Research Square

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Deep Imaging of Cleared Brain by Confocal Laser-Scanning Microscopy

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Method Article

Keywords: Tissue clearing, Urea, Refractive index, Laser-scanning microscopy

Posted Date: March 24th, 2016

DOI: https://doi.org/10.1038/protex.2016.019

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Abstract

The Sca_l_eS technique for clearance of biological tissue enables us to perform high-resolution three-dimensional \(3D) reconstructions of entire structures in fixed, unsectioned brain samples where specific cell types are labeled with fluorescent proteins genetically or with fluorophore-conjugated antibodies. While Sca_l_eA2 and Sca_l_eU2, the previously reported urea-based clearing solutions have refractive indices \(RIs) of 1.38 and 1.40, respectively, Sca_l_eS solutions containing sorbitol in addition to urea have higher RIs around 1.47. Importantly, Sca_l_eS solutions preserve fluorescence signals and tissue fine structures, allowing quantitative three-dimensional reconstructions. Here we provide a basic protocol describing the procedures for solution preparation, sample clarification and immobilization for observation using confocal laser-scanning microscopy. We emphasize the importance of RI matching by showing results of comparative experiments that deep imaged YFP-expressing neurons within cleared brains by Sca_l_eS, Sca_l_eA2, or Sca_l_eU2 and utilized objective lenses with long working distances, sufficiently high numerical apertures, but different RIs. Minor edits requested by the author were made to the formatting of the protocol on 5th April 2016. The original version of the protocol is available as an attachment. Katharine Barnes, Managing Editor, Nature Protocols, 5/4/16

Reagents

MICE Transgenic mice expressing YFP in the cytoplasm of a subpopulation of neurons \(YFP-H mouse line) C57BL6/J mouse line **REAGENTS and MATERIALS** i) Fixation Paraformaldehyde \(PFA), granular \(Nacalai, 02890-45) HBSS \(Gibco, Invitrogen) Novo Heparin 5,000 units/ 5 ml \(Mochida Pharmaceutical Co. Ltd., Japan) ii) Clearing D\(−)-sorbitol \(Wako Pure Chemical Industries, 199-14731) Urea \(Wako Pure Chemical Industries, 217-00615) Triton X-100 \(Nacalai Tesque, 35501-15) Glycerol \(Sigma, G9012) Dimethyl sulfoxide \(DMSO) \(Wako Pure Chemical Industries, 043-07216) N-acetyl-L-hydroxyproline \(Skin Essential Actives, Taiwan) γ-cyclodextrin \(Wako Pure Chemical Industries, 037-10643) Methyl-β-cyclodextrin \(Tokyo Chemical Industry Co. Ltd., M1356) Ca^{2+}, Mg^{2+}-free phosphate-buffered saline \[PBS\(−)] 30 ml centrifuge tube \(SARSTEDT, # 62.543) \(**Figure 1a**) 13 ml centrifuge tube \(SARSTEDT, # 60.541.545) \(**Figure 1b**) **CAUTION** N-acetyl-L-hydroxyproline must be from Skin Essential Actives \(Taiwan). The manufacture's product appears to be animal skin origin. Sometimes, this amino acid derivative is difficult to obtain. If so, it must be omitted. Do not replace it with other products such as those provided by fermentation manufactures. Far from being ineffective, the products of microbial origin seem to affect tissue clarification. iii) Immobilization Agarose \(TAKARA, Japan, LO3) Alon alpha A \(Daiichi-Sankyo, Japan) Parafilm M® \(Bemis Company Inc.) Pasteur pipette \(length: 146 mm, diameter: 7 mm, Iwaki, Japan) Bubble-remover: Bend a Pasteur pipette in flame and fire-polish the tip \(**Figure 1c**). Polyvinyl pipette \(3-ml, Copan Italy, 200C) Bubble-flusher: Bend a polyvinyl pipette with heat \(**Figure 1d**). Plastic cup \(height: 5–6 cm, diameter: 8–10 cm, SANPLATEC Corp., Japan) \(**Figure 1e**, center) Kim Wipe \(Kimberly, S-200) **REAGENT SETUP** i) Fixation **4% \(wt/vol) PFA/PBS\(−) \(pH 7.6–7.8)** Dissolve 4 g of PFA in approximately 80 ml of Milli-Q water completely by stirring on a hot plate. Add 10 ml of 10× PBS\(−). Adjust pH to 7.6–7.8 by adding 1 N

