Supporting Information

An Activity-Based Methionine Bioconjugation Approach to Developing Proximity-Activated Imaging Reporters

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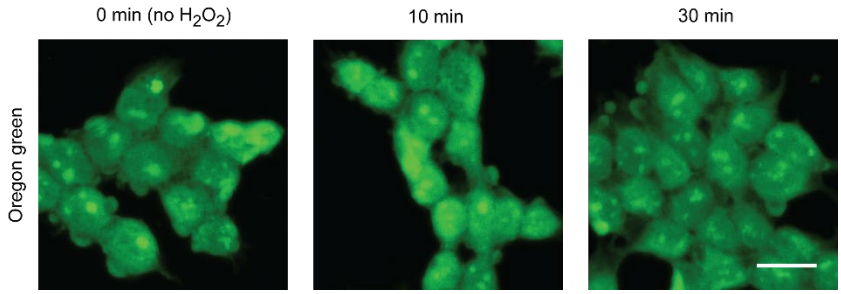


Figure S1. Confocal images for Oregon green channel of the Met-PAIR experiment on actin corresponding to Figure 2b. Scale bar: 20 µm.

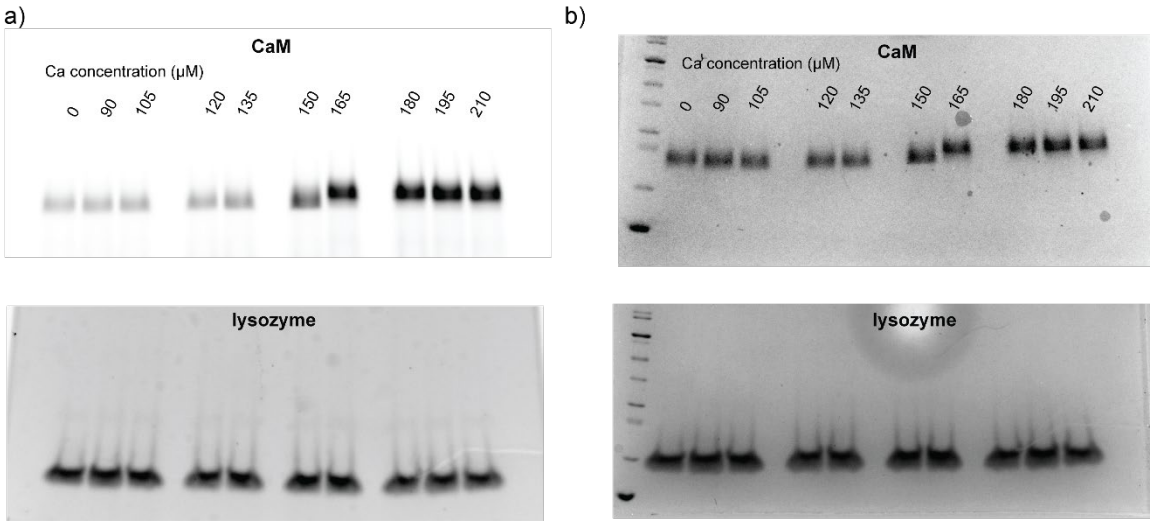
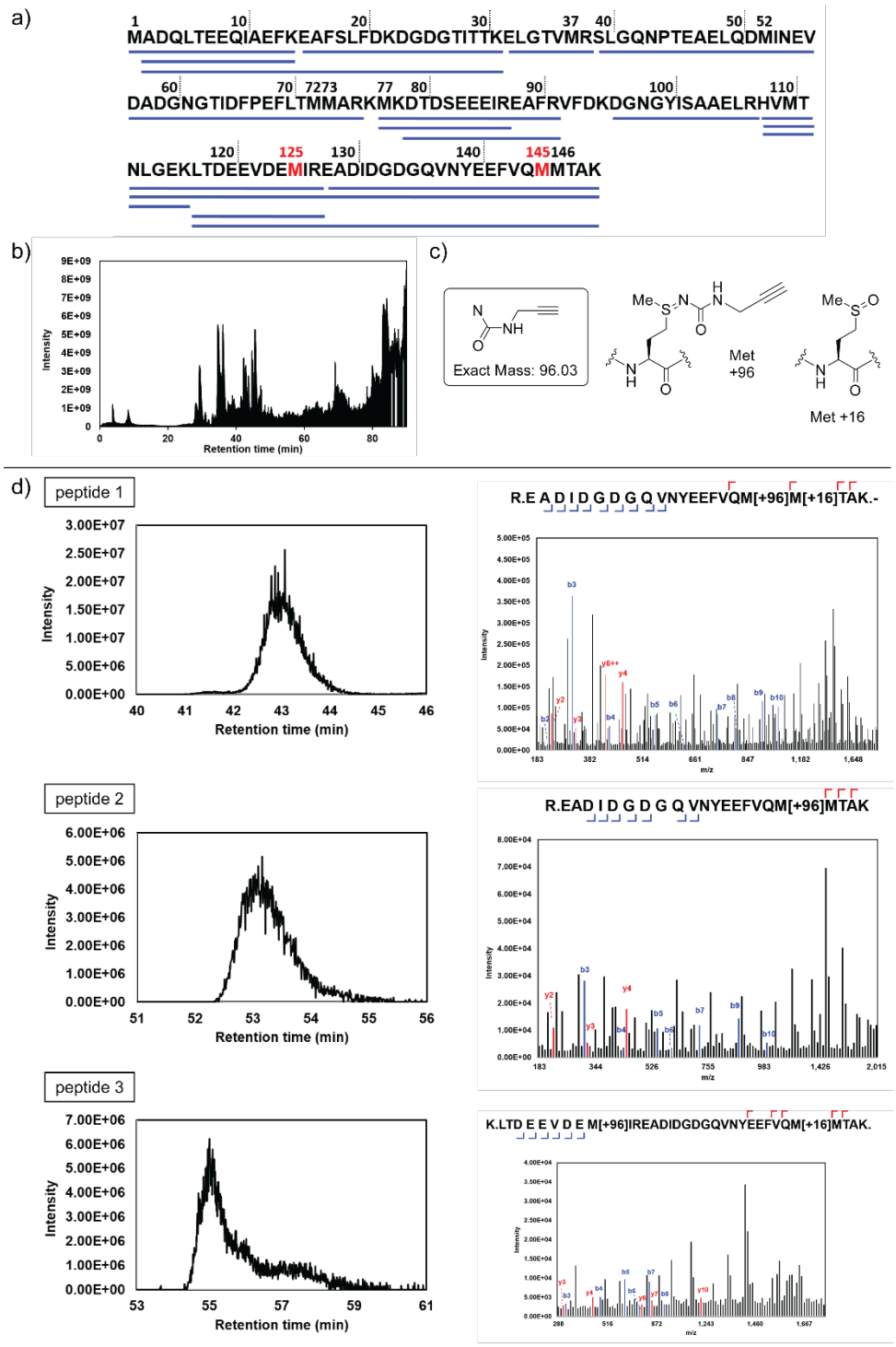


Figure S2. Representative gel images for in vitro CaM (top) or lysozyme (bottom, control protein) labeling with different Ca dose corresponding to Figure 3c. a) Fluorescence gel image. b) Coomassie total staining image of a).



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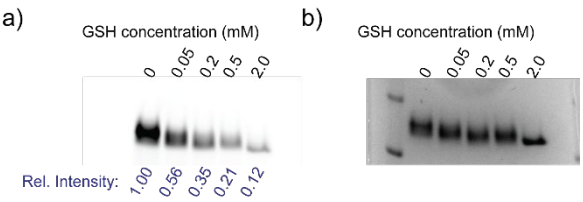


Figure S4. Gel images for in vitro CaM labeling in the presence of glutathione (GSH, 0–2.0 mM) Reaction conditions: CaM (2.75 μM), **Ox4** (15 μM), $CaCl_2$ (210 μM), and GSH (shown amount) at rt for 5 min, and the reaction mixture was subjected to click chemistry protocol described in *Ca dose experiment (Figure 3c)*. a) Fluorescence gel image. Relative fluorescence intensity was obtained by ImageJ software. b) Coomassie total staining image of a). The data show that the Ox reagents can label protein targets even in the presence of competing thiols at 1000-fold excess.

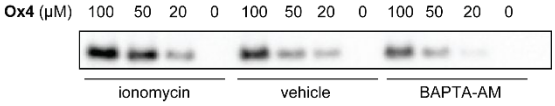
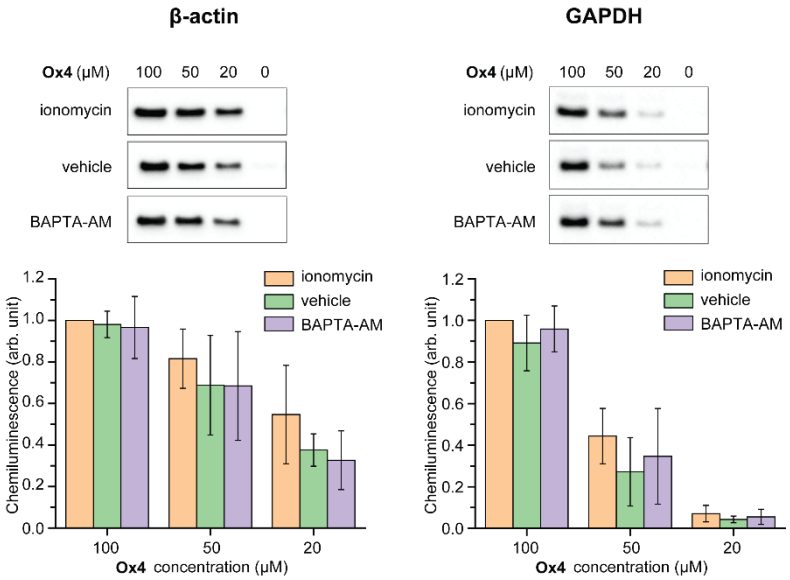


Figure S5. Full-width anti-CaM western blot image corresponding to Figure 3e.



