhibition of neuronal excitability[3]. Ca^{2+} influx accompanied by massive neuronal excitation induces the dispersion of synaptic $GABA_AR$ clusters, whereas persistent Ca^{2+} release from the ER promotes the clustering of synaptic $GABA_ARs$. Other groups have also reported that the tuning direction of growth cones is critically dependent on the source of the Ca^{2+} signal: Ca^{2+} influx induces repulsion, while Ca^{2+} release guides the attraction of the neuronal growth cone[4]. Therefore, to fully understand the Ca^{2+} signaling pathways underlying specific cellular outputs, it is important to identify the source of Ca^{2+} signals by describing Ca^{2+} signals at the subcellular resolution.

In this protocol, we describe a Ca^{2+} imaging method to report Ca^{2+} signals at the subcellular resolution, which allows the estimation of the Ca^{2+} signal sources (**Figure 1**). Ca^{2+} microdomains just beneath the plasma membrane are successfully monitored by genetically encoded Ca^{2+} indicators (GECIs) targeted to the plasma membrane via the attachment of the plasma membrane-localization signal Lck within Src kinase to the N-termini of GECIs[5]. To detect the Ca^{2+} signal pattern in the vicinity of the ER at a better spatial and temporal resolution, we recently developed OER-GCaMP6f, in which GCaMP6f[6] targets the ER outer membrane, using the ER transmembrane protein. OER-GCaMP6f can sensitively report Ca^{2+} release from the ER at a better spatiotemporal resolution than conventional nontargeted GCaMP6f in COS-7 cells[7] and HEK293 cells[8], by avoiding the diffusion of Ca^{2+} and GECIs. We also confirmed that the spontaneous Ca^{2+} elevation in cultured hippocampal astrocytes reported by OER-GCaMP6f showed a different spatiotemporal pattern compared to that monitored by plasma membrane-targeted GCaMP6f (Lck-GCaMP6f)[7,9], indicating that Ca^{2+} imaging with OER-GCaMP6f in combination with Lck-GCaMP6f contributes to the dissection of Ca^{2+} signals at the subcellular resolution to identify their sources.

Presently, we detail the protocol for the Ca^{2+} signal dissection in HeLa cells and neuron-astrocyte

mixed cultures plated on glass coverslips. The Ca^{2+} imaging technique with GECIs indicated here, Lck-GCaMP6f, plasma-membrane-targeted RCaMP2[10] (Lck-RCaMP2), and OER-GCaMP6f (**Figure 1**) are applicable to all cells in which these GECIs can be expressed.

PROTOCOL:

All the experiments described here were approved by the RIKEN safety committee and animal experiment committee, according to the guideline issued by the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

1. Preparation of cells

1.1. Preparation of poly(ethyleneimine)-coated coverslips

NOTE: Poly(ethyleneimine) (PEI) coating is recommended for the glass apparatus, as it allows neurons and astrocytes to attach tightly to the coverslips without preventing their development. However, other coating methods (e.g., poly-ornithine, poly L-lysine, laminin coating) are also available, if necessary, for glass-bottom dishes.

1.1.1. Place an 18 mm-diameter glass coverslip in each well of a 12-well plate. Prepare 0.04% PEI solution (12.5 mL/12-well plate) using sterilized water.

1.1.2. Add 1 mL of 0.04% PEI solution to each well. Ensure that there are no bubbles underneath the coverslips.

1.1.3. Incubate the plates in a CO_2 incubator overnight at 37 °C.

1.1.4. The next day, wash the coated coverslips 3x with 1 mL of sterilized water. Remove the PEI solution with an aspirator, add 1 mL of sterilized water to each well, and shake the 12-well plate so that the PEI solution between the coverslip and the plate can be washed out thoroughly. As the remaining PEI is toxic for cells, ensure that the water after the final wash is aspirated completely.

1.1.5. Dry and sterilize the coverslips inside the hood with ultraviolet (UV) light for at least 15 min. The PEI-coated dish can be stored at 4 °C for up to 2 months. Illuminate the dishes with UV light for 15 min just before use.

1.1.6. Add 5 mL of sterile distilled water in the space between the wells to prevent evaporation of the culture medium.

1.2. Plating cell lines

NOTE: This protocol provides just one example for transfection into cells from mammalian cell lines, such as HeLa cells and COS-7 cells. Users can apply other transfection protocols that are optimized for their experiments. In this section, we will describe the HeLa cell culture protocol,

which is also applicable to COS-7 cells.

1.2.1. On the day before the transfection, culture the cells in a 10 cm culture dish until they attain 70%–90% confluence.

1.2.2. Prewarm the culture medium (see **Table of Materials**) to 37 °C.

1.2.3. Wash the cells 2x with phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS [-]).

1.2.4. Aspirate the PBS(-), add 1 mL of 0.5% trypsin-ethylenediaminetetraacetic acid (EDTA) solution, and incubate the cells at 37 °C for 90 s until they attain a round shape.

1.2.5. Add 9 mL of prewarmed culture medium to stop the trypsinization. Dilute the cells with culture medium at a ratio of 1:6.

1.2.6. Seed 1 mL of the diluted cells on PEI-coated coverslips in the 12-well plates.

1.3. Preparation of hippocampal neuron-astrocyte mixed culture from rats or mice

NOTE: Sections 1.3 and 1.4 must first be reviewed and approved by an Institutional Animal Care and Use Committee and must follow officially approved procedures for the care and use of laboratory animals. The flowchart of the neuronal culture protocol is shown in **Figure 2**.

1.3.1. Prepare all reagents under the laminar flow hood. Place Dulbecco's modified Eagle's medium (DMEM) into two 100 mm culture dishes (approximately 20 mL/dish) that are in an icebox.

1.3.2. Prepare 50 mL of the dissection medium composed of DMEM and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (see **Table of Materials**) and dispense the medium into three 60 mm culture dishes (approximately 7 mL/dish) and eight 35 mm culture dishes (approximately 2 mL/dish). Place the dishes in another icebox.

1.3.3. Prepare 50 mL of the incubation saline, composed of Hanks' balanced salt solution supplemented with 20 mM HEPES (see **Table of Materials**), and place 8 mL of the saline in a 15 mL conical tube on ice.

1.3.4. Prepare the plating medium with minimum essential medium (MEM) supplemented with B-27, glutamine, and penicillin-streptomycin (see **Table of Materials**). Maintain this medium at room temperature (20–28 °C).

1.3.5. Sterilize the surgical instruments with 70% ethanol.

1.3.6. Place a paper towel in a glass jar with a lid and 1 mL of isoflurane. Let the isoflurane evaporate for 1 min.

1.3.7. Place a pregnant rat or a mouse in the jar prepared according to step 1.3.6 and keep the animal in the jar until it is deeply anesthetized (approximately 30 s to 1 min).

1.3.8. Take the anesthetized animal out of the jar and disinfect the animal and the dissection equipment by spraying them with 70% ethanol. Cut the ventral midline with standard dissecting scissors and tweezers and extract the uterus from the pregnant rat or mouse.

1.3.9. Extract E18–19 embryos from the uterus of an anesthetized female rat or mouse, using delicate dissecting scissors, and place the extracted embryos with a ring forceps into ice-cold DMEM in a 10 cm dish for cold anesthesia.

1.3.10. Decapitate the embryo with fine dissection scissors and place the head in ice-cold DMEM in a 10 cm dish.

1.3.11. Extract the brain from each embryo with a 13 cm curved Semken forceps and forceps with fine tips. Keep the brain in the ice-cold dissection medium in a 60 mm dish.

1.3.12. Remove the hippocampi, using two forceps with fine tips, in the ice-cold dissection medium in 35 mm dishes and maintain the isolated tissue in the incubation saline placed in a 15 mL conical tube on ice.

