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bio-101 www.bio-protocol.org/e4123 Bio-101: e4123. DOI:10.21769/BioProtoc.4123

Clearing of the Mouse Brain for Optical Imaging Using CUBIC

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[Abstract] Here, we describe a simple and efficient tissue clearing method, CUBIC (Clear, Unobstructed Brain/Body Imaging Cocktails and Computational analysis), which was first introduced in 2014 (Susaki *et al*., 2014). In the present study, the hemispheres of wild-type adult mice were cleared by standard procedures and were browning free with good transparency and no significant changes in tissue shape or volume over a short time frame. Further, in combination with immunofluorescence staining, 3D high-resolution images of tyrosine hydroxylase-positive dopaminergic neurons in the substantina striatum region were obtained by rotating-disc confocal microscopy, in addition to images of mouse cortical and hippocampal neurons. Considering the limitations of existing brain tissue removal methods, such as CLARITY, 3DISCO, and SeeDB, CUBIC boasts simple operation, high fluorescent protein preservation, and good antibody penetration. Moreover, CUBIC variants and optimizations are available for the 3D imaging of rodent, large primate, and human tissues to meet researchers' specific requirements, which could make it a popular option among research labs. Our study demonstrates the use of the CUBIC protocol for understanding brain structure and function in model animals with neurological diseases.

Graphic abstract:

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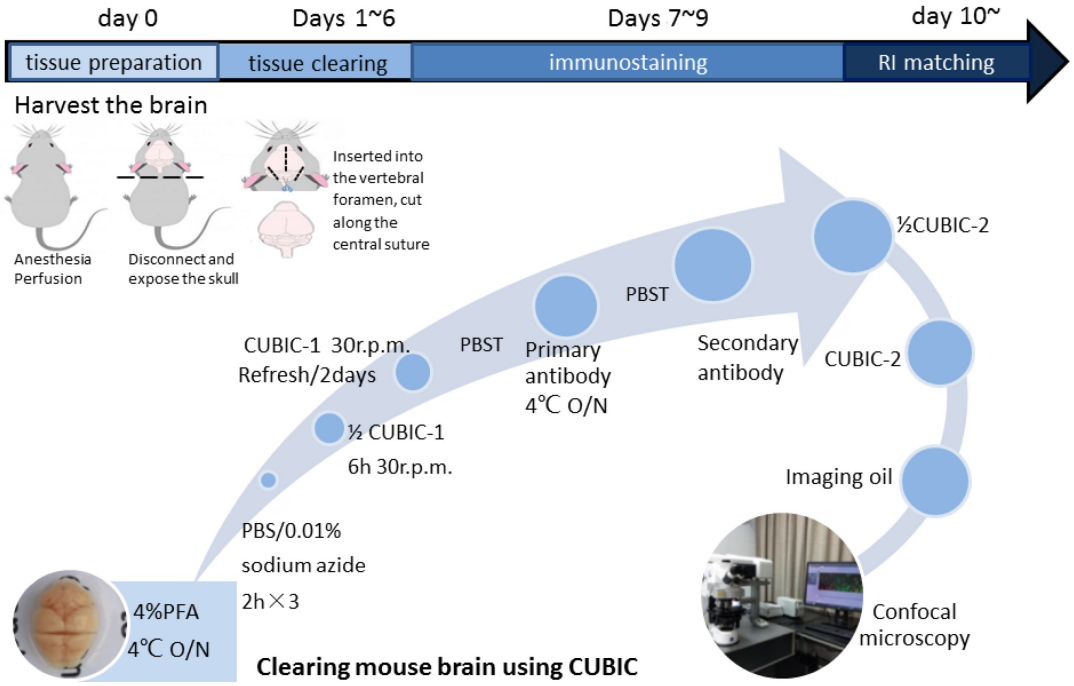