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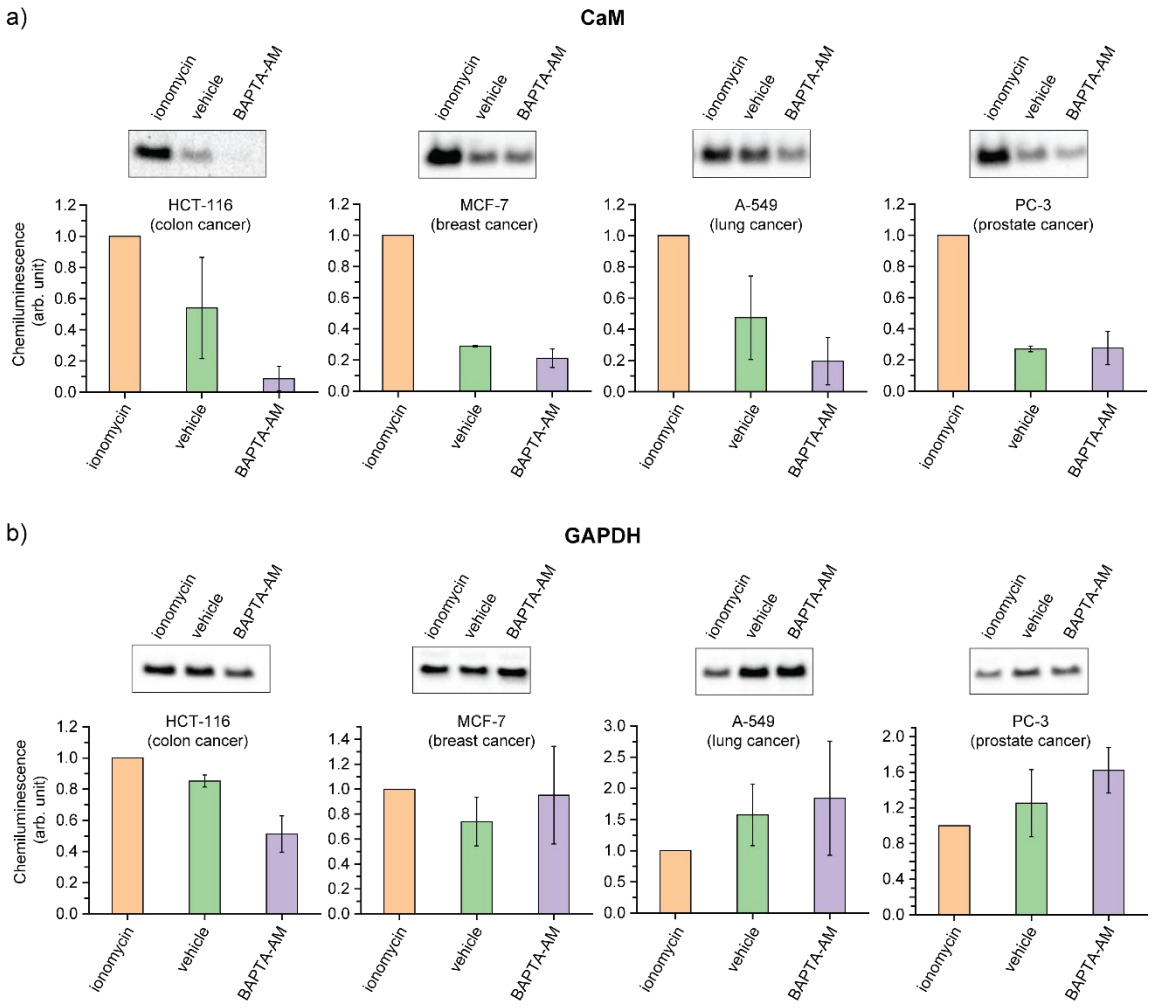

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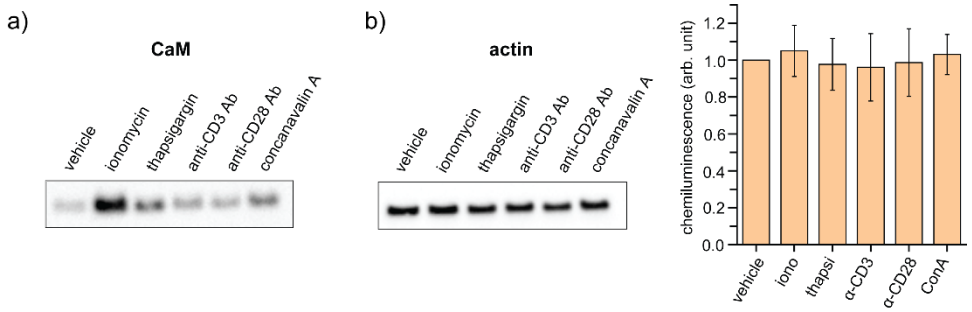

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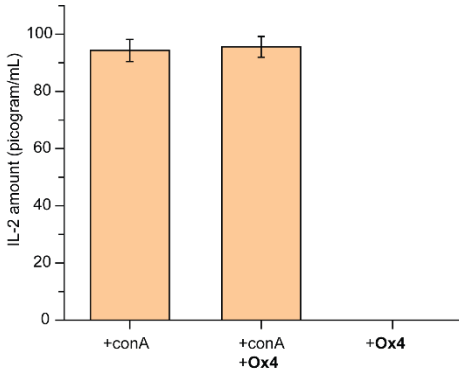

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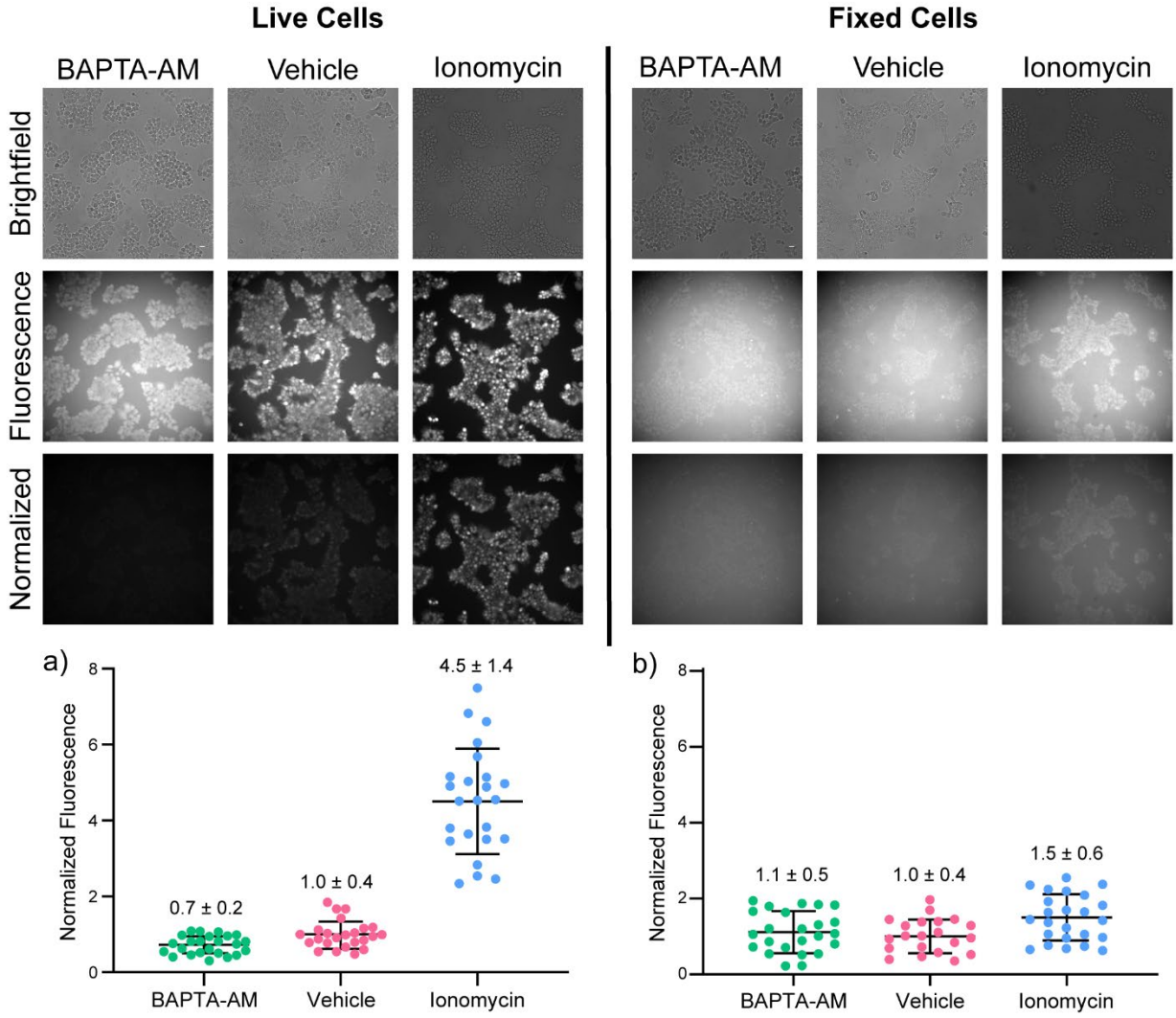


Figure S10. Brightfield and fluorescence images of HEK cells before and after fixation that have been stained with 1 μM Oregon Green 488 BAPTA-AM treated with one of the following HBSS solutions; 10 μM ionomycin, 30 μM BAPTA-AM (Ca^{2+}, Mg^{2+} free HBSS) and 0.1% DMSO vehicle. 20 μm scale bar. a) Live cell average background subtracted

cellular fluorescence normalized to a vehicle average. 3 ROIs per area, 4 areas per coverslip, 2 coverslips per condition. n = 24 for each condition. n represents an ROI. b) Fixed cell average background subtracted cellular fluorescence normalized to vehicle average. 3 ROIs per area, 4 areas per coverslip, 2 coverslips per condition. n = 24 for each condition except vehicle (n = 21). n represents an ROI.

