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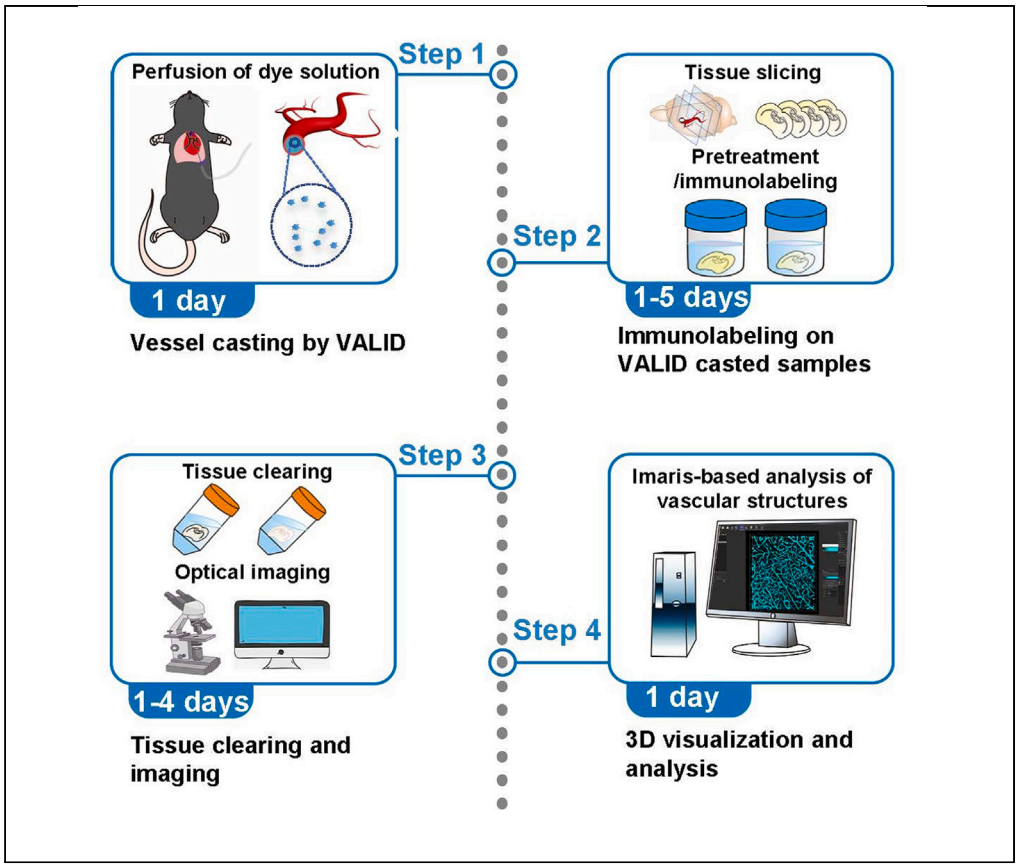
Protocol for fine casting, imaging, and analysis of murine vascular networks with VALID



The majority of fluorescent vessel labeling techniques currently available are limited by their expense, incomplete labeling, or complexity. Here, we present VALID (Vessel lAbeling via geLatin-based lIpophilic Dye solution)—a protocol for complete labeling of different vascular networks. We describe steps for preparing different dye hydrogels, murine vascular casting and tissue harvesting, immunolabeling, tissue clearing, and imaging, as well as detailed analysis of the vascular networks. This protocol is helpful for evaluating vascular lesions in studying different vessel-associated diseases.

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

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Highlights

A vessel casting protocol using lipophilic dye hydrogel

Effective for labeling complete vascular networks within different organs

Applied along with 3D immunolabeling and tissue clearing

Supports 3D visualization and analysis of different vasculature

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Protocol for fine casting, imaging, and analysis of murine vascular networks with VALID

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SUMMARY

The majority of fluorescent vessel labeling techniques currently available are limited by their expense, incomplete labeling, or complexity. Here, we present VALID (*v*essel l*a*beling via ge*l*atin-based l*i*pophilic *d*ye solution)—a protocol for complete labeling of different vascular networks. We describe steps for preparing different dye hydrogels, murine vascular casting and tissue harvesting, immunolabeling, tissue clearing, and imaging, as well as detailed analysis of the vascular networks. This protocol is helpful for evaluating vascular lesions in studying different vessel-associated diseases.

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

BEFORE YOU BEGIN

Recently, 3D imaging of entire vascular networks via optical imaging techniques have been widely employed in numerous studies.[2] The combination of vessel labeling, tissue clearing and optical imaging techniques allow 3D mapping of the vasculature within different tissues/organs. As the first and critical step, efficient and complete labeling of the entire vascular networks with bright fluorescence largely guarantees the quality of the reconstructed vascular structures. Though there are already numerous vessel labeling methods ready to use, they are still restricted by certain limitations such as insufficient signal-to-background ratio, incomplete labeling for large vessels, potential dye leakage or high cost[3,4]. Here, we present VALID (**V**essel l**A**beling via ge**L**atin-based l**I**pophilic **D**ye solution), an efficient, easy-handling and cost-effective protocol that enables complete and uniform labeling of different vascular networks with bright fluorescence.

The execution of VALID pipeline is relatively easy and flexible. In brief, a hydrogel-based lipophilic dye solution is first prepared and transcardially perfused into the animal body for casting the entire vascular networks. After tissue fixation, the desired tissues/organs are optionally counterstained by specific antibodies, the tissues/organs are then gone through the tissue clearing procedure followed by imaging with a light sheet microscope. This protocol provides detailed guidelines for the major steps of VALID, including: 1) Preparation of lipophilic dye stocking/working solution, 2) Vascular labeling and tissue fixation, 3)2D and 3D immunolabeling, 4) Tissue clearing, 5) Light sheet imaging, 6) 3D analysis of vascular structures via Imaris.