1.3.13. Wash the hippocampi with incubation saline and incubate with trypsin (1.25 mg/mL) and DNase I (0.25 mg/mL) in the incubation saline for 5 min at 37 °C. The recommended incubation volume is 2.7 mL of the incubation saline, 150 µL of 20x stock trypsin, and 150 µL of 20x stock DNase (see **Table of Materials**).

1.3.14. Wash the hippocampi 3x with ice-cold incubation saline.

1.3.15. Aspirate the incubation saline and add 1 mL of the plating medium containing DNase I (10 µL of stock solution; see **Table of Materials**). Suspend the tissue by pipetting no more than 20x and measure the density of viable cells, using a cell counter and Trypan blue assay.

1.3.16. Dilute the hippocampal cells to a density of 1.4×10^5 viable cells/mL for rats and 2.5×10^5 viable cells/mL for mice. Seed 1 mL of the diluted cell suspensions onto the PEI-coated coverslips in 12-well culture plates.

1.3.17. Maintain the cells at 37 °C in a CO_2 incubator for 2–3 days.

1.3.18. Remove the plating medium. Do not let the cells dry out. Gently and quickly add the prewarmed maintenance medium (see **Table of Materials**).

1.4. Preparation of rat cortical neuron-astrocyte mixed culture and frozen cells, and the revival of frozen cultures

NOTE: A cryopreservation method for cortical cells was described previously[11]. Here, a modified protocol in which cortical cells can be stored at -80 °C for at least 3 months is provided. The flowchart for this protocol is shown in **Figure 2**.

1.4.1. Prepare DMEM, dissection medium, incubation saline, and plating medium as indicated in steps 1.3.1–1.3.4, and the **Table of Materials**.

1.4.2. Prepare the wash medium constituted of DMEM, heat-inactivated fetal bovine serum, and penicillin-streptomycin (see **Table of Materials**), if necessary.

1.4.3. Extract E18–19 embryos from the uterus of an anesthetized female rat or mouse, using delicate dissection scissors, and use ring forceps to place each extracted embryo into ice-cold DMEM for cold anesthesia.

1.4.4. Remove the brains from the embryos and keep them in ice-cold dissection medium. Remove the cortexes and maintain them in the incubation saline placed in a 15 mL conical tube on ice.

1.4.5. Wash the cortexes with incubation saline and incubate the cortexes with trypsin (1.25 mg/mL) and DNase I (0.25 mg/mL) in incubation saline for 5 min at 37 °C. The recommended incubation volume for 12 cortexes is 5.4 mL of incubation saline, 300 µL of 20x stock trypsin, and 300 µL of 20x stock DNase I.

1.4.6. Wash the cortexes 3x with ice-cold incubation saline.

1.4.7. Remove the supernatant and add 2 mL of plating medium supplemented with 150 µL of DNase I stock. Dissociate the cells by pipetting less than 20 strokes, and filter the cells using a cell strainer with a pore size of 70 µm.

1.4.8. Wash the cell strainer with 20 mL of the plating medium for plating. For the preparation of frozen cell stock, wash the cells with 20 mL of the wash medium (see **Table of Materials**)

1.4.9. Measure the density of the viable cells, using a cell counter and the Trypan blue method.

1.4.10. Dilute the cortical cells to a density of 1.4×10^5 viable cells/mL with the plating medium and add 1 mL of the diluted cell suspension to PEI-coated coverslips in the 12-well culture plates.

1.4.11. Maintain the cells at 37 °C in a CO_2 incubator for 2–3 days and change the culture medium to the maintenance medium.

1.4.12. After step 1.4.9, prepare the frozen cortical cell stock by centrifuging the cells at 187 x *g* for 3 min, using a swing rotor.

1.4.13. Aspirate the supernatant and add the cryopreservation medium (see **Table of Materials**) kept at 4 °C, to obtain a cell density of 1 x 10^7 cells/mL. Aliquot 1 mL of the cell suspension into cryogenic tubes.

1.4.14. Place the tubes in a cell freezing container with a freezing rate of -1 °C/min, until a temperature of -80 °C is reached, and transfer the freezing container to a -80 °C freezer. The cells can be stored for at least 3 months at -80 °C.

1.4.15. To revive frozen cells, prewarm the wash medium (approximately 13 mL for each cryogenic tube) and maintenance medium for frozen cortical cells (see **Table of Materials**).

1.4.16. Thaw the frozen cells rapidly at 37 °C in a water bath.

1.4.17. Dilute the thawed cells gently with prewarmed wash medium. Centrifuge the cells at 187 x *g* for 3 min, using a swing rotor.

1.4.18. Suspend the pellet in 1 mL of wash medium and measure the viable cell density.

1.4.19. Dilute the cells with the maintenance medium for frozen cortical cells to yield a cell density of 3.0 x 10^5 viable cells/mL, and seed 1 mL of the cell suspension in the PEI-coated 12-well plates.

2. Expression of membrane-targeted GECIs

2.1. Transfection of cells

2.1.1. Add 250 ng of the GECI plasmid (i.e., Lck-GCaMP6f, Lck-RCaMP2, or OER-GCaMP6f with CMV promoter)[7–9] to 100 µL of the reduced serum medium (see **Table of Materials**) per well. For the cotransfection of Lck-RCaMP2 and OER-GCaMP6f, use 250 ng of each plasmid in 100 µL of reduced serum medium in each well.

2.1.2. Add 0.5 µL of transfection reagent (see **Table of Materials**) per well into the plasmid-reduced serum medium mixture. For the cotransfection of Lck-RCaMP2 and OER-GCaMP6f, add 0.5 µL of transfection reagent per well.

2.1.3. Incubate the mixture for 30 min at room temperature (20–28 °C).

2.1.4. Add 100 µL of the mixture to each coverslip in a drop-wise manner.

2.1.5. Incubate the cells for 48–72 h in a CO_2 incubator at 37 °C to allow the expression of the GECIs.

2.2. Transfection and adeno-associated virus infection of hippocampal or cortical neurons

NOTE: Transfection for 3–5 days in vitro (DIV) results in a higher transfection rate for neurons. Transfection at 6–8 DIV is preferred for the optimal expression of GECIs in astrocytes. For the expression of GECIs in dissociated culture neurons after 9 DIV, the infection of the adeno-associated virus (AAV) vectors provides a better expression efficacy. AAV vectors for the expression of Lck-GCaMP6f, Lck-RCaMP2, and OER-GCaMP6f under the EF1a promoters were prepared as described previously, using HEK293 cells[12] (see **Table of Materials**).

2.2.1. For transfection 3–8 days after plating, label two tubes, one for plasmid DNA and the other for transfection reagent.

2.2.2. Add 50 µL of reduced serum medium (see **Table of Materials**) per well to each tube.

2.2.3. Add 0.5 µg of plasmid DNA per coverslip and 1 µL of supplement accompanied by transfection reagent for neurons (see **Table of Materials**) per well to the plasmid DNA tube. For the cotransfection of Lck-RCaMP2 and OER-GCaMP6f, 0.5 µg of each plasmid and 1 µL of supplement are mixed in 50 µL of reduced serum medium per well.

2.2.4. Add 1 µL of transfection reagent (see **Table of Materials**) per well in the transfection reagent tube. The same amount of transfection reagent is used for cotransfection.

2.2.5. Vortex both tubes for 1–2 s.

2.2.6. Add the transfection reagent mixture (from step 2.2.4) to the DNA mixture (from step 2.2.3). Mix by pipetting gently and incubate the mixture (100 µL per coverslip) for 5 min at room temperature.

2.2.7. Load this mixture onto the cells in a drop-wise manner.

2.2.8. Incubate the cells in a CO_2 incubator for 2–3 days until the marker proteins are expressed.

2.2.9. For AAV infection, add 3 µL of AAV per well to the mixed neuron-astrocyte culture. Mix gently by rocking the dish. For the double infection of Lck-RCaMP2 and OER-GCaMP6f, 3 µL of each AAV is introduced per well. In case the numbers of cells expressing GECIs are insufficient, the optimal AAV amount for infection should be determined.