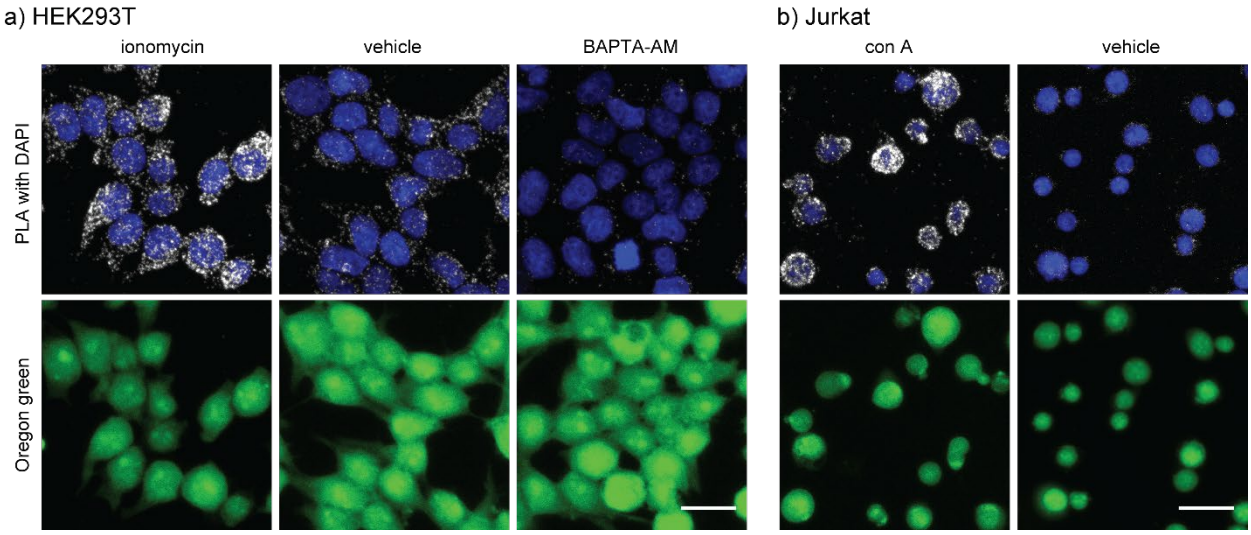
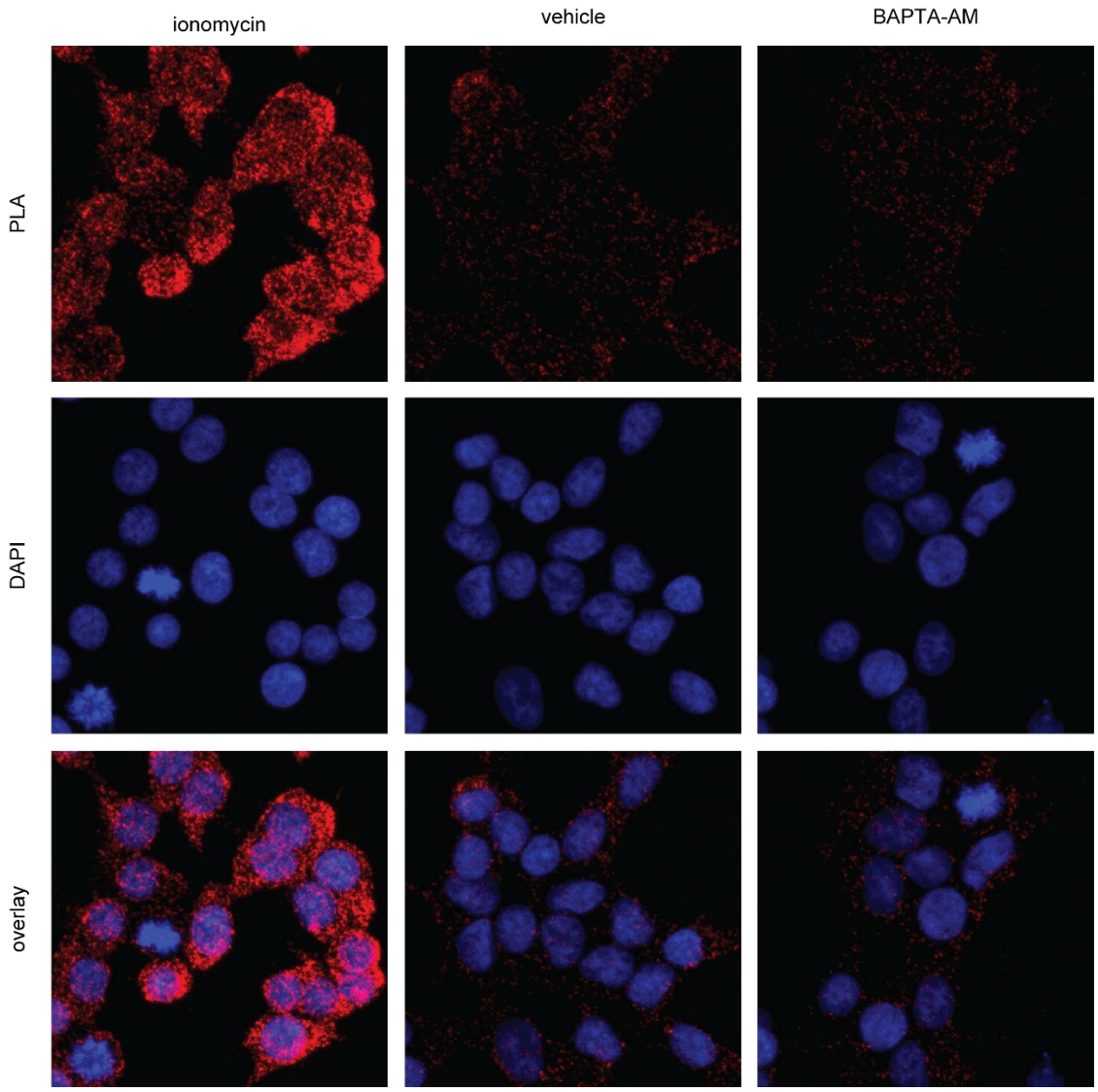


Figure S11. Confocal images for Oregon green channel of the Met-PAIR experiment on CaM, corresponding to Figure 4: a) HEK293T and b) Jurkat cells. Scale bar: 20 µm.



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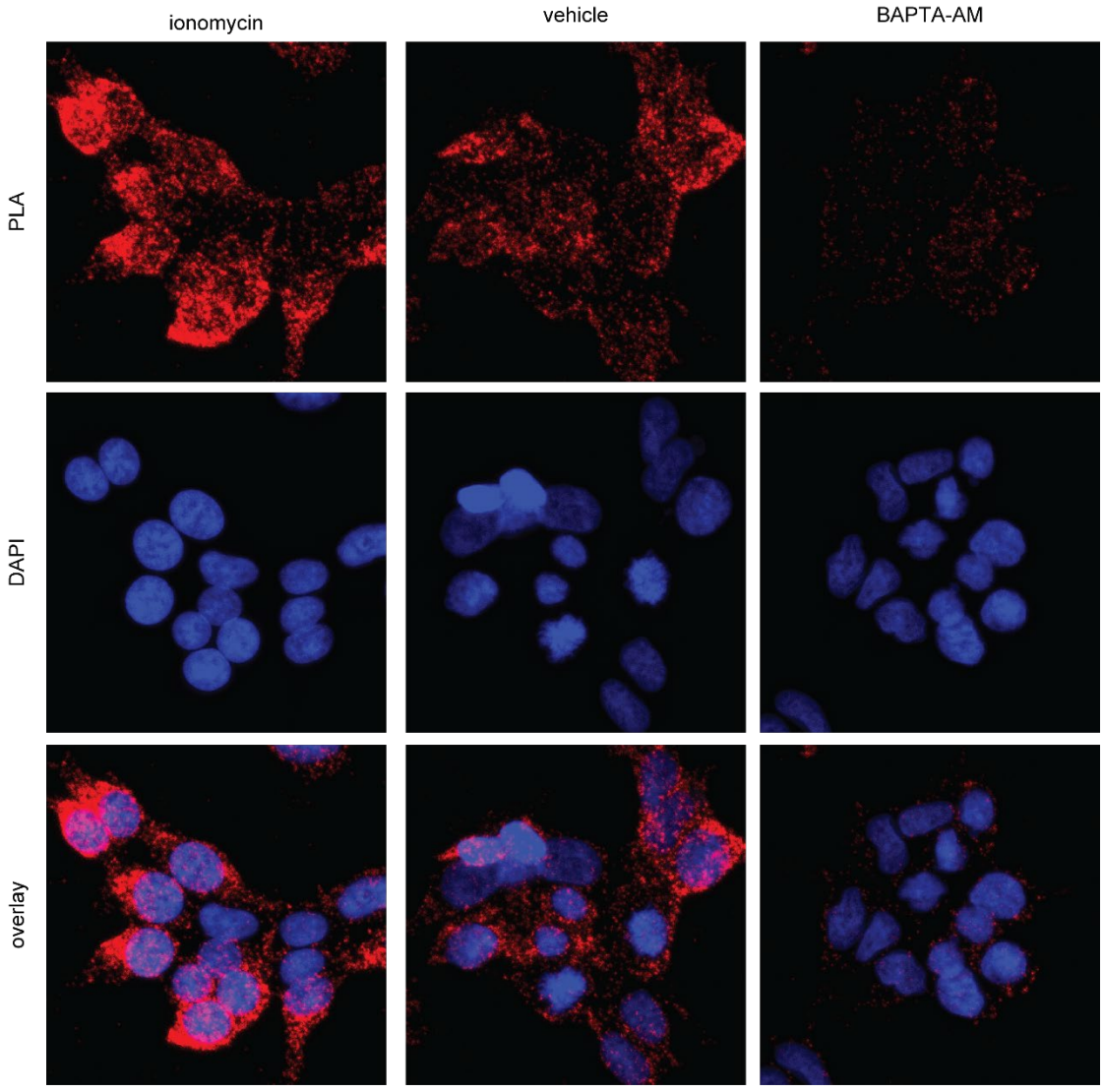

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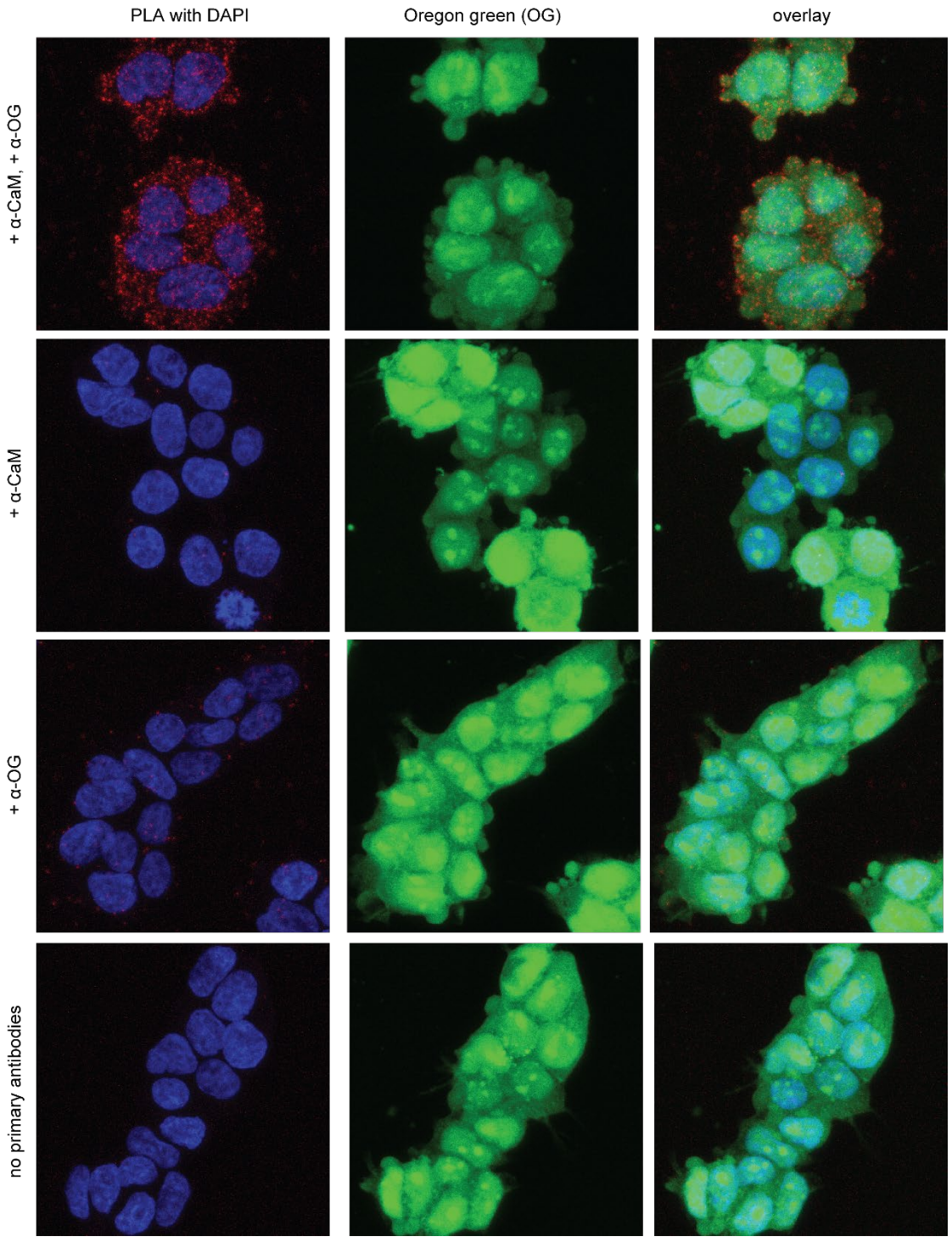