Institutional permissions

Wild-type mice (C57BL/6J, 8–10 weeks old) are used in this study. Animals are housed in a specific-pathogen-free (SPF) animal house under a 12/12 h light/dark cycle with unrestricted access to food

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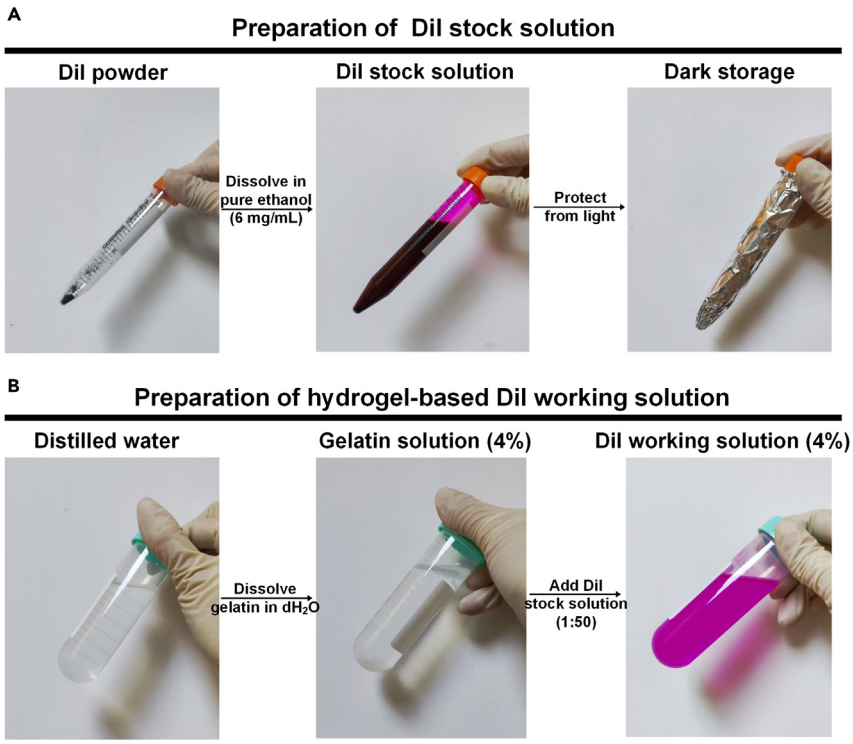

Figure 1. Procedures for preparation of different dye solutions involved in VALID

(A) Preparation of DiI stock solution.

(B) Preparation of hydrogel-based DiI working solution.

and water. All animal experiments are performed under the Experimental Animal Management Ordinance of Hubei Province, P. R. China, and the guidelines from the Huazhong University of Science and Technology and were approved by the Institutional Animal Ethics Committee of Huazhong University of Science and Technology.

Preparation of lipophilic dye working hydrogel

Timing: 1–2 h

1. Preparation of dye stock solution.
   a. Dissolve lipophilic dye powder in 100% ethanol with 15 mL centrifuge tube at a concentration of 6 mg / mL for 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), 1 mg / mL for 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) and 1,1′-dioctadecyl-3,3,3′3′-tetramethylindodicarbocyanine perchlorate (DiD), respectively, as shown in Figure 1A.
   b. Put the centrifuge tube on a table concentrator with gently shaking under room temperature to make the dye powder fully dissolve.

Note: The prepared stock solution can be stored in dark at room temperature up to one year.

2. Preparation of dye working solution.
   a. Dissolve the pig skin gelatin powder in distilled water to prepare the 2%/4% (w/v) gelatin solution with 50 mL centrifuge tube, then place the tube in a 50°C water bath to ensure complete dissolution.
   b. The dye working hydrogel is prepared by diluting 200 μL dye stock solution in 10 mL prepared gelatin solution with gently shaking. The final dye working hydrogel is shown in Figure 1B.

Note: The prepared dye working hydrogel should be stored at 40°C to prevent polymerization before use, and should be used as soon as possible after preparation.

Other preparations before vessel casting

Timing: 1–2 h

3. Prepare 4% PFA solution.
   a. Dissolve PFA powder in 0.01 M PBS solution, prepare 4% PFA solution, stir and heat it in a water bath at 58°C until completely dissolved, then cool the solution to room temperature. Store the 4% PFA solution at 4°C.

   Note: When preparing 4% PFA solution, latex gloves and a mask should be worn to avoid direct contact with chemicals. Please perform dissolution operations in a chemical fume hood.

   b. After cooling, filter the 4% PFA solution to remove the impurities.
4. Prepare a box of ice standby. Ensure warm temperature of the perfusion table (The ideal temperature for casting vessels is 25°C–35°C). If the weather is cold, it is necessary to turn on the air conditioner in advance.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
GFAP antibody; dilution 1:500	Proteintech	Cat#16825-1-AP, RRID:AB_2109646
CD31 Monoclonal antibody; dilution 1:500	Proteintech	Cat#66065-2-Ig, RRID: AB_2918476
Anti-Tyrosine Hydroxylase Rabbit pAb; dilution 1:500	Servicebio	Cat#GB11181, RRID: AB_2921651
NeuN polyclonal antibody; dilution 1:500	Proteintech	Cat#26975-1-AP, RRID:AB_2880708
Parvalbumin polyclonal antibody; dilution 1:500	Proteintech	Cat#29312-1-AP, RRID: AB_2918280
MAP2 antibody; dilution 1:500	Proteintech	Cat# 7490-1-AP, RRID:AB_2137880
Iba1 antibody; dilution 1:500	Abcam	Cat#ab5076, RRID:AB_2224402
Goat Anti-Rabbit secondary antibody Alexa Fluor 488; dilution 1:500	Abcam	Cat#ab150077, RRID:AB_2630356
Goat Anti-Mouse secondary antibody CoraLite 594; dilution 1:500	Proteintech	Cat#SA00013-3, RRID:AB_2797133
Chemicals, peptides, and recombinant proteins		
PBS	Sigma-Aldrich	Cat#P3813
Paraformaldehyde	Sigma-Aldrich	Cat#158127
Pork skin gelatin	Sigma-Aldrich	Cat#V900863
1,1′-dioctadecyl-3,3,3′, 3′-tetramethylindocarbocyanine perchlorate (DiI)	Aladdin	Cat#D131225
3,3′-dioctadecyloxacarbocyanine perchlorate (DiO)	Aladdin	Cat#D131213
1,1′-dioctadecyl-3,3, 3′3′-tetramethylindodicarbocyanine perchlorate (DiD)	Thermo Fisher Scientific	Cat#D7757
MeOH	Sinopharm Chemical Reagent Co. Ltd.	Cat#10014118
EtOH	Sinopharm Chemical Reagent Co. Ltd.	Cat#10009218
Tert-butanol	Sigma-Aldrich	Cat#360538
Tetrahydrofuran	Sigma-Aldrich	Cat#186562
DBE	Sigma-Aldrich	Cat#108014