2.2.10. Maintain the culture for 1–2 weeks until the GECIs are expressed.

3. Ca^{2+} imaging

3.1. Simultaneous imaging of cells expressing Lck-RCaMP2 and OER-GCaMP6f

NOTE: To simultaneously record the Lck-RCaMP2 and OER-GCaMP6f signals, image-splitting optics are required. The optics enable the separation of RCaMP2 and GCaMP6f and their projection onto the same photographic frame of the camera (**Figure 3A**). Simultaneous imaging

also requires (1) light sources that can simultaneously emit excitation light in the blue (450–490 nm) and green (500–560 nm) spectra, (2) double-band filter and dichroic mirror sets in the microscope, and (3) emission filters for RCaMP2 and GCaMP6f. For details, refer to the **Table of Materials**.

3.1.1. Turn on the imaging devices and computers at least 30 min before the recording. Prewarm the microscope heating chamber to 37 °C. Set up the image-splitting device, filters, and light source. Align the image-splitting optics so that the same field of view appears on the camera. Choose the appropriate objective lens (see **Table of Materials**).

3.1.2. Mount the coverslip containing the cells transfected with Lck-RCaMP2 and OER-GCaMP6f in the recording chamber, add the appropriate imaging medium or buffer (400 µL for 18 mm coverslips) in the chamber, and place it on the microscope stage. Place a lid on the recording chamber to avoid the evaporation of the medium.

3.1.3. Locate the cells expressing both Lck-RCaMP2 and OER-GCaMP6f by fluorescent imaging. Minimize the excitation light intensity to prevent photobleaching and phototoxicity.

3.1.4. Remove the lid and start a time-lapse recording at 10 Hz. During this recording, add the agonist to the chamber to evoke Ca^{2+} responses (e.g., histamine for HeLa cells, ATP for COS-7 cells).

3.1.5. Save the time-lapse data in the hard disk drive (HDD).

3.1.6. Analyze the data using image analysis software.

3.2. Recording spontaneous activities of astrocytes expressing Lck-RCaMP2 and OER-GCaMP6f

NOTE: Without image-splitting optics, the Ca^{2+} signals at the plasma membrane and those around the ER can be monitored in the same cell. Here, the sequential recording of Lck-RCaMP2 and OER-GCaMP6f in the same astrocytes is described. An oil-immersion objective with a numerical aperture larger than 1.3 is highly recommended for spontaneous Ca^{2+} activity.

3.2.1. Turn on the microscope, camera, light source, and the microscope heating chamber at least 30 min before recording.

3.2.2. Mount the coverslip containing the cells transfected with Lck-RCaMP2 and OER-GCaMP6f in the recording chamber and add 400 µL of the imaging medium. Place a lid on top of the chamber.

3.2.3. Choose the filter set for GCaMP6f and the light source (blue excitation light, e.g. 470– 490 nm; see **Table of Materials**). Locate the astrocytes expressing OER-GCaMP6f.

3.2.4. Choose a filter set and the light source for RCaMP2 (green excitation light, e.g., 510–560 nm; see **Table of Materials**) and confirm whether Lck-RCaMP2 is expressed in the same astrocytes. Avoid long exposure to the light source to prevent photobleaching.

3.2.5. Record time-lapse images of Lck-GCaMP2 at 2 Hz for 2 min. Save the imaging data on the HDD.

3.2.6. Change the filter set to that for GCaMP6f. Record time-lapse images of OER-GCaMP6f in the same field of view, at 2 Hz for 2 min. Save the data on the HDD.

3.2.7. Analyze the data using the image analysis software.

3.3. Recording spontaneous neuronal activity and induced Ca^{2+} elevation in neurons

NOTE: The microscope setup for neuronal imaging is the same as that described in section 3.2. Here, the imaging of spontaneous Ca^{2+} elevation due to Lck-GCaMP6f and Ca^{2+} elevation induced by mGluR activation due to OER-GCaMP6f is described.

3.3.1. To record spontaneous neuronal activity, mount the coverslip containing the cells expressing Lck-GCaMP6f in the recording chamber and add 400 µL of imaging medium. Place a lid on top of the chamber.

3.3.2. Set the filter and the light source (blue excitation, e.g. 470–790 nm; see **Table of Materials**) to those for GCaMP6f. Find the neurons expressing Lck-GCaMP6f and showing spontaneous activity.

3.3.3. Acquire images at 2 Hz or faster. Save the data on the HDD.

3.3.4. Analyze the data using the image analysis software.

3.3.5. To record induced Ca^{2+} elevation, mount the coverslips containing the cells expressing OER-GCaMP6f with 400 µL imaging medium. Place a lid on top of the chamber.

3.3.6. Using the filter set for GCaMP6f, find the neurons expressing OER-GCaMP6f.

3.3.7. Remove the lid. Start the time-lapse recording at 2 Hz or faster. During the recording, add the agonists for a G_q-protein-coupled receptor (e.g., mGluR5 agonist [RS]-3,5-dihydroxyphenylglycine [DHPG]) to evoke a Ca^{2+} release from the ER.

3.3.8. Save the time-lapse images on the HDD and analyze the data.

REPRESENTATIVE RESULTS:

Lck-RCaMP2 and OER-GCaMP6f were expressed in HeLa cells, and both signals were recorded simultaneously using image-splitting optics, 24 h after transfection (**Figure 3A** and **Video 1**). The

images were acquired at 10 Hz. Histamine (His, 1 µM), which induces Ca^{2+} release from the ER, was added during the recording. Upon the application of His, the signal intensity of Lck-RCaMP2 and OER-GCaMP6f increased, as shown by the pseudocolor display of $\Delta F/F_0$, which represents the change from the initial fluorescence intensity (**Figure 3B**). The time courses of Ca^{2+} elevation ($\Delta F/F_0$) reported by Lck-RCaMP2 and OER-GCaMP6f were compared in the same region of interest (ROI) (**Figure 3C**). The $\Delta F/F_0$ values were normalized to their peak values to enable the time course comparison between the two different GECIs, which have different expression levels and distributions. Both sensors reported an oscillation-like Ca^{2+} elevation. Lck-RCaMP2 and OER-GCaMP6f showed the same time course for Ca^{2+} elevation in two cells among the five cell types examined (**Figure 3C**, ROI 1 and 3). However, Ca^{2+} elevations shown by Lck-RCaMP2 remained at a higher level compared to that shown by OER-GCaMP6f (**Figure 3C**, ROI 2, 4, and 5). 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.

Spontaneous Ca^{2+} signals from astrocytes in the neuron-astrocyte mixed culture from rat hippocampi (**Figure 4A**) and cortexes (**Figure 4B**) were shown by Lck-RCaMP2 and OER-GCaMP6f (**Figure 4**, **Video 2**, **Video 3**, **Video 4**, and **Video 5**). Cortical cultures were revived from the frozen stock that was prepared as described in this protocol. Lck-RCaMP2 and OER-GCaMP6f signals were sequentially recorded at 2 Hz from the same cells. Three ROIs were selected in the area that showed Ca^{2+} elevation by each GECI, and the time course of $\Delta F/F_{base}$ (i.e., the fluorescence intensity) changed from the average fluorescence intensity during the entire recording period (F_{base}). When the baseline fluorescence is stable and Ca^{2+} elevations are less frequent, F_{base} becomes a useful baseline to detect Ca^{2+} elevation events. Spontaneous Ca^{2+} elevations were visible only at the astrocytic process, not at the cell body. This result is consistent with the previous reports on astrocytic spontaneous Ca^{2+} signals by other GECIs visualized in vitro[13] and in vivo[14]. In both hippocampal and cortical astrocytes, Ca^{2+} elevations shown by Lck-RCaMP2 (top) were more frequent than those shown by OER-GCaMP6f. This result is consistent with our previous demonstration that Ca^{2+} elevations in astrocytes due to Lck-GCaMP6f were more frequently detected than those due to OER-GCaMP6f[7] and suggests that this notion is also applicable at the single-cell level.