NaOH. Add Milli-Q water to make 100 ml. ii) Clearing **Triton X-100 stock solution:** 10% \(wt/vol) Triton X-100 Dissolve 10 g of Triton X-100 in approximately 80 ml of Milli-Q water by stirring. Add Milli-Q water to make 100 ml and stir until well mixed. Store at 4 ºC. **Sca_I_eS1, Sca_I_eS2, Sca_I_eS3, and Sca_I_eS4** Prepare Sca_I_eS solutions as follows \(1–liter solutions). 1| Dissolve all the solutes \(see **Table 1**) in approximately 100 ml of Milli-Q water in a 1–liter beaker. 2| Mix with a large spatula for hydration of both urea crystals and sorbitol powder \(**Figure 2a**). 3| Add Milli-Q water to make approximately 950 ml. 4| Heat the mixture in a microwave oven \(500 W) for 4–5 min. 5| Cool down the mixture for 1 hr while stirring well \(**Figure 2b**). 6| Add Milli-Q water to make 1,000 ml and stir until well mixed. **CAUTION** Sca_I_eS1–S3 solutions each contains a high concentration of urea. Efficient hydration of a large quantity of urea crystals needs stirring with a spatula \(**Figure 2a**). **CAUTION** Sca_I_eS1–S3 solutions are supposed to work for tissue clarification. In contrast, Sca_I_eS4 solution is used not only for clearing but also as a mounting/storage medium. There are two types of Sca_I_eS4 with different concentrations of DMSO: Sca_I_eS4D25 and Sca_I_eS4D2.5, which contain 25% and 2.5% \(vol/vol) DMSO, respectively. Sca_I_eS4D25 exerts high performance in tissue clearing. Immediately after preparation particularly at a high temperature, however, it may show misty appearance \(**Figure 2c**, center). The mist gradually becomes invisible in about a week in a storage bottle. The mist is not a problem when the solution is used as a mounting medium for two-photon excitation microscopy or common confocal laser-scanning microscopy \(CLSM). In contrast, the mist may affect observation using selective plane illumination microscopy \(SPIM), which requires the maximum transparency of the medium. The alternative solution Sca_I_eS4D2.5 always looks transparent \(**Figure 2c**, right) and can be used as a mounting medium for SPIM. Equilibration with Sca_I_eS4D2.5 takes several hours. Because Sca_I_eS4D2.5 is much less potent in tissue clearing, it should not be used for the purpose of tissue clarification. Also, Sca_I_eS4D2.5 cannot keep tissue clarification well; transparent samples in Sca_I_eS4D2.5 become turbid in several days. It is therefore recommended that the SPIM observation be performed between several hours and a few days after substitution of Sca_I_eS4D2.5. **CAUTION** pH-adjustment is not required. Neither sterilization nor addition of preservative is required. **CAUTION** All Sca_I_eS solutions must be degassed for 5 hrs at approximately 25 ºC by a vacuum pump combined with bath-sonication \(240 W, 46 kHz) \(**Figure 2d**). iii) Immobilization **Sca_I_eS4D25\(0):** Sca_I_eS4D25 without Triton X-100 **1.5–2.5% \(wt/vol) agarose in Sca_I_eS4D25\(0)** Add Sca_I_eS4D25\(0) to 1.5–2.5 gram agarose powder to make a 100 ml mixture in a 150–200-ml glass bottle. Melt the agarose powder by heating the mixture in a microwave oven \(500 W) for 4 min. Use fresh preparation; reuse of stored melted agarose solution causes generation of air bubbles prior to observation.

Equipment

EQUIPMENT Microwave oven with a rated high frequency output 500 W Bath-sonicator \(Output: 240 W, Frequency: 23 kHz) \(ASONE, Japan) \(**Figure 2d**) Vacuum chamber \(**Figure 2d**) Vacuum pump \(ULVAC KIKO. Inc., Japan) \(**Figure 2d**) Constant-temperature incubator with a shaker \(TAITEC) \(**Figure 2e**) Orbital shaker \(TAITEC) \(**Figure 2f**) Olympus FV1200 CLSM system

Objective lens: 25× \(Olympus, XLSLPLN25XGMP, RI = 1.41–1.52, NA = 1.0, WD = 8 mm) \(**Figure 1e**, left) Objective lens: 25× \(Olympus, XLSLPLN25XSVMP, RI = 1.33–1.40, NA = 0.95, WD = 8 mm) \(**Figure 1e**, right) Leica MZ16F Fluorescence stereomicroscope with Zeiss AxioCam 506 color Objective: 1× objective lens \(Leica, PLANAPO, NA = 0.141)