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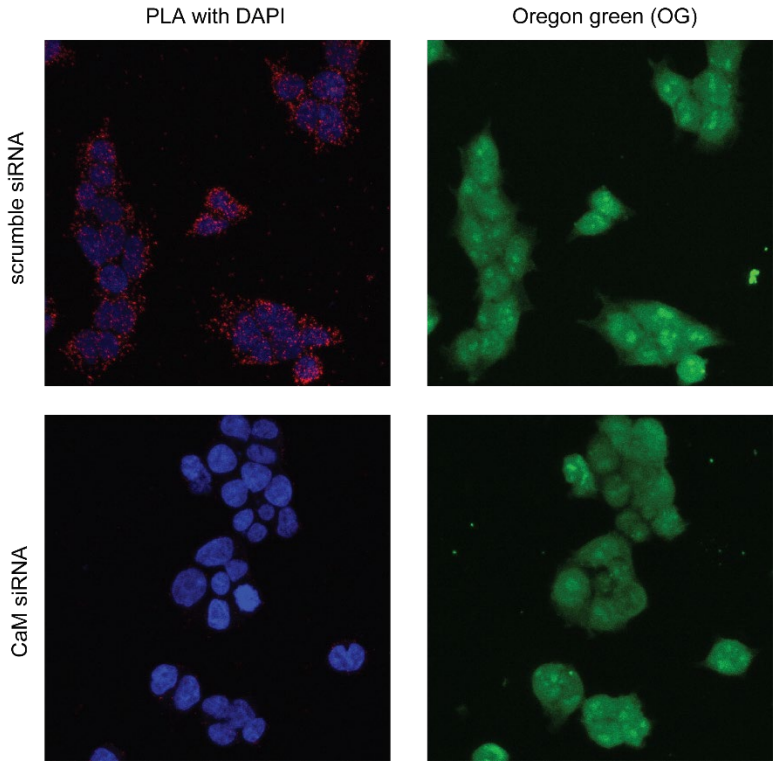

Figure S15. Confocal images for Met-PAIR on CaM for HEK293T cells with siRNA-mediated knockdown of CaM (bottom) or scramble siRNA as a positive control (top) in the same conditions as Figure 4b vehicle. The images are taken as 8-bit and processed through maximum intensity projection of the z stack images. HEK293T cells were transfected with siRNA (50 μM) using Lipofectamine RNAiMAX for 72 h. Red: PLA. Blue: DAPI nuclear staining.

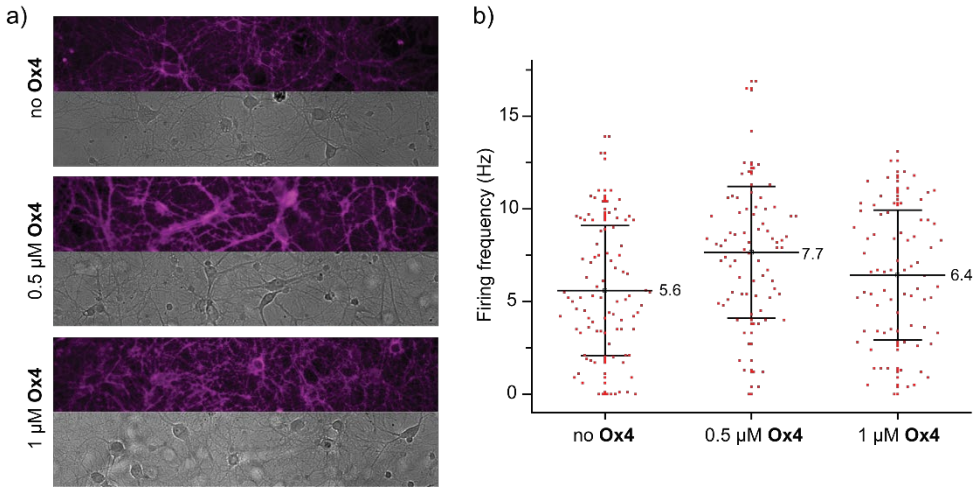

Figure S16. Fluorescence analysis of voltage response of rat hippocampal neuron stained by a voltage-sensitive fluorophore, BeRST1 in the presence or absence of **Ox4**. 0.1% dimethylformamide was applied for the "no **Ox4**" conditions. a) Representative neuron images for the experiments. BeRST1 fluorescence (top) and DIC channel (bottom) are shown in magenta and gray, respectively. b) Whisker plots for the confocal images in a). Each dot represents the firing frequency of the cell bodies of each neuron. Whisker and center line represents the standard deviation and mean intensity, respectively. The mean value is shown near the center line. The quantification was conducted with imaging of 4 regions in each of 2 independent biological replicates (total 8 images). The number of neurons quantified per condition: 112 (no **Ox4**), 93 (0.5 μM **Ox4**), 91 (1 μM **Ox4**).

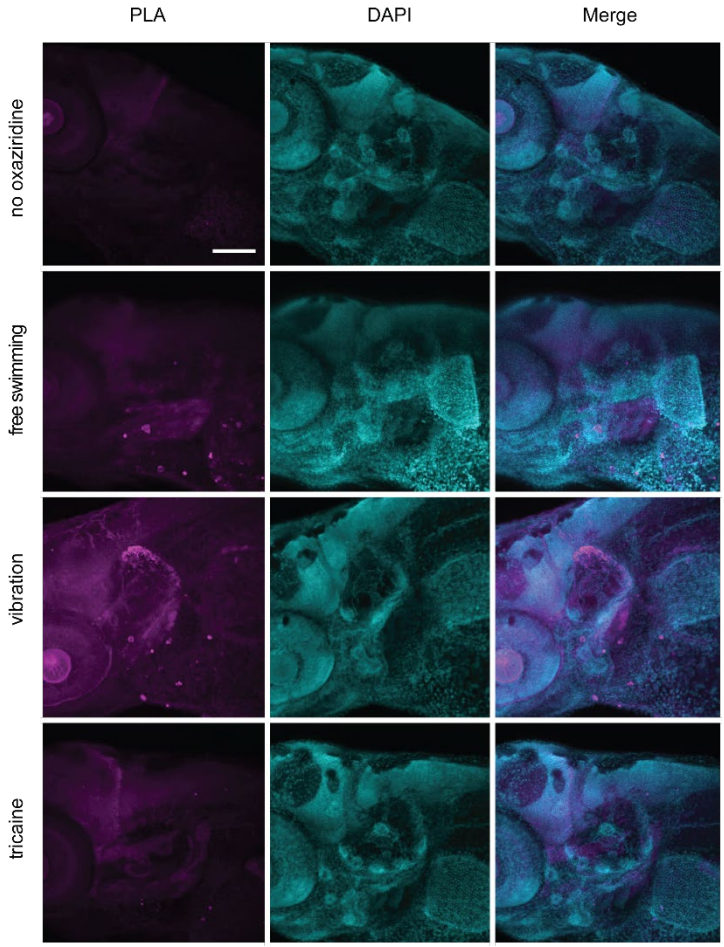

Figure S17. Confocal images for Met-PAIR on CaM for zebrafish (3 day post-fertilization) with mouse anti-CaM antibody and mouse anti-biotin antibody, corresponding to Figure 5b. Magenta: PLA signal. Cyan: DAPI nuclear staining. Scale bar: 100 μm.

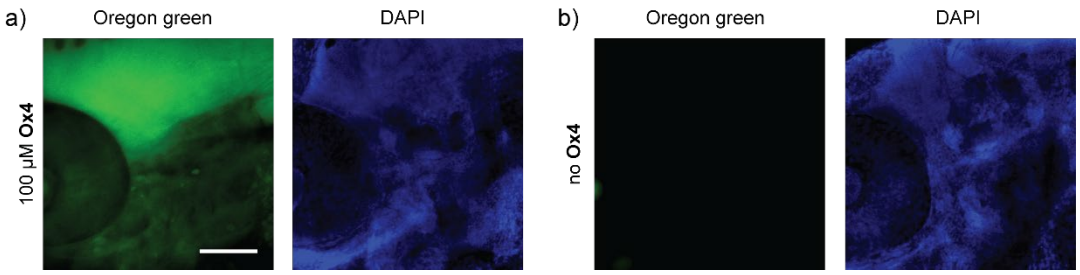

Figure S18. Confocal images for head of zebrafish (3 day post-fertilization) fixed and treated with Oregon green-azide to visualize and confirm the in vivo **Ox4** labeling. Green: Oregon green signal. Blue: DAPI nuclear staining. a) **Ox4**-treated zebrafish images (100 μM). b) Negative control zebrafish images. Scale bar: 100 μm.

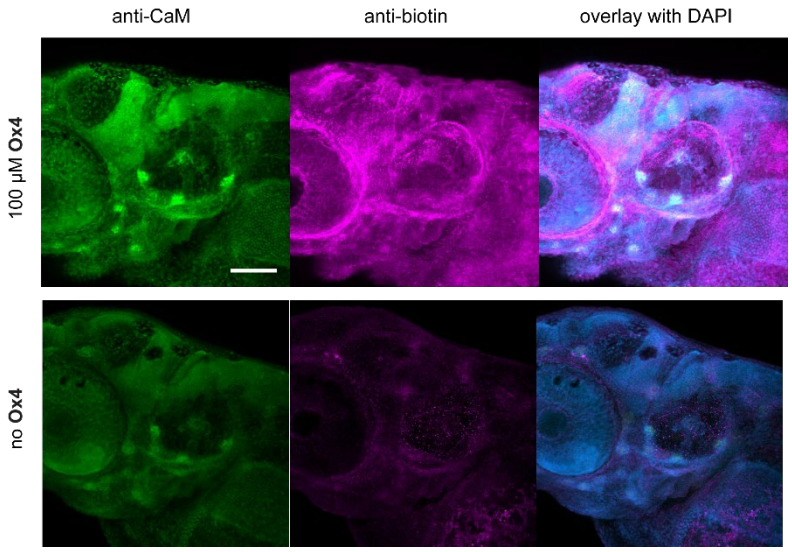

Figure S19. Confocal images for immunofluorescence of head of zebrafish (3 day post-fertilization) after the in vivo **Ox4** labeling. Green: mouse anti-CaM primary antibody with donkey anti-mouse secondary antibody–AlexaFluor488 conjugates. Magenta: rabbit anti-biotin primary antibody with donkey anti-rabbit secondary antibody–AlexaFluor594 conjugates. Top: **Ox4**-treated larvae (100 µM). Bottom: Negative control larvae. Scale bar: 100 µm.