Spontaneous Ca^{2+} elevations by Lck-GCaMP6f in immature rat hippocampal neurons (10 DIV) were seen at 2 Hz (**Figure 5A** and **Video 6**). The time courses of $\Delta F/F_0$ in five different ROIs suggest that these Ca^{2+} elevations are locally confined to the subcellular domains. **Figure 5B** (**Video 7**) shows the Ca^{2+} responses in mature mouse hippocampal neurons (30 DIV) infected with OER-GCaMP6f-expression AAV vectors. Neurons were stimulated with 100 µM DHPG, which is the agonist for metabotropic glutamate receptors, inducing Ca^{2+} release. DHPG-induced Ca^{2+} release due to OER-GCaMP6f was detected.

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram showing membrane-targeted GECIs. Schematic diagram of plasma membrane-targeted GECIs (Lck-GCaMP6f and Lck-RCaMP2) and outer ER membrane-targeted GCaMP6f (OER-GCaMP6f).

Figure 2: Flowchart for hippocampal and cortical cell preparation, plasmid transfection, and AAV infection. The microscopic images are representative, freshly plated DIV-6 cortical cells (left) and revived cells from the frozen stock (right). The scale bar = 100 µm.

Figure 3: Example of the simultaneous imaging of Lck-RCaMP2 and OER-GCaMP6f in HeLa cells. (**A**) Schematic representation of signal separation with image-splitting optics. The same field of view for Lck-RCaMP2 and OER-GCaMP6f is simultaneously projected on the camera. A representative recording acquired at 10 Hz by a CMOS camera is provided in **Video 1,** and one frame of this recording is shown in panel **A** (right). (**B**) Pseudo-color images of $\Delta F/F_0$ for Lck-GCaMP2 (top) and OER-GCaMP6f (bottom). Histamine (His, 1 µM) was added at 0 s. (**C**) Representative normalized $\Delta F/F_0$ time course of Lck-GCaMP2 (magenta) and OER-GCaMP6f (green). Data were normalized to the maximum $\Delta F/F_0$ value for each plot. The gray bars indicate the timing of His application. Data were analyzed with a custom-made software TI Workbench[15]. The scale bar in the microscopic image = 50 µm.

Figure 4: Spontaneous Ca^{2+} elevation in astrocytes monitored for Lck-RCaMP2 and OER-GCaMP6f expression. (**A**) Representative hippocampal and (**B**) cortical astrocytes transfected with Lck-RCaMP2 (top) and OER-GCaMP6f (bottom). Cortical cells were revived from frozen stock cultures. Lck-RCaMP2 and OER-GCaMP6f images were sequentially acquired in the same cell, at 2 Hz, with an EM-CCD camera. The plots on the left show the time courses of $\Delta F/F_{base}$ measured in the ROIs indicated in the microscopic image. Data were analyzed with TI Workbench. Actual movies are provided in **Video 2**, **Video 3**, **Video 4**, and **Video 5**. The scale bar in the microscopic image = 20 µm. The baseline drift suggests the changes in the global Ca^{2+} level in the cell.

Figure 5: Examples of Ca^{2+} imaging in neurons with Lck-GCaMP6f and OER-GCaMP6f. (**A**) Representative rat hippocampal neurons expressing Lck-GCaMP6f at DIV 10 (left) and plots showing the time courses of $\Delta F/F_0$ measured in the ROIs (yellow circles) have been indicated in the image (right). The numbers in the time course correspond to the ROI numbers in the image. Note that the temporal pattern of Ca^{2+} elevation is different among the various regions of interest. The baseline drift suggests the increase in the global Ca^{2+} level in this neuron. (**B**) An example of mature mouse hippocampal neurons (DIV 30) infected with OER-GCaMP6f expression AAV vectors (left). The time course plot of $\Delta F/F_0$ measured shows the Ca^{2+} response to 100 µM (RS)-3,5-dihydroxyphenylglycine (DHPG) applied at the timing shown by the gray bar. Yellow circles show the position of ROIs where the time course was obtained. The images were acquired at 2 Hz with a cooled-CCD camera (panel **A**) or an EM-CCD camera (panel **B**) and analyzed with TI Workbench. The scale bar in the microscopic image = 20 µm.

Video 1: Example of simultaneous imaging of Lck-RCaMP2 and OER-GCaMP6f in HeLa cells. Representative recording acquired at 10 Hz and presented in **Figure 3**. The scale bar = 50 µm.

Video 2: Spontaneous Ca^{2+} transient observed in Lck-RCaMP2 in hippocampal astrocyte. Representative recording acquired at 2 Hz (**Figure 4A**), recorded in the same field of view as **Video 3**. The scale bar = 20 µm.

Video 3: Spontaneous Ca^{2+} transient observed in OER-GCaMP6f in a hippocampal astrocyte. Representative recording acquired at 2 Hz (**Figure 4A**), in the same field of view as **Video 2**. The scale bar = 20 μm.

Video 4: Spontaneous Ca^{2+} transient observed in Lck-RCaMP2 in a cortical astrocyte Representative recording acquired at 2 Hz (**Figure 4B**), recorded in the same field of view as **Video 5**. The scale bar = 20 μm.

Video 5: Spontaneous Ca^{2+} transient observed in OER-GCaMP6f in a cortical astrocyte. Representative recording acquired at 2 Hz (**Figure 4B**), in the same field of view as **Video 4**. The scale bar = 20 μm.

Video 6: Example of Ca^{2+} imaging in a rat hippocampal neuron (DIV 10) by Lck-GCaMP6f. Example of neuronal Ca^{2+} signals recorded at 2 Hz (**Figure 5A**). The scale bar = 20 μm.

Video 7: Ca^{2+} release in a mouse hippocampal neuron (DIV 30) expressing OER-GCaMP6f. Example of neuronal Ca^{2+} signals recorded in a mouse hippocampal neuron infected with OER-GCaMP6f expression AAV vectors (**Figure 5B**). The neuron was stimulated with 100 μM dihydroxyphenylglycine (DHPG) applied at 30 s to evoke Ca^{2+} release from the ER. The scale bar = 20 μm.

DISCUSSION:

Diverse biological outputs are initiated by Ca^{2+} signals. Ca^{2+} is a versatile intracellular signaling messenger. Decoding Ca^{2+} signals to evoke specific outputs has been a fundamental biological question, and Ca^{2+} imaging techniques to describe the diversity of Ca^{2+} signals are required. The presently detailed protocol enables the detection of distinct Ca^{2+} signals at the plasma membrane and ER (**Figure 3** and **Figure 4**) and local Ca^{2+} microdomains inside a cell (**Figure 4** and **Figure 5**). This contributes to describing the diversity of intracellular Ca^{2+} signals. The temporal resolution of Ca^{2+} signals was also improved by targeting GECIs in the plasma membrane and ER because it can avoid the effect of a three-dimensional diffusion of the Ca^{2+} and GECIs themselves, and it has the potential to detect the very moment of Ca^{2+} influx or Ca^{2+} release, which occurs on the membrane.

