Chapter 1

Tissue Clearing and 3-D Visualization of Vasculature with the PEGASOS Method

Dian Jing, Yi Men, and Hu Zhao

Abstract

Tissue clearing techniques turn tissue transparent through a series of chemical and physical treatments. They have provided a useful tool for three-dimensional (3-D) imaging to study tissue spatial organization and interactions. Many tissue clearing methods have been developed in recent years. Each method has its own application range depending on the purposes of the study. Three criteria for selecting an appropriate clearing method include clearing transparency, fluorescence preservation, and broad tissue applicability. PEG-associated solvent system (PEGASOS) emerged recently as a solvent-based tissue clearing method capable of rendering diverse tissues highly transparent while preserving fluorescence. Combined with vascular labeling techniques, PEGASOS method enables 3-D visualization of vasculature in whole tissues at subcellular resolution. Here, we describe the standard PEGASOS passive immersion protocol and several compatible vascular labeling techniques. Methods of 3-D imaging, data processing, and annotations are also briefly introduced.

Key words Tissue clearing, 3-D imaging, Vasculature, PEGASOS

1 Introduction

Tissue opacity is mainly caused by light scattering and light absorption. The heterogeneous optical properties of different tissue components lead to refractive index (RI) mismatches, which in turn cause light scattering [1]. Moreover, endogenous pigments including heme, melanin, lipofuscin, and cytochrome strongly absorb light [2]. The calcified minerals in hard tissues also block the light transmission. In order to achieve transparency, both chemical and physical approaches are, therefore, applied in various tissue clearing methods to eliminate light scattering components and to achieve uniform internal RI, known as RI matching. Achieving of RI matching enables images being acquired even at depth of several millimeters into tissues.

Xu Peng and Warren E. Zimmer (eds.), *Cardiovascular Development: Methods and Protocols*, Methods in Molecular Biology, vol. 2319, https://doi.org/10.1007/978-1-0716-1480-8_1,

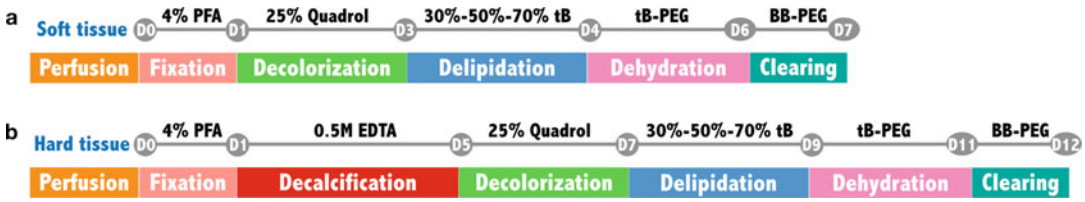

Fig. 1 PEGASOS passive immersion procedure for both soft and hard tissues. (**a**) Treatment steps and timetables of PEGASOS passive immersion procedure for clearing soft tissues. (**b**) For hard tissues, perform decalcification between fixation and decolorization

Over the last decade, numerous tissue clearing techniques have been developed, and they all followed above principles to turn tissue transparent. These clearing methods can be classified into three major categories: (1) organic solvent-based tissue clearing techniques, including the DISCO series [3–5], fluoclear BABB [6], and polyethylene glycol (PEG)-associated solvent system (PEGASOS) [7]; (2) Aqueous reagent-based tissue clearing techniques, such as SeeDB2 [8], the CUBIC series [9, 10], and Ce3D [11]; (3) hydrogel-based TC techniques, including CLARITY [12], PACT [13], and various modified protocols [14, 15].

Here, we introduce the PEGASOS tissue clearing method developed by our lab, which is a solvent-based clearing method capable of turning both soft and hard tissues highly transparent with excellent fluorescence preservation [7]. The standard PEGASOS workflow includes the following steps: perfusion, decalcification, decolorization, delipidation, dehydration, and clearing. Performing whole-body PEGASOS via recirculation system renders the whole mouse transparent, whereas the PEGASOS passive immersion procedure is suitable for individual organs (Fig. 1). We will describe the passive immersion protocol in the following section.