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
m-Xylylenediamine (MXDA)	Tokyo Chemical Industry	Cat#D0127
Sorbitol	Sigma-Aldrich	Cat#85529
Triton X-100	Sigma-Aldrich	Cat#T8787
Sodium azide	Sigma-Aldrich	Cat#S2002
Experimental models: Organisms/strains		
Mouse: C57BL/6J, male and female, 8–10 weeks old	Jackson Laboratories	Cat# JAX:000664, RRID:IMSR_JAX:000664
Software and algorithms		
Matlab	MathWorks	https://www.mathworks.com/products/matlab.html
Imaris	Bitplane	https://www.bitplane.com/imaris/imaris
Fiji	Schindelin et al.[5]	https://imagej.nih.gov/ij/
Other		
Conical tubes (50 mL)	Corning	Cat#430828
Conical tubes (15 mL)	Corning	Cat#430790
Syringe pump	Leadfluid	Cat#TFD04
Peristaltic pump	Leadfluid	Cat#BT100L

MATERIALS AND EQUIPMENT

PBST

Reagent	Final concentration	Amount
1× PBS	99.9%	999 mL
Triton X-100	0.1%	1 mL
Total	**N/A**	**1000 mL**

Storage: Store at room temperature. We typically prefer to add 0.2% sodium azide to prevent microbial growth. We usually use PBST within 3 months of preparation.

PTwH

Reagent	Final concentration	Amount
1× PBS	99.8%	998 mL
Tween-20	0.2%	2 mL
Heparin	10 μg/mL	10 mg
Total	**N/A**	**1000 mL**

Storage: Store at room temperature. We typically prefer to add 0.2% sodium azide to prevent microbial growth. We usually use PTwH within 3 months of preparation.

Permeabilization solution

Reagent	Final concentration	Amount
DMSO	20%	50 mL
Glycine	0.3 M	5.625 g
Triton X-100	0.2%	0.5 mL
1× PBS	79.8%	199.5 mL
Total	**N/A**	**250 mL**

Storage: Store at room temperature. We typically prefer to add 0.2% sodium azide to prevent microbial growth. We recommend up to one month storage for this solution.

Blocking solution		
Reagent	Final concentration	Amount
1× PBS	83.8%	41.9 mL
DMSO	10%	5 mL
Goat serum	6%	3 mL
Triton X-100	0.2%	0.1 mL
Total	**N/A**	**50 mL**

Storage: Store at room temperature. We typically prefer to add 0.2% sodium azide to prevent microbial growth. We recommend up to one month storage for this solution.

Antibody diluent buffer for 2D immunolabeling		
Reagent	Final concentration	Amount
1×PBS	96.8%	48.4 mL
Triton X-100	0.2%	0.1 mL
Goat serum	3%	1.5 mL
Total	**N/A**	**50 mL**

Storage: We typically prefer to add 0.2% sodium azide to prevent microbial growth. This solution is recommended freshly made before use.

Antibody diluent buffer for 3D immunolabeling		
Reagent	Final concentration	Amount
PTwH	92%	46 mL
Goat serum	3%	1.5 mL
DMSO	5%	2.5 mL
Total	**N/A**	**50 mL**

Storage: We typically prefer to add 0.2% sodium azide to prevent microbial growth. This solution is recommended freshly made before use.

MACS-R0		
Reagent	Final concentration	Amount
MXDA	20% (vol/vol)	10 mL
D-sorbitol	15% (wt/vol)	7.5 g
ddH_2O	67.3% (vol/vol)	33.65 mL
Total	**N/A**	**50 mL**

Storage: Store in dark at room temperature for up to one month. Protect from light.

MACS-R1		
Reagent	Final concentration	Amount
MXDA	40% (vol/vol)	20 mL
D-sorbitol	30% (wt/vol)	15 g
1×PBS	38.3% (vol/vol)	19.13 mL
Total	**N/A**	**50 mL**

Storage: Store in dark at room temperature for up to one month. Protect from light.

MACS-R2		
Reagent	Final concentration	Amount
MXDA	40% (vol/vol)	20 mL
D-sorbitol	50% (wt/vol)	25 g
ddH_2O	21.3% (vol/vol)	10.64 mL
Total	**N/A**	**50 mL**

Storage: Store in dark at room temperature for up to one month. Protect from light.

⚠ CRITICAL: When preparing MACS solutions, latex gloves should be worn to avoid direct contact with chemicals. Though the MXDA is hardly volatile, a ventilated environment is recommended.

STEP-BY-STEP METHOD DETAILS

Vessel casting via perfusion of lipophilic dye hydrogel and tissue harvesting

Timing: 1–2 d

This step describes the detailed procedure for how to cast the vessels using the prepared lipophilic dye hydrogel and how to harvest and fix the desired tissues/organs after labeling.

1. Prepare the materials and equipment used for perfusion, as shown in Figures 2A–2E.
2. Warm the 0.01 M PBS to 40°C using a water bath. Turn on the pump and drain the air with warm PBS (Figure 2A).

   Note: Before perfusion, ensure that the pump pipe used for perfusion (Figure 2A) is filled with warm PBS. Existence of air will lead to insufficient perfusion, resulting in potential damage to the vascular structures.

3. Deeply anesthetize the mice with a mixture of ketamine/xylazine (100/20 mg/kg) via intraperitoneal injection. Then transcardially perfuse the mice with 0.01 M PBS to wash out the blood.

   Note: It is observed that the liver turns white, and the right atrial appendage flows clear PBS, indicating that the blood is sufficiently washed out.

   ⚠ CRITICAL: There may be a little difference in labeling the lung vasculature. The users should insert the perfusion needles into the right ventricle and cut the left atrial appendage to ensure that the dye solution go through the pulmonary circulation.

4. Turn off the perfusion pump, change PBS into the prepared lipophilic dye solutions. Perfuse 10–15 mL of the dye solution into the mouse body under warm conditions, as shown in Figure 2F.

   ⚠ CRITICAL: During perfusion, the entire mouse body will experience continuous convulsions, which indicates that the labeling works well. If there is no convulsion happened, or the mouse lungs are swelling along with water outflowing the nasal cavity of mouse, it indicates that the vessel labeling is largely failed. This is probably because the syringe needle is inserting too deep into the right ventricle. In this case, gently move the needle outward without dropping out of the heart. If there is a general convulsion happened soon afterwards, the vessel casting can be continued and completed. Or the vessel casting is failed and should be repeated on another mouse.

