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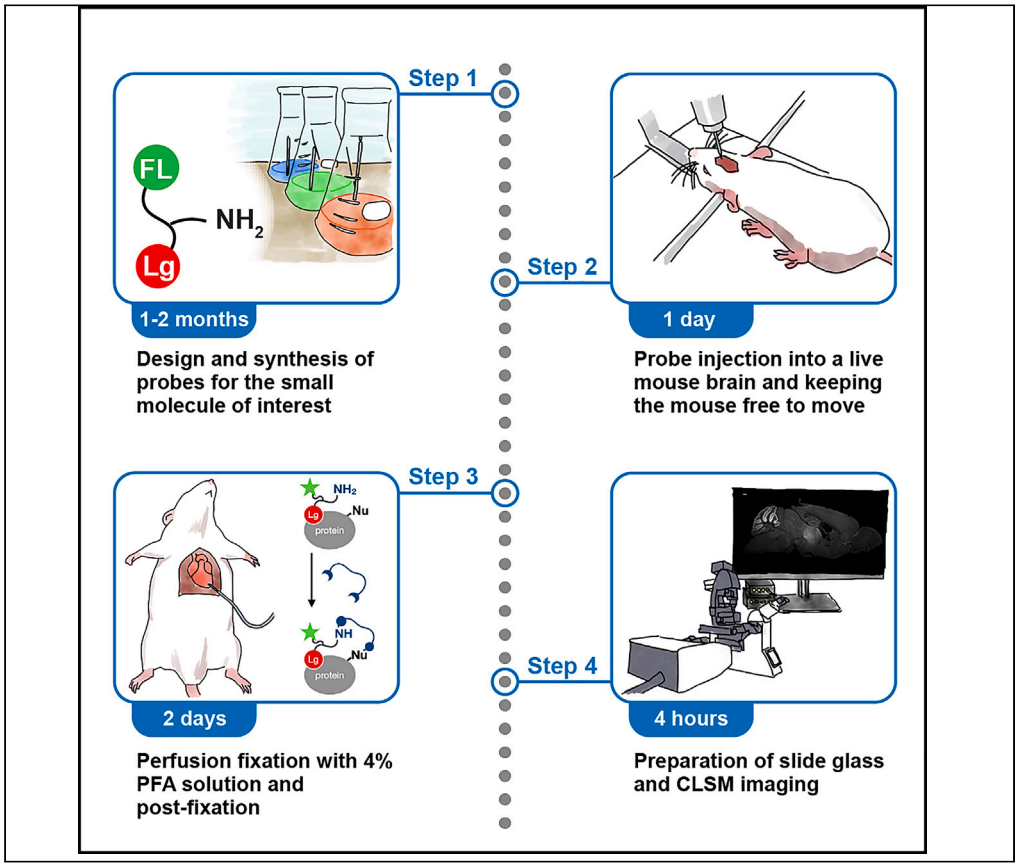
Protocol to visualize the distribution of exogenously administered small molecules in the mouse brain



Here, we present fixation-driven chemical crosslinking of exogenous ligands, a protocol to visualize the distribution of exogenously administered small molecules in the mouse brain. We first describe the probe design of the small molecules of interest and the probe microinjection into a live mouse brain in detail. We then detail procedures for paraformaldehyde-perfusion fixation. This approach is especially useful for imaging-based evaluation of the small-molecule ligands distribution in mouse brain tissue relying on their interaction with endogenous proteins.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for imaging exogenously administered small molecules of interest in the brain

A chemical strategy developed by revisiting the traditional tissue-fixation chemistry

Immobilization of target small molecules in tissues based on ligand-protein interaction

Step-by-step guide for visualizing the distribution changes of molecules of interest

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Protocol to visualize the distribution of exogenously administered small molecules in the mouse brain

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SUMMARY

Here, we present fixation-driven chemical crosslinking of exogenous ligands, a protocol to visualize the distribution of exogenously administered small molecules in the mouse brain. We first describe the probe design of the small molecules of interest and the probe microinjection into a live mouse brain in detail. We then detail procedures for paraformaldehyde-perfusion fixation. This approach is especially useful for imaging-based evaluation of the small-molecule ligands distribution in mouse brain tissue relying on their interaction with endogenous proteins.