Vascular system forms complicated spatial pattern within organs. 3-D imaging based on tissue clearing provides a powerful research tool. Many methods have been used to label the vasculature. Transgenic mouse models are the most popular and recommended method for vasculature labeling. With proper transgenic mouse strain, vasculature components can be efficiently labeled with GFP or other fluorescent proteins [7, 16–18]. Dye injection and dye perfusion are conventional labeling approaches. Commonly used dyes include GS-IB_4, dextran sulfate, and DiI [19–21]. However, not all the dyes are compatible with every clearing method. PEGASOS method is compatible with GS-IB_4 and dextran sulfate but not DiI. Solvent components including tert-butanol and benzyl benzoate (BB) dissolve DiI. In addition, the vasculature labeling efficiency of dye is usually lower than using transgenic model and can vary significantly among different animals. Traditional immunostaining with antibodies is also a valuable

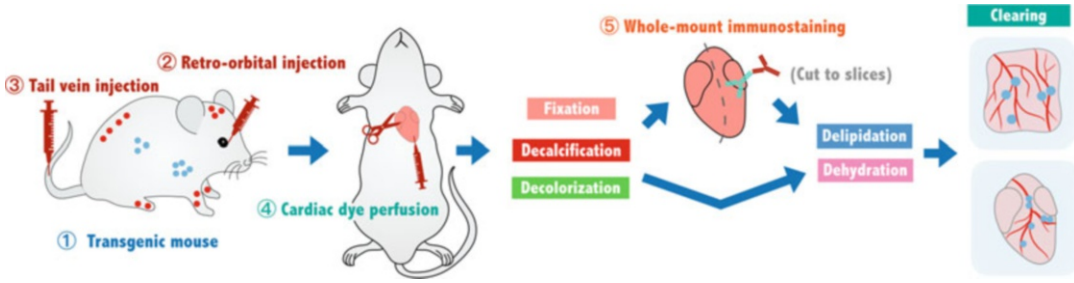

Fig. 2 Combination of vascular labeling with the clearing process. Overview of the PEGASOS tissue clearing method and compatible vascular labeling methods. Transgenic mice with endogenous vascular fluorescence are most commonly used with this tissue clearing method (①). Retro-orbital injection and tail vein injection provide effective means of labeling the vasculature (②, ③) before sacrifice. In transcardial-circulatory perfusion, dye can be also perfused via the vascular system (④). For dissected thick tissue slices, whole-mount immunostaining is another labeling approach that can be applied before the delipidation process (⑤)

tool for labeling vasculature. Penetration of antibodies into deep regions is the major challenge. Antibody incubation conditions should be optimized to improve staining efficiency [7, 16, 19]. In our experience, whole-mount immunostaining with regular antibodies should be applied for soft tissue slices of no more than 500 μm thickness. Our efforts of whole-mount immunohistochemical staining for hard tissue organs or thick slices were never successful. Combined with these vascular labeling techniques, we demonstrated PEGASOS method is able to visualize vasculature within different organs and tissues in 3-D [7, 22, 23] (Fig. 2).

Here, we describe the protocol of the PEGASOS passive immersion method in detail. Four compatible vessel labeling methods, including transgenic mouse models, retro-orbital dye injection, postmortem dye perfusion, and whole-mount immunostaining, are introduced. Technical details for confocal imaging and data processing are briefly introduced.

2 Materials

2.1 Animals

1. Transgenic mice of 4–8-week-old with endogenous vascular labeling. Commonly used mouse strains for labeling vasculatures: Cre strains for labeling endothelium include *Tie2-Cre* (JAX #008863) and *Cdh5-Cre*^{ERT2} [23]. *aSMA-CreERT* [7] mouse can be used for labeling smooth muscle tissue to distinguish arteries from veins and capillaries. *NG2-DsRed* (JAX #008241), *NG2-CreER*™ (JAX #008538), or *Leptin-Cre* (JAX #008320) mouse strains can be used for labeling pericytes. Commonly used reporter strains include *tdTomato* (*Ai14*) (JAX #007908), *tdTomato* (*Ai9*) (JAX #007909), *EYFP* (*Ai3*) (JAX #007903), and *ZsGreen* (*Ai6*) (JAX #007906). To generate *Tie2-Cre, Ai14* mice, male *Tie2-Cre*

mice were bred with female *Ai14* mice. PEGASOS method is compatible with fluorescent proteins used in above reporter mouse strains.

2. Dye labeling or immunohistochemical staining strategy is applicable to mice of any genotype.
3. All animal experiments should be approved by the relevant institutional animal care and performed in accordance with guidelines from the NIH or the corresponding national entity governing such procedures.