   Note: The volume of dye solution for mice is largely depend on how the perfusion performs. If the perfusion is good, 10 mL dye solution is enough. If the perfusion is not very good (but not failed), more dye solution will be needed (15 mL).

   Note: The two concentrations are designed for different organ types. Lower concentration (2%) is suitable for organs with large cavity, such as heart. And higher concentration (4%) is suitable for other organs.

5. Stop the perfusion, pull out the needle, and turn off the pump. Quickly transfer the perfused mouse into ice box for rapid cooling, as shown in Figures 2G and 2H.

Figure 2. Detailed method for perfusion, vessel casting, tissue harvesting and fixation

(A) Overview of the perfusion table for vessel casting.

(B) Surgical instruments used for perfusion.

(C)Peristaltic pump used for perfusion and vessel casting.

(D) Shaker.

(E) Preparation of a box fulfilled with ice.

(F–H) (F) Restrict the mouse on the table by use of the needles, exposing the chest cavity, and starting perfusion with 1×PBS through the left ventricle followed by vessel casting. The mouse body after vessel casting was quickly transferred to the ice box (G) and submerged into the ice (H).

(I) Harvested tissues were then post-fixed by 4% PFA in 4°C.

Note: After 1–2 h cooling in the ice box, transfer the mouse body to the 4°C refrigerator overnight.

6. After sufficient cooling, collect the desired mouse organs and tissues carefully from the mouse body.

⚠ CRITICAL: Cooling of the mouse body is important for the dye solution to form stable hydrogels within the blood vessels, therefore, successful execution of this step is very critical for the final labeling performance of vascular networks.

Note: Because the tissue is not fixed by 4% PFA, it is relatively soft and fragile during tissue harvesting. Therefore, operation of tissue extraction should be careful and gentle. The harvest mouse tissues/organs are put into a 50 mL tube containing 4% PFA, and store it in a 4°C refrigerator overnight for post fixation, as shown in Figure 2I.

Note: Do not put too many tissue samples in one tube for post fixation, which may affect tissue morphology. Add enough 4% PFA to fully immerse the tissue for good fixation. The volume ratio of the sample and the 4% PFA solution should be larger than 1:10.

2D and 3D immunolabeling of VALID-casted samples (optional)

Timing: 1–5 d

In some cases, simultaneous investigation of other cell types with vascular networks assisted by immunolabeling is needed, such as parvalbumin (PV), glial fibrillary acidic protein (GFAP), tyrosine Hydroxylase (TH), NeuN, Ionized calcium binding adapter molecule 1 (Iba1) and microtubule-associated protein 2 (MAP2). VALID protocol has been proved to be compatible with immunolabeling. This step describes the detailed procedure for how to perform 2D and 3D immunolabeling on VALID-casted samples.

7. Tissue embedding and sectioning.
   a. Wash the fixed samples with 0.01 M PBS with gently shaking, refresh the PBS every 1 h for at least three times to wash out the residual PFA.
   b. Add agarose powder in pure water at the concentration of 4%. Heating the turbid liquid with a microwave oven until the powder is fully dissolved.
   c. Cool the agarose solution and immerse the desired samples into the solution until solidification of agarose.
   d. Slice the embedded samples into tissue sections with desired thickness using a commercial vibratome.
8. 2D immunolabeling for VALID-casted samples.
   a. Blocking. Fully immerse the thin tissue sections in the blocking solution for 3–6 h with gently shaking at 37°C.
   b. Primary antibody labeling. Transfer the tissue sections into the primary antibody solution (1:500 diluted in the antibody diluent buffer) for 0.5–1 d with gently shaking at 37°C. Make sure that the samples are fully immersed.
   c. Wash the samples with PBST solution for at least 6 times (20 min/ per time) to remove the residual primary antibodies.
   d. Secondary antibody labeling. Transfer the tissue sections into the secondary antibody solution (1:500 diluted in the antibody diluent buffer) for 0.5–1 d with gently shaking. Make sure that the samples are fully immersed.

   Note: The concentration of primary and secondary antibodies can be varied depending on the user guide from different manufacturer.

   e. Wash the samples with PBST solution for 6 times (20 min/ per time) to remove the residual primary antibodies.

   Note: All steps are carried out at room temperature. Protect the samples from light during the entire procedure. The immunolabeled samples are stored in PBS at 4°C before imaging

9. 3D immunolabeling for VALID-casted samples.

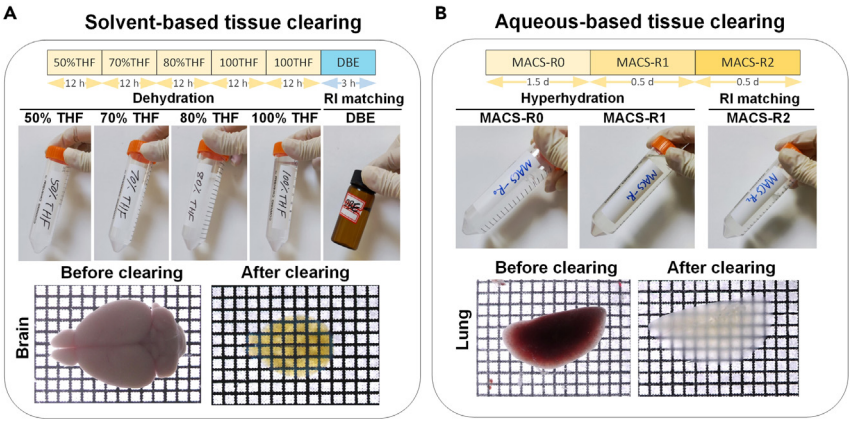

Figure 3. Examples for tissue clearing using two different types of clearing method

(A) Tissue clearing with a solvent-based clearing method, using THF for dehydration and DBE for RI matching.