For complete details on the use and execution of this protocol, please refer to Nonaka et al.[1]

BEFORE YOU BEGIN

From basic research to medical diagnosis, a lot of small molecules were widely used as functional probes. In order to evaluate the function of such small molecules and develop new ones, it should be critically important to know their spatial distribution *in vivo*. However, there have been limited methods for analyzing the small molecule distribution and its temporal changes in the biological tissue and *in vivo*.

Here we introduce a detailed protocol of the ***FixEL*** method to visualize the distribution of exogenously administered small molecules in the mouse brain (H. Nonaka et al., *Chem* **9** (2023)). *FixEL* was developed by chemically twisting the well-accepted conventional PFA tissue fixation method. PFA tissue fixation allows for immobilizing the protein distribution, whereas small molecules are generally NOT fixed to the tissue. *FixEL* allows the immobilization of small molecules in biological tissues. In *FixEL*, the molecule of interest (MOI) is modified with amino groups that can react with PFA and fluorescent dyes (Figure 1A). The amino group-modified MOI probe is administered to the animal, distributed *in vivo*, and then immobilized in the tissue by the PFA tissue fixation (Figure 1B). Immobilization of the MOI in the tissue allows us to visualize the distribution of MOI by fluorescence microscopy. This protocol describes the specific steps for the design of probes, surgery including probe injection and perfusion fixation, and imaging. We have also used this protocol to obtain snapshot imaging of temporal changes in the distribution of MOI.

Institutional permissions

All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Use Committees of Kyoto University.

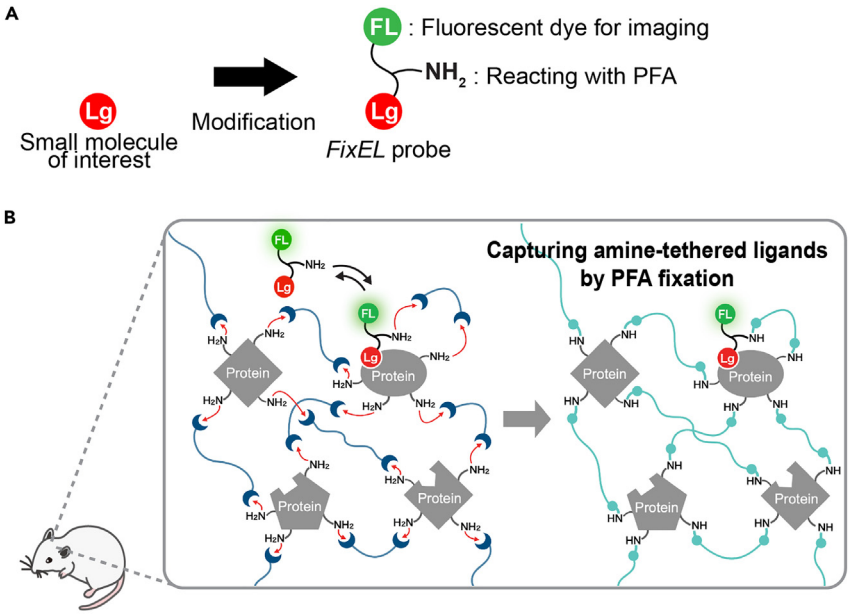

Figure 1. *FixEL* visualizes the distribution of target small molecules in the biological tissues

(A) Modification of small molecules of interest with primary amine for immobilization of their distribution in the tissue.

(B) Illustrated scheme of FixEL. The amine-modified small molecule is immobilized in the tissue simultaneously as tissue fixation by PFA.