2.2 Anesthesia System

1. Isoflurane.
2. Oxygen cylinders.
3. Ketamine (80 mg/kg body weight).
4. Xylazine (10 mg/kg body weight).

2.3 Retro-Orbital Injection

1. Dyes for vascular labeling:
   (a) Fluorescein isothiocyanate–dextran (2000 kDa, Sigma-Aldrich FD2000S). Dilute FITC-Dextran in PBS with a concentration of 75 mg/mL.
   (b) DyLight 594 labeled Griffonia Simplicifolia Lectin I (GSL I) isolectin B_4 (GS-IB_4) (Vector Lab DL1207). Dilute 0.5 mg GSL I-B_4 in PBS or saline water to reach a final concentration of 1 mg/mL.
2. 28-gauge insulin syringe.

2.4 Perfusion and Fixation

1. Heparin PBS: 10 U/mL heparin sodium in PBS.
2. 4% paraformaldehyde (PFA) (pH 7.4).
3. 22-gauge needle.
4. 25 mL syringe.
5. Silicone tube.
6. Dissecting straight scissors.
7. Surgical scissors.
8. Straight tweezers.
9. 50 mL centrifuge tubes.
10. Foam plate and pins.
11. Blood container.

2.5 PEGASOS Passive Immersion Procedure

1. Decalcification solution: 0.5 M ethylenediaminetetraacetic acid (EDTA). Add sodium hydroxide to dissolve EDTA and adjust the pH to 7.0.
2. Decolorization solution: 25% N,N,N′,N′-Tetrakis(2-Hydroxypropyl) ethylenediamine (Quadrol) (*see* **Note 1**).

3. Delipidation solution: Dilute pure tert-butanol (tB) (Sigma-Aldrich 360,538) with dH_2O to prepare 30% tB, 50% tB, and 70% tB (v/v). Add pure Quadrol (3% w/v final concentration) to adjust the pH to above 9.5 (*see* **Note 2**).
4. Dehydration solution: tB-PEG, a mixture of 70% v/v tB, 30% v/v PEG methacrylate Mn 500 (PEGMMA500) supplemented with 3% w/v Quadrol.
5. Clearing medium: BB-PEG, a mixture of 75% v/v BB and 25% v/v PEGMMA500 supplemented with Quadrol (3% w/v final concentration). The RI of BB-PEG is 1.543.
6. 50 mL centrifuge tubes.
7. A temperature-controlled shaker.

2.6 Whole-Mount Immunostaining

1. Blocking buffer composed of 10% dimethyl sulfoxide, 0.5% IGEPAL CA-630, and 1× casein buffer in PBS.
2. Primary antibody: Anti-α-smooth muscle antibody (dilution 1:500) is commonly used for artery labeling. Anti-CD31 antibody (dilution 1:100) is commonly used for vessel endothelium labeling. Isolectin B_4 ($GS\text{-}IB_4$) can also be used for endothelium staining. Optimal dilution should be determined individually, and 1:100 is a good starting dilution to test.
3. Alexa Fluor conjugated secondary antibody.
4. 1.5 mL Eppendorf tubes.
5. Aluminum foil paper.
6. A temperature-controlled shaker.

2.7 3-D Imaging Acquisition for Cleared Samples

1. Depression slide.
2. Cover glass.
3. Imaging system: confocal microscopy/two-photon microscopy/light-sheet fluorescent microscopy.

2.8 Data Processing

1. ImageJ software (National Institutes of Health).
2. Imaris software (Bitplane).
3. High performance computer workstation. The optimum configuration includes: 128 GB RAM, dual CPU, high-end graphic card (Nvidia GTX 1080 Ti or AMD Radeon 580), and large-storage hard drives (>5 TB).