(B) Tissue clearing with an aqueous-based clearing method, MACS.

a. Sample pre-treatment. Treat the samples with a sequence of methanol solution: 20%, 40%, 60%, 80%, 100%, 80%, 60%, 40% and 20%, 1 h for each step with gently shaking, then washed with PBST for 1 h.

b. Tissue permeabilization and blocking. Incubate the pre-treated samples in permeabilization solution at 37°C overnight, then transfer the samples to the blocking solution at 37°C for 1 d. Wash the samples by PTwH overnight. Refresh the PTwH solutions for at least 6 times.

c. Primary antibody labeling. Incubate the treated samples in the primary antibody dilution at 37°C with gentle shaking for 2–3 d. Wash the samples for 1 d with PTwH. Refresh the PTwH solutions for at least 6 times.

d. Secondary antibody labeling. Incubate the samples in the secondary antibody dilution at 37°C with gentle shaking for 2–3 d.

e. Wash the tissues in PTwH for 1 d. Refresh the PTwH solutions for at least 6 times.

Note: It should be noticed that if 3D immunolabeling needs to be performed, the step 8 should be skipped and the step 7 is optional (Sometimes thick tissue slices are also used for 3D immunolabeling). During the entire 3D immunolabeling process, sodium azide must be added to all used solutions to prevent the sample from decaying. All steps are performed with gently shaking.

Pause point: The immunolabeled tissues can be stored at this time in PBST/PTWH for one week before clearing and imaging. The immunolabeled samples should be stored at 4°C until use.

Tissue clearing for VALID-casted samples

Timing: 1–4 d

The VALID-casted samples with/without immunolabeling should be cleared before 3D imaging by optical tomography. This step will provide sufficient details on how to perform tissue clearing for VALID-casted samples.

10. Solvent-based tissue clearing,[6–8] as shown in Figure 3A.
    a. Preparation of gradient dehydration solutions. Use distilled water to prepare gradient methanol (MeOH), ethanol (EtOH) and tetrahydrofuran (THF) solution at concentrations of 50%, 70%, 80%, 100%, 100%. Prepare the Tert-butanol (TB) solution at concentrations of 50%, 70%, 80%, 90%, 100%, 100%.

b. Dehydrate the prepared samples using the certain gradient solutions described above. For example, dehydrate the samples by THF solutions at concentration gradients of 50%, 70%, 80%, 100%, 100%. The time for each dehydration step is 4 h (tissue slices)/12 h (whole organs).

Note: All the steps are performed with gently shaking. The volume ratio of the sample and the 4% PFA solution should be larger than 1:10.

Note: Here we provide four dehydration reagents in this step, users may only choose one for their own research. In our experience, we recommend THF for dehydration due to its relative high efficiency and robustness.

c. Transfer the dehydrated samples to pure dibenzyl ether (DBE) for refractive index matching until the samples become fully transparent.

Note: When clearing hard bones, the samples should be firstly decalcified by 20% EDTA solution for 1–2 d, the decalcified bones are then processed with tissue clearing procedure. The signals of vasculature labeled by VALID are usually very bright, the autofluorescence of samples can be ignored in most instances. If the autofluorescence within samples is very strong, such as muscle samples, it may be effective by bleaching the samples in 1%–2% H_2O_2 for 30 min to reduce autofluorescence.

11. Aqueous-based tissue clearing (MACS),[9] as shown in Figure 3B.
    a. Preparation of MACS solution. MACS-R0 contains 20 vol% MXDA and 15% (w/v) sorbitol mixed with distilled water, MACS-R1 contains 40 vol% MXDA and 30% (w/v) sorbitol dissolved in 1 × PBS. MACS-R2 contains a mixture of 40 vol% MXDA and 50% (w/v) sorbitol in distilled water.

Note: Proper heating with a water bath could promote the dissolution of sorbitol.

b. MACS clearing. Incubate the prepared samples in 20–30 mL MACS-R0, MACS-R1, and MACS-R2 solutions in 50 mL conical tubes with gentle shaking at room temperature.

Note: The time needed for each step depends on tissue size. We use 1 h, 1 h, 0.5 h for tissue slices and 1.5 d, 0.5 d, 0.5 d for whole organs. The volume ratio of the sample and the 4% PFA solution should be larger than 1:10.

Note: Users can refer to the original publications of MACS method for further details. Additionally, other aqueous-based clearing methods like Ce3D[10] and Sca*l*eS,[11] which are detergent-free, could also be employed. Note that VALID is not compatible with aqueous-based clearing methods involving large concentration detergents.

3D imaging and analysis of the vascular networks

Timing: 1–6 h

This step will describe the procedure for fluorescence imaging with confocal or light sheet microscope, as well as the detailed pipeline for 3D analysis of vascular structures via Imaris software.

12. Fluorescence imaging.
    a. Confocal imaging. Use a laser-scanning confocal microscope (LSM710, Zeiss, Germany) for imaging tissue slices.

Figure 4. Setup for light sheet imaging
(A) Overview of the LiTone XL light sheet microscope.
(B and C) Direct view of the imaging module with (B) and without (C) head cover.
(D) Magnified view of the imaging room.
(E) The sample chamber.
(F) The sample holder.
(G) Example images for cleared samples mounting in the sample holder.
(H) Interface for the acquisition software for the light sheet system.

Note: LSM 710 is equipped with a 5× objective lens (FLUAR, NA = 0.25, WD = 12.5 mm) and a 10× objective lens (FLUAR, NA = 0.5, WD = 2 mm). The Zen 2011 SP2 software (Version 8.0.0.273, Carl Zeiss GmbH, Germany) is used to collect data.

b. Light sheet imaging. Image the cleared samples (e.g., brain, spinal cord, kidney, liver, etc.) with a light sheet microscope (LiTone XL, Light Innovation Technology, China) equipped with a 4× objective lens (NA = 0.28, working distance (WD) = 20 mm).

Note: The detailed information of light sheet microscope used in this study is shown in Figure 4.

Note: In this step, we just provide the basic information of fluorescence imaging systems used in this study for reference. Users are able to employ different imaging systems in their lab to image the fluorescent-labeled vasculature.

