

***In vivo* Fluorescence Imaging of Extracellular ATP in the Mouse Cerebral Cortex with a Hybrid-type Optical Sensor**

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[Abstract] Adenosine 5'-triphosphate (ATP) works as an extracellular signaling molecule for cells in the brain, such as neurons and glia. Cellular communication via release of ATP is involved in a range of processes required for normal brain functions, and aberrant communication is associated with brain disorders. To investigate the mechanisms underlying these cellular processes, various techniques have been developed for the measurement of extracellular ATP. To monitor the dynamics of extracellular ATP signaling with high spatiotemporal resolution, we recently developed a hybrid-type ATP optical sensor (ATPOS) that enables *in vivo* fluorescence imaging of extracellular ATP dynamics in the brain. ATPOS is synthesized by labeling an ATP-binding protein, *Bacillus* FoF₁-ATP synthase ε subunit, with a small-molecular fluorescent dye Cy3. Injection of ATPOS into the cerebral cortex of living mice enables visualization of the wave-like propagation of extracellular ATP release in response to electrical stimulation. The protocol described here should be useful for visualizing ATP signaling in diverse processes involved in intercellular communication in the brain.

Keywords: ATP, Extracellular signaling molecule, *In vivo* imaging, Fluorescent sensor, Brain

[Background] Adenosine 5'-triphosphate (ATP), which is well known as the intracellular energy currency, works as an extracellular signaling molecule in various organs and tissues of the body (Zimmermann, 2016). In the brain, ATP is released from neurons and glia (Pankratov et al., 2006; Butt, 2011), and is involved in neurotransmission (Burnstock, 2007), neuron–glia interaction (Fields and Burnstock, 2006; Khakh and North, 2012), and regulation of blood flow (MacVicar and Newman, 2015). Release of ATP also occurs during pathological neuronal activity known as cortical spreading depression (CSD) (Schock et al., 2007; Heinrich et al., 2012), which is associated with migraine and cerebral ischemia (Lauritzen et al., 2011). Thus, extracellular ATP signaling is considered to play key roles not only in normal brain functions but also in brain disorders.

To better understand the roles of ATP signaling, it is important to visualize the spatiotemporal changes of extracellular ATP. Various techniques have been developed for monitoring extracellular ATP; for example, bioluminescence assays combined with microdialysis and electrophysiological methods using enzyme-coated electrodes can quantitate extracellular ATP in the brain of living animals (Gourine et al.,

2005; Melani *et al.*, 2005). However, these techniques provide little spatial information. In contrast to these non-imaging techniques, fluorescence imaging using optical sensors is a promising tool for monitoring the dynamics of a target molecule with high spatiotemporal resolution (Giepmans *et al.*, 2006). To date, a variety of ATP sensors based on fluorescent proteins have been reported (Berg *et al.*, 2009; Imamura *et al.*, 2009; Tantama *et al.*, 2013; Yaginuma *et al.*, 2015; Arai *et al.*, 2018; Lobas *et al.*, 2019). However, their application in *in vivo* imaging of extracellular ATP remains difficult (Conley *et al.*, 2017; Lobas *et al.*, 2019) because they are not sensitive enough to detect ATP released into the extracellular space (Yegutkin, 2008). Moreover, these ATP sensors exhibit pH-dependent fluorescence changes due to the pH sensitivity of the fluorescent proteins used in the sensors (Tsien, 1998; Chudakov *et al.*, 2010). This pH dependence is unfavorable for application in the brain, where the tissue pH fluctuates during neuronal activity (Chesler and Kaila, 1992; Magnotta *et al.*, 2012).

We recently developed a hybrid-type ATP optical sensor, ATPOS, which consists of an ATP-binding protein, *Bacillus* FoF1-ATP synthase ϵ subunit, with a cysteine point mutation at glutamine-105 (ATPBP-Q105C) and a small-molecular fluorescent dye Cy3 bearing a cysteine-reactive maleimide group (Kitajima *et al.*, 2020). ATPOS shows a large fluorescence response to ATP (~2 fold) with high affinity ($K_d = \sim 150$ nM), pH independence, and high selectivity, and is therefore suitable for fluorescence imaging of extracellular ATP dynamics (Kitajima *et al.*, 2020). For application of ATPOS to extracellular ATP imaging in the brain, we used BoNT/C-Hc, a nontoxic subunit of *Clostridium botulinum* type C neurotoxin (Tsukamoto *et al.*, 2005), through which ATPOS can be anchored to the surface of neuronal cell membranes. In addition, we adopted Alexa Fluor 488 (Alexa488) as a reference fluorophore, so that ATPOS can be used for dual-color ratiometric imaging. Accordingly, ATPOS is applied as a form of molecular complex with BoNT/C-Hc and Alexa488-labeled streptavidin (Figure 1). After delivery into the cerebral cortex of living mice, the ATPOS complex can aid visualization of wave-like extracellular ATP dynamics during CSD evoked by electrical stimulation (Kitajima *et al.*, 2020). Here, we describe the protocols for the production of ATPOS and its application to fluorescence imaging of extracellular ATP in the mouse brain. These protocols will be useful for studying the roles of extracellular ATP signaling in various physiological and pathological processes in the brain.