3 Methods

(For endogenously labeled mice, skip methods in Subheading 3.1 and proceed to methods in Subheading 3.2)

3.1 Retro-Orbital Injection of Vasculature Dye (See Note 3)

1. Anesthetize a mouse using 3% isoflurane and 4% oxygen. Alternatively, a cocktail of ketamine (80 mg/kg body weight) and xylazine (10 mg/kg body weight) could be intraperitoneally administered (*see* **Notes 4** and **5**).
2. Prepare one of the vascular labeling dyes in a 28-gauge insulin syringe: 200 μL (a) 75 mg/mL FITC-Dextran or (b) 1 mg/mL GS-IB_4.
3. Place the mouse in left recline position with the head facing to the right. Apply gentle pressure with fingers to the skin around the right eye to slightly protrude the eye from the eye socket (*see* **Note 6**).
4. Carefully introduce the needle via the medial canthus at an angle of 30° with bevel down (*see* **Note 7**). The bone beneath can be felt as a guide. Once the needle tip is at the base, slowly and smoothly depress the plunger to deliver the dye (*see* **Notes 8–10**).
5. Slowly withdraw the needle after injection. Wait for 5–30 min until dye circulates through the whole body before sacrificing the animal. Optimal circulation time for different organs can vary and should be determined individually (*see* **Note 11**).

3.2 Perfusion (Including Postmortem Dye Perfusion) and Tissue Preparation

1. Prepare the surgical setup including 50 mL cold heparin PBS (*see* **Note 12**), 25 mL cold 4% PFA, and surgical instruments. Connect a 22-gauge needle to a 25 mL syringe using a silicone tube. Eliminate the bubbles in the injection system.
2. Limbs of the anesthetized mouse can be directly fixed on a foam plate. All the following steps must be performed in a biosafety hood.
3. Open the chest and abdominal cavity with surgical scissors, then insert the 22-gauge needle into the mouse's left cardiac ventricle with bevel up (*see* **Note 13**). Inject about 2 mL heparin PBS to fill the heart and cut an incision on the right atrium immediately using a surgical scissor. The right atrium incision provides the outlet for the perfusion fluid.
4. Inject 50 mL heparin PBS to flush out as much blood as possible (*see* **Note 14**).
5. Postmortem dye perfusion (optional): Vasculature labeling dyes can also be perfused through the circulation at this stage. After flushing out the blood, inject dye solution to label the vessels: Dilute 200 μL (a) 75 mg/mL FITC-Dextran or (b) 1 mg/mL GS-IB_4 in 3 mL PBS for perfusion (*see* **Note 15**). Wait for 5 min for complete staining and proceed to the next step.
6. Perfuse 25 mL 4% PFA transcardially for fixation (*see* **Note 14**).
7. Dissect carefully the target samples.

8. Immerse the dissected samples in 4% PFA at 4 °C overnight. On the following day, wash samples with PBS three times for 20 min each on a shaker.

3.3 PEGASOS Passive Immersion Procedure (Fig. 1)

1. Decalcification (only for hard tissues): immerse samples in 0.5 M EDTA (pH 7.0) at room temperature (RT) on a shaker at 60 rpm for around 4 days. Change the decalcification solution daily. Wash samples with pure H_2O for 30 min to elute excessive EDTA.
2. Decolorization: place samples in 25% Quadrol for 1–2 days at 37 °C under constant shaking at 60 rpm. Change medium daily until the medium does not turn yellow anymore (*see* **Note 16**).
3. Delipidation: treat samples with gradient delipidation solutions of 30%, 50%, and 70% tB at 37 °C under constant shake at 60 rpm. The treatment time depends on the tissues volume. Suggested durations for different tissues are summarized (Table 1). This step takes approximately 2 days.
4. Dehydration: immerse samples into tB-PEG solution at 37 °C on a shaker at 60 rpm for 2 days. Change the medium at least once. After dehydration, the samples should be kept away from any water containing reagent.
5. Clearing: immerse samples in the final BB-PEG clearing medium for 1 day on a shaker at 37 °C until tissues turn transparent (*see* **Note 17**) (Fig. 3a). Cleared tissue can be preserved in the BB-PEG solution at room temperature (RT).

3.4 Whole-Mount Immunostaining Within PEGASOS Passive Immersion Procedure

The following protocol is based on the whole-mount immunohistochemical performed on brain slice of 500 μm thickness.

1. Decalcification and decolorization are the same as in Subheading 3.3.
2. Wash samples with PBS solution for 30 min before blocking.
3. Blocking: prepare the blocking buffer in a 1.5 mL Eppendorf tube containing blocking solution. Tissue sections are placed into 1 mL blocking buffer and incubated overnight at RT on a shaker (60 rpm) (*see* **Note 18**).