Procedure

PROCEDURE i) Fixation Uniform fixation of thick samples, such as the whole brain, is imperative for large-scale quantitative 3D reconstructions. **1|** Anesthetize an adult \(6–40 weeks old) mouse via intraperitoneal injection of pentobarbital \(Somnopentyl) \(60 mg/kg body weight). **2|** Transcardially perfuse the animal with 20 ml of ice-cold HBSS supplemented with heparin \(10 units/ml) in 1 min. The clearing of the liver indicates a good systemic perfusion. **3|** Transcardially perfuse the animal with 40 ml of ice-cold 4% \(wt/vol) PFA/PBS\(−) in 2 min. Care should be taken not to introduce air bubbles while transferring from HBSS to this fixative. The hardening of the liver indicates a good systemic fixation. **CAUTION** Freshly prepared 4% \(wt/vol) PFA/PBS\(−) should be used for efficient fixation. Check its pH prior to use. It should be neutral or slightly alkaline \(pH 7.6–7.8). **CAUTION** The perfusion flow rate should be not less than 20 ml/min for a rapid and uniform fixation. Slow perfusion may result in tissue autolysis due to post-mortem effects. **4|** Dissect out the brain and postfix it with 25–30 ml of 4% \(wt/vol) PFA/PBS\(−) \(pH 7.6–7.8) for 8–72 hrs at 4 ºC with mild shaking. **5|** Store the sample in PBS\(−) at 4 ºC until use but for not more than 1 week. **CRITICAL STEP** The apparent fluorescence of the brain sample is reduced during postfixation, but can be recovered substantially after several hour incubation in PBS\(−). At this stage, it is recommended that fluorescent pictures of the brain are taken using a fluorescence stereomicroscope for records. These images may be compared with those after deSca_l_ing for assessment of fluorescence preservation during treatments with Sca_l_eS0, Sca_l_eS1, Sca_l_eS2, and Sca_l_eS3. ii) Clearing It may be hard to clear the whole brain of older mice \(> 20 weeks old) across the pia mater. For older mice, it is recommended to split the brain into two pieces. For example, unless commissural connections are examined, cut the brain at mid-plane into two cerebral hemispheres. The following procedure assumes that the hemisphere or a 1–3-mm-thick slice of the mouse brain is processed as a sample to be cleared. The clearing procedure should be properly used according to the type of tissue to be cleared \(**Table 2a–c**). **1|** Transfer the sample into Sca_l_eS0 solution in a centrifuge tube \(**Figure 1a, b**), and incubate it at 37 ºC with gentle shaking \(~70 rpm/min) \(**Figure 3a**) for 12 hrs. **2|** Replace Sca_l_eS0 solution with Sca_l_eS1. Incubate the sample in Sca_l_eS1 at 37 ºC with gentle shaking \(~70 rpm/min) \(**Figure 3a**) for 12 hrs. **3|** Replace Sca_l_eS1 solution with Sca_l_eS2. Incubate the sample in Sca_l_eS2 at 37 ºC with gentle shaking \(~70 rpm/min) \(**Figure 3a**) for 12 hrs. **4|** Replace Sca_l_eS2 solution with Sca_l_eS3. Incubate the sample in Sca_l_eS3 at 37 ºC with gentle shaking \(~70 rpm/min) \(**Figure 3a**) for 12 hrs. **CRITICAL STEP** It is important to use a container \(see-through vial) where you can easily assess sample integrity and transparency. **CAUTION** Much care should be taken not to damage or destroy the sample. Use a dispensing spoon to hold the tissue during solution replacement \(**Figure 3b**). If the sample is fragile, decrease the shaking speed. **CAUTION** Spilled Triton X-100 may deteriorate the cap