Flowchart of the CUBIC clearing and imaging protocol. A. Tissue preparation. The adult mouse was first perfused with cold PBS/PFA solution. The harvested brain tissue was fixed with 4% PFA on day 0. B. Tissue clearing. The tissue was incubated with 1/2-water-diluted CUBIC-1, resulting in the removal of hemochrome and lipid membranes. It usually takes 1-6 days to become more transparent. C. Immunostaining. Tissues (2-mm brain slice) were blocked and then stained with directly conjugated anti-TH diluent (1:100) and FITC diluent (1:500) on days 7-9. D. RI matching. Tissues were immersed in 1/2-PBS-diluted CUBIC-2 on day 10 to match the refractive index (RI). Following immersion in imaging oil for 2 h, the cellular position within tissues was photographed by confocal microscopy.

Keywords: Tissues Clearing, CUBIC, 3D imaging, Rotating-disc confocal imaging

[Background] Tissue optical clearing (TOC) enables the visualization of regions of interest in deep tissue and is now widely established. In recent years, a variety of methods have been developed based on organic solvents, water-based non-gels, and hydrogels (Ertürk *et al*., 2011; Chung and Deisseroth, 2013; Ke *et al*., 2013); however, both the CLARITY and 3DISCO protocols are associated with significant drawbacks: 3DISCO loses endogenous protein fluorescence and involves toxic and flammable substances (Matsumoto *et al*., 2019), while CLARITY relies on dedicated equipment for electrophoresis. In addition, acrylamide has carcinogenicity and toxicity in its aqueous form (Hogervorst *et al*., 2007). Compared with these other brain-clearing techniques, CUBIC is safe, simple to operate, and efficiently achieves transparency. This method clears fixed brain samples in a few days to render the brain transparent, thus meeting the quality requirements of optical imaging of the sample. Using the standard CUBIC tissue clearing procedure, we subject mouse brain samples to transcardial perfusion, clearing,

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immersion in antibody solution, and refractive index matching. The aminoalcohol used can effectively remove hemochrome from hemoglobin and myoglobin, which not only reduces light scattering but also lessens the absorption of light to achieve further decolorization and transparency. When combined with rotating-disc confocal microscopy, CUBIC facilitates comprehensive and quantitative analyses for understanding the brain structure on the millimeter-scale level. It boasts a simple clearance procedure (Muntifering *et al.*, 2018), excellent clearance effect in a short time frame (approximately 11 days) (Wan *et al.*, 2018), and outstanding fluorescence preservation (Tainaka *et al.*, 2018), making it a popular option among research labs.

After performing the CUBIC protocol, the tissues are optically transparent (Richardson and Lichtman, 2015), which meets the quality requirements for the optical imaging of samples. In addition, it is compatible with traditional immunofluorescence methods (Xu *et al.*, 2019) and can be used by researchers to explore the effects of immunolabeling. Combined with confocal, two-photon microscopy or light-sheet microscopy, CUBIC can easily obtain high-resolution (millimeter level) 3D brain images (Isogai *et al.*, 2017; Irie *et al.*, 2018) to provide information about the type, position, and activities of cells and cellular networks (Tomer *et al.*, 2014), promoting research on the relationship between brain structure and function. CUBIC provides an integrative platform to advance scalable and collaborative 3D image analysis (Matsumoto *et al.*, 2019) that enables a wide range of users to perform experiments targeting cellular and organ layers with multiple samples (Mano *et al.*, 2020). This technology can solve developmental and fundamental neuroscience problems (Mano *et al.*, 2018) and answer scientific questions associated with Alzheimer's disease (AD) (Liebmann *et al.*, 2016) and other neurodegenerative diseases (Ueda *et al.*, 2020).