The protocol has some limitations. Users should keep in mind that the detected signals are the summation of "the moment of Ca^{2+} influx or release" and "Ca^{2+} diffused out from the original Ca^{2+} source", especially for large Ca^{2+} signals. For example, although His stimulation in HeLa cells evokes Ca^{2+} release from the ER, its resultant Ca^{2+} signals are detected not only by ER-targeted OER-GCaMP6f but also by plasma-membrane-targeted Lck-RCaMP2 (**Figure 3**). Another limitation is that the spatiotemporal pattern of Ca^{2+} signals may not be the only determinant of the output of Ca^{2+} signals. The distribution of downstream effector proteins (such as Ca^{2+}-dependent kinases and phosphatases) may also be a determining factor[2]. To completely decode the intracellular Ca^{2+} signals, analysis of downstream enzyme behavior, which is not covered in this protocol, is absolutely necessary.

One of the most critical aspects for successful Ca^{2+} imaging is the imaging setup and image acquisition conditions, as well as for other live-imaging studies. We previously showed that Ca^{2+} responses in the cell are highly dependent on the duration and intensity of excitation and on image acquisition conditions, including exposure time and acquisition frequency[16]. Excitation illumination power is the most critical factor, as it can cause light toxicity and photobleaching of GECIs. The recording conditions of exposure time, recording frequency, excitation light intensity, and duration of recording should be optimized according to the purpose of the experiment. We recommend reducing the exposure time and the excitation light intensity as much as possible to avoid photobleaching and phototoxicity 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 avoid photobleaching and phototoxicity also. 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. Another important factor is the expression level of the GECIs. GECIs have a Ca^{2+}-buffering effect as they are Ca^{2+}-binding proteins. Therefore, the overexpression of GECIs results in the buffering of Ca^{2+}, which is physiologically necessary for the cells. The amount of GECI expression should be minimized to avoid imaging cells expressing high amounts of GECIs.

In conclusion, the dissection of Ca^{2+} signals at a subcellular resolution is one of the most important steps for decoding intracellular Ca^{2+} signals that determine the output biological phenomenon. This protocol provides a new method for the dissection of Ca^{2+} signals to describe the diversity among these signals. Presently, this technique is limited for in vitro experiments. However, Lck-GCaMP6f is already being used for in vivo Ca^{2+} imaging in mice[17], and OER-GCaMP6f was confirmed to monitor Ca^{2+} signals in vivo in the VD motor neurons in *C. elegans*[7]. Therefore, targeting GECIs in the subcellular compartment has the potential to be expanded to in vivo imaging in the future, thus enabling Ca^{2+} dissection in vivo.

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DISCLOSURES:

The authors have nothing to disclose.

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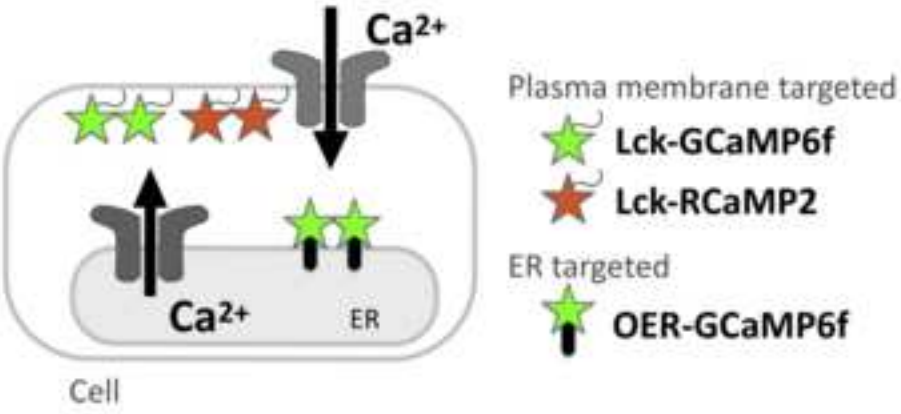

Figure 1

Figure 2

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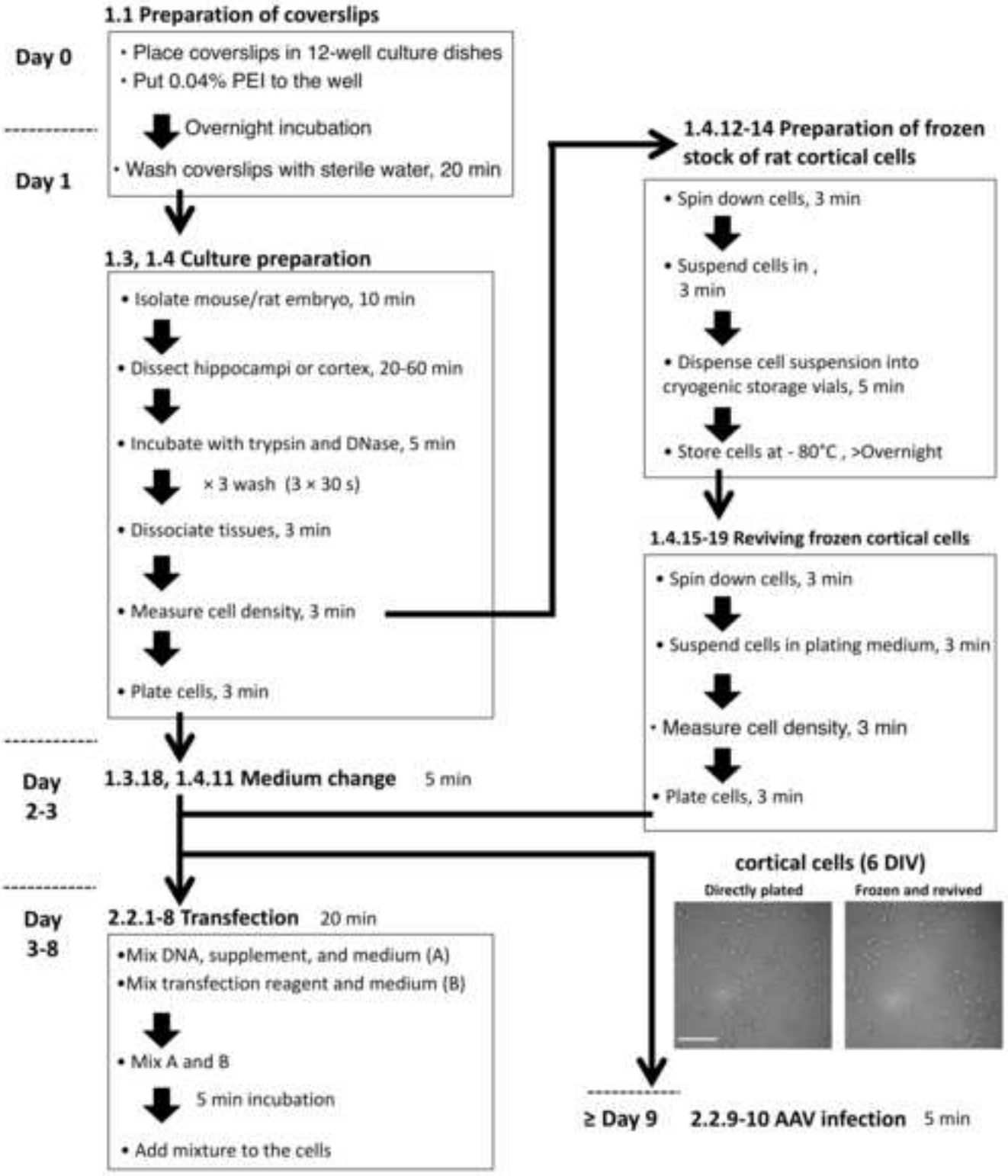