Table 1
Time schedule of delipidation for different tissues

Delipidation	Tissue organs at 4–6 weeks age	Tissue organs at 8 weeks age	Tissue sections <2 mm
30% tB	4 h	6 h	2 h
50% tB	6 h	8 h	4 h
70% tB	1 day	1.5 days	4 h

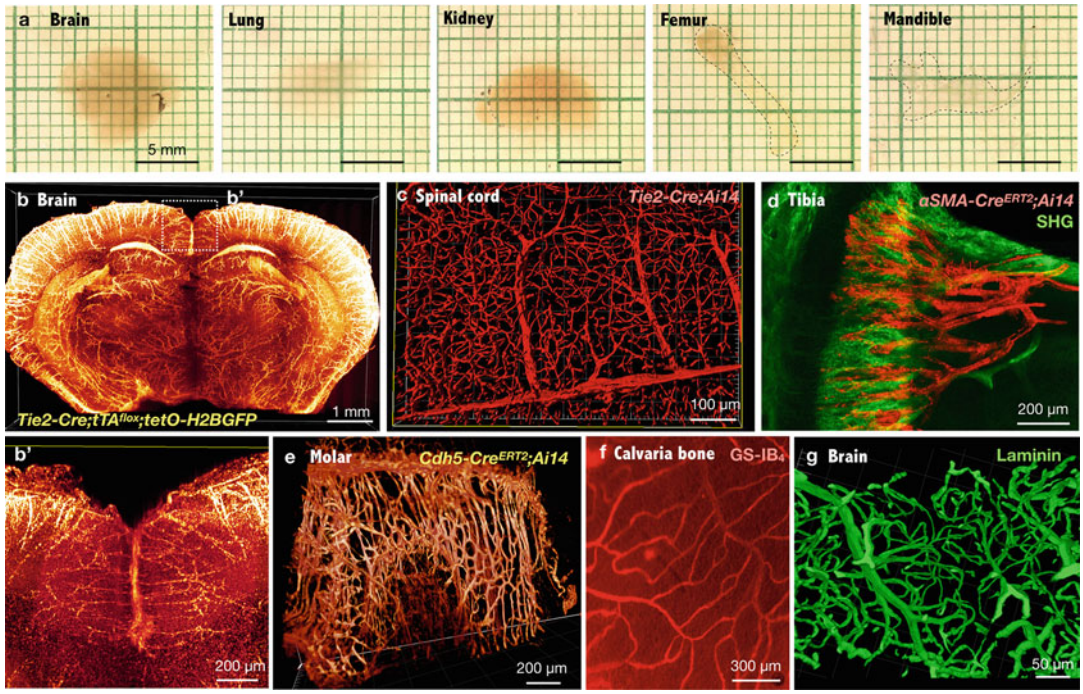

Fig. 3 Clearing and 3-D imaging of vasculature of various organs labeled with different approaches. (**a**) Both soft and hard tissue organs achieved high transparency after PEGASOS passive immersion method. (**b**) The whole brain of a *Tie2-Cre*; *tTA*^{flox}; *tetO-H2BGFP* mouse was cleared and imaged with a light-sheet microscope. Boxed area is enlarged in (**b′**). (**c**) The vasculature of the spinal cord of a *Tie2-Cre*; *Ai14* mouse was imaged through the vertebrae bone using confocal microscopy. (**d**) The arteries (red) of bone marrow near the metaphysis region of *αSMA-Cre*^{ERT2}; *Ai14* mouse was imaged using two-photon microscopy, the second harmonic generation (SHG) signals (green) outline the trabecular bone. (**e**) The vasculature within and surrounding the mouse molar of *Cdh5-Cre*^{ERT2}; *Ai14* mouse. (**f**) The vasculature underneath calvarial bone was labeled by GS-IB$_4$ dye tail vein injection and imaged with a stereo fluorescence microscopy. (**g**) Whole-mount immunofluorescent staining using antilaminin antibody was performed for a brain slice

4. After blocking, stain samples with 0.5–1 mL primary antibody diluted with the blocking buffer for 3 days at 4 °C on a shaker at 60 rpm. Refer to the dilution ratio for slice staining in the instructions (*see* **Note 19**).
5. Wash samples with PBS solution for 1 day at RT on a shaker at 60 rpm.
6. Stain samples with the 0.5–1 mL secondary antibody diluted with the blocking solution for another 3 days at 4 °C on a shaker at 60 rpm (*see* **Note 19**).
7. Wash samples with PBS solution for 1 day at RT on a shaker at 60 rpm.
8. Move stained samples back to a 50 mL centrifuge tube and proceed for delipidation, dehydration, and clearing processes (same as in Subheading 3.3).