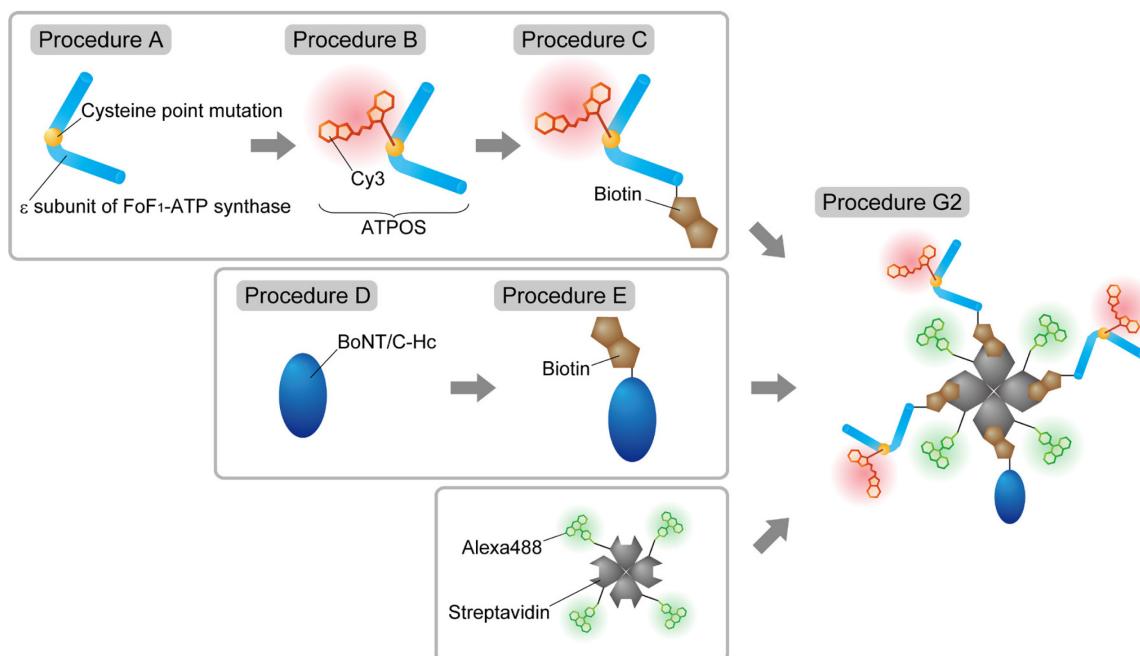


Figure 1. Schematic illustration of the experimental workflow for the production of the ratiometric ATPOS complex. Procedure A: The ϵ subunit of FoF₁-ATP synthase with a cysteine point mutation at glutamine-105 is expressed in bacterial cells. Procedure B: ATPOS is produced by labeling the ϵ subunit with Cy3 bearing a cysteine-reactive maleimide group. Procedure C: Biotin is covalently attached to ATPOS. Procedure D: BoNT/C-Hc is expressed in bacterial cells. Procedure E: Biotin is covalently attached to BoNT/C-Hc. Procedure G2: Biotinylated ATPOS, biotinylated BoNT/C-Hc, and Alexa488-labeled streptavidin are mixed at a molar ratio of 3:1:1 to assemble the ratiometric ATPOS complex via biotin-streptavidin linkage.

Materials and Reagents

1. HiTrap TALON crude 1-ml column (BD Biosciences, catalog number: 29048565)
2. TALON metal affinity resin (Clontech, catalog number: 635503)
3. PD-10 desalting column (GE Healthcare, catalog number: 17085101)
4. Empty disposable PD-10 column (GE Healthcare, catalog number: 17043501)
5. Amicon Ultra-0.5 centrifugal filter unit (10 kDa cutoff) (Merck, catalog number: UFC5010)
6. Amicon Ultra-0.5 centrifugal filter unit (30 kDa cutoff) (Merck, catalog number: UFC5030)
7. Dental acrylic (GC, Fuji LUTE BC)
8. Glass capillary (Harvard Apparatus, catalog number: 30-0053)
9. 10-ml syringe (Terumo, catalog number: SS-10LZ)
10. Plastic tube (Imamura, catalog number: 01605)
11. Monopolar tungsten microelectrode (FHC, catalog number: UEWMGCSEKNNM)
12. *Escherichia coli* BL21 (DE3)
13. Wild-type mice (C57BL/6NCrSlc, male, postnatal 1-2 months old)
14. Plasmid encoding ATPBP-Q105C (Kitajima *et al.*, 2020)