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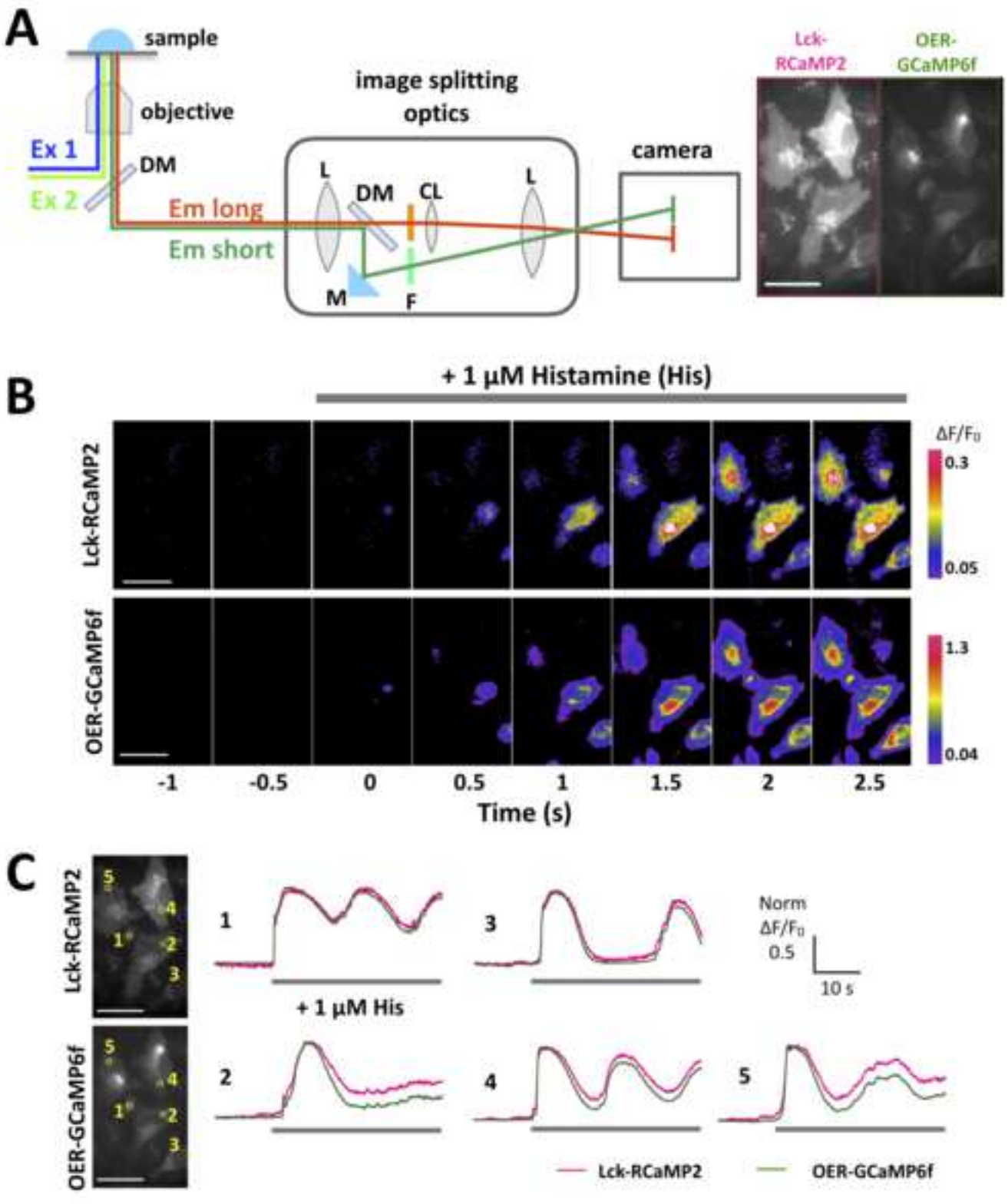

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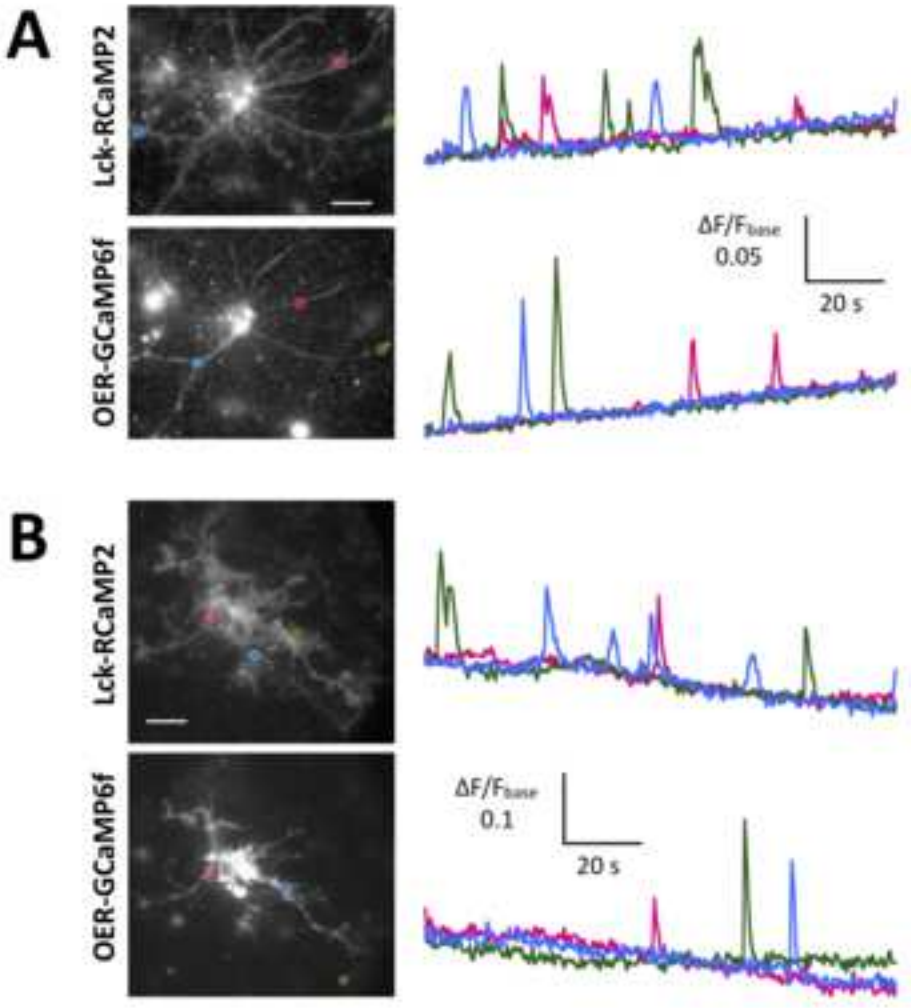