13. Imaris-based 3D visualization and analysis of vascular networks, as shown in Figure 5.

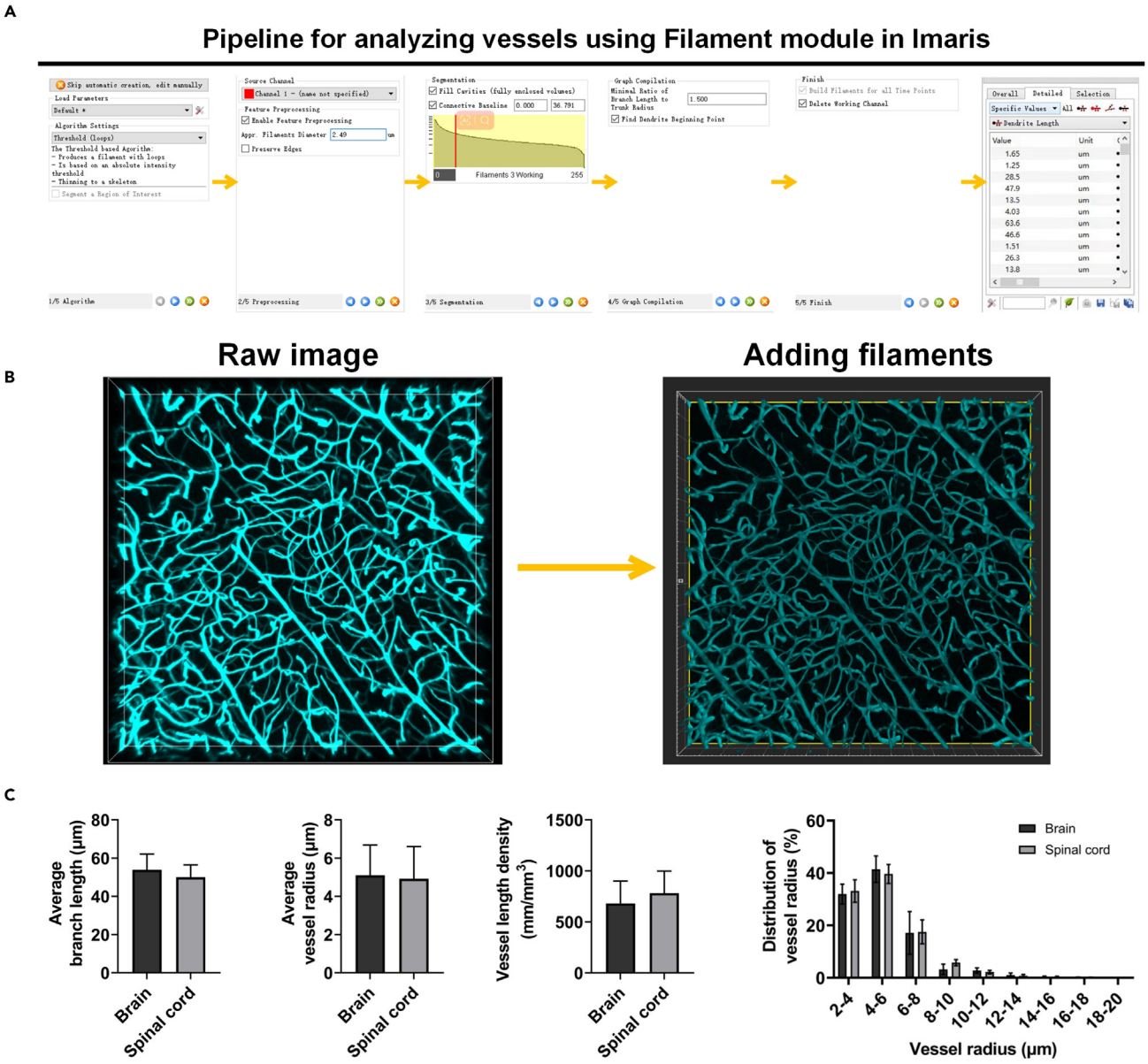

Figure 5. Visualization and quantification of blood vessels using Imaris

(A) Pipeline for analyzing vascular structures with Filament module in Imaris.

(B) Visualization and rendering of vascular imaging datasets of mouse cerebral cortex by Filaments module.

(C) Quantitative analysis of vascular structures within mouse brain and spinal cord (n = 3). All values are presented as the mean ± SD.

a. Convert acquired imaging data files (.tif, .czi) to Imaris™ file (.ims) format by dragging and dropping the files into 'Imaris™ File Converter', or directly import the .tif files into Imaris and save as .ims file.

b. Open the vascular data files in 3D view within Imaris, set the image properties based on the parameters of imported image data (e.g., resolution for X, Y, Z axis). Rotate the 3D image in the navigate function, obtain the snapshot image by clicking the 'snapshot' button at desired resolution.

c. Use the 'filament' function to perform quantitative analysis of the vascular networks.

i. Add a 'filament' to the current opened dataset, the software will direct you to choose different algorithms and parameters to conduct the analysis.
ii. We use the "Threshold (loops)" algorithm in filaments module to trace and quantify blood vessels.
iii. The parameter for filaments diameter is automatically set by the algorithm according to the voxel size. The voxel size of our data is 0.83 μm × 0.83 μm×2 μm. Threshold for extracting vascular information was manually adjusted to ensure the complete recognition of all blood vessels.
iv. After reconstruction of the vascular network, obtain the local vessel density, average radius, and the vessel segment length using the "statistics-selection" in filaments module. Export the desired datasets as excel files.

Note: Optionally, users can also choose the 'autopath (no loops)' algorithm in filaments module to trace and quantify blood vessels. In this case, users can choose tracing method including 'auto network', 'auto path' and 'manual'. And we prefer the 'auto network' because it is the easiest. However, if the data quality is not very high, the 'manual' could be selected to avoid too much error tracing.

Note: Recently, a novel open-source software, called VesselExpress, was published in Cell Reports Methods that enabled efficient analysis of vessel trees in 3D data of various organs.[12] This software can provide more comprehensive analysis of vascular networks than Imaris. It may become a powerful alternative to analyze different vascular networks in 3D.

EXPECTED OUTCOMES

Complete and uniform labeling of vascular networks with bright fluorescence with VALID

Efficient and complete labeling of vascular networks down to capillary level is a rather important step for 3D visualization and quantification of vascular structures for health and disease.[2,13,14] However, this could be still challenging with vessel labeling methods currently available. The VALID protocol offers a versatile and reliable tool for complete and uniform vascular labeling. Proper execution of VALID can result in fine labeling of the entire vascular structures within various tissues/organs at high signal-to-background ratios. The completeness of VALID-casted vascular structures was first validated by anti-CD31 antibodies. As expect, VALID finely overlie vasculature stained with CD31 (Figure 6A). Figures 6B and 6C shows the vessel labeling performance of VALID on different mouse tissue/organ sections, including mouse brain and other organs such as heart, kidney, liver, intestine, etc. It can be seen that the vascular structures are finely labeled and clearly visualized.