Table 2
Examples of objectives used for cleared specimens with high RI

Inc.	Objective name	NA	Working distance (mm)	Immersion
Leica	HCX APO L20×/0.95 IMM	0.95	1.95	BABB
ZEISS	LD LCI Plan-Apochromat 25×/0.8 Imm Corr DIC M27	0.8	0.57	Water, silicone oil, glycerin, oil
	C Plan-Apochromat 40×/1.3 Oil DIC M27	1.3	0.22	Oil
	EC Epiplan-Neofluar 50×/1.0 Oil Pol M27	1.0	0.4	Oil
Nikon	CFI Plan Apochromat 10XC Glyc	0.5	Upright: 5.50 Inverted: 2.00	Medium with RI ~1.44–1.50
	CFI90 20XC Glyc	1.0	8.2	

3.5 3-D Imaging Acquisition and Reconstruction

1. Set the cleared sample in a depression slide with clearing medium, then cover it with cover glass (*see* **Notes 20** and **21**).
2. Confocal microscope parameter settings are mainly dependent on the objectives and desired imaging quality (*see* **Note 22**). Commonly used objectives available for cleared specimens' deep imaging are listed for reference (Table 2) (*see* **Note 23**).
3. Start imaging and collect image files.
4. Image J is an open-source software and contains modules for most 3-D imaging processing functions including deconvolution, stitching, alignment, and quantification. Imaris is a commercial software with powerful modules for 3-D rendering and many other advanced functions (Fig. 3b–g) (*see* **Note 24**).

4 Notes

1. Quadrol is highly viscous. Weighing is more convenient than measuring volume, so weight/volume is used for convenience. It is miscible with water. To facilitate flowability, Quadrol container can be prewarmed with hot water around 60° C. Avoid boiling during the preparation.
2. Pure tB is colorless solid at RT. Its melting point is 26 °C. Heat the container with hot water before use.
3. Retro-orbital injection is much easier to perform than tail vein injection for administration of dye or compounds. Both routes are equally effective [24].
4. Isoflurane anesthesia is the recommended anesthetic method for perfusing animal. The mice can be anesthetized within 3–5 min. Intraperitoneal injection of anesthetic can be used when retro-orbital sinus dye injection or vein tail dye injection is to be performed.

5. Pinch the mouse digits and check response to confirm the anesthesia outcome before retro-orbital sinus injection.
6. The jugular veins and carotid artery run along the ventral cervical area, so excessive pressure to the area may affect blood circulation and perfusion efficiency [25].
7. The needle bevel should face toward the bone of the orbit to avoid damaging the eyeball [25].
8. Successful retro-orbital injection leads to a bulging or blanching of the eye without solution leaking out.
9. Injected volume should not exceed 0.2 mL, and no more than two injections should be performed for one mouse at one time. Excessive injection volume may cause the animal to die immediately, probably due to disrupted electrolytes balance.
10. Evans blue dye can be used for practicing retro-orbital injection. Successful injection can be indicated by tail and digits turning blueish.
11. The staining time is variable, depending on the tissues' volume and density. The large blood vessels in various organs can be stained immediately (within 5 min). More time is needed for minor blood vessels in distal regions including limbs and guts. Staining time should not be more than 30 min.
12. EDTA is an alternative anticoagulant that acts by binding calcium in blood. 0.05 M EDTA can also be used to wash out blood in place of heparin.
13. In order to control the insertion depth, the needle bevel should face toward the operator. Stop inserting the needle when the tip bevel just gets into the left ventricle. Excessive needle insertion can damage the cardiac valve and cause failure of perfusion.
14. An indication of a successful transcardiac perfusion is the liver turning pale rapidly. Try perfuse with more heparin PBS if the color of the liver remains unchanged. Another indicator of success is the limb muscles turning stiff after PFA perfusion.
15. FITC-Dextran is much cheaper, and its fluorescence is also stronger than GS-IB_4. However, Dextran may leak out of capillaries and cause nonspecific staining in some organs. Labeling efficiency of postmortem dye perfusion is lower than retro-orbital dye injection, especially for postcapillaries veins in distal regions.
16. Quadrol is a strong chelating reagent, which releases heme from hemoglobin. Its color turns slightly yellow during decolorization process. Different organs have different levels of heme, and their decolorization processing time may vary.