Methods and experimental details

General methods

Materials and Reagents

All chemicals and proteins were purchased from commercial suppliers and used without any purification unless otherwise noted. **Ox4** was synthesized according to a previous report,[1] and 100 or 1000X solution in DMF were used for cell experiments and stored in –20 °C. For zebrafish experiment, an aliquot of 1000X DMSO stock solution stored in –80 °C was used. Comparable performance of a few month old stock solution of **Ox4** to fresh stock was confirmed by repeating fluorescence gel experiment (corresponding to Figure 3c). All of anti-β-actin blot and β-actin PLA experiments were conducted with mouse anti-β-actin antibody (Santa Cruz, monoclonal, sc-69879). All anti-CaM blot and calcium PLA experiments were conducted with mouse anti-CaM antibody (Millipore, monoclonal, 05-173) unless otherwise noted. Rabbit anti-CaM antibody was purchased from Abcam (monoclonal, ab45689). Rabbit anti-fluorescein/Oregon green antibody was purchased from ThermoFisher (polyclonal, A-889). Rabbit anti-biotin antibody and mouse anti-biotin antibody were purchased from Bethyl (polyclonal, A150-109A) and Invitrogen (monoclonal, 03-3700), respectively. Mouse anti-GAPDH antibody was purchased from Abcam (monoclonal, ab8245). Mouse anti-MAP2A antibody–AlexaFluor™488 conjugate was purchased from Novus Biologicals (monoclonal, NB120-11267AF488). Duolink PLA red starter kit was purchased from Sigma-Aldrich. Blocking One was purchased from Nacalai Tesque (03953-95). Three sets of CaM siRNA (SI02224215, SI02758413 and SI02622060) or scrambled siRNA (SI03650318) were obtained from Qiagen, and the knockdown experiment was conducted according to literature precedents.[2,3]

Gel and blot analysis

Gel and blot samples were prepared by addition of 4X LDS sample buffer (Invitrogen #NP0007) and 0.2 mM DTT solution in PBS to a sample. After heating at 95 °C for 5 min, the samples were loaded to Tris-Glycine gel (Invitrogen, #XP04205BOX), and the electrophoretic separation was conducted for 50 min, followed by protein transfer to blot membrane (Biorad, TransBlot Turbo LF PVDF membrane). The membrane was blocked with Blocking One at room temperature (rt) for 15 min, and incubated with primary antibody solution (1:2000) in Blocking One at rt for 1 h. The membrane was washed with TBST buffer 3 times, incubated with anti-mouse antibody–HRP conjugates (1:2500) in Blocking One at rt for 1h, washed with TBST buffer 3 times, and imaged by ChemiDoc imaging system (Biorad). For anti-CaM blot, after the protein transfer process, the membrane was briefly rinsed with PBS, incubated in 0.2% glutaraldehyde solution in PBS at rt for 30 min, washed with TBST buffer 3 times, and subjected to the blocking process and the rest of the antibody procedures as described above.

MS analysis

Electrospray ionization (ESI) MS was conducted on an Agilent 6224 time of flight (TOF) mass spectrometer equipped with an Agilent 1260 Infinity series high-performance liquid chromatography system. The protein was eluted on a 50 mm ProSwift™ RP-4H LC Column

(Thermo Fisher Scientific, # 069477) using a water/acetonitrile gradient up to 95% acetonitrile (with 0.1% formic acid) over 8 min.

LC-MS/MS analysis was performed as previously described.[4,5] Tryptic-digested peptides were pressure-loaded onto a 250 μm inner diameter deactivated fused silica capillary tubing packed with 4 cm of Aqua C18 reverse-phase resin (Phenomenex, #04A-4299) pre-equilibrated using a gradient from 0–100% buffer B in buffer A over 10 min, followed by a 5 min wash with 100% buffer B and a 10 min wash with 100% buffer A (buffer A 95:5 water/acetonitrile, 0.1% formic acid; buffer B 80:20 acetonitrile/water, 0.1% formic acid). The samples were then attached using a MicroTee PEEK 360 μm fitting (Thermo Fisher Scientific, #P-888) to a 13 cm laser pulled column (100 μm inner diameter deactivated fused silica capillary) packed with either (a) 10 cm Aqua C18 reverse-phase resin and 3 cm of strong-cation exchange resin or (b) 13 cm Aqua C18 reverse-phase resin. Samples were analyzed using a Q Exactive Plus mass spectrometer (Thermo Fisher) coupled to an Agilent 1260 Infinity series high-performance liquid chromatography system and using the Xcalibur software (Thermo Fisher, version 4.0.27.19). Samples eluted using columns containing strong-cation exchange resin (version a) were analyzed using a 5-step Multidimensional Protein Identification Technology (MudPIT) program, using 0%, 25%, 50%, 80%, and 100% salt bumps of 500 mM aqueous ammonium acetate and using a gradient of 5–55% buffer B in buffer A. Samples eluted using a C18-only column (version b) were analyzed using a 0–45% buffer B in buffer A gradient over 70 min, followed by a 20 min wash with 100% buffer B. Data was collected in data-dependent acquisition mode with dynamic exclusion enabled (60 s). One full MS (MS1) scan (400-1800 m/z) was followed by 15 MS2 scans of the nth most abundant ions. Heated capillary temperature was set to 200 °C and the nanospray voltage was set to 2.75 kV. Data analysis was conducted using the Byonic software.[6] Methionine residues were searched with a variable modification of 96.0324 (labeled by **Ox4**) or adduct for methionine oxidation. Peptides were required to have at least one tryptic end.

Cell culture

HEK293T, HCT-116, MCF7, A-549, and PC-3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax and 10% fetal bovine serum (FBS). Jurkat cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with Glutamax and 10%FBS. For HEK293T and MCF7 cells, 1% non-essential amino acids were added to the medium. In addition, for MCF7 and Jurkat cells, 1% sodium pyruvate was added to each medium. For the pull down assay experiments, the cells were cultured in a 6-well plate (Corning, #3516) with 100% confluency (1×10^6 cells for Jurkat cells) without poly-L-lysine coating. For Met-PAIR experiments of HEK293T cells, 35,000–40,000 cells were prepared in a 12-well chambered slide (Ibidi, # 81201) pre-coated with poly-L-lysine at rt for 15 min (50 mg/mL). For Met-PAIR experiment of Jurkat cells, the 3,000,000 cells were washed with HBSS (–Ca, –Mg) once and re-suspended in 1 mL of the HBSS. Then, 50 μL of the cell suspension (150,000 cells) was put onto a coverslide pre-delineated by a hydrophobic pen (Vector Laboratories, #H-4000) as well as pre-coated with the poly-L-lysine. And then the slide was allowed to sit at rt for 5 min, gently washed with the HBSS, and subjected to the oxaziridine labeling described in *Met-PAIR experiment*. Each well area of the Jurkat experiment for the hydrophobic pen delineation was ~0.56 cm^2 similar to the 12-well chambered slide.

Hippocampi were dissected from embryonic day 19 Sprague Dawley rats (Charles River Laboratory) in cold sterile HBSS (no divalent cations). All dissection products were supplied by Invitrogen, unless otherwise stated. Hippocampal tissue was treated with trypsin (2.5%) for 15 min at 37 °C. The tissue was triturated using fire polished Pasteur pipettes, in minimum essential media (MEM) supplemented with 5% fetal bovine serum (FBS; Thermo Scientific), 2% B-27, 2% 1M dextrose (Fisher Scientific) and 1% GlutaMax. The dissociated cells were plated onto 12 mm diameter coverslips (Fisher Scientific) pre-treated with poly-D-lysine (1 mg/mL, Sigma-Aldrich) at a density of 20,000–25,000 cells per coverslip in MEM supplemented media (as above). Neurons were maintained at 37 °C in a humidified incubator with 5 % CO_2. After 1 day in vitro (DIV) half of the MEM supplemented media was removed and replaced with Neurobasal media containing 2% B-27 supplement and 1% GlutaMax. Functional imaging was performed on mature neurons 13–20 DIV. Treatment with **Ox4** and fixation of neurons were done after 12–15 DIV.

Confocal imaging

Fluorescence imaging was performed on a Zeiss laser scanning microscope 710 with a 40X water-immersion (Immersol™ W2010, Zeiss) objective lens (numerical aperture 1.4) for all cell experiments and with a 20X (Plan-Apochromat, Zeiss, 420650-9901) objective lens (numerical aperture 0.8) for zebrafish experiments. Excitation at 405 nm (DAPI), 488 nm (Oregon green), and 594 nm (Texas red for PLA) were used with filter settings 410–496 nm, 492–550 nm, and 599–690 nm, respectively. Images in each fluorescence channel were acquired separately to minimize fluorescence emission overlap. 20~30 Z stack slices were taken for cell experiments (16 bit) and 70~150 slices for zebrafish experiments (8 bit). Fluorescence images were processed by using the ImageJ software after average intensity projection (see *Quantification of fluorescence images* for the detail).

In vitro experiments

Preparation of calcium free, purified calmodulin for the in vitro experiments

Powder of purchased calmodulin (1 mg, G-Biosciences, #786-1243) was dissolved in PBS (250 µL, 4 mg/mL, 240 µM). To an aliquot (150 µL) of the protein solution, PBS (210 µL, fin. protein concentration: 100 µM) and EDTA (fin. concentration: 5 mM) was added. After 45-min incubation, the solution was passed through a pre-equilibrated desalting column (PD MiniTrap G-25 desalting column, GE Healthcare, #28918007), and the eluted solution was concentrated to ~150 µL by centrifugal filter (3k MWCO). The protein concentration was determined by Direct Ditect Infrared Spectrometer (Millipore). The obtained solution was aliquoted into PCR tubes, frozen with liquid nitrogen, and stored in –20 °C.

Ca dose experiment (Figure 3c)

CaM (2.75 µM) was incubated with **Ox4** (15 µM) and $CaCl_2$ (given conc) in PBS (total reaction volume 8 µL) at rt for 5 min. The reaction was quenched with acetylmethionine (0.5 mM), followed by incubation at rt for 1 h with click chemistry reagents: TBTA (0.1 mM), TAMRA-azide (0.2 mM, Sigma Aldrich, #760757), $CuSO_4$ (1 mM), and sodium ascorbate (2 mM). The reaction was quenched with EDTA (20 mM) and analyzed by fluorescence gel imaging. DTT and heating treatment process described in *Gel and blot analysis* was omitted for this experiment.

MS analysis (Figure 3d)

CaM (2.75 µM) was incubated with **Ox4** (15 µM) and $CaCl_2$ (0 or 180 µM) in PBS (total 50 µL reaction volume) at rt for 5 min. The reaction mixture was quenched with acetylmethionine (150 µM) and EDTA (1.5 mM). The solution was passed through a desalting column (Bio-Rad, #7326227) with Tris buffer (50 µL), and analyzed by ESI-MS.

Proteomics analysis for the CaM labeling (Figure S3)

CaM (2.75 µM) was incubated with **Ox4** (15 µM) and $CaCl_2$ (180 µM) in PBS (total 200 µL reaction volume) at rt for 5 min. The reaction mixture was quenched with acetylmethionine (150 µM) and EDTA (1.5 mM). Cold methanol (1.8 mL, –20 °C) was added, and the mixture was placed at –80 °C overnight to precipitate the protein. The mixture was centrifuged (16 g, 4 °C, 10 min). The supernatant was removed and the pellet was washed with cold acetone (–20 °C) three times. The pellet was then resuspended in 30 µL 8M urea/PBS and 30 µL of a 1X ProteaseMax™ (Promega, #V2072) solution (a 5X solution was prepared by addition of 100 µL of 100 mM ammonium bicarbonate buffer to the provided lyophilized powder and subsequently diluted to 1X) and vortexed for 15 s. 40 µL ammonium bicarbonate (100 mM), 120 µL PBS, and 1.2 µL of 5X ProteaseMax solution were then added and the mixture was vortexed. Sequencing grade Trypsin (Promega, # V5111) was reconstituted in 40 µL of the supplied Trypsin buffer and 1 µL of the resulting solution was added to the sample. The mixture was incubated at 37 °C overnight in a shaking incubator. The following day, the mixture was acidified with a final concentration of 5% formic acid (12 µL), centrifuged at maximum speed for 30 min, and the supernatant transferred to a low-adhesion tube and stored at –80°C until the MS analysis.