15. Plasmid encoding BoNT/C-Hc (Takikawa *et al.*, 2014)
16. Cy3 maleimide (GE Healthcare, catalog number: PA13131)
17. Alexa488-labeled streptavidin (Thermo Scientific, catalog number: S11223)
18. NHS-PEG₄-biotin (Thermo Scientific, catalog number: A39259)
19. Ampicillin (Nacalai Tesque, catalog number: 02739-32)
20. Kanamycin (Nacalai Tesque, catalog number: 19839-44)
21. Isopropyl- β -D-thiogalactopyranoside (IPTG) (Nacalai Tesque, catalog number: 19742-81)
22. Lysozyme (Nacalai Tesque, catalog number: 19499-91)
23. Triton X100 (Nacalai Tesque, catalog number: 12967-45)
24. DNase I (Takara, catalog number: 2270A)
25. Imidazole (Nacalai Tesque, catalog number: 19004-35)
26. Medetomidine (Nippon Zenyaku, Domitor)
27. Midazolam (Sandoz, Midazolam Injection 10 mg [SANDOZ])
28. Butorphanol (Meiji Seika Pharma, Vetorphale)
29. Mineral oil (Sigma, catalog number: M8410)
30. Dimethyl sulfoxide (DMSO) (Nacalai Tesque, catalog number: 13408-64)
31. Luria Bertani (LB) agar plates (see Recipes)
32. 2 \times YT media (see Recipes)
33. LB media (see Recipes)
34. Phosphate-buffered saline (PBS) (see Recipes)
35. 10 \times PBS (see Recipes)
36. Artificial cerebrospinal fluid (aCSF) (see Recipes)
37. HEPES-buffered saline (HBS) (see Recipes)
38. Anesthetic solution (see Recipes)

Equipment

1. 1-L flask
2. French press (Aminco, model: FA-080B)
3. Fast protein liquid chromatography system (GE Healthcare, model: AKTA purifier 10)
4. Spectrophotometer (Eppendorf, model: Eppendorf BioPhotometer Plus)
5. Heating pad (Bio Research Center, model: BWT-100A)
6. Metal frame and frame holder (custom-made, Figures 2A and 2B)
7. Dental drill (Nakanishi, model: ULTIMATE XL)
8. Pipette puller (Sutter Instrument, model: P-97)
9. Beveller (PRIME TECH, model: EZP-75)
10. Wide-field microscope (Leica, model: M165FC)
11. Objective lens (Leica, Plan APO 1.0 \times , catalog number: 10450028)
12. Filter set (Leica, Filter set ET GFP3/CY3, catalog number: 10450612)

13. High-speed scanning polychromatic light source (Hamamatsu Photonics, model: C7773)
14. EM-CCD camera (TELEDYNE Photometrics, model: Evolve512)
15. Stereotaxic manipulator (Luigs and Neumann, model: SM-5)
16. Stimulus isolator (AMPI, model: ISO-Flex)

Software

1. ImageJ/Fiji (NIH, version: 1.50e, <https://imagej.net/Fiji>)
2. MetaMorph (Molecular Devices)

Procedure

A. Expression and purification of ATPBP-Q105C

1. Transform *Escherichia coli* BL21 (DE3) cells with the plasmid encoding ATPBP-Q105C (see Note 1) as follows. Add 100 ng plasmid to 30 μ l competent cells in a microtube, gently mix, and incubate on ice for 5 min. Heat the cells at 37°C for 3 min, incubate on ice for 5 min, and add 300 μ l LB media.
2. Plate the cells on an LB agar plate containing 100 μ g/ml ampicillin and incubate overnight at 37°C.
3. Inoculate 5 ml 2× YT media containing 100 μ g/ml ampicillin in a 50-ml tube with a single colony grown on the plate, and grow the culture at 37°C with shaking at 200 rpm for 6 h.
4. Transfer 2 ml culture to a 1-L flask with 250 ml 2× YT media containing 100 μ g/ml ampicillin, and grow the culture at 37°C with shaking at 200 rpm until the OD₆₀₀ reaches 0.5-0.8.
5. Add 1 ml 100 mM IPTG to the culture and incubate at 25°C with shaking at 160 rpm for 16 h.
6. Collect the cells by centrifugation at 4°C for 15 min at 2,070 \times g, and store the cell pellet at -80°C.
7. Resuspend the cells in 12.5 ml PBS on ice. Add 1.3 ml PBS containing 10 mg/ml lysozyme and perform three freeze-thaw cycles. Add 150 μ l 10% Triton X100, 150 μ l 1 M MgCl₂, and 9 μ l 5 U/ μ l DNase I, and incubate at 4°C for 15 min.
8. Centrifuge the cell lysate at 4°C for 30 min at 15,000 \times g and collect the supernatant in a new tube.
9. Equilibrate a HiTrap TALON crude 1-ml column with 5 ml PBS, and transfer the supernatant into the column.
10. Apply 25 ml PBS containing 10 mM imidazole to the column for washing.
11. Apply 20 ml PBS containing 150 mM imidazole to the column, and collect the eluate in 1 ml fractions in new tubes.
12. Perform SDS-PAGE analysis to identify the fractions containing ATPBP-Q105C. Measure the protein concentration by the Bradford protein assay using BSA as a standard, and calculate the molar concentration of ATPBP-Q105C (15.9 kDa) (see Note 2).