sealing of the centrifuge tube, leading to solution leakage during long hours of shaking. Wipe the solutions well off the cap screw. **5|** deSca_l_ing. Replace Sca_l_eS3 solution with PBS\(−) and incubate the sample at 4 ºC for 6 hrs with gentle shaking \(30–40 rpm/min) on the orbital shaker. **CRITICAL STEP** Confirm turbid appearance of the deSca_l_ed sample. It should look like it used to be before clearing. See i) step 5|. Take pictures of the sample using a fluorescence stereomicroscope under the same conditions as before. If the sample is not yet turbid, repeat deSca_l_ing with fresh PBS\(−). **6|** Replace PBS\(−) with Sca_l_eS4D25 and incubate the sample at 37 ºC with gentle shaking \(~70 rpm/min) for 12 hrs \(**Figure 3a**). **TROUBLESHOOTING** If the sample cannot be clarified, prolong the incubation, refresh the incubation with new Sca_l_eS4D25, or do deSca_l_ing over again. **PAUSE POINT** DeSca_l_ed samples can be kept at 4 ºC for a long period. The transparent sample can be stored in Sca_l_eS4D25 at 4 ºC for >1 year. iii) Immobilization The Sca_l_eS4D25-treated tissue floats in the mounting solution, such as Sca_l_eS4D25 and Sca_l_eS4D2.5, and needs to be immobilized in a container for high-resolution fluorescence observation. Here the sample is supposed to be immobilized onto a culture dish for epi-fluorescence observation using CLSM, which is common laboratory equipment. Also, the immobilization is designed for an upright microscope with dipping objectives that have long working distances \(**Figure 1e**), and therefore requires a tall plastic dish. The following 2 methods assume an experiment, where the hemisphere or a 1–3-mm-thick slice of the YFP-H mouse brain is imaged deep for a high-resolution 3D reconstruction of YFP-expressing neurons. In many cases, furthermore, the experiment aims at wide imaging to generate such 3D reconstructions at multiple neighboring xy positions. Accordingly, the experiment usually spans several hours, and during the time period the sample must not move or expand/shrink. \(1) Agarose method **1|** Melt 1.5–2.5% \(wt/vol) agarose in Sca_l_eS4D25\(0) using a microwave oven \(500 W) for 4 min and cool down to 35–40 ºC. **2|** Place the sample on the bottom of a plastic cup \(depth: 4–5 cm, diameter: 8–10 cm). **3|** Wipe up excessive Sca_l_eS4D25 solution around the sample with Kim Wipe \(**Figure 4a**). Leave the sample in the air for 10–15 min at room temperature to lightly dry the sample surface. **4|** Drop the agarose solution on the top of the sample using a polyvinyl pipette. A mountain-like skirt of agarose is made that covers the entire sample \(**Figure 4b**). **CAUTION** Much care should be taken to verify that the agarose solution is cooled down enough. **5|** Leave the agarose skirt for hardening in the air for a few hours or in a refrigerator when in a hurry. **6|** Trim away the edges of the agarose skirt using a micro-spatula \(**Figure 4c**). Remove the agarose fragments or debris completely by Kim Wipe \(**Figure 4d**). Wipe out the cup surface with a wet Kim Wipe and leave it in the air for 5 min. **7|** Attach the rim of the agarose gel onto the cup surface by glue \(Alon alpha A). Adhesion at multiple points is required \(**Figure 4e**). Wait for approximately 10 min for complete fixation of the whole sample, which can be checked by slanting the cup. **8|** Gently pour an appropriate volume \(1/4 vol of the cup capacity) of Sca_l_eS4D25 solution into the cup \(**Figure 4f**). **TROUBLESHOOTING** If the sample with agarose gel is detached from the cup bottom, move the sample to another cup and repeat the steps 1|–8|. **9|** Equilibrate the sample to Sca_l_eS4D25 by gentle shaking for 1 hr at room temperature \(**Figure 4g**). **10|** Substitute fresh Sca_l_eS4D25 or Sca_l_eS4D2.5 solution for observation. This sample system with Sca_l_eS4D25 can be stored at 4 ºC for at least 2 months. **11|** Immerse a dipping objective lens in Sca_l_eS4D25 or Sca_l_eS4D2.5 and make it approach to the sample

slowly \(**Figure 4h**). **CAUTION** Before objective immersion, check by eyes that there are no air bubbles on the sample surface. Bubbles can be eliminated by gently scraping the surface with the bubble-remover \(**Figure 1c, 5a**). After objective immersion, check tiny air bubbles on the sample surface through the microscope and remove them by the bubble-remover. On the other hand, air bubbles trapped on the tip of the objective should be removed by the bubble-flusher \(**Figure 1d, 5b**). **CAUTION** CLSM observation for a long time \(> 12 hrs) results in water evaporation of the mounting medium. This is prevented by covering the surface of the medium with a thin plastic film made of saran \(trade name Saran Wrap) \(**Figure 5c**). \(2) Parafilm method **1|** Place the sample on the bottom of a plastic cup \(depth: 4–5 cm, diameter: 8–10 cm). **2|** Wipe up excessive Sca_l_eS4D25 solution around the sample with Kim Wipe. Leave the sample in the air for 10–15 min at room temperature to lightly dry the sample surface. **3|** Make a hole \(diameter: 5–6 mm) on a Parafilm sheet \(5–6 cm square). **4|** Put the Parafilm sheet over the sample while positioning the hole to the target area \(**Figure 6a, b**). **CAUTION** Much care should be taken not to damage the sample. **5|** Attach the hem of the Parafilm sheet and the bottom surface by glue \(Alon alpha A). **6|** Gently pour an appropriate volume \(1/4 vol of the cup capacity) of Sca_l_eS4D25 solution into the cup. **7|** Equilibrate the sample to Sca_l_eS4D25 by gentle shaking for 1 hr at room temperature. **8|** Substitute fresh Sca_l_eS4D25 or Sca_l_eS4D2.5 solution for observation. This sample system with Sca_l_eS4D25 can be stored at 4 ºC for at least 2 months. **9|** Immerse a dipping objective lens in Sca_l_eS4D25 or Sca_l_eS4D2.5 and make it approach to the sample slowly. **CAUTION** Before objective immersion, check by eyes that there are no air bubbles on the sample surface. Bubbles can be eliminated by gently scraping the surface with the bubble-remover \(**Figure 1c, 6c**). After objective immersion, check tiny air bubbles on the sample surface through microscopy and remove them by the bubble-remover. On the other hand, air bubbles trapped on the tip of the objective should be removed by the bubble-flusher \(**Figure 5b**). **CAUTION** CLSM observation for a long time \(> 12 hrs) results in water evaporation of the mounting medium. This is prevented by covering the surface of the medium with a thin plastic film made of saran \(trade name Saran Wrap).