In cell experiments

Pull-down assay of live-cell oxaziridine labeling (Figure 3e,f)

From a well of cells cultured in a 6-well plate (Corning, #3516) without poly-L-lysine coating at 100% confluency, culture medium was taken out and washed with HBSS (–Ca and –Mg), and cells were transferred to 1.7-mL Eppendorf tubes and collected by centrifugation (200 rcf, 10 min). For HCT-116, MCF-7, A-549, and PC-3 cells, cells were incubated in 5 mM EDTA HBSS solution (–Ca and –Mg) at rt for 30 min before the transfer process to facilitate the removal of cells from the plate, and washed with HBSS (–Ca and –Mg) 3 times after the transfer and centrifugation processes.

In the Eppendorf tube, the cells were incubated with **Ox4** (given concentration for HEK293T. 20 μM for other cell lines) in HBSS (with Ca and Mg except for BAPTA-AM experiment) at rt for 20 min in the presence of one of following reagents: ionomycin (10 μM, except for Jurkat with 2 μM, Cayman Chemical, #11932), DMSO (1% v/v), BAPTA-AM (30 μM, TCI Chemicals, #T2845), thapsigargin (1 μM, Adipogen, #AG-CN2-0003-M), anti-CD3 mouse antibody (10 μg /mL, ThermoFisher, monoclonal, #14-0037-82), anti-CD28 mouse antibody (5 μg/mL, ThermoFisher, monoclonal, #16-0289-81), or concanavalin A (0.1 mg/mL, Sigma Aldrich, #C2010). After the addition of HBSS containing 0.5 mM acetylmethionine, the cells were incubated at rt for 2 min, separated from the buffer by centrifugation, placed in –80 °C for 1 h, and lysed in RIPA buffer (80 μL) containing 0.2 mM acetylmethionine and EDTA-free protease inhibitor (cOmplete Tablets, Roche #04-693-159-001).

After aspirating and dispensing to maximize the lysis process, cell debris was separated by centrifugation (16.1k g, 30 min, 4 °C), followed by incubation with TBTA (0.2 mM), biotin-PEG-azide (0.6 mM, Click Chemistry Tools, #AZ104), $CuSO_4$ (1 mM), and Na ascorbate (2 mM) at rt for 1 h. The click chemistry reaction was quenched by EDTA (10 mM). Cold methanol (1.4 mL) was added, and the suspension was placed in –80 °C for >4 h. The precipitate was collected by centrifugation (16.1k g, 30 min, 4 °C), washed with methanol (2 × 0.8 mL), air-dried at rt for 15 min, and reconstituted in 2% SDS PBS (40 μL). 1~2 μL of the solution was taken, and total protein amount was quantified by BCA assay (Pierce, #23223). The rest of the solution was diluted with 0.1% triton PBS (760 μL) and loaded to pre-equilibrated streptavidin beads (~80 μL of actual resin volume rather than suspension, Pierce, #20349). The beads suspension was shaken at rt for 2 h, and the supernatant was removed. After the beads were washed with 0.1% triton PBS (2 × 0.8 mL), proteins were eluted with 0.2 mM DTT 1X LDS sample buffer in 0.1% triton PBS (80 μL) by heating at 95 °C for 5 min. The eluted solution was analyzed according to *Gel and blot analysis* described above with the loading amount to gel adjusted based on the BCA assay result (~20 μL injection).

Met-PAIR experiment (Figure 2 and 4)

For PAIR experiment of the actin oxidation, cultured HEK293T cells were washed with HBSS (+Ca, +Mg), treated in HBSS containing H_2O_2 (1 mM) at rt for given time (0, 10, or 30 min), washed with HBSS (+Ca, +Mg) three times, and incubated with **Ox4** (20 μM) in HBSS at rt for 20 min. The cells were washed with the HBSS containing acetylmethionine (0.2 mM) once, and then fixed with 4% formaldehyde in PBS at rt for 15 min.

For PAIR experiment of CaM, cells in culture medium or buffer were washed with HBSS (+Ca, +Mg) once and incubated with **Ox4** (20 μM) in HBSS ((+Ca, +Mg except for the BAPTA-AM experiment) at rt for 20 min in the presence of one of the reagents listed in *Pull-down assay of live-cell oxaziridine labeling*. For neuron, HBSS (+Ca, +Mg) containing KCl (90 mM) or tetrodoroxin (10 μM) were used, and neuron were incubated at 37 °C for 5 min. The cells were washed with the HBSS containing acetylmethionine (0.2 mM) once, and then fixed with 4% formaldehyde in PBS at rt for 15 min.

The fixed cells were washed with PBS once and permeabilized in PBS containing triton-100X at rt (HEK293T: 0.5% triton for 30 min, Jurkat: 0.2% triton for 30 min, and neuron: 0.25% triton for 5 min). After wash with PBS twice, the cells were incubated at rt for 30 min in TBS buffer containing $CuSO_4$ (4 mM), Na ascorbate (10 mM), and either Oregon green azide (50 μM, Click Chemistry Tools, #1264) or biotin-PEG-azide (50 μM, Click Chemistry Tools, #AZ104). The cells were washed with PBS once, incubated at rt in PBS containing 5 mM EDTA at least for 15 min, and washed twice again with PBS. For HEK293T cells in a 12-well chambered slide, the chamber was removed after the permeabilization process, and each well was delineated with a hydrophobic pen. For neuron, after the click chemistry process, each coverslip was taken from a 6-well plate, and its edge was delineated with the hydrophobic pen (neuron on the edge was briefly removed by kimwipe beforehand to facilitate the delineation process). The drawn hydrophobic boundary was dried at rt for 5~10 min (minimum amount of PBS was applied to cells during this drying process to prevent dryness of cells), and the entire slide or coverslip was washed with PBS once. See Figure S21 for more details.

After the whole delineation process, 20 μL incubation volume per well was used for the rest of the Met-PAIR procedures. The cells were blocked in 4:1 TBST buffer/Blocking One for 15 min at rt (HEK293T and Jurkat) or Duolink blocking medium for 1 h at 37 °C (neuron) and incubated with anti-protein antibody (1:500) and anti-Oregon green or anti-biotin antibody (1:500) in 19:1 TBST buffer/Blocking One at rt for 1 h (HEK293T and Jurkat) or in Duolink antibody diluent at 37 °C for 1 h (neuron), followed by wash with PBS at rt for 5 min twice. Secondary antibody incubation with anti-mouse PLUS antibody (1:5) and anti-rabbit MINUS antibody (1:5) were conducted in 19:1 TBST buffer/Blocking One at 37 °C for 1 h (HEK293T and Jurkat) or in Duolink antibody diluent at 37 °C for 1 h (neuron). After wash with Duolink wash A buffer, ligation and polymerization processes as well as mounting process were performed following the Duolink PLA protocol from Sigma Aldrich. For neuron, neuronal staining was performed at rt for 30 min in Duolink antibody diluent containing anti-MAP2A antibody–AlexaFluor™488 conjugate (1:100) after the polymerization process. After the mounting process, the coverslip was sealed with nail polish, air-dried at least for 20 min, and analyzed by confocal microscopy. No unexpected or unusually high safety hazards were encountered.

Effect of Ox4 on secretion of interleukin-2 (IL-2) from Jurkat cells (Figure S8)

This concanavalin A activation experiment was based on a previous report.[7] Jurkat cells were treated with con A (0.1 mg/mL) and incubated with **Ox4** (0 or 20 μM) or 60 min in HBSS at 37 °C. After 60 min, the cells were pelleted and the supernatants were analyzed for amounts of IL-2 using ELISA. Briefly, 100 μL of the supernatants were obtained before and after addition of con A and the IL-2 amounts were analyzed using an IL-2 ELISA kit as per manufacturer's recommendations (Invitrogen, # 88-7025-22).

Ca imaging with Oregon green-BAPTA (OGB) in HEK cells (Figure S10)

HEK293T cells were plated onto 12 mm glass coverslips, which were pre-incubated in poly-D-lysine for 1 h. 75,000–70,000 cells were plated onto each coverslip and imaged 2 days later. Each coverslip was incubated for 20 minutes at 37 ºC in HBSS solution with 1 µM Oregon Green 488 BAPTA-AM (OGB-AM), 0.01% pluronic F-127, and one of the following reagents: 10 µM ionomycin, 30 µM BAPTA-AM (Ca^{2+}, Mg^{2+} free HBSS) and 0.1% DMSO vehicle. After incubation each coverslip was placed into an imaging dish filled with HBSS solution containing the one of the reagents (ionomycin, BAPTA-AM or DMSO). Images of four regions on each coverslip, 2 coverslips per condition, were taken on a wide field epifluorescence microscope, AxioExaminer Z-1 (Zeiss), equipped with a Spectra-X Light engine LED light (Lumencor) and controlled with MicroManager (Studio Version 1.4.22). Imaging data was acquired with a W-Plan-Apo 20x/1.0 water objective (20x, Zeiss) and an OracFlash4.0 sCMOS camera (Hamamatsu). OGB was illuminated with a cyan LED (475/34 nm bandpass, 44.1 mW/mm^2, 250 ms exposure) and emission was collected using a QUAD emission filter set which is comprised of a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm long pass) combined with a quadruple emission filter (430/32 nm, 508/14 nm, 586/30 nm, 708/98 nm). Cells were fixed in a 4% formaldehyde solution and images retaken as before. The average background subtracted cellular fluorescence for each of the images was quantified, normalized to the vehicle and plotted. Three regions of interest (ROIs) were selected for each image.

Effect of Ox4 on neuron activity with voltage-sensitive fluorophore BeRST1 (Figure S14)

Neurons were incubated at 37 ºC with a 500 nM BeRST1 solution in HBSS for 20 minutes. BeRST1 is a fluorescent voltage indicator whose fluorescence changes in response to changes in cellular membrane potential.[8] The coverslip was transferred to an imaging dish containing one of the following HBSS solutions; 500 nM **Ox4**, 1 µM **Ox4** or 0.1% DMF. Spontaneous neuronal activity was recorded optically for approximately 10 seconds using an epifluorescence microscope, AxioExaminer Z-1 (Zeiss), equipped with a Spectra-X Light engine LED light (Lumencor) and controlled with MicroManager (Studio Version 1.4.22). Imaging data was acquired with a W-Plan-Apo 20x/1.0 water objective (20x, Zeiss) and an OracFlash4.0 sCMOS camera (Hamamatsu) at an optical sampling rate of 500 Hz. BeRST1 was excited with a red LED (631/28 nm bandpass, 4.64 W/cm^2) and emission was collected with a quadruple emission filter (430/32, 508/14, 586/30, 708/98 nm) after passing through a quadruple dichroic mirror (432/38, 509/22, 586/40, 654 nm LP). A total of 4 areas per coverslip were imaged. Spiking frequency was determined by counting spikes from ROIs associated with cells bodies.