B. Fluorophore labeling

1. Add 985 μ l PBS to each of two new microtubes. Transfer 10 μ l eluate containing 1 mM ATPBP-Q105C into each of the microtubes. Add 5 μ l 10 mM Cy3 maleimide dissolved in DMSO to each microtube, gently mix, and incubate at 25°C for 45 min.
2. Equilibrate a PD-10 desalting column with 25 ml PBS and transfer 2 ml mixture into the column.
3. Apply 700 μ l PBS to the column and discard the eluate.
4. Apply 3 ml PBS to the column and collect the eluate containing the Cy3-labeled ATPBP-Q105C (ATPOS) in a new tube. Determine the concentration of ATPOS by the Bradford protein assay using BSA as a standard (see Note 3).

C. Biotinylation of ATPOS

1. Transfer 1,000 μ l 6 μ M ATPOS into a microtube containing 497 μ l PBS. Add 3 μ l 5 mM NHS-PEG₄-biotin dissolved in DMSO, gently mix, and incubate at 25°C for 1 h.
2. Equilibrate a PD-10 desalting column with 25 ml PBS and transfer 1.5 ml mixture into the column.
3. Apply 1.2 ml PBS to the column and discard the eluate.
4. Apply 3 ml PBS to the column and collect the eluate containing biotinylated ATPOS in a new tube.
5. Transfer 500 μ l eluate into an Amicon Ultra-0.5 centrifugal filter unit (10 kDa cutoff) and concentrate the solution by centrifugation at 4°C for 5 min at 14,000 \times g. Apply the rest of the eluate to the centrifugal filter unit and repeat the centrifugation. Determine the concentration of biotinylated ATPOS by the Bradford protein assay using BSA as a standard (see Note 4); store at -80°C.

D. Expression and purification of BoNT/C-Hc

1. Transform *Escherichia coli* BL21 (DE3) cells with the plasmid encoding BoNT/C-Hc (see Note 1) as follows. Add 100 ng plasmid to 30 μ l competent cells in a microtube, gently mix, and incubate on ice for 5 min. Heat the cells at 37°C for 3 min, and incubate on ice for 5 min. Add 100 μ l LB media and incubate at 37°C for 20 min. Plate the cells on an LB agar plate containing 20 μ g/ml kanamycin and incubate overnight at 37°C.
2. Inoculate 5 ml LB media containing 20 μ g/ml kanamycin in a 50-ml tube with a single colony grown on the plate, and grow the culture at 37°C with shaking at 200 rpm for 4 h.
3. Transfer 2 ml culture into a 1-L flask with 250 ml 2 \times YT media containing 20 μ g/ml kanamycin, and grow the culture at 37°C with shaking at 200 rpm until the OD₆₀₀ reaches 0.5-1.0.
4. Add 250 μ l 100 mM IPTG to the culture and incubate at 25°C with shaking at 200 rpm for 24 h. Collect the cells by centrifugation at 4°C for 15 min at 2,070 \times g and store the cell pellet at -80 °C.
5. Resuspend the cells in 30 ml PBS on ice and lyse the cells under a pressure of 1,200 psi with a French press. Centrifuge the cell lysate at 4°C for 30 min at 15,000 \times g and collect the supernatant in a new tube.

6. Equilibrate 2 ml TALON metal affinity resin with 2 ml 10× PBS in a 50-ml tube, add the supernatant, and agitate the suspension on ice for 30 min.
7. Transfer the suspension into an empty disposable PD-10 column and apply 50 ml PBS containing 5 mM imidazole to the column for washing.
8. Apply 5 ml PBS containing 150 mM imidazole to the column and collect the eluate in 1 ml fractions in new tubes. Measure the protein concentration by the Bradford protein assay using BSA as a standard; determine the fraction containing the highest concentration of the protein (designated 1st fraction), the fraction containing the second highest concentration of the protein (designated 2nd fraction), and the fraction containing the third highest concentration of the protein (designated 3rd fraction) (see Note 5).
9. Equilibrate a PD-10 desalting column with 25 ml PBS and transfer 2 ml 1st and 2nd fractions and 500 µl 3rd fraction into the column.
10. Apply 500 µl 3rd fraction and 2.6 ml PBS to the column and collect 3.1 ml eluate containing BoNT/C-Hc in a new tube. Determine the concentration of BoNT/C-Hc by the Bradford protein assay using BSA as a standard and calculate the molar concentration of BoNT/C-Hc (51.6 kDa) (see Note 6).