Materials and Reagents

1. Blades (LEICA, catalog number: 819)
2. Microcentrifuge tubes, 2 ml (Biosharp, catalog number: 1810411)
3. Eppendorf tubes, 10 ml (Biosharp, catalog number: BS-100-M)
4. Intravenous (i.v.) injection needle, 21-G, butterfly type (GeneRulor, catalog number: SH12154)
5. Disposable syringes, 1 ml and 20 ml (NCS Testing Technology, catalog numbers: 20190629, 20191029)
6. Tinfoil (Heavy Duty, catalog number: 2345345)
7. 24-well plates (Corning, catalog number: 3527)
8. Animals

   Wild-type C57BL/6 (6-8 weeks of age) mice kept on a standard 12 h light/dark cycle; food and water were provided *ad libitum*.

   Note: All experimental procedures and housing conditions were approved by the Animal Care and Use Committee of Guangdong Medical University.
9. Urea (Sangon Biotech, catalog number: A610148-0500)
10. Sucrose (Sangon Biotech, catalog number: A610498-0500)

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11. N,N,N',N',-Tetrakis (2-hydroxypropyl) ethylenediamine (TCI, catalog number: T0781)
12. Triethanolamine (Sangon Biotech, catalog number: A690031)
13. Triton X-100 (Beyotime, catalog number: ST795)
14. PFA (MACKLIN, catalog number: 30525-89-4)
15. Tween 20 (Sigma-Aldrich, catalog number: P1379)
16. Sodium azide (Sigma-Aldrich, catalog number: ZY26628)
17. Heparin sodium (Tocris, catalog number: 2812/100)
18. HCl (Xilong Scientific, catalog number: 7647-01-0)
19. NaOH (GHTECH, catalog number: 1310-73-2)
20. Sodium azide (Nacalai Tesque, catalog number: 31208-82)
21. Anti-tyrosine hydroxylase antibody (Abcam, catalog number: ab112)
22. Goat anti-rabbit IgG H&L (FITC antibody) (Abcam, catalog number: ab6717)
23. Mineral oil (RI = 1.467; Sigma-Aldrich, catalog number: M8410)
24. Goat serum (Sigma-Aldrich, catalog number: G6767)
25. Phosphate-buffered saline (PBS) (1 M, pH 7.4) containing 0.1% Triton X-100 (PBST) (see Recipes)
26. 4% (w/v) paraformaldehyde (PFA) solution (see Recipes)
27. Clearing solution (see Recipes)
28. CUBIC tissue removal reagent (see Recipes)

Equipment

1. Dissection tools required for standard mouse perfusion, *e.g.*,
   a. Ophthalmic surgical scissors, 10 cm (straight) (Guangzhou Life Technology Co., catalog number: LG01-107-4H)
   b. Standard tweezers (Guangzhou Life Technology Co., catalog number: LG01-105-3)
2. pH meter (Mettler Toledo, catalog number: FE28)
3. Magnetic stirrer (Tuhua, catalog number: 79-1)
4. Rocking bed hammock (Qilinbeier, catalog number: TS-3D)
5. 2-8°C refrigerator (Haier, catalog number: SC-412)
6. Rodent Brain Matrices (TOW-INT TECH, catalog number: ST-2275)
7. Round-edged spoon (Guangzhou Life Technology Co., catalog number: LG01-111-2H)
8. Confocal microscope (Olympus, catalog number: FLUOVIEW FV3000)
   Note: In this study, we used the FLUOVIEW FV3000 from Olympus for imaging objective lenses:
   4× (NA = 0.16, WD = 13 mm)
   10× (NA = 0.4, WD = 3.1 mm)
   20× (NA = 0.7, WD = 1.8 mm)

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Software

The following software is used as part of this bio-indentation protocol and data analysis process:

1. Microsoft Excel (Office 2016)
2. FIJI ImageJ (https://imagej.net/Fiji) (Version, 64-bit)

Procedure

A. Preparation of the solutions
   1. Prepare phosphate-buffered saline (PBS) solution (1 M, pH 7.4) containing 0.1% Triton X-100 (PBST) (see Recipe 1).
   2. Prepare 4% (w/v) paraformaldehyde (PFA) solution (see Recipe 2).
   3. Prepare the clearing solution (see Recipe 3).