Anticipated Results

ANTICIPATED RESULTS To provide a proof of concept of the methods described above, we performed CLSM observation using the brain hemispheres of YFP-H mice \(27 weeks old) that had been cleared by the Sca_l_eS technique \(**Figure 7a**), equilibrated with Sca_l_eS4D25, and immobilized by the Parafilm method. The observation employed an Olympus CLSM system \(FV1200) and long working distance \(WD) objectives to obtain the 3D reconstructions of YFP-expressing neurons that extended from the cerebral surface to the thalamus. To show the effects of refractive index \(RI) matching, we used two 25× dipping objectives that cover different RI ranges as follows. XLSLPLN25XGMP \(Olympus, RI = 1.41–1.52, NA = 1.0, WD = 8 mm) XLSLPLN25XSVMP \(Olympus, RI = 1.33–1.40, NA = 0.95, WD = 8 mm). These two objectives were basically designed for a multi-photon excitation microscopy system \(Olympus FV1000MPE); their imaging characteristics are guaranteed in the infra-red range. However, we have found that they both can be used satisfactorily with the CLSM system. In the experiment of **Figure

7b**, we cleared a hemisphere \(left half) of a YFP-H mouse \(27 weeks old) by Sca_l_eS technique. After incubation in Sca_l_eS4D25 for 24 hrs, the transparent hemisphere was immobilized on the cup using the Parafilm method. YFP-expressing neurons were imaged using FV1200 with the 25× objective \(XLSLPLN25XGMP). This objective supports for relatively high RIs \(1.41–1.52) and is therefore applicable for Sca_l_eS4D25 \(RI = 1.47). The correction collar was adjusted so that the images at a depth of 4 mm \(dendate gyrus) became clear, and a clear 3D reconstruction \(a long quadratic prism) was generated. Then, the other 25× objective \(XLSLPLN25XSVMP) was substituted. This objective supports for low RIs \(1.33–1.40) and should have RI mismatching with Sca_l_eS4D25. After a variety of adjustments by the correction collar, in fact, the best 3D reconstruction was not acceptable. Next, the mounting medium \(Sca_l_eS4D25) was replaced with Sca_l_eA2 solution. The Parafilm immobilization permits exchange of mounting media multiple times. When the sequential observations using the two objectives were performed, successful 3D reconstruction was generated with only XLSLPLN25XSVMP, which again verified the effect of RI matching. A similar comparative experiment was performed using a hemisphere \(left half) of another YFP-H mouse \(27 weeks old) for Sca_l_eS4D25 and Sca_l_eU2 \(**Figure 7c**). As was anticipated, when equilibrated with Sca_l_eS4D25, XLSLPLN25XGMP gave better signal-to-noise ratio than XLSLPLN25XSVMP. On the other hand, after equilibration with Sca_l_eU2, XLSLPLN25XSVMP provided better 3D reconstruction than XLSLPLN25XGMP.

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Acknowledgements

ACKNOWLEDGEMENTS The authors thank H. Sakurai for general assistance, RIKEN BSI-Olympus Collaboration Center for technical support, R. Takahashi for technical assistance of animal care. This work was supported in part by grants from the Japan Ministry of Education, Culture, Sports, Science and Technology Grant-in-Aid for Scientific Research on Priority Areas and the Human Frontier Science Program, and the Brain Mapping by Integrated Neurotechnologies for Disease Studies \(Brain/MINDS) from Japan Agency for Medical Research and development, AMED.