E. Biotinylation of BoNT/C-Hc

1. Add 750 µl PBS to each of two new microtubes. Transfer 250 µl eluate containing 40 µM BoNT/C-Hc into each of the microtubes. Add 1 µl 200 mM NHS-PEG₄-biotin dissolved in DMSO to each microtube, gently mix, and incubate on ice for 45 min.
2. Equilibrate a PD-10 desalting column with 25 ml PBS and transfer 2 ml mixture into the column.
3. Apply 500 µl PBS to the column and discard the eluate.
4. Apply 3.1 ml PBS to the column and collect the eluate containing biotinylated BoNT/C-Hc in a new tube.
5. Transfer 500 µl eluate into an Amicon Ultra-0.5 centrifugal filter unit (30 kDa cutoff) and concentrate the solution by centrifugation at 4°C for 5 min at 14,000 × g. Apply the rest of the eluate to the centrifugal filter unit in 500 µl aliquots and repeat the centrifugation. Determine the concentration of biotinylated BoNT/C-Hc by the Bradford protein assay using BSA as a standard (see Note 7); store at -80°C.

F. Surgery

1. Anesthetize a male C57BL/6 mouse by intraperitoneally injecting 0.1 ml/10 g body weight of anesthetic solution. Wait for 5-10 min and then assess the depth of anesthesia by tail pinch. Place the mouse under a wide-field microscope and maintain the rectal temperature at 37°C with a heating pad.
2. Remove the scalp covering the skull with scissors, attach a custom-made metal frame to the exposed skull with dental acrylic (Figure 2A), and fix the metal frame with a custom-made frame holder (Figure 2B).

3. Make a groove in the skull with a dental drill along the circumference of a 4-mm-diameter circle centered 3 mm posterior to the bregma and 3 mm lateral to the midline. Remove the bone flap within the circle with forceps, and leave the dura intact. Cover the surface of the exposed cortex with aCSF.

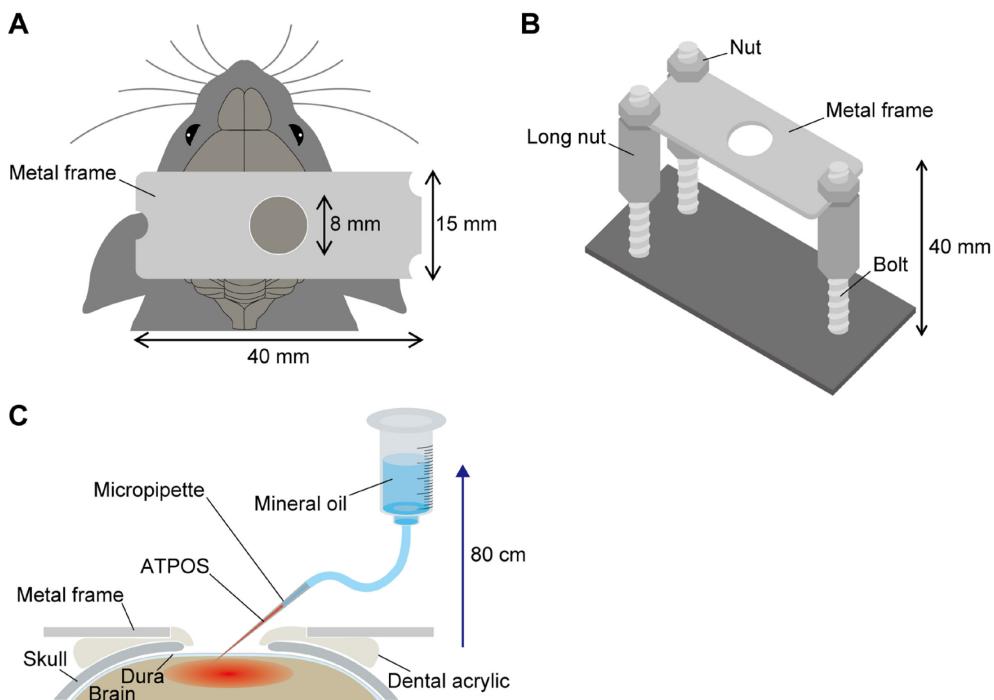


Figure 2. Schematic illustration of the experimental set-up for the application of ATPOS to the mouse cerebral cortex. A. A custom-made metal frame is attached to the mouse skull. B. A custom-made frame holder. The metal frame is clamped at three positions by the upper nuts and the lower long nuts. C. ATPOS is pressure-injected into the cerebral cortex with a micropipet through the craniotomy by raising the cylinder containing mineral oil by 80 cm vertically from the pipet tip.

G. Injection of ATPOS

1. Prepare a glass micropipet as follows. Pull a glass capillary on a P-97 pipet puller and bevel the tip of the micropipet at an angle of 35 degrees relative to a grinding plate until the inner tip diameter reaches 10 μ m.
2. Prepare the ATPOS complex solution as follows. Transfer 3 μ l 40 μ M biotinylated ATPOS into a microtube containing 15.5 μ l HBS, and add 1 μ l 40 μ M biotinylated BoNT/C-Hc. Add 0.5 μ l 83 μ M Alexa488-labeled streptavidin dissolved in PBS and gently mix.
3. Load 20 μ l ATPOS complex solution into the micropipet from its back end with a microloader pipet tip, and fill the rest of the inner space of the micropipet with 10 μ l mineral oil. Attach the external cylinder of a 10-ml syringe to a 1.5-m-long plastic tube and add 5 ml mineral oil to the cylinder to fill the plastic tube. Connect the other end of the plastic tube to the back end of the micropipet (see Note 8).