B. Harvesting tissues
   1. Deeply anesthetize an adult male wild-type C57BL/6 mouse by intraperitoneal injection of pentobarbital (0.35 mg/kg) solution.
      Note: Once the mouse shows no toe-pinch response, the mouse is properly anesthetized.
   2. Use pins to fix the stretched out mouse limbs to a foam board.
   3. Expose the heart by an incision on the xiphoid process through the chest, skin, and bones using scissors.
   4. Cut off the mouse's right auricle and perfuse the left ventricle with 20 ml precooled 1× PBS (pH 7.4) at 10 ml/min. Subsequently, perfuse with 20 ml 4% PFA.
      Note: Wait until the mouse is observed to have a white liver and clear, transparent fluid from the right auricle.
   5. Cut off the mouse’s head, cut away the skin, and carefully break the skull between the eyes using scissors and tweezers so as not to damage the sample. Remove pieces of the skull using small, curved forceps. Take out the brain and other desired organs.
   6. Immerse the brain tissue in 10 ml 4% PFA. Post-fix overnight with shaking ~30 rpm at 4°C.
      Caution: PFA solution is toxic. The transcardial perfusion and all subsequent mouse handling must be conducted in a fume hood and with personal protective equipment.
   7. Wash the brain tissue 3 times (each 2 h) with 10 ml PBS/0.01% sodium azide at room temperature.

C. Clearing on days 1-6
   1. Immerse the sample in 6 ml 1/2-water-diluted CUBIC-1 for 6 h with shaking ~30 rpm at room temperature.
   2. Incubate in 6 ml CUBIC-1 until clear and transparent; change the solution every two days. Depending on the degree of transparency, the organization clearance time should be adjusted

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appropriately. In this experiment, 2 days was required for 2-mm brain slices, and 4-6 days was required for hemispheres.

3. Wash the sample once with 10 ml PBS/0.01% sodium azide for about 2 h at room temperature before immunolabeling.
   Note: Immerse the tissue sample in 3 volumes of reagent. The shaker speed for 2-mm brain slices should be slow (recommended 30 rpm) and for half or whole brain should be about 60 rpm.

D. Immunostaining on days 7-9
   1. Wash the brain tissue twice in PBST at room temperature with shaking.
   2. Incubate the tissue with primary antibodies in PBST at 4°C with shaking overnight. In the work presented here, the primary antibodies are against rabbit anti-tyrosine hydroxylase diluted 1:100 in PBST.
   3. Wash the sample 3 times with PBST (each 2 h).
   4. Incubate the brain with the desired secondary antibodies in PBST for 6 h at room temperature on a shaker, protected from light. In the work presented here, the secondary antibodies are FITC-labeled anti-rabbit, diluted 1:500 in PBST.
   5. Wash the sample 3 times with PBST (each 2 h).

E. RI matching on days 10-11
   1. Immerse the sample in 6 ml 1/2-PBS-diluted CUBIC-2 for 6 h with shaking ~30 rpm at room temperature.
   2. Place the sample in 6 ml CUBIC-2 with gentle shaking at room temperature overnight.
   3. Place the tissue on a paper with gridlines to check its visual clarity by assessing whether the gridlines can be seen. Once the tissue has been fully cleared, it can be imaged.

F. Confocal microscopy imaging
   Place the labeled tissue on a 24-well plate and add mineral oil until it is covered. The tissue is carefully secured to prevent floating and displacement. Use a confocal microscope to image the tissue under different objective lenses (4×, 10×, 20×).