Successful immunolabeling on VALID-casted samples

Immunolabeling has been considered as a powerful tool for molecular labeling of various tissues in numerous life science research. Simultaneous visualization of other cell types with vascular networks assisted by immunolabeling techniques is sometimes indispensable for investigation of different vascular complications in vessel-associated diseases. We have proved that VALID reveals excellent compatibility with immunolabeling, both for traditional 2D immunolabeling and recently-developed 3D immunolabeling techniques. We employed a variety of antibodies, including parvalbumin (PV), glial fibrillary acidic protein (GFAP), tyrosine Hydroxylase (TH), NeuN, Ionized calcium binding adapter molecule 1 (Iba1) and microtubule-associated protein 2 (MAP2). Figures 7A and 7B shows the representative dual-color images containing GFAP-positive neurons and VALID-casted blood vessels. Additionally, VALID can also be combined with the latest whole-mount immunolabeling pipeline for 3D visualization of vascular structures and distribution of other cell types, such as NeuN-positive cells within mouse brain, as shown in Figure 7C.

3D imaging of the labeled vascular networks

In our previous publications,[1] we have demonstrated that VALID successfully restrained elution against organic solvents and were finely retained without apparent loss after clearing, therefore it is suitable for

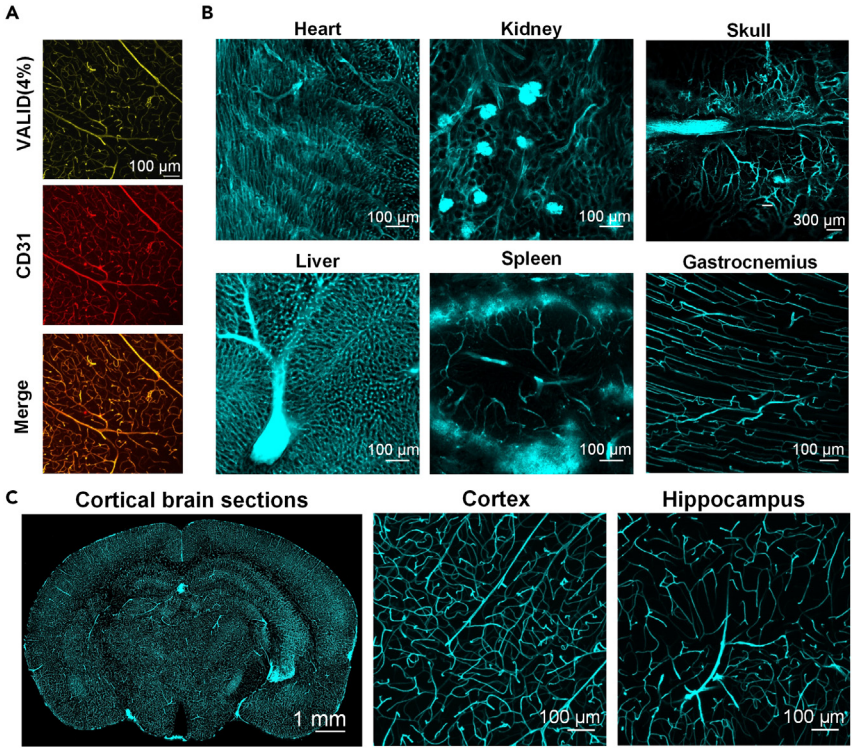

Figure 6. The vessel labeling performance of VALID on different organ types

(A) Validation of VALID with CD31 antibodies.

(B)Fluorescence images of vascular architectures labeled by VALID with DiI for different organs, including heart, kidney, skull, liver, spleen and gastrocnemius.

(C) Fluorescence images of vascular architectures labeled by VALID with DiI for mouse brain.

different tissue clearing protocols. Combining VALID with tissue clearing and light sheet microscopy allows 3D imaging and analysis of different vascular networks under physiological and pathological state.

Figure 8 displays the 3D visualization of vascular networks within mouse spinal cord.

LIMITATIONS

Though VALID proves to be an effective vessel labeling method, it does have several limitations that will potentially restrict its applications in biomedical studies.

First, the typical concentration of gelatin (2% and 4%) in the working solution for mouse have been proved to be effective for mouse and rat. When applied to high order mammalian animals, the concentrations of gelatin for the VALID protocol should be higher due to with larger size of blood vessels. However, higher concentration gelatin would lead to increased viscosity of the solution and quicker polymerization, which would potentially lead to insufficient perfusion, resulting in unexpected poor casting of blood vessels.

Second, though we find that VALID greatly enhances the compatibility of lipophilic dye signals with organic solvents-based tissue clearing methods, it does not achieve similar positive results for aqueous-based methods containing high concentration detergents. We find the formed hydrogel will be dissolved by the commonly-used detergents, leading to dye accumulation outside the tissue. Therefore, current VALID protocol is only suitable for aqueous-based methods without large proportion of detergents.

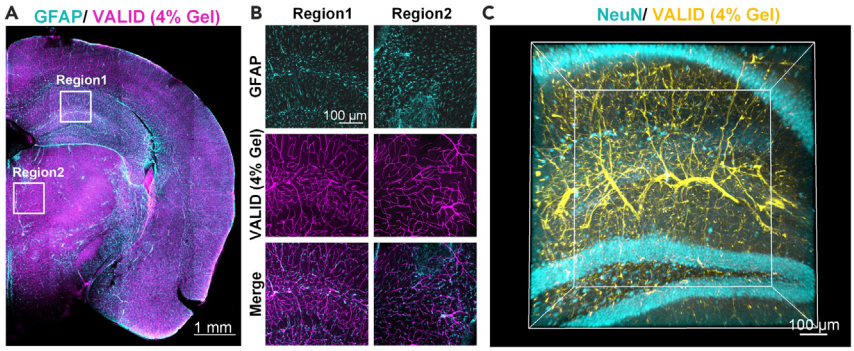

Figure 7. Example images for immunolabeling on VALID-casted brain samples

(A) 2D Fluorescence images of GFAP antibodies co-labeled with VALID.

(B) Magnified images of specific regions marked in (A).

(C) 3D imaging results of NeuN positive cells co-labeled with VALID in mouse brain tissue.

Third, the compatibility of VALID labeling with solvent-based methods varies across different organs. For example, the brain vasculature will demonstrate more robust compatibility than other organs. We speculate that this is probably because the brain vasculature possesses more strict permeability due to the existence of blood brain barrier. Additionally, the mice labeled with VALID are not perfused with PFA for immediate fixation, all tissues were post-fixed after harvesting for complete preservation of labeled fluorescence signals, which may lead to loss of RNA and immediate-early genes.