4. Fix the micropipette in a stereotaxic manipulator at an angle of 45 degrees relative to the brain surface and insert the pipette tip to a depth of 300 μ m from the brain surface.
5. Inject the ATPOS complex solution at 1 psi by raising the cylinder 80 cm vertically from the pipet tip for 20-30 min (Figure 2C).
6. After the injection is finished, withdraw the micropipet from the brain.

H. *In vivo* ATP imaging

1. Find an appropriate imaging area by observing the fluorescence of ATPOS under 555-nm illumination, and focus on the brain surface by observing a brightfield image of the imaging area.
2. Acquire Cy3 and Alexa488 fluorescence images with an EM-CCD camera at 2.5 Hz sequentially (200 ms duration for each fluorescence) by alternating 555-nm and 490-nm illumination for excitation of Cy3 and Alexa488, respectively (see Note 9).
3. For drug application, load an appropriate volume of the drug dissolved in aCSF into a glass micropipet, and fill the rest of the inner space of the micropipet with mineral oil (see Note 10). Connect the micropipet with a plastic tube filled with mineral oil and inject the drug at 1 psi by raising the cylinder attached to the plastic tube 80 cm vertically from the pipet tip.
4. To evoke CSD by electrical stimulation, fix a monopolar tungsten microelectrode in a stereotaxic manipulator at an angle of 45 degrees relative to the brain surface and place the tip of the electrode below the dura. Attach a ground wire to the custom-made metal frame for head fixation, and deliver a train of 100 μ s pulses at 200 Hz lasting for 1 s with an intensity of 10 mA with a stimulus isolator.

Data analysis

1. Open a series of acquired images using the ImageJ/Fiji software (ver. 1.50e), and split the images into a stack from the Cy3 fluorescence channel and a stack from the Alexa488 fluorescence channel (Figure 3A).
2. Calculate the background intensity for each fluorescence channel as the mean pixel values in the cortical area that is not stained with the ATPOS complex, and subtract the background intensity from the stack of each fluorescence channel (Figure 3B).
3. Process the stacks using a Gaussian spatial filter with a radius of 2 pixels (pixel size = 8 μ m) (Figure 3C), and create a stack of ratiometric images by dividing the stack of the Cy3 fluorescence channel by that of the Alexa488 fluorescence channel (Figure 3D).
4. Calculate the ratiometric fluorescence response of ATPOS (R/R_0) as follows. Create an image showing the baseline of the fluorescence ratio (R_0) by averaging the ratiometric images acquired before drug application or electrical stimulation (Figure 3E). Divide the stack of ratiometric images by the averaged image (Figure 3F) and plot a time-course of the mean fluorescence response in regions of interest (ROIs) selected in the cortical area stained with the ATPOS complex (Figure 4).