Data analysis

CUBIC is a widely applicable, user-safe, and sample-friendly tissue cleaning procedure. Molecular and structural studies of deep tissues are obtained by imaging immunofluorescently labeled tissue samples. To achieve the best imaging quality, careful preparation of the sample and the transparency step is crucial. Building on past research, we demonstrate the suitability of CUBIC for rapid, high-resolution structural studies of rodent (Figure 1E-1H) tissue samples. We measured the refractive indices of the permeabilized solutions, CUBIC-1 (RI: 1.4468) and CUBIC-2 (RI: 1.4787),

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which are within the reference refractive index range of the CUBIC reagents (1.38-1.48) (Tainaka *et al*., 2018), using an Abbe refractometer. Compared with uncleared tissue (Figure 1A-1D), delipidized and RI-matched tissues have enhanced optical transparency, allow immunofluorescent labeling of proteins at the molecular level, and yield high-resolution images (Figure 2A-2D).

In summary, we validated the ability of CUBIC to prepare brain tissue samples for imaging via rotating-disc confocal microscopy. According to the actual photographs of our transparent samples, this solution is more suitable for objective lenses with a numerical aperture of about 0.4, but it does not rule out the use of other commercial high numerical aperture microscope objectives since the larger the numerical aperture, the higher the image resolution. Using this method, researchers may tackle the broad spectrum of neuroscientific demands from the meticulous analysis of single-cell morphology to the comprehensive study of the whole organ.

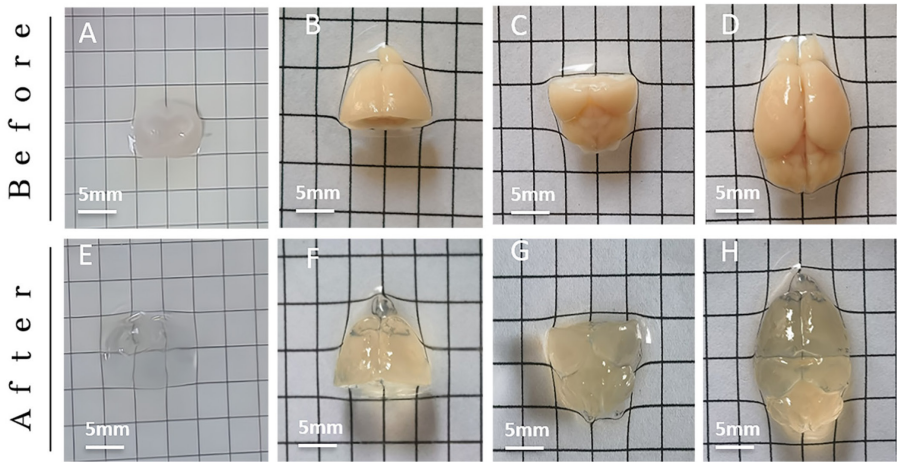

Figure 1. Transparency can be evaluated by eye under the light source. We successfully cleared the thick brain block and the mouse hemisphere using the CUBIC protocol. The brain of C57BL/6 male mice (8 weeks of age) was used. (A, E) Images show 2 mm-thick coronal sections of mouse brain; note the black lines in the cleared section (E) that were once poorly resolved in the uncleared section (A). The uncleared anterior half (B) and posterior half of the brain (C) respond to the cleared F and G, respectively. Compared with the uncleared hemisphere (B, C), the cleared hemisphere (F, G) was remarkably transparent and highly uniform, with a slightly elevated volume. (D, H) Image shows no significant change in the size or shape of the glassy hemisphere (H) as compared with the uncleared splicing hemisphere (D). Scale bar, 5 mm (A-H). At least three different samples were examined for CUBIC.