Safety statement for tetrodotoxin

Tetrodotoxin (TTX) is a voltage-gated sodium channel blocker. Subcutaneous exposure or exposure via inhalation or ingestion is extremely toxic. All TTX treated samples should be treated with bleach before being disposed of as chemical waste. Wear nitrile gloves while handling samples and wash hands thoroughly after experiments involving TTX.

https://ehs.berkeley.edu/sites/default/files/sops/TTX%20in%20animals%20SOP%20template.docx

Zebrafish experiments

Zebrafish husbandry

Zebrafish of the AB strains was raised and bred at 28.5°C on a 14 h light/10 h dark cycle. Embryos were produced by natural crosses and staged by hours, or days post fertilization (hpf, or dpf) following AUP (AUP-2019-04-12034) approved by the UC Berkeley ACUC.

In vivo zebrafish labeling with Ox4

Zebrafish embryos were incubated in E3 medium containing phenylthiourea (PTU, 0.003%). The larvae at 3 day post-fertilization was washed with E3 medium, manually dechorionated, transferred to a cell culture 6-well plate, and incubated in E3 medium containing **Ox4** (0 or 100 μM), DMSO (1%), and Tris (pH 7.4, 4 mM) at rt for 20 min under vibration (100 Hz frequency) or anesthesia (tricaine, 920 μM) conditions. The medium was taken, washed with E3 medium containing acetylmethionine (0.6 mM) and DMSO (1%) twice, and fixed with 4% paraformaldehyde solution in PBS ar rt for 2 h or at 4 °C overnight. The fixed larvae were transferred to Eppendorf tubes and put in methanol at –20 °C at least overnight.

Azide-alkyne cycloaddition for Ox4-modified zebrafish

The larvae after the methanol process were rehydrated by wash with 60% methanol (5 min), 30% methanol (5 min), and 0% methanol (3 × 5 min) in PBS with 0.1% tween 20 (PBST buffer), treated with proteinase K (10 μg/mL, Roche #03-115-828-001) in PBS at rt for 45 min (no shaking), and re-fixed in 4% paraformaldehyde in PBS at rt for 2 h (no shaking). After wash with the PBST buffer (1X PBS with 0.1% tween 20) five times, the larvae were incubated under azide-alkyne cycloaddition conditions[9] at rt for 1 h and then at 4 °C overnight: THPTA (0.2 mM), TCEP (0.5 mM), biotin-PEG-azide or Oregon green-azide (10 μM), $CuSO_4$ (0.2 mM), DMSO (1%), tween 20 (0.1%) in TBS. The larvae were washed with PBST buffer containing 0.5 mM EDTA five times, and used for fluorescence imaging (Oregon green) or PLA experiment.

Zebrafish PLA experiment

For the PLA experiment, the biotin-labeled larvae were transferred to PCR tubes (incubation volume for each step: 160 μL) and incubated at rt for at least 2 h in blocking buffer: 5% donkey serum (Jackson Immuno Research, 017-000-121), 0.05% triton-100X, 1% DMSO, and 0.2% w/w BSA in PBS. Primary antibody incubation with mouse anti-CaM and rabbit anti-biotin antibody (1:500 dilution each) in the blocking buffer were performed at 4 °C overnight. After wash with the blocking buffer (5 × 5 min), secondary antibody incubation with anti-mouse PLUS antibody (1:5 dilution) and anti-rabbit MINUS antibody (1:5) were conducted in the blocking buffer at 37 °C for 5 h. The larvae were washed with Duolink wash A buffer (5 × 5 min), ligated at 37 °C for 5 h in the same dilution as the manufacture protocol, washed with Duolink wash A buffer (5 × 5 min), and incubated in the amplification conditions at 37 °C overnight. The larvae were washed with PBST buffer once and incubated with DAPI in PBST buffer at rt for 1 h. The larvae were washed with Duolink wash B buffer (3 × 15 min at least) and 0.01X wash B buffer once (15 min), stored in 7:3 glycerol/PBS mixture at 4 °C at least for 30 min, and mounted on a coverslip for imaging. All of the incubation and wash processes after the methanol treatment were conducted with a rotator (Fisher Scientific, #05-450-127) except for the wash B processes

with a rocker (Labnet, #S2025-B), as the larvae's tendency to stick to Eppendorf tubes and break down during wash B process with the rotator.

Met-PAIR protocol for cellular Ca imaging

Live-cell chemical labeling with Ox4 and cell fixation (timing 1.5 h)

1. Dulbecco's Modified Eagle Medium (DMEM) was removed from HEK293T cells in a 12-well chambered slide (70~80% confluency).
2. Hanks balanced salt solution (HBSS) buffer (100 µL) was added to each well, and the slide was rocked gently for 20 sec. (**Note**: HBSS buffer without Ca was used for the BAPTA-AM experiment.)
3. The buffer was removed, and HBSS buffer (total 100 µL) containing **Ox4** (20 µM) and the desired reagent (ionomycin, DMSO, or BAPTA-AM) was added. (**Note**: HBSS buffer, **Ox4**, and one of the reagents were mixed in an Eppendorf tube before the addition to the cells.)
4. The slide was placed on the bench at rt for 20 min.
5. The buffer was removed, and HBSS buffer (total 100 µL) containing acetylmethionine (0.2 mM) was added.
6. The buffer was removed, and 4% formaldehyde in PBS was added. The slide was sit at rt for 15 min.
7. The formaldehyde solution was removed.
8. Phosphate buffered saline (PBS) was added. The slide was rocked for 20 sec. Then the buffer was removed.
9. Step 8 was repeated twice. (**Note**: The fixed cells can be stored in PBS for several weeks in 4 °C as long as sufficient amount of buffer is present in each well.)

Permeabilization, chamber removal, and click chemistry (timing 2 h)

10. PBS was removed, and PBS containing 0.5% v/v triton-100 was added (100 µL).
11. The cells were incubated in the buffer at rt for 30 min.
12. The buffer was removed, PBS (100 µL) was added.
13. Step 12 was repeated twice.
14. The rubber chamber was removed from the slide manually, according to the manufacturer:s instructions.
15. Individual wells were delineated using a hydrophobic pen, and the slide was dried at rt for 3 min. (**Note**: Minimum amount of PBS (5~20 µL) was added to each well to prevent cells from drying. Excess amount of buffer would not be ideal for the drawn boundary to be dried.)
16. The slide was dipped into a coplin staining jar containing PBS (~40 mL) at rt for 2 min. (**Note**: Remaining buffer in each well after this wash process was removed by pipetting carefully from the corner of each well.)
17. Tris-buffered saline (TBS) buffer (20 µL) containing $CuSO_4$ (4 mM), sodium ascorbate (10 mM), and Oregon green azide (50 µM) was added. (**Note**: The stock solution of sodium ascorbate needs to be freshly prepared every time, as the reagent tends to be oxidized to an inactive form upon storage in solution.)
18. The cells were incubated at rt for 30 min, and the buffer was removed.
19. The slide was dipped into a staining jar containing PBS (~40 mL) at rt for 5 min.
20. PBS (20 µL) containing EDTA (5 mM) was added, and incubated at rt for 15min.

21. The remaining buffer was removed by pipetting, and the slide was dipped into a staining jar containing PBS (~40 mL) at rt for 5 min.
22. Step 21 was repeated once again. (**Note**: The cells after the click chemistry and EDTA quenching processes can be stored in PBS for a few days at 4 °C as long as sufficient amount of buffer is present in each well. The authors did not find significant evaporation of 40 µL of PBS in each well after overnight storage).

Proximity-ligation assay (timing 6 h)

23. PBS was removed, and a mixture of TBST (TBS with 0.1% v/v tween20) and Blocking One (total 20 µL, 4:1 v/v) was added.
24. The cells were incubated at rt for 15 min.
25. After the buffer was removed, a mixture of TBST/Blocking one (19.92 µL, 19:1 v/v) containing anti-calmodulin antibody (0.04 µL, 1:500 dilution) and anti-Oregon green antibody (0.04 µL, 1:500 dilution) was added. (**Note**: Always prepare fresh solution of the antibodies.)
26. The cells were incubated at rt for 60min.
27. The solution was removed from each well, and the slide was dipped into a staining jar containing PBS (~40 mL) at rt for 5 min.
28. Step 27 was repeated once again.
29. After the remaining buffer was removed, a mixture of TBST/Blocking one (12 µL, 19:1 v/v) containing anti-mouse PLUS antibody (4 µL, 1:5 dilution) and anti-rabbit MINUS antibody (4 µL, 1:5 dilution) was added. (**Note**: Always prepare fresh solution of the antibodies.)
30. The cells were incubated at 37 °C for 60 min. (**Note**: In order to prevent evaporation of the incubation buffer at 37 °C, use of a humidified chamber is recommended during this step as well as the following PLA steps. See Figure S20.)
31. The solution was removed from each well, and the slide was dipped into a staining jar containing Wash A buffer (~40 mL) at rt for 5 min.
32. Step 31 was repeated once again.
33. After the buffer was removed, a mixture of water (15.5 µL)/ligation buffer (4 µL) containing ligase (0.5 µL, 1:40 dilution) was added. (**Note**: Make sure to completely thaw the stock solution of the ligation buffer by using vortex before diluting with water. Always prepare fresh solution of the ligase. Use a benchtop cooler for ligase stock solution to minimize loss of the enzymatic activity.)
34. The cells were incubated at 37 °C for 30 min. (**Note**: As the commercially available ligation buffer contains dithiothreitol as a reductant, incubation for more than 30 min is not recommended.)
35. The solution was removed from each well, and the slide was dipped into a staining jar containing Wash A buffer (~40 mL) at rt for 5 min.
36. Step 35 was repeated once again.
37. After the buffer was removed, a mixture of water (15.75 µL)/amplification buffer (4 µL) containing polymerase (0.25 µL, 1:80 dilution) was added. (**Note**: Always prepare fresh solution of the polymerase.)

38. The cells were incubated at 37 °C for 100 min. (**Note**: As the commercially available amplification buffer contains a photosensitive fluorophore, the slides should be covered with foil to protect them from light during amplification and subsequent wash processes.)
39. The solution was removed from each well, and the slide was dipped into a staining jar containing Wash B buffer (~40 mL) at rt for 10 min.
40. Step 39 was repeated once again.
41. The solution was removed from each well, and the slide was dipped into a staining jar containing a mixture of water (49.5 mL) and Wash B buffer (0.5 mL, 1:100 dilution) at rt for 1 min.
42. After the remaining buffer was removed, Duolink mounting medium containing DAPI (6 µL/well) was added.
43. Coverslip was carefully placed onto the slide to avoid inclusion of air bubbles.
44. The excess mounting from the coverslip and slide was gently removed with Kimwipes (**Note**: Excessive mounting medium would hamper the sealing material to be solidified in the next step. It should be noted that Duolink mounting medium itself does not solidify.)
45. The coverslip was sealed by a nail polish.
46. The sealed slide was dried at rt at least for 20 min.
47. The slide can be imaged at this point or should be stored in 4 °C for a short term and –20 °C for a long term.