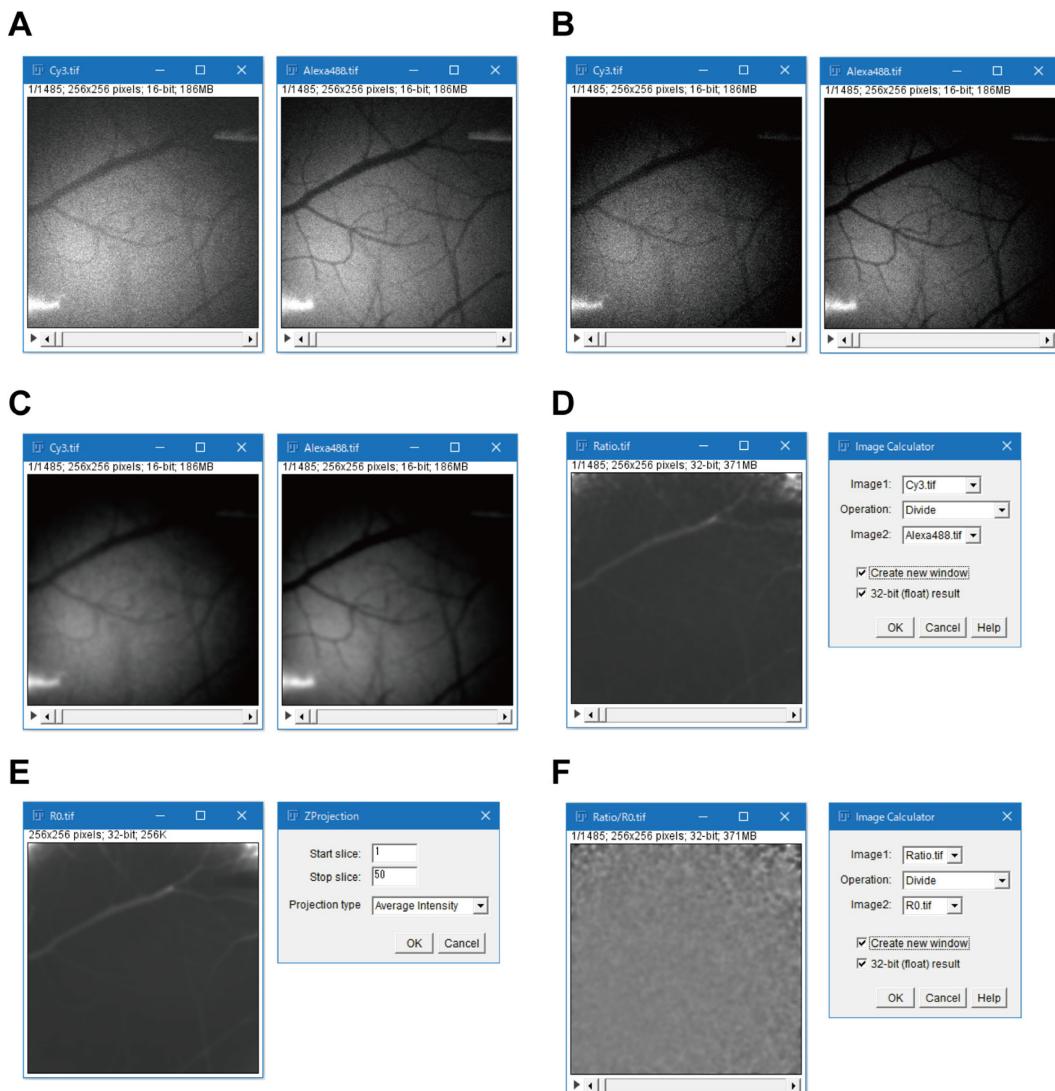


Figure 3. Image processing with the ImageJ/Fiji software. A. Stacks of Cy3 (left) and Alexa488 (right) fluorescence channels obtained by splitting channels using menu commands (Image > Stacks > Tools > Deinterleave). B. Stacks of Cy3 (left) and Alexa488 (right) fluorescence channels obtained by subtraction of the background fluorescence using menu commands (Process > Math > Subtract). C. Stacks of Cy3 (left) and Alexa488 (right) fluorescence channels obtained by Gaussian-spatial filtering using menu commands (Process > Filters > Gaussian Blur). D. A stack of ratiometric images (left) obtained by division of the stack of the Cy3 fluorescence channel by that of the Alexa488 fluorescence channel (right) using menu commands (Process > Image Calculator). E. A ratiometric image (R_0) (left) obtained by averaging frames acquired during the baseline period (right) using menu commands (Image > Stacks > Z Project). Enter frame numbers at the start and end of the baseline period in the 'Start slice' and 'Stop slice' boxes, respectively. In this example, the baseline period is 20 s (400 ms per frame \times 50 frames). F. A stack of images (R/R_0) (left) obtained by dividing the stack of ratiometric images by the averaged image (right) using menu commands (Process > Image Calculator).