Normally, the maximum effect is achieved within 3 days for long bone and liver, which are highly most heme-enriched organs.

17. One day after placing samples into the BB-PEG clearing medium, open the container and expose the medium to the air for the next 2 days and then seal it. Somehow, we noticed this operation improves the transparency outcome. Samples in BB-PEG can be stored at 4 °C or RT for over a year without significant loss of endogenous fluorescence signals.
18. The ability of antibodies to penetrate whole organs is limited. It remains nearly impossible to stain hard tissues. We recommend whole-mount immunostaining to be performed on samples of no more than 0.5 mm thickness.
19. Antibody incubation at RT results in higher background than at 4 °C.
20. Samples are kept within the BB-PEG clearing medium for preservation and imaging. Air bubbles should be avoided.
21. A depression slide is sufficient in size to contain most tissue samples for imaging. For even larger samples, a plastic cryoembedding mold (EMS Tissue-Tek Cryomold) can be used by placing a glass coverslip on top.
22. When selecting proper objective for 3-D imaging, the objective numerical aperture (NA) number and working distance are two major considerations. The NA number determines both lateral and axial resolutions. The working distance determines the image depth that can be acquired.
23. An immersion objective matched with the RI of the cleared sample achieved much better resolution than nonimmersion objective or RI nonmatched objectives. Leica BABB objective (HCX APOL 20X0.95 IMM) is our favorite choice when imaging.
24. Imaris provides powerful function modules for 3-D rendering, 3-D analysis, image segmentation, region of interest selection, neuro tracing and video creation, etc. The price is very expensive. ImageJ is a freeware and should be updated regularly to include most up-to-date plugins.

Acknowledgments

This work was supported by the start-up funding from the Texas A&M University, to Hu Zhao and National Natural Science Foundation of China (No. 81771031, 81901041) to Dian Jing and Yi Men.