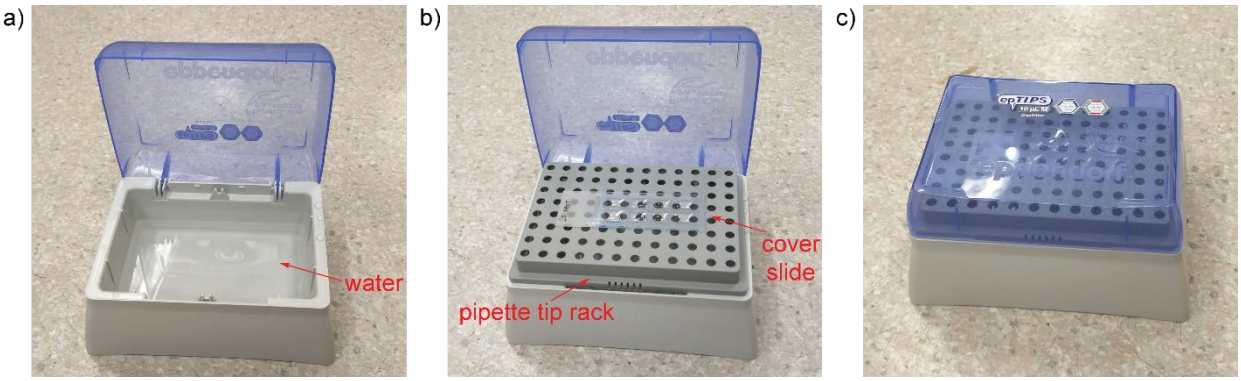

Figure S20. Images for a setup of a humidified chamber for a cover slide. a) An image for a pipette tip box half filled with water. b) An image for a pipette tip with a tip rack and cover slide placed inside. c) An image for a closed pipette tip box after a) and b).

Figure S21. Images for hydrophobic-pen delineation of a cover slip. a) An image showing removal of cells on edge of a cover slip with Kimwipe. Top and left side of the cover slip was stained with the hydrophobic pen for a visual help. b) An image showing cover slips before and after the hydrophobic pen delineation process. c) An image showing the placement of the delineated cover slip into a 24-well culture dish for the PLA process.

Notes for troubleshooting Met-PAIR experiments.

Below is a list of troubleshooting tips for optimizing Met-PAIR and related PLA imaging methods.

1. If low PLA signal is observed, perform a control experiment to verify that all materials (**Ox4**, click reagents, antibodies, ligase, and polymerase) have undergone degradation; success of **Ox4** labeling and click chemistry can be confirmed by fluorescence imaging (Oregon green) after the click chemistry, and quality of PLA reagents can be checked by repeating reported PLA protocols or use commercially available control kits (Sigma-Aldrich, #DUO92202). Increasing the amplification time will be helpful to increase the PLA signal.

2. To optimize signal-to-noise responses in PLA signal by stimulation (e.g. ionomycin vs vehicle), lowering the concentration of Ox4 or decreasing the live-cell labeling time, since Ca-unbound form of CaM could be labeled with extended labeling, could be pursued.

3. If unexpected PLA signal is observed, conduct immunofluorescence experiments with each antibody to verify whether proper antibodies are used, antibody incubation conditions are optimal, and/or blocking conditions are optimized for a particular cell type and antibody. If an antibody of interest has not been used in a literature before, it is recommended to test its specificity (e.g. siRNA knockdown experiment to see reduction of the PLA or immunofluorescence signal).

Quantification of fluorescence images

1. *Z stack image projection and preparation of thresholded images for the PLA channel*. Quantification of cell images was conducted by ImageJ software. Z stack slices were combined through average intensity projection ([Image]-[Stacks]-[Z Project] from the main window). The stacked images were split into each channel ([Image]-[Stacks]-[Stack to images]). The 16-bit red channel image (PLA signal) was thresholded ([Image]-[Adjust]-[Threshold]). After the threshold process, the image becomes converted to 8-bit automatically by the software. (It should be noted that brightness/contrast of a red channel image shown right after the average intensity projection might not have been adjusted consistently with another red channel image, as the ImageJ automatically change the brightness/contrast after the projection. The brightness of the image after the projection can be checked from [Image]-[Show Info]-[Display range], and this value was adjusted consistently between the same channel images in [Image]-[Adjust]-[Brightness/Contrast] for the presentation of images in the manuscript.)

2. *Identification of single cells in the green channel*. Definition of each cell was conducted using the Oregon green fluorescence channel as a proxy for cytosol staining or MAP2A staining for the neuron experiment. The green channel image after the projection was thresholded by the same way as the red channel. Cell boundary was delineated either manually or by watershed function ([Process]-[Binary]-[Make Binary] then [Process]-[Binary]-[Watershed]). For the manual delineation, paintbrush function (Figure S22, its button with a brush shape can be found in the main window) was used to draw the cell boundary by erasing the pixel. If the background is white (black cells), the image color should be inverted ([Image]-[Lookup Tables]-[Invert LUT]), so that the paintbrush function serves as an eraser to define the cell boundary. Overlapped cells or cells partially present in the image were excluded for the analysis, judged by DAPI and Oregon green channels. The obtained green channel image was converted to 8 bit ([Image]-[Type]-[8-bit]), and re-thresholded (there is no need to change the threshold value after the delineation process. However, the Analyze Particle function described later requires an 8-bit thresholded image, and this re-threshold process is often necessary). Each cell in the processed green channel image was analyzed by Analyze Particle function ([Analyze]-[Analyze Particles]). Typical Analyze Particles settings are as follows: size 20~6000 (These arbitrary values are subject to change if necessary. Make sure all cells delineated were found after Analyze Particle.), circularity 0.00-1.00, show outlines, Add to Manager and In situ Show boxes checked. After the particle analysis, “Show All” box in ROI Manager was unchecked.

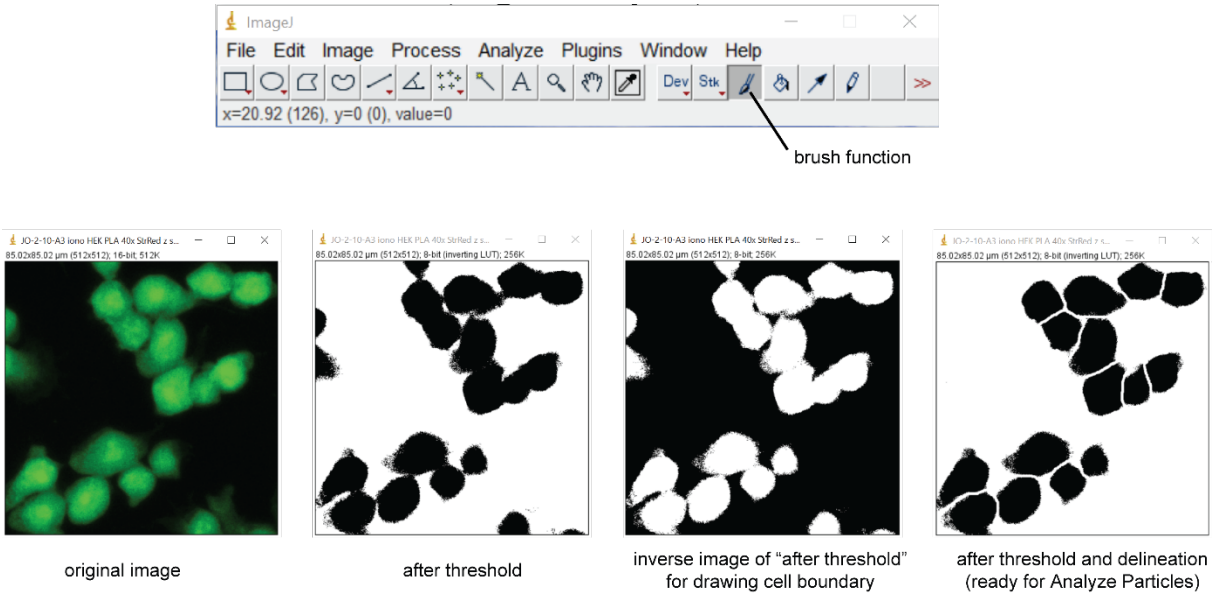

Figure S22. Representative images for the cell boundary delineation process in the ImageJ software.

3. *Quantification of each cells in the PLA channel by the green channel*. With the ROI Manager open after the particle analysis, the processed red channel image was selected, and "Show All" box was checked (Figure S23, the cell boundary defined in the green channel should be displayed in the red channel at this point), followed by "Measure" in the ROI Manager to give the mean intensity of PLA fluorescence signal in each cell defined by Oregon green channel in the Results window. If the mean intensity is not shown in the Results window, go to [Results]-[Set Measurements] in the Results window and check the Mean gray value box.

The obtained PLA intensity for each cell was plotted using Origin software. Statistical analysis was also conducted by the same software.

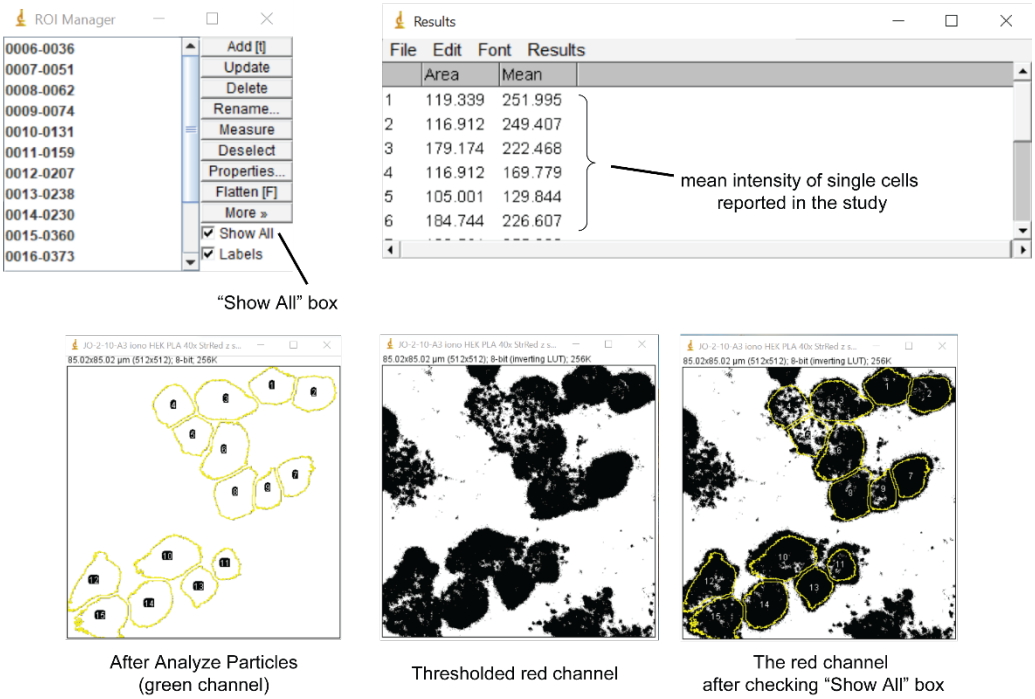

Figure S23. Representative images for the process of measurement of fluorescence intensity of single cells in the ImageJ software.

Some of these quantification processes were performed using Macros function in ImageJ to facilitate the processes ([Plugins]-[Macros]-[Record]). The recorded processes can be usable by hitting "create" button in the Recorder window, and a new window "Macro.ijm" would show up. With the window selected, Ctrl+R would conduct the command written (in Windows). Following sentences are examples for the average intensity projection and image staking function.

```
run("Z Project...", "projection=[Average Intensity]");

run("Stack to Images");
```

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