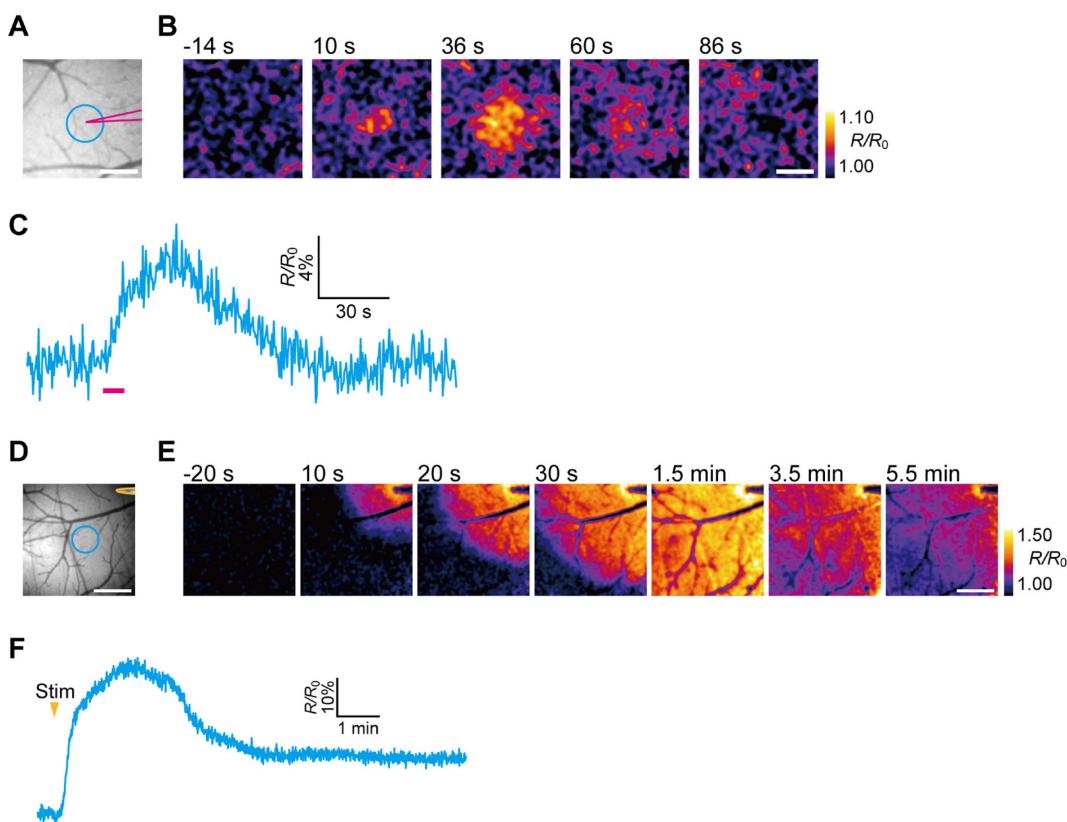


Figure 4. *In vivo* fluorescence imaging of extracellular ATP in the cerebral cortex. A-C. Visualization of the elevation of extracellular ATP levels after application of 10 mM ATP. A. The position of the micropipet for ATP injection (magenta) and the location of an ROI with a 15-pixel radius (blue). B. Representative images showing the ratiometric fluorescence response of ATPOS (R/R_0) upon ATP injection. The extracellular ATP levels are increased around the micropipet. Time after the start of injection is presented above the images. Scale bar, 250 μ m. C. A time-course of the ratiometric fluorescence response of ATPOS (R/R_0) extracted from the ROI depicted in A. The magenta bar indicates ATP injection. D-F. Visualization of wave-like extracellular ATP dynamics during CSD evoked by electrical stimulation. D. The position of the stimulating electrode (yellow) and the location of an ROI with a 25-pixel radius (blue). E. Representative images showing the ratiometric fluorescence response of ATPOS (R/R_0) upon electrical stimulation. A wave-like extracellular ATP release propagates from the stimulation site. Time after the initiation of electrical stimulation is presented above the images. Scale bar, 500 μ m. F. Time-course of the ratiometric fluorescence response of ATPOS (R/R_0) extracted from the ROI depicted in D.

Notes

1. The bacterial expression construct is based on pET vectors in which the recombinant protein is expressed under the control of a T7 promotor. Therefore, one has to use a host strain bearing DE3 lysogen, such as BL21(DE3), with which IPTG induces the expression of T7 polymerase,

leading to activation of the T7 promotor.

2. The molar concentration of the purified ATPBP-Q105C is typically around 1 mM.
3. The molar concentration of ATPOS is typically around 6 μ M.
4. The molar concentration of biotinylated ATPOS is typically around 40 μ M.
5. The protein concentration in the 1st, 2nd, and 3rd fraction is typically around 3 mg/ml, 2 mg/ml and 1 mg/ml, respectively.
6. The molar concentration of BoNT/C-Hc is typically around 40 μ M.
7. The molar concentration of biotinylated BoNT/C-Hc is typically around 40 μ M.
8. It is important to prevent air bubbles getting into the micropipet or plastic tube.
9. The obtained images have a dimension of 256 \times 256 pixels, with a pixel size of 8 μ m \times 8 μ m at the object plane.
10. For application of ATP, load 20 μ l 10 mM ATP dissolved in aCSF into a glass micropipet and fill the rest of the inner space of the micropipet with 10 μ l mineral oil.

Recipes

1. LB agar plates
10 g tryptone
5 g yeast extract
5 g NaCl
1 g glucose
15 g agar
Add Milli-Q H₂O to 1 L
2. 2 \times YT media
16 g tryptone
10 g yeast extract
5 g NaCl
Add Milli-Q H₂O to 1 L
3. LB media
10 g tryptone
5 g yeast extract
5 g NaCl
1 g glucose
Add Milli-Q H₂O to 1 L
4. PBS (pH 7.4)
8 g NaCl
0.2 g KCl
0.2 g KH₂PO₄
2.9 g Na₂HPO₄·12H₂O

Add Milli-Q H₂O to 1 L

5. 10× PBS (pH 7.4)
80 g NaCl
2 g KCl
2 g KH₂PO₄
29 g Na₂HPO₄·12H₂O
Add Milli-Q H₂O to 1 L

6. aCSF
7.3 g NaCl
0.3 g KCl
0.2 g NaH₂PO₄·2H₂O
2.2 g NaHCO₃
0.3 g CaCl₂·2H₂O
0.2 g MgCl₂·6H₂O
3.6 g glucose
Add Milli-Q H₂O to 1 L

7. HBS
2.4 g HEPES, 8.8 g NaCl, 0.2 g KCl. Add Milli-Q H₂O to 1 L

8. Anesthetic solution
1.9 ml medetomidine (1 mg/ml)
2.0 ml midazolam (5 mg/ml)
2.5 ml butorphanol (5 mg/ml)
Add 0.9% NaCl to 25 ml

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Competing interests

The authors declare no conflicts of interest.

Ethics

All experimental procedures used in animal experiments have been approved by the Animal Welfare Committee of the University of Tokyo.

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