Figures

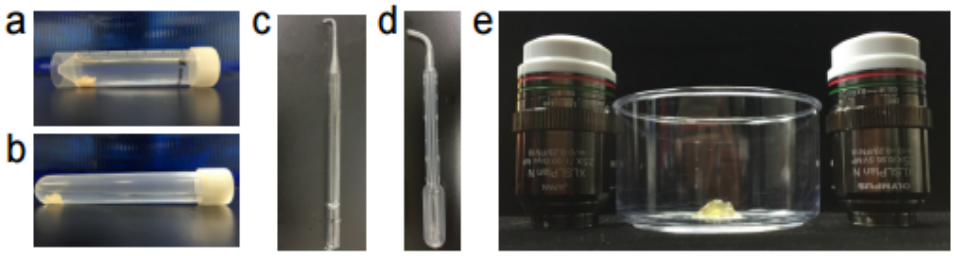


Figure 1

Figure 1

Materials used in the Sca_l_eS protocol. (a) A 30 ml centrifuge tube (SARSTEDT, # 62.543). (b) A 13 ml centrifuge tube (SARSTEDT, # 60.541.545). (c) A bubble-remover. (d) A bubble-flusher. (e) Two long WD objectives and a plastic cup with a transparent mouse hemisphere. _left_, 25× (Olympus, XLSLPLN25XGMP, RI = 1.41–1.52, NA = 1.0, WD = 8 mm). _right_, 25× (Olympus, XLSLPLN25XSVMP, RI = 1.33–1.40, NA = 0.95, WD = 8 mm).

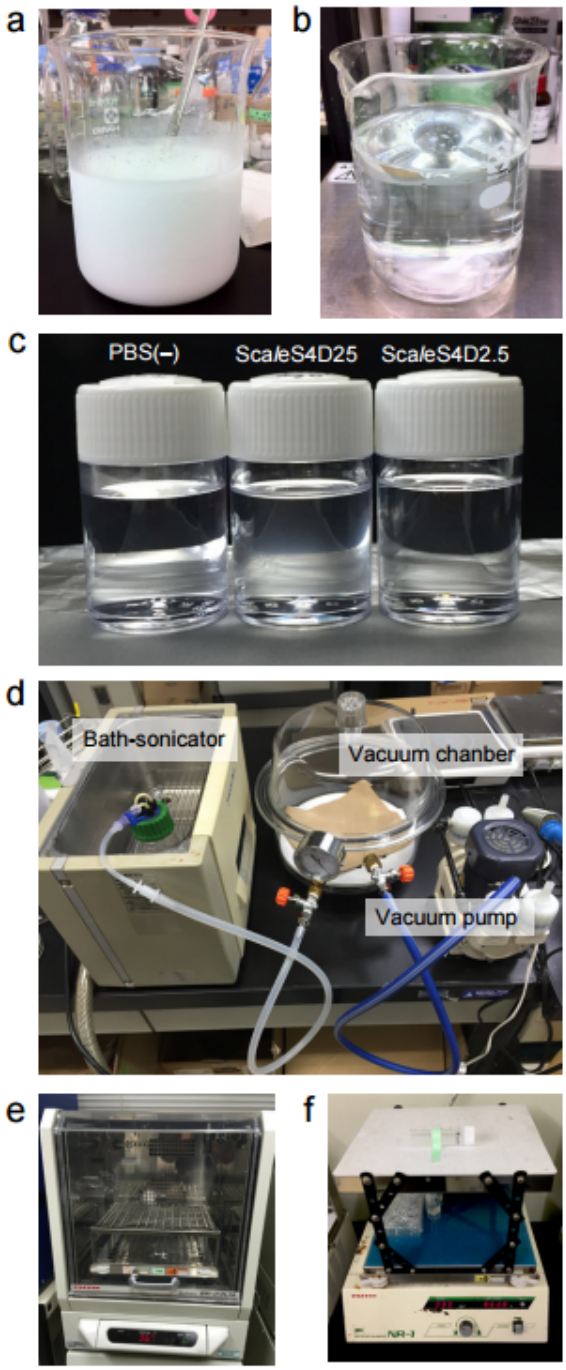


Figure 2

Figure 2

Preparation of Sca_l_eS solutions. (a) A large spatula necessary for hydrating urea crystals and sorbitol powder. (b) The solution shown in (a) becomes transparent after heating. (c) Misty appearance of Sca_l_eS4D25 (middle) compared with PBS(−) (left) and Sca_l_eS4D2.5 (right). (d) A degassing system composed of a vacuum pump and a bath-sonicator. (e) A constant-temperature incubator with a shaker for incubation with Sca_l_eS solutions. (f) An orbital shaker.