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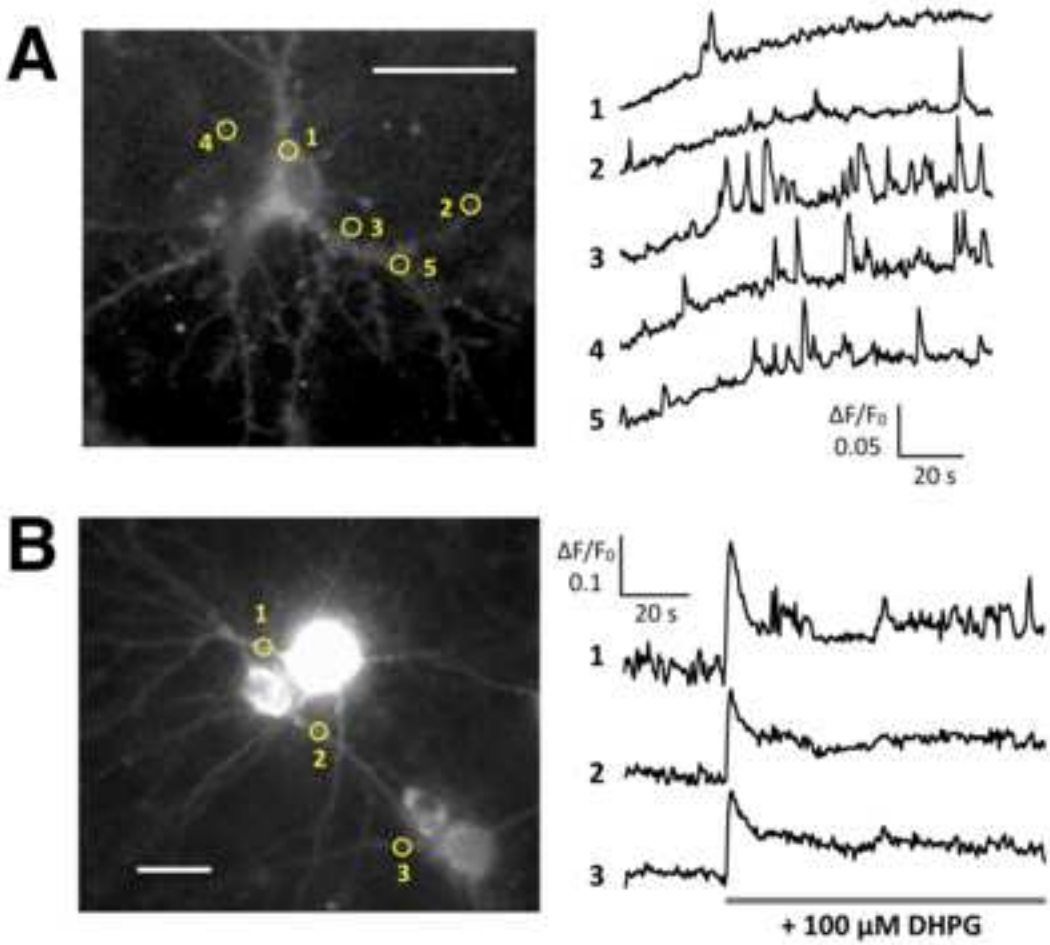
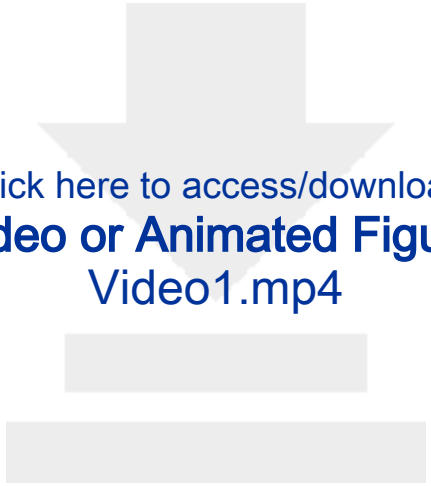


Figure 5

Video1



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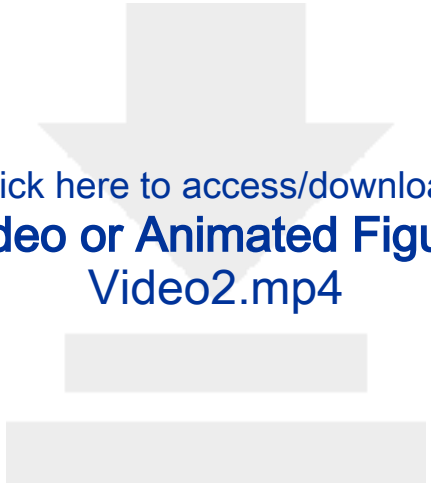
Video2



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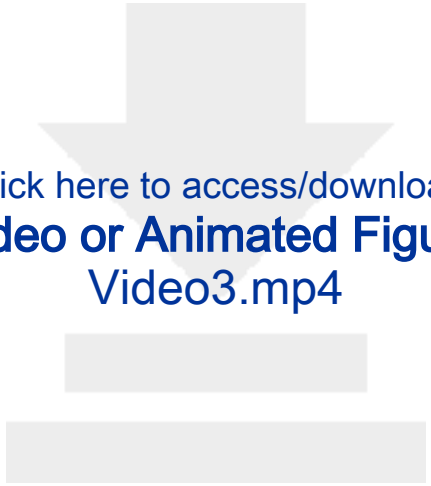

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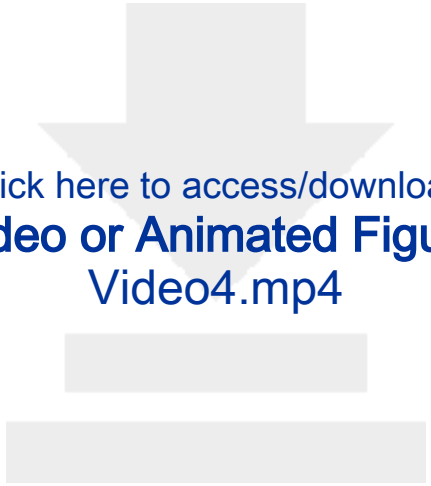

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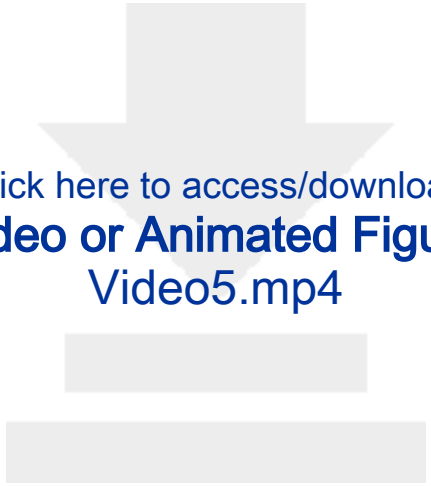

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Video7

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
(RS)-3,5-Dihydroxyphenylglycine (DHPG)	Tocris	#0342	
0.5% DNase I stock solution	Sigma-Aldrich	#11284932001	Prepare 0.5% DNase I (w/v) in Hanks' Balanced Salt Solution supplemented with 120 mM MgSO4. Prepare 160 µL aliquots and store at -30°C.
0.5% Trypsin-EDTA solution	Thermo Fisher Scientific	#25300054	
100 mM L-glutamine (×100 stock)	Thermo Fisher Scientific	#25030081	Preparing small aliquots of 250--750 µL and store at -30°C.
100 mM Sodium pyruvate (×100 stock)	Thermo Fisher Scientific	#11360070	Aliquots (10 mL) can be stored at -20°C. After thawing, the solution can be maintained at 4°C for 2 months.
12-Well multiwell culture plates with low-evaporation lid	Falcon	#353043	Low-evaporation lid is critical for culturing neuron-glia mixed culture. For cell line cells, alternative culture dishes can be used.
18-mm diameter circular coverslips	Karl Hecht "Assistent"	#41001118	Thickness 1, 18-mm diameter circular coverslips; alternative coverslips can be used.
1M HEPES	Thermo Fisher Scientific	#15630080	pH 7.2 - 7.6

2.5% Trypsin stock solution (×20 stock)	Sigma-Aldrich	#T4674	Prepare 150 µL aliquot and store at -30°C.
50% Poly(ethyleneimine) (PEI) solution	Sigma-Aldrich	#P3143	Prepare 2% (V/V) PEI stock solution (×50) with distilled water sterilized by filtration. Store stock solution at -30°C after preparing small aliquots of 250-750 µL. Prepare 0.04% PEI solution with distilled water on the day of coverslip coating.
70% Ethanol			Kept in a spray bottle to be used for surface disinfection.
Adeno-associated virus (AAV) for Lck-GCaMP6f, Lck-RCaMP2, and OER-RCaMP2 expression under the direction of the EF1a promoter			AAV can be prepared using AAV Helper Free System (Agilent Technologies) and HEK293 cells, or alternative methods. pAAV.EF1a.Lck-GCaMP6f, pAAV.EF1a.Lck-RCaMP2, and pAAV.EF1a.OER-GCaMP6f are available upon request.