TROUBLESHOOTING

Problem 1

Poor vessel casting performance caused by various improper perfusion operation, including.

- Do not fully drain the air in the pump pipe during the entire perfusion pipeline.
- The syringe needle inserts too deep into the right ventricle.
- The syringe needle drops during perfusion.
- The dye working solution is not kept at appropriate temperature or the environment is too cold during perfusion.

Potential solution

For example, keep a watchful eye on the pump pipe during the entire perfusion pipeline to avoid air from entering, especially at the beginning of perfusion and the time point for changing perfusion buffer. It is necessary to ensure strict operation procedure and suitable environment to guarantee the success of vessel casting.

Problem 2

The fluorescent labels for vascular structures are not complete and uniform with obvious loss of vessel information, or even the blood vessels are discontinuous or broken.

Potential solution

After eliminating possible problems during perfusion, which have been already discussed in Problem 1, this problem is largely caused by the improper fixation or tissue harvesting. Prolong the cooling time after perfusion to ensure complete polymerization of the dye hydrogel or the dye may be washed out by the post fixation procedure. Given that the mouse bodies are not fixed when harvesting, researchers should be gentle and careful when harvesting desired tissues/organs, or the vascular structures within tissues/organs will be easily deformed and even damaged.

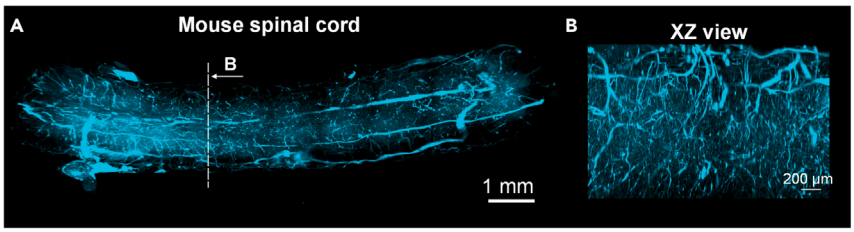

Figure 8. 3D visualization of vasculature within cleared mouse spinal cord

(A) Overview of the reconstructed vascular networks within the mouse spinal cord.

(B) XZ view of the vascular structures at location marked in (A).

Problem 3

Antibodies do not work on VALID-casted samples via 2D and 3D immunolabeling procedures.

Potential solution

It is a rather frustrating process to select suitable antibodies, because in many cases the antibodies do not work well due to unknown reasons. We recommend a pre-validation of the selected antibodies by a conventional 2D immunolabeling pipeline on freshly prepared VALID-casted samples. If the antibodies do not work, do not hesitate to use the same kind antibodies with a different product ID or produced by another company. After the validations of antibodies by 2D immunolabeing, it is also necessary to test whether the antibodies are appropriate for 3D immunolabeling. In our experience, a large proportion of antibodies that work well on VALID-casted samples under conventional conditions continue to work in the 3D immunolabeling and tissue clearing pipeline. However, we still recommend performing a quick 3D immunolabeling and tissue clearing pipeline on thin tissue slices by shorten the pre-treatment time to confirm that the antibodies are also capable for 3D immunolabeling. Careful validation of antibodies is expected to save a lot of time and cost for staining much larger samples.

Problem 4

The entire tissues/organs are not fully transparent after the tissue clearing procedure.

Potential solution

It will appear sometimes that the samples are weakly cleared after the tissue clearing procedure. In our experience, this problem may occurred under several typical conditions, and we recommend potential solutions that may improve the transparency.

Solvent-based clearing.

- The tissues are not sufficiently dehydrated. Prolong the dehydration time (For example, 1.5-fold–2-fold time) for each step, especially for the final 100% step, to achieve better clearing performance.
- The tissue contains large proportion of lipids. Immerse the fully dehydrated tissue into the Dichloromethane (DCM) for about 0.5 h–1 h as additional lipid removal step, then change the refractive index matching solution.

Aqueous-based clearing (MACS):

- The tissues are not sufficiently swelled in the first step. Increase the incubation time for tissues in MACS-R0.
- The tissues contains residual PFA. If the PFA solution is not washed out from the tissues, it will greatly influence the clearing performance.

Problem 5

The fluorescent signals of antibodies labeled by 3D immunolabeling does not penetrate to the central region of the tissue, resulting in a dark region in the center of tissues, or obviously decreased signal intensity as compared to the edges of the tissues.

Potential solution

In our experience, there are several potential factors that lead to insufficient antibody penetration, and we now list them one by one and provide potential solutions.

- The samples are not fully immersed in the buffer containing antibodies. We recommend a free-floating condition with gently shaking for the samples in the staining buffer, incomplete immersion will greatly contribute to the poor labeling performance.
- The concentration of the antibodies is too low. It may be necessary to increase the concentration of primary antibody or secondary antibody if the penetration depth is limited with weak fluorescent signals. Increase of antibody concentration could ensure there is enough antibodies to diffuse into the deep tissue without being depleted by the superficial antigen.
- The time for pretreatment during 3D immunolabeling procedure is not enough. Pretreatment of the samples with a sequence of methanol solution is critical for facilitating the antibody penetration. Properly prolong the pretreatment time for large tissue may get a better labeling results.
- The incubation time of samples in antibody buffer is too short. If the above potential problems are excluded but the expected deep structure is still not observed, it may suggest that the samples are not incubated in the primary and secondary antibody solutions for enough time. The antibodies does not reach deep tissues. Therefore, prolong the incubation time in the primary and secondary antibody buffer, for example from 2 d to 4 d, is an effective way to deal with this problem.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dan Zhu (dawnzh@mail.hust.edu.cn).

Materials availability

This study did not generate new unique materials or reagents.

Data and code availability

The data of this study are available from the corresponding authors upon reasonable request.

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AUTHOR CONTRIBUTIONS

D.Z. and J.Z. designed the study. J.Z. and X.L. performed most of the experiments. X.L. and J.D. contributed to animal preparation and data collection. J.Z., X.L., and Y.D. contributed to vessel labeling of samples. J.Z., Z.L., and J.X. contributed to data analysis. J.Z. prepared the figures and wrote the manuscript. D.Z. and T.Y. supervised the project and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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