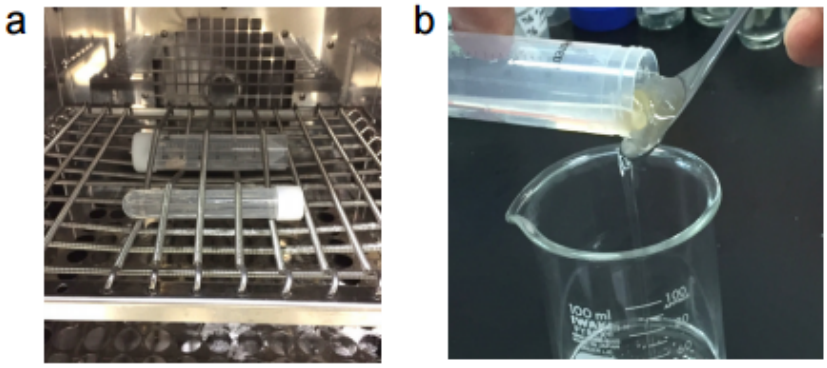


Figure 3

Figure 3

Handling of mouse brain tissues in Sca_l_eS solutions. (a) Centrifuge tubes containing samples and Sca_l_eS solutions are placed horizontally on the shaker of the constant-temperature incubator. (b) A dispensing spoon useful for replacing solutions.

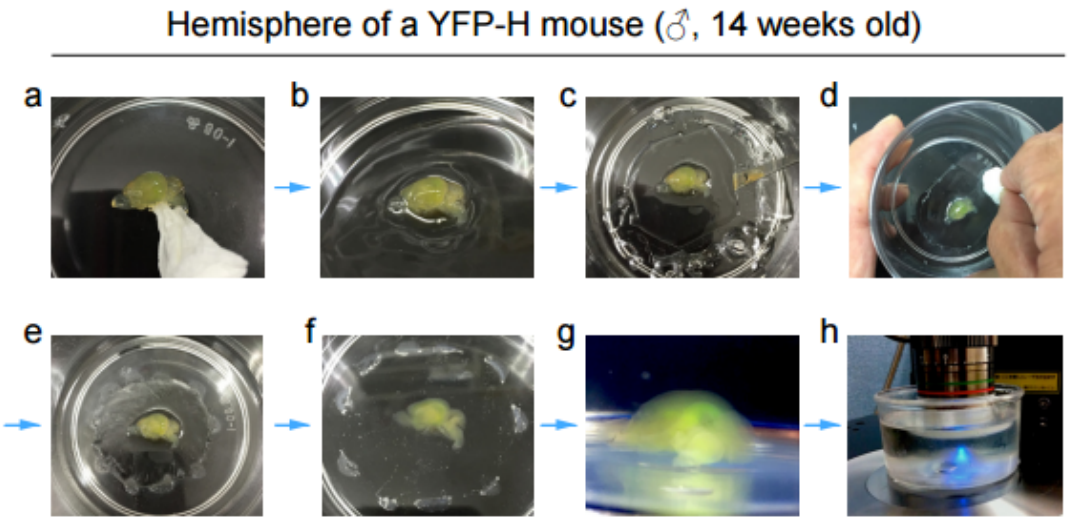


Figure 4

Figure 4

Agarose method for mounting a cleared hemisphere with a Sca_I_eS4 solution in the plastic cup. (a) Wiping up excessive Sca_I_eS4 solution. (b) Pouring the agarose solution. (c) Trimming away the edges of the agarose skirt. (d) Removing the agarose fragments or debris. (e) Gluing the rim of the agarose gel. (f) Pouring a Sca_I_eS4 solution into the cup.

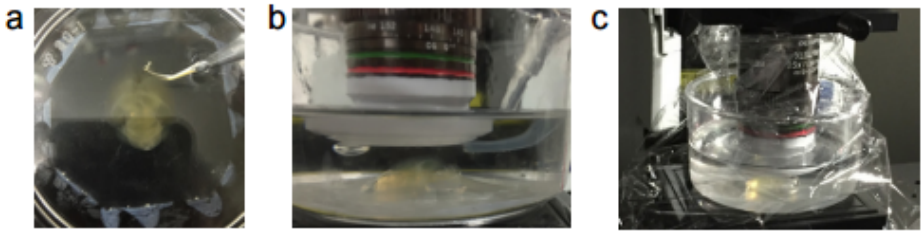


Figure 5

Figure 5

Preparations prior to observation. (a) Removing air bubbles before objective immersion. (b) Removing air bubbles beneath the lens tip. (c) Saran Wrap covering.