Design and synthesis of probes

Timing: 1–2 months

1. Design probes.
   a. Select a molecule of interest (MOI).
   b. Design the structure of chemical probes. The probe contains primary amine and fluorescent dye with a linker (Figure 1A). For specific design examples, see H. Nonaka et al. (2023).[1]
      i. If the selected MOI is known or assumed to bind to specific proteins or other molecules, the linker structure should not affect such binding as much as possible. Structure-activity relationship (SAR) studies are valuable in determining the site of linker introduction. Co-crystal structure information of the MOI and protein is also helpful. Several structures must be designed and synthesized with linkers introduced at different sites for *in vitro* assays if there is no information on SAR.
      The fluorescent dye and amine must be far away from the ligand to not interfere with ligand binding. In contrast, the distance between the fluorophore and the amine is not critical.
      ii. Polyethylene glycol would be a good choice for the linker structure to ensure the hydrophilicity of the probe.
      iii. Fluorescent dyes should not cause nonspecific binding in living animal tissues. Hydrophilic dyes (e.g., Alexa Fluor 555/647) are preferred to meet this requirement.
      iv. Structures with a C4 linker between the main linker and the amino group are available in *FixEL*. Our previous studies used C4 linkers based on the Lys backbone (H. Nonaka et al., 2023).
2. Synthesize probes.
   a. Chemically synthesize the probes designed above.
3. *In vitro* assay of probe functionality.
   a. If the selected MOI is a ligand, verifying whether the synthesized probe retains binding ability and selectivity in an *in vitro* assay using purified proteins and cell lines is necessary.

Set up for surgery

Timing: 30 min

Before completing surgery, ensure proper compliance with the regulatory guidelines from your institution for rodent survival surgery.

4. Gather supplies for surgery.
   a. Supplies for probe injection.
      i. Disposable gloves.
      ii. Paper towel and wiper.
      iii. 70% EtOH.
      iv. Shaving knife.
      v. Syringes.
      vi. Anesthetic solution.
      vii. Anesthetic antagonist solution.
      viii. Stereotactic apparatus for mouse injection.
      ix. Surgical scissor.
      x. CCD camera and monitor.
      xi. Ruler.
      xii. Hand drill for skull.
      xiii. Glass capillary.
      xiv. Paraffin liquid.
      xv. Microinjector.
      xvi. Tweezer.
      xvii. Clips for suturing mouse skin.
   b. Supplies for perfusion fixation.
      i. Paper towel.
      ii. Sealed cage.
      iii. Isoflurane.
      iv. Corkboard.
      v. Bat.
      vi. Needle to pin.
      vii. Surgical scissors.
      viii. Needles to insertion.
      ix. Peristaltic pump and tube.
      x. Tweezer.
      xi. Ice.
5. Prepare experimental animals.
   a. Ensure that your mouse is at the appropriate age and weight for survival surgery with anesthetic and that your regulatory institution has approved the procedure.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Isoflurane	FUJIFILM Wako Pure Chemical Corporation	Cat# 099-06571; CAS: 26675-46-7
Medetomidine hydrochloride (Domitor)	Nippon Zenyaku Kogyo Co., Ltd.	CAS: 86347-15-1
Midazolam (Sandoz)	Sandoz K.K.	Cat# 614243022; CAS:59467-70-8
Butorphanol tartrate (Vetorphale)	Meiji Seika Pharma Co., Ltd.	CAS: 58786-99-5
Saline (Otsuka Normal Saline)	Otsuka Pharmaceutical Factory, Inc.	Cat# 035081517
Atipamezole hydrochloride (ANTISEDAN)	Nippon Zenyaku Kogyo Co., Ltd.	Cat# 05913; CAS: 104075-48-1

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dulbecco's PBS (–) powder	Nissui Pharmaceutical Co., Ltd.	Cat# 05913
Paraffin liquid	Nacalai Tesque	Cat# 26114-75
Paraformaldehyde	FUJIFILM Wako Pure Chemical Corporation	Cat# 162-16065; CAS: 30525-89-4
Sucrose	FUJIFILM Wako Pure Chemical Corporation	Cat# 196-00015; CAS: 57-50-1
Tissue-Tek OCT compound	Sakura Finetek	Cat# 25608-930
Experimental models: Organisms/strains		
5-week-old male C57BL/6N mice	Shimizu Laboratory Supplies Co., Ltd.	Cat# C57BL/6NCrSlc
Software and algorithms		
Leica LAS X software (version 3.5.3)	Leica Microsystems	RRID: SCR_013673
Other		
Glass capillaries, 1.14 mm 3.5″	World Precision Instruments	Cat# 504949
Puller	Narishige	Cat# PC-100
Stereotactic apparatus for mouse injection	Muromachi Kikai Co., Ltd.	Cat# MA-625
Heating pad	Vivaria Co., Ltd.	Cat# MP-916
Hybrid Leutor (hand drill)	Nihon Seimitsu Kikai Kosaku Co., Ltd.	Cat# LHBM-12
Nanoliter 2010 (microinjector)	World Precision Instruments	Current successor: Cat# 300704
SMARTouch (microinjector controller)	World Precision Instruments	Cat# MICRO2T
Wound clips	Roboz Surgical Instrument	Cat# RS-9255
Wound clip applier	Roboz Surgical Instrument	Cat# RS-9250
NARCOBIT-E (anesthetic machine)	Natsume Seisakusho Co., Ltd.	Cat# KN-1071
Peristaltic pump	AS ONE	Cat# SMP-21AS
Tissue-Tek Cryomold	Sakura Finetek	Cat# 4557
Cryostat	Leica Biosystems	Cat# CM1950
Platinum PRO glass slide	Matsunami Glass Ind., Ltd.	Cat# SPRO-04
Micro cover glass (22 × 22 no. 1)	Matsunami Glass Ind., Ltd.	Cat# C022221
Fluoromount/Plus	Diagnostic BioSystems Inc.	Cat# K048
Confocal laser scanning microscope TCS SP8	Leica Microsystems	N/A