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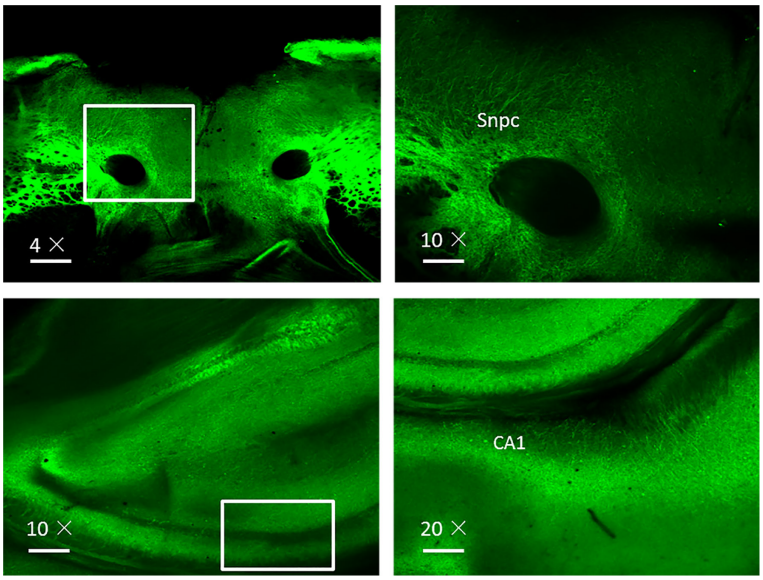

Figure 2. Fluorescence imaging of adult mouse brain slices. (A) A 2 mm-thick brain section from a C57BL/6 mouse (8-week-old, male) was stained with a fluorescein isothiocyanate (FITC)-conjugated anti-tyrosine hydroxylase antibody. The white inset at the right midbrain nigra pars compacta (SNpc) is magnified next to the image (B), which can be detected in the distribution area of DA neurons. Confocal images of the hippocampal region (C) were acquired using a 10× objective (Olympus, 10×, NA = 0.40, working distance = 3.1 mm). (D) An enlargement of the box area in (C), showing the detailed anatomy of CA1 neurons in the hippocampal region of mice.

Recipes

1. Phosphate-buffered saline (PBS) solution (1 M, pH 7.4) containing 0.1% Triton X-100 (PBST)
   Prepare 1 L 10× PBS stock solution by mixing:
   80 g NaCl
   2 g KCl
   36.3 g $Na_2HPO_4 \cdot 12H_2O$
   2.4 g $KH_2PO_4 \cdot 12H_2O$
   Add dH_2O to 1 L and stir for about 2 h
   Adjust to pH 7.4 with NaOH and store at room temperature
   Dilute this 10× stock solution 1:10 using dH_2O and then add 0.1% Triton X-100. This solution can be stored at room temperature (18-25°C) for several months.
2. Prepare 4% (w/v) paraformaldehyde (PFA) solution:
   a. Dissolve 8 g PFA in 200 ml (total) 1× PBS.
   b. Add a few NaOH pellets and heat the solution to 50-60°C to facilitate dissolution.
   c. Readjust the pH to 7.4 using HCl.
   d. PFA for use as a fixative can be stored at 4°C for up to 1-2 months.

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Note: PFA is a very toxic reagent. Avoid inhalation or contact with skin and eyes.

3. Clearing solution

   CUBIC-1 is a mixture of urea (25 wt% final concentration), Quadrol (25 wt% final concentration), Triton X-100 (15 wt% final concentration), and dH_2O.

   Prepare 100 g CUBIC-1 solution:

   a. Dissolve 25 g urea and 24.96 g N,N,N',N'-Tetrakis (2-hydroxypropyl) ethylenediamine in 28.8 g dH_2O using a magnetic stirrer and heat (not exceeding 75°C).
   b. After complete dissolution, keep stirring the mixture at room temperature and add 15 g Triton X-100.
   c. CUBIC-1 can be stored at room temperature for up to 1 month.

   CUBIC-2 is a mixture of urea (25 wt% final concentration), sucrose (50 wt% final concentration), 2,2',2''-nitrilotriethanol (10 wt% final concentration), and dH_2O.

   Prepare 100 g CUBIC-2 solution:

   a. Dissolve 25 g urea and 50 g sucrose in 15 g dH_2O using a magnetic stirrer and heat (not exceeding 75°C).
   b. After complete dissolution (typically 10-15 min), cool the mixture at room temperature, add 10 g 2,2',2''-nitrilotriethanol, and stir further.
   c. CUBIC-2 can be stored at room temperature for up to 2 weeks.