References

1. Seo J, Choe M, Kim SY (2016) Clearing and labeling techniques for large-scale biological tissues. Mol Cells 39(6):439–446. https://doi.org/10.14348/molcells.2016.0088.
2. Susaki EA, Ueda HR (2016) Whole-body and whole-organ clearing and imaging techniques with single-cell resolution: toward organism-level systems biology in mammals. Cell Chem Biol 23(1):137–157. https://doi.org/10.1016/j.chembiol.2015.11.009.
3. Erturk A, Becker K, Jahrling N, Mauch CP, Hojer CD, Egen JG et al (2012) Three-dimensional imaging of solvent-cleared organs using 3DISCO. Nat Protoc 7(11):1983–1995. https://doi.org/10.1038/nprot.2012.119.
4. Pan C, Cai R, Quacquarelli FP, Ghasemigharagoz A, Lourbopoulos A, Matryba P et al (2016) Shrinkage-mediated imaging of entire organs and organisms using uDISCO. Nat Methods 13(10):859–867. https://doi.org/10.1038/nmeth.3964
5. Cai R, Pan C, Ghasemigharagoz A, Todorov MI, Foerstera B, Zhao S et al (2018) Panoptic vDISCO imaging reveals neuronal connectivity, remote trauma effects and meningeal vessels in intact transparent mice. bioRxiv. https://doi.org/10.1101/374785
6. Schwarz MK, Scherbarth A, Sprengel R, Engelhardt J, Theer P, Giese G (2015) Fluorescent-protein stabilization and high-resolution imaging of cleared, intact mouse brains. PLoS One 10(5):e0124650. https://doi.org/10.1371/journal.pone.0124650.
7. Jing D, Zhang S, Luo W, Gao X, Men Y, Ma C et al (2018) Tissue clearing of both hard and soft tissue organs with the PEGASOS method. Cell Res 28(8):803–818. https://doi.org/10.1038/s41422-018-0049-z.
8. Ke MT, Nakai Y, Fujimoto S, Takayama R, Yoshida S, Kitajima TS et al (2016) Super-resolution mapping of neuronal circuitry with an index-optimized clearing agent. Cell Rep 14(11):2718–2732. https://doi.org/10.1016/j.celrep.2016.02.057
9. Tainaka K, Kubota SI, Suyama TQ, Susaki EA, Perrin D, Ukai-Tadenuma M et al (2014) Whole-body imaging with single-cell resolution by tissue decolorization. Cell 159(4):911–924. https://doi.org/10.1016/j.cell.2014.10.034.
10. Tainaka K, Murakami TC, Susaki EA, Shimizu C, Saito R, Takahashi K et al (2018) Chemical landscape for tissue clearing based on hydrophilic reagents. Cell Rep 24(8):2196–210.e9. https://doi.org/10.1016/j.celrep.2018.07.056
11. Li W, Germain RN, Gerner MY (2017) Multiplex, quantitative cellular analysis in large tissue volumes with clearing-enhanced 3D microscopy (Ce3D). Proc Natl Acad Sci U S A 114(35):E7321–E7330. https://doi.org/10.1073/pnas.1708981114
12. Chung K, Deisseroth K (2013) CLARITY for mapping the nervous system. Nat Methods 10(6):508–513. https://doi.org/10.1038/nmeth.2481
13. Treweek JB, Chan KY, Flytzanis NC, Yang B, Deverman BE, Greenbaum A et al (2015) Whole-body tissue stabilization and selective extractions via tissue-hydrogel hybrids for high-resolution intact circuit mapping and phenotyping. Nat Protoc 10(11):1860–1896. https://doi.org/10.1038/nprot.2015.122.
14. Greenbaum A, Chan KY, Dobreva T, Brown D, Balani DH, Boyce R et al (2017) Bone CLARITY: clearing, imaging, and computational analysis of osteoprogenitors within intact bone marrow. Sci Transl Med 9(387):eaah6518. https://doi.org/10.1126/scitranslmed.aah6518.
15. Reveles Jensen KH, Berg RW (2017) Advances and perspectives in tissue clearing using CLARITY. J Chem Neuroanat 86:19–34. https://doi.org/10.1016/j.jchemneu.2017.07.005
16. Cao Y, Wang H, Wang Q, Han X, Zeng W (2018) Three-dimensional volume fluorescence-imaging of vascular plasticity in adipose tissues. Mol Metabol 14:71–81. https://doi.org/10.1016/j.molmet.2018.06.004
17. Jung B, Arnold TD, Raschperger E, Gaengel K, Betsholtz C (2018) Visualization of vascular mural cells in developing brain using genetically labeled transgenic reporter mice. J Cereb Blood Flow Metab 38(3):456–468. https://doi.org/10.1177/0271678X17697720
18. Damisah EC, Hill RA, Tong L, Murray KN, Grutzendler J (2017) A fluoro-Nissl dye identifies pericytes as distinct vascular mural cells during in vivo brain imaging. Nat Neurosci 20(7):1023–1032. https://doi.org/10.1038/nn.4564
19. Bryson JL, Coles MC, Manley NR (2011) A method for labeling vasculature in embryonic mice. J Vis Exp 56. https://doi.org/10.3791/3267

20. Lugo-Hernandez E, Squire A, Hagemann N, Brenzel A, Sardari M, Schlechter J et al (2017) 3D visualization and quantification of microvessels in the whole ischemic mouse brain using solvent-based clearing and light sheet microscopy. J Cereb Blood Flow Metab:271678X17698970. https://doi.org/10.1177/0271678X17698970
21. Li Y, Song Y, Zhao L, Gaidosh G, Laties AM, Wen R (2008) Direct labeling and visualization of blood vessels with lipophilic carbocyanine dye DiI. Nat Protoc 3(11):1703–1708. https://doi.org/10.1038/nprot.2008.172
22. Jing D, Yi Y, Luo W, Zhang S, Yuan Q, Wang J et al (2019) Tissue clearing and its application to bone and dental tissues. J Dent Res 98 (6):621–631
23. Yi Y, Men Y, Jing D, Luo W, Zhang S, Feng JQ et al (2019) 3-dimensional visualization of implant-tissue interface with the polyethylene glycol associated solvent system tissue clearing method. Cell Prolif 52:e12578. https://doi.org/10.1111/cpr.12578
24. Steel CD, Stephens AL, Hahto SM, Singletary SJ, Ciavarra RP (2008) Comparison of the lateral tail vein and the retro-orbital venous sinus as routes of intravenous drug delivery in a transgenic mouse model. Lab Anim (NY) 37 (1):26–32. https://doi.org/10.1038/laban0108-26
25. Yardeni T, Eckhaus M, Morris HD, Huizing M, Hoogstraten-Miller S (2011) Retro-orbital injections in mice. Lab Anim (NY) 40(5):155–160. https://doi.org/10.1038/laban0511-155.