MATERIALS AND EQUIPMENT

Anesthetic solution		
Reagent	Final concentration	Amount
Medetomidine hydrochloride (1 mg/mL)	7.5 μg/ 100 μL	1.875 mL
Midazolam (5 mg/mL)	40 μg/ 100 μL	2 mL
Butorphanol tartrate (5 mg/mL)	50 μg/ 100 μL	2.5 mL
Saline	N/A	18.625 mL
Total	**N/A**	**25 mL**

The prepared stock is stored at 4°C and used within two months.

- Anesthetic antagonist solution: add 0.15 mL of atipamezole hydrochloride (5 mg/mL) to 9.85 mL of saline. The prepared stock is stored at 4°C and used within two months.

4% paraformaldehyde (PFA) solution		
Reagent	Final concentration	Amount
Ultrapure water	N/A	~ 950 mL
Paraformaldehyde	4%	40 g
NaOH aq. (2 M)	N/A	~ 200 μL
Dulbecco's PBS (–) powder	1×	9.6 g
Total	**N/A**	**1 L**

Add PFA and NaOH aq. to ultrapure water under heat (60°C). After PFA is dissolved, add Dulbecco's PBS (–) powder. Adjust the volume to 1 L with additional ultrapure water. The prepared stock is stored at 4°C and used within one week.

- 30% Sucrose solution: add 150 g sucrose in 350 mL PBS(–).

The prepared stock is stored at 4°C.

⚠ CRITICAL: Paraformaldehyde (PFA) is acutely toxic and irritates the skin and eyes. In addition, formaldehyde from PFA is carcinogenic and may cause sensitization by skin contact. All preparation of 4% paraformaldehyde solution should be done under a fume hood with goggles and rubber gloves.

STEP-BY-STEP METHOD DETAILS

Probe injection into a live mouse brain and keeping the mouse free to move

Timing: 1 day

The probe of MOI is injected into a live mouse brain under anesthesia.

1. Craniotomy.
   a. Inject 200 μL of anesthetic solution into the mouse intraperitoneally.
   b. Wait for the mouse to be anesthetized. Ensure the loss of the pedal withdrawal response.
   c. Remove hairs around the head moistened with 70% EtOH with a shaving knife.
   d. Fix the head to the stereotactic apparatus.
   e. Make an incision in the skin of the head using small surgical scissors.
   f. Place the mouse under the CCD camera.
   g. Determine the target position on the skull using a CCD camera, monitor, and ruler.
   h. Make a small hole in the target area of the skull using a hand drill.

⚠ CRITICAL: Apply the drill gently, and be careful not to damage anything other than the skull.

2. Microinjection.
   a. Fill the glass capillary with paraffin liquid.
   b. Using a microinjector, load the 4.5 μL of probe solution into the glass capillary.
      i. PBS (–) and saline are available as solvents for probe injection.
   c. Insert the glass capillary into the target position.
   d. Inject the whole amount of probe solution into the brain with a microinjector at 600 μL/min.
3. Recovery from anesthesia.
   a. Remove the mouse from the stereotactic apparatus.
   b. Close the scalp with clips.
   c. Inject an anesthetic antagonist solution intraperitoneally.
   d. Let the mouse move freely in the cage.