4. CUBIC tissue removal reagents (Table 1).

Table 1. The proportions of various components of the CUBIC tissue removal reagents. Accordingly, the reagent dosage can be modified according to your needs.

Solution	Chemical	Recipe (wt%)
CUBIC-1	Urea	25
	N,N,N′,N′-Tetrakis (2-hydroxypropyl) ethylenediamine	25
	Triton X-100	15
	dH_2O	35
CUBIC-2	Urea	25
	Sucrose	50
	2,2′,2″-Nitrilotriethanol	10
	dH_2O	15
Imaging Oil	TSF 4300	50
	Mineral oil	50

Notes

A. Poor PFA perfusion

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1. Possible reason
   a. Insufficient cooling of PBS and PFA.
   b. Wrong position of the tip of the needle.
   c. Insufficient pressure for perfusion.
2. Solution
   a. Keep PBS and PFA on ice immediately before perfusion.
   b. Make sure that the tip of the needle is in the left ventricle of the heart.
   c. Make sure that the perfusion outlet only occurs at the cut in the liver.

B. Poor organ clearing during CUBIC-1 treatment
1. Possible reason
   a. Insufficient incubation in CUBIC-1.
   b. Alkaline pH of PFA.
   c. Too long a fixation time.
2. Solution
   a. Increase delipidation time, exchange CUBIC-1 one or two times.
   b. Adjust the pH of PFA to ~7.4.
   c. Stop post-fixation within 24 h. Over-fixation causes both lower clearing efficiency and autofluorescence.

C. Poor organ clearing during CUBIC-2 steps
1. Possible reason
   a. Insufficient incubation in CUBIC-1.
   b. Insufficient incubation in CUBIC-2.
   c. Incomplete dissolution or precipitate in CUBIC-2.
2. Solution
   a. Increase CUBIC-1 treatment time.
   b. Use more CUBIC-2 and exchange it one or two times.
   c. Make sure that no precipitate exists in the prepared/stocked CUBIC-2.

D. Poor transparency
1. Possible reason
   Inefficient penetration of the solution into the tissues.
2. Solution
   a. Trim away unnecessary portions of your sample to increase penetration.
   b. Shaking of samples, step-wise soaking, timing, and temperature are all important for successful optical clearing.

E. Weak or undetectable fluorescence

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1. Possible reason
   a. Temperature during clearing.
   b. Inappropriate microscopy settings.
   c. Insufficient fluorescence signal.
   d. Poor antibody permeability.
2. Solution
   a. Higher temperatures during clearing may decrease fluorescence signals. Try to incubate at room temperature rather than at 37°C.
   b. Check the laser power, filter, and exposure time settings.
   c. Select a bright fluorescent protein with a strong promoter, such as small-molecule nanobodies or fluorescent molecular probes. The fluorescent secondary antibody incubation and all subsequent steps should be protected from light.
   d. Increase the concentration of the antibody and extend the incubation time.

Acknowledgments

This project was supported by the PhD Start-up Fund of Guangdong Medical University (B2019016); the Administration of Traditional Chinese Medicine of Guangdong Province (20201180); the Administration of Traditional Chinese Medicine of Guangdong Province (20211223); the Science and Technology Special Project of Zhanjiang (2019A01009); the Basic and Applied Basic Research Program of Guangdong Province (2019A1515110201); the Key Program of Marine Economy Development (Six Marine Industries) Special Foundation of the Department of Natural Resources of Guangdong Province (GDNRC[2020]038); and the Fund of Southern Marine Science and Engineering Guangdong Laboratory (Zhanjiang) (ZJW-2019-007).

Competing interests

There are no conflicts of interest or competing interests.

Ethics

Animals received care in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, and the experimental procedures were approved by the Animal Care and Use Committee (Guangdong Medical University).

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