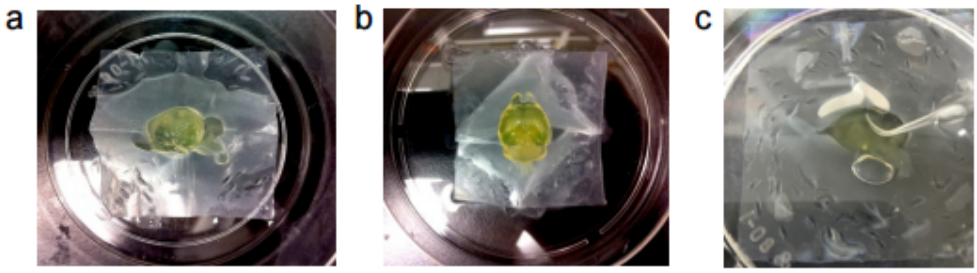


Figure 6

Figure 6

Parafilm method for mounting a cleared hemisphere with a Sca_l_eS4 solution in the plastic cup. (a) A left hemisphere with the Parafilm cover. (b) A whole brain with the Parafilm cover. (c) Removing air bubbles trapped under the cover.

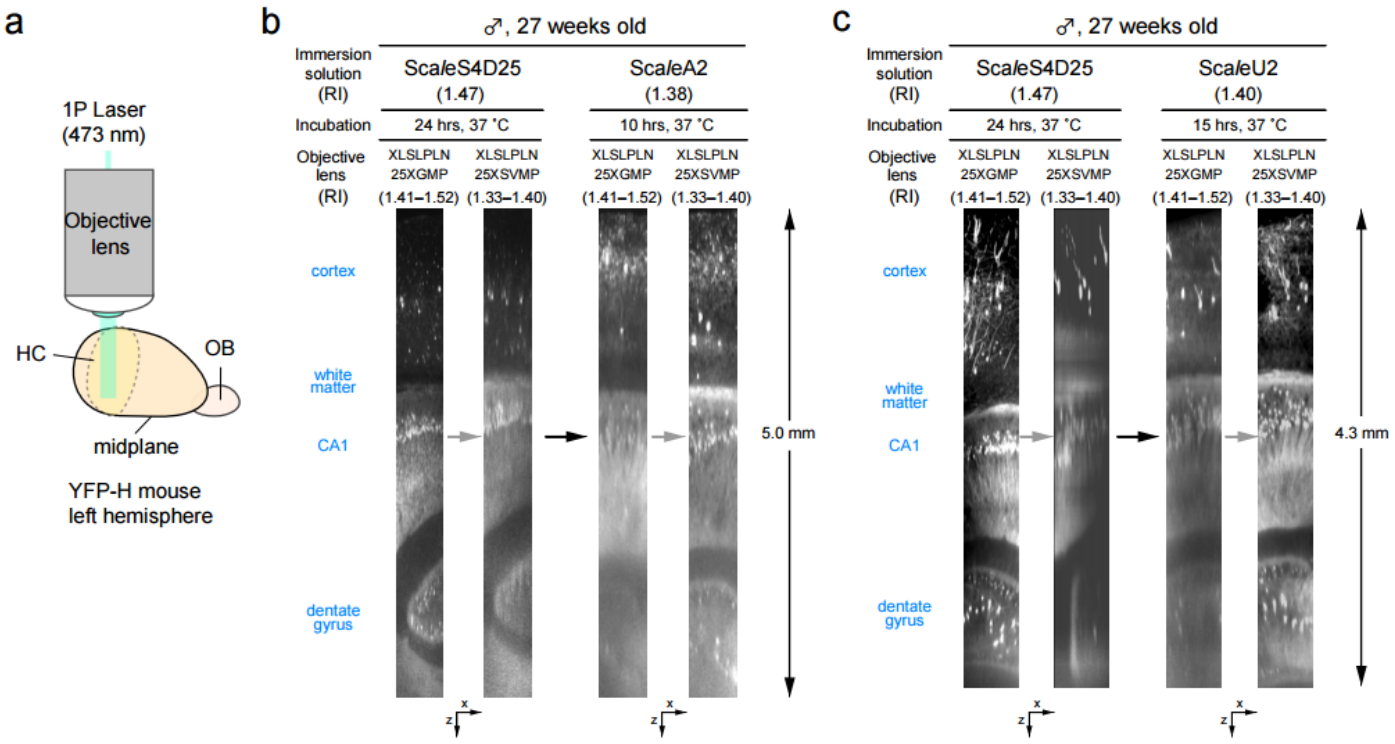


Figure 7

Figure 7

Refractive index matching for choosing right objectives. (a) Schematic diagram illustrating how YFP-expressing neurons in a cleared hemisphere from a 27-week-old YFP-H mouse were imaged by CLSM. Z-step, 7 μm. 1P: single photon, HC: hippocampus, OB: olfactory bulb. (b, c) The x-z planes of the reconstructed prisms are shown. Spherical aberration was compensated at the depth of 2.5 mm (CA1 region) for each observation. Gray arrows indicate the exchange of objective lenses. Black arrows indicate the exchange of the mounting solutions from Sca_l_eS4D25 to Sca_l_eA2 (b) or Sca_l_eU2 (c).

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