⚠ CRITICAL: Keep the mouse on the heating pad until it wakes up and moves around on its own. Monitor and keep the mouse in the cage.

Note: To inject a probe into the mouse brain, orient the capillary perpendicular to the surface of the brain, drill a hole in the skull, and insert the capillary following the information in the mouse brain atlas according to the age of the week.[2,3]

Perfusion fixation with 4% PFA solution

Timing: 30 min

PFA penetrates the mouse body to fix tissues and simultaneously immobilize small molecules of interest.

4. Anesthesia.
   a. Place the mouse in a sealed cage filled with 4% isoflurane.
   b. Wait for the mouse to be anesthetized. Ensure the loss of the pedal withdrawal response.
5. PFA perfusion (must be done in a fume hood).
   a. Place the mouse on its back on a corkboard in a bat.
   b. Attach an inhaler to the head and flush 4% isoflurane.
   c. Fix both hands and one leg to the corkboard with needles.
   d. Perform a thoracotomy to access the heart.
   e. Insert a needle connected to a peristaltic pump into the left atrium of the mouse.
   f. Make an incision in the right atrial appendage and perfuse with ice-cold 4% PFA 60 mL (three volumes of mouse body weight) in 10 min.
   g. Ensure that the entire body of the mouse is rigid.
   h. Isolate the whole brain using surgical scissors and transfer to 4 mL of ice-cold 4% PFA solution in a 5 mL-size tube.

Post-fixation and sucrose substitution

Timing: 2 days

6. Soak the tissue in 4% PFA solution overnight at 4°C.
7. Replace with 30% sucrose solution and soak until the tissue is submerged.

Slice preparation with the cryostat

Timing: 2 h

8. Immerse tissue block into Tissue-Tek OCT compound in Tissue-Tek Cryomold.
9. Freeze the sample in the cryostat.
10. Slice the sample with 20 μm of thickness.

CLSM imaging

Timing: 2 h

11. Put brain slices on a glass slide and enclose them with cover glass and Fluoromount/Plus.
12. Image slides with confocal laser scanning microscope.

EXPECTED OUTCOMES

This protocol allows us to readily visualize the exogenously administered small molecules in animal tissues and to monitor their distribution changes using conventional confocal fluorescence microscopy.

We used this method to evaluate how small molecular ligands for mGlu1 administered from the lateral ventricles reach the cerebellum. The cerebellum is a region where mGlu1 is abundantly expressed. Most of the mGlu1 ligand was distributed around the lateral ventricles immediately after administration, but 6 h later, the distribution was shifted to the molecular layer of the cerebellum, mainly due to the selective interaction with mGlu1 abundantly expressed in the cerebellum (Figures 2A and 2B). In addition, we succeeded in capturing by imaging with high resolution, the gradual penetration of the small mGlu1 ligand from the cerebrospinal fluid into the deep cerebellum after administration to the lateral ventricles (Figure 2C).