B-27 supplement (×50 stock)	Thermo Fisher Scientific	#17504044	This can be replaced by B-27 plus supplement (Thermo Fisher Scientific; #A3582801) or MACS NeuroBrew-21 (Miltenyi Biotec, Bergisch Gladbach, Germany; #130-093-566).
B57BL/6	Japan SLC, Inc.		
Camera for microscopic image recording			The following cameras were available for use: cooled-CCD camera (e.g., Hamamatsu Photonics, OECA-ER), EM-CCD camera (e.g., Hamamatsu Photonics, ImagEM; Andor, iXon) or CMOS camera (e.g., Hamamatsu Photonics ORCA-Flash4.0)
Cell freezing container	Sarstedt K.K.	#95.64.253	Alternative cell freezing container can be used.
Cell strainer	Falcon	#352350	
CO_2 incubator			Maintain at 37°C, 5% CO_2.
Cryogenic tube	Corning	#430661	Alternative cryogenic tubes can be used.
Cryopreservation medium	Zenoaq		"CELLBANKER1"

Culture medium (for HeLa cells)			Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, and penicillin-streptomycin solution (final concentration: Penicillin 100 units/mL and Streptomycin 100 µg/mL)
Dissection medium			One milliliter of 1 M HEPES (final concentration 20 mM) to 49 mL DMEM
DMEM	Nacalai	#08456-65	Alternative DMEM can be used.
DMEM	Nacalai	#08456-65	Low glucose
DNA transfection reagent	Sigma-Aldrich	#6366244001	"X-tremegene HP DNA transfection reagent" Alternative transfection reagents can be used.
Glass jar with a lid			500 mL jar (for mouse) or 1500 mL jar (for rat) to anesthetize the animal

HBSS	Thermo Fisher Scientific	#14170161	HBSS free of calcium and magnesium
Heat inactivated bovine serum	Thermo Fisher Scientific	#10100147	
HeLa cells	RIKEN BioResource Center	#RCB0007	
Histamine	Sigma-Aldrich	#H7125	
Image analysis software			Such as Metamorph (Molecular Devices), Image J (NIH), and TI Workbench14 (custom made)
Image splitting optics	Hamamatsu Photonics	#A12801-01	W-view GEMINI
Image splitting optics dichroic mirror	Semrock	#FF560-FDi01-25×36	For separation of green fluorescent protein/red fluorescent protein (GFP/RFP) signal
Image splitting optics emission filters	Semrock	#FF01-512/25-25, #FF01-630/92-25	For emission of GFP/RFP signal, respectively

Imaging medium and buffer			Use optimal medium or buffer for the experiment. When medium is used, medium without phenol red is desirable to reduce background fluorescence. Add 20 mM HEPES to maintain pH outside of CO_2 incubator.
Incubation saline			Add 1 mL of 1 M HEPES (20 mM) to 49 mL HBSS
Inverted fluorescence microscope			Such as IX73 (Olympus) or Eclipse TI (Nikon Instech)
Isoflurane	Pfizer		Used for anesthesia
Maintenance medium (for 4 × 12 well dishes)			48.5 mL Neurobasal-A medium supplemented with 1 mL B-27, 500 µL of L-glutamine stock and 25 µL Penicillin-Streptomycin solution.
Maintenance medium for frozen cortical cells (for 1 × 12 well dishes)			12.2 mL Neurobasal plus medium supplemented with 250 µL B-27, 125 µL of L-glutamine stock and 6.2 µL Penicillin-Streptomycin solution.
MEM (Minimum Essential Medium)	Thermo Fisher Scientific	#11090-081	

Microscope filter set for GCaMP6f imaging			Appropriate filter for GFP (excitation, 480 ± 10 nm; emission, 530 ± 20 nm)
Microscope filter set for RCaMP2 imaging			Appropriate filter for RFP (excitation, 535 ± 50 nm; emission, 590 nm long pass)
Microscope filter sets for double imaging of RCaMP2 and GCaMP6f	Semrock	#FF01-468/553-25, #FF493/574-FDi01-25×36, #FF01-512/630-25	Dual excitation filter, Dual dichroic mirror, and emission filter for GFP/RFP imaging.
Microscope heating system			A heating system to maintain cells at 37°C during the imaging. To avoid drift caused by thermal expansion, heating systems covering the entire microscope itself (e.g., Tokai Hit, Thermobox) are recommended.

Microscope light source for excitation			Mercury lamp (100 W), xenon lamp (75 W), Light-emitting diode (LED) illumination system (e.g., CoolLED Ltd., precisExcite; Thorlabs Inc., 4-Wavelength LED Source; Lumencor, SPECTRA X light engine). In case of mercury lump and xenon lamp, use ND filter to reduce the excitation intensity.
Microscope objective lens			Plan-Apochromat oil immersion objective with numerical aperture higher than 1.3 is highly recommended for the recording of spontaneous Ca2+ activity in neurons and astrocytes.
Neurobasal plus medium	Thermo Fisher Scientific	#A3582901	
Neurobasal-A Medium	Thermo Fisher Scientific	#10888022	Neurobasal plus medium (Thermo Fisher, A3582901) can be used instead of Neurobasal-A medium.

PBS(-): Phosphate-buffered saline free of Ca2+ and Mg2+	Fujifilm Wako Pure Chemical Cooperation	#164-23551	The absence of Ca2+ and Mg2+ is critical not to inhibit the trypsin activity. An alternative to PBS(-) can be used.
PC and image acquisition software			Such as Metamorph (Molecular Devices); Micromanager; TI Workbench[14].
Penicillin-Streptomycin solution	Thermo Fisher Scientific	#15140122	Penicillin 10,000 units/mL and Streptomycin 10,000 µg/mL
Plasmid for Lck-GCaMP6f, Lck-RCaMP2, and OER-RCaMP2 expression under cytomegalovirus promoter [7-9]			Available upon request
Plating medium (for 4 × 12 well dishes)			48 mL MEM supplemented with 1 mL B-27 supplement, 500 µL L-glutamine stock (final concentration: 2 mM), 500 µL of sodium pyruvate stock (1 mM) and 25 µL Penicillin-Streptomycin solution (penicillin 5 u/mL, streptomycin 5 µg/mL). This concentration of Penicillin-Streptomycin, which is 1/20 of the concentration recommended by the manufacturer, is critical for neuronal survival.

Recording chamber	Elveflow	Ludin Chamber	This recording chamber is for 18 mm diameter round coverslips.
Reduced serum media	Thermo Fisher	#11058021	Opti-MEM
Stereomicroscope			Used to dissect hippocampi. Olympus SZ60 or equivalent stereomicroscopes are available.
Surgical instruments			Standard dissecting scissors to cut the abdomen of a mouse or a rat, tweezers to pinch the uterus, delicate dissecting scissors to cut the uterus and the head of embryo, ring forceps to pinch the embryos, 13-cm curved Semken forceps (Fine Science Tools #11009-13) to extract brains, 3 forceps with fine tips (Dumont Inox #5)

Transfection reagent for neuron	Thermo Fisher Scientific	#L3000008	"Lipofectamine 3000" reagent. It is composed of the the "supplement (P3000)" that should be mixed with plasmid DNA in the step 2.2.3, and the "transfection reagent (lipofectamine 3000)" used in the step 2.2.4.
Trypan blue (0.4%)	Thermo Fisher Scientific	#15250061	
Wash medium for frozen cortical cells			25 mL DMEM, supplemented with 250 µL heat-inactivated fetal bovine serum + 12.5 µL Penicillin Streptomycin.
Wistar rats	Japan SLC, Inc		Pregnant rats (E18)

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Author(s):	Hiroko Bannai, Matsumi Hirose, Fumihiro Niwa, Katsuhiko Mikoshiba

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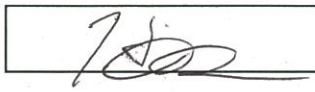
Signature:	
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Point-by-point response to comments from the editor and reviewers

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.

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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.