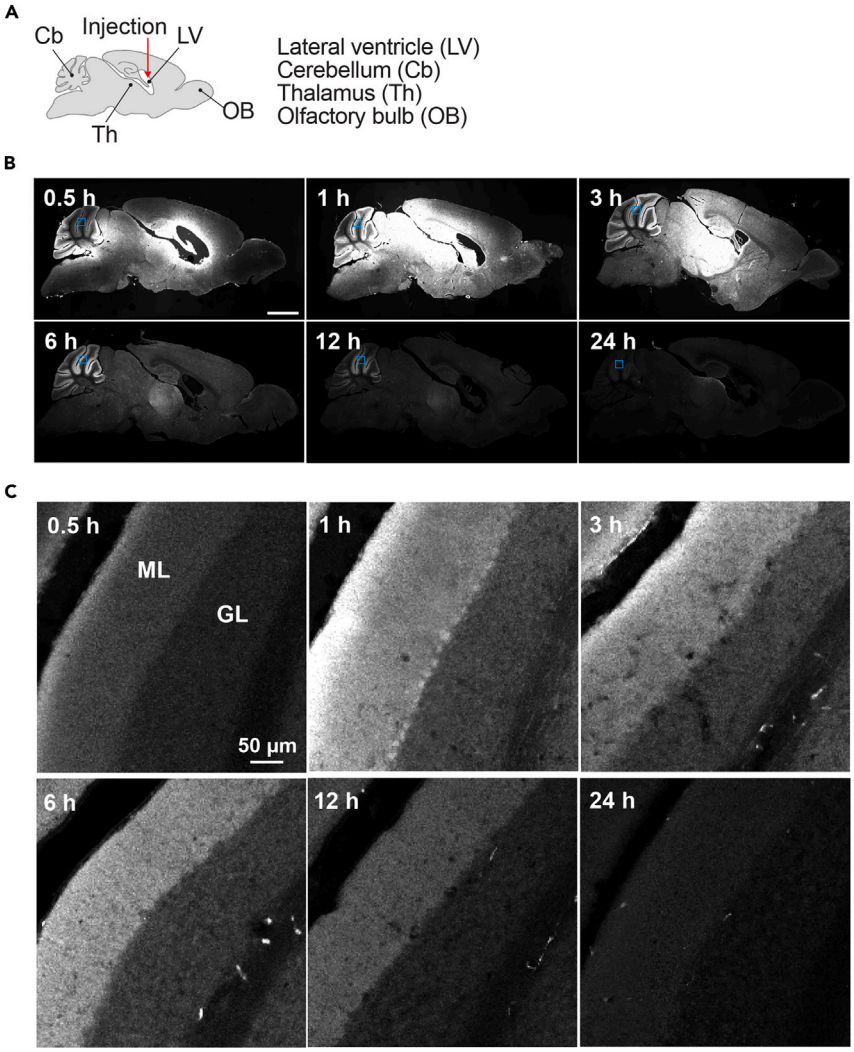

Figure 2. Visualization of the distribution of mGlu1 ligand (CNITM)

(A) An illustrated sagittal slice of a mouse whole brain. Red arrows indicate the probe administration position.

(B) Temporal changes in ligand distribution. Scale bar: 2 mm.

(C) Enlarged view of the area enclosed by the sky-blue square in B. ML: Molecular layer. GL: Granular layer.

LIMITATIONS

FixEL requires chemical modification of the ligand in order to attach a primary amine and fluorescent dye, which may affect its affinity, membrane permeability, and tissue adsorption. However, MOI we tested in H. Nonaka et al., 2023 exhibited the spatial distribution of the probes was consistent with the expression areas of the corresponding target proteins. This result suggests that the chemical decoration of the ligand has minimal effect on the properties of the corresponding ligand.

TROUBLESHOOTING

Problem 1

Backflow occurs when injecting the probe (related to Step 2).

Potential solution

- The hole made in the skull may be too large. Make the hole the minimum size to insert the capillary.

- When injecting the probe into the lateral ventricle, wait 1–2 min after the end of injection to reduce the backflow seen when removing the capillary.

Problem 2

Abnormal mouse behavior occurs after probe administration (related to Step 3).

Potential solution

- Investigate and verify in advance that the target small molecule is non-toxic.

Problem 3

The tissue does not harden after perfusion (related to Step 5).

Potential solution

- PFA solution prepared for several weeks will not provide adequate fixation. Use a fresh solution.
- If the fresh PFA solution does not resolve the problem, the needle may not have penetrated the left atrium properly during perfusion. Do not insert the needle too deeply.

Problem 4

Extensive scarring of tissue slices is observed (related to Step 10).

Potential solution

Temperature control of the Cryostat may be insufficient. Do not lower the temperature too much.

Problem 5

Cross-linking between the target small molecule ligand and protein does not occur (related to Step 12).

Potential solution

- Depending on the position of amino groups or fluorescent dyes introduced, the affinity of the target ligand may be significantly reduced. Be sure to check the probe's affinity in advance with an *in vitro* assay.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Itaru Hamachi (ihamachi@sbchem.kyoto-u.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate nor analyze new datasets.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

T.M. wrote the first draft of the manuscript. H.N., S.S., and I.H. contributed toward writing the manuscript. T.M., H.N., S.S., and I.H. were involved in editing.

DECLARATION OF INTERESTS

I.H. has filed a patent application (WO2019/168125).

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