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ab243298
Tissue Clearing Kit - hydrophobic

View Tissue Clearing Kit - hydrophobic datasheet:

www.abcam.com/ab243298

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For clearing tissue easily and quickly

This product is for research use only and is not intended for diagnostic use.

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1. Overview

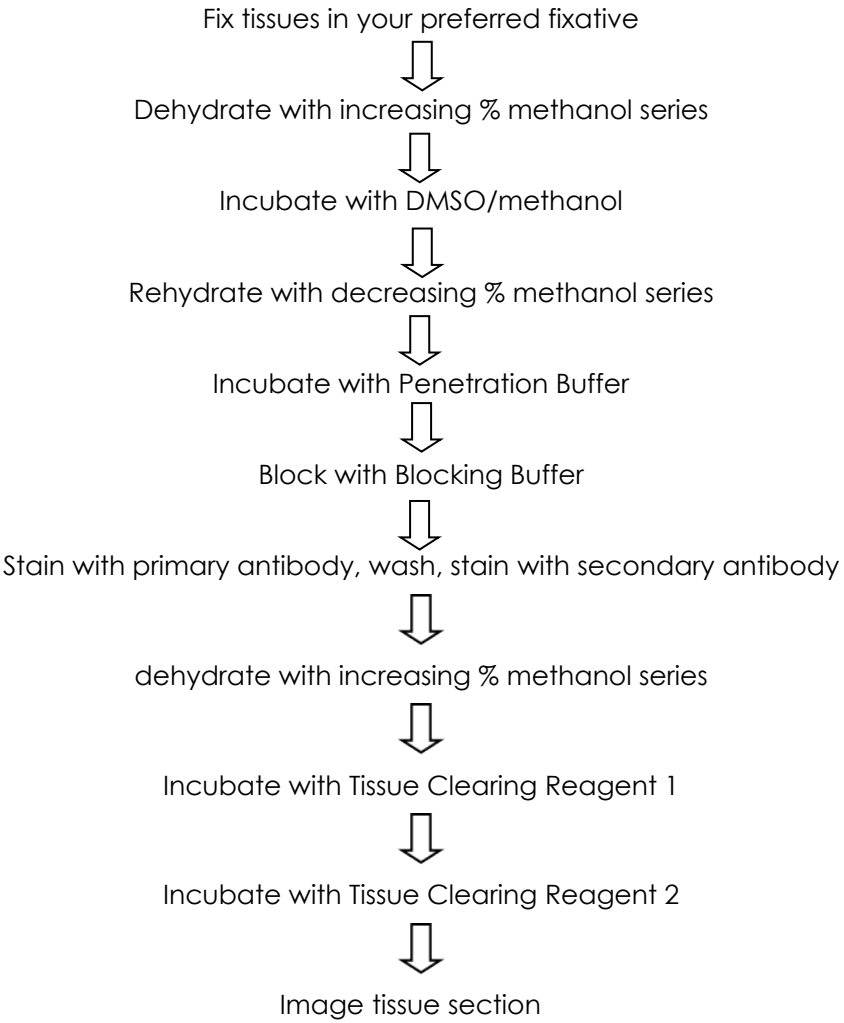
Tissue Clearing Kit - hydrophobic (ab243298) enables you to set up tissue clearing easily, and clear tissue quickly. The protocols are simple, and use standard laboratory equipment, and can be used with immunostaining, fluorescent proteins and chemical dyes. Clearing is reversible so that you can section your tissue for conventional H&E or IHC staining after 3D imaging.

The tissue clearing reagents were developed with Visikol Inc, who developed the Visikol® HISTO™ tissue clearing technology, which is the basis of our reagents.

To help you get started with tissue clearing, we have tissue clearing validated antibodies for use with this kit. Validated antibodies are for key markers, such as NeuN, Ki67, GFAP, Nestin, and are tested with 1 mm mouse brain sections, and neuronal models, HepG2, and cancer spheroid models (depending on the marker).

If you are using this kit with one of our tissue clearing validated antibodies, then please check the antibody datasheet for optimized notes for that antibody.

2. Protocol Summary



3. Materials Supplied

3.1 Contents and storage of the kits

Item	Tissue Clearing Kit - hydrophobic (ab243298)		Tissue Clearing Reagent 1 and 2 combo (ab243316)		
	30 mL size	15 mL size	10 mL size	30 mL size	100 mL size
Tissue Clearing Reagent 1	30 mL	15 mL	10 mL	30 mL	100 mL
Tissue Clearing Reagent 2	30 mL	15 mL	10 mL	30 mL	100 mL
Tissue Clearing Antibody Buffer	30 mL	15 mL	-	-	-
Tissue Clearing Blocking Buffer	30 mL	15 mL	-	-	-
Tissue Clearing Penetration Buffer	30 mL	15 mL	-	-	-
Tissue Clearing Washing Buffer 10X	70 mL	35 mL	-	-	-
Tissue Clearing Permeabilization Buffer	30 mL	15 mL	-	-	-

Table 1: Contents and storage of the kits.

- Tissue Clearing Reagent 1 and Tissue Clearing Reagent 2 should be stored at room temperature in a dry environment. Do not freeze. When stored as directed, the products are stable for 24 months from the date of receipt.
- The Tissue Clearing buffers should be stored at room temperature except for the Antibody Buffer and the Blocking Buffer which should be stored in a refrigerator (2–8°C) upon receipt and are stable for 12 months from the date of receipt.

3.2 Contents and storage of the stand-alone reagents and buffers

Item	Product Code	Sizes	Notes
Tissue Clearing Reagent 1	ab243300	30 mL and 100 mL	Refractive index of 1.50
Tissue Clearing Reagent 2	ab243301	30 mL and 100 mL	Refractive index of 1.50
Tissue Clearing Antibody Buffer	ab243303	30 mL and 100 mL	PBS with 0.2% Tween, heparin, 3% donkey serum, and 5% DMSO
Tissue Clearing Blocking Buffer	ab243304	30 mL and 100 mL	PBS with 0.2% Triton-X-100, 6% donkey serum and 10% DMSO
Tissue Clearing Penetration Buffer	ab243305	30 mL and 100 mL	PBS with 0.2% Triton-X-100, 0.3M glycine and 20% DMSO
Tissue Clearing Washing Buffer 10X	ab243306	70 mL and 200 mL	PBS with 2% Tween and 0.01% heparin
Tissue Clearing Permeabilization Buffer	ab243307	30 mL and 100 mL	-

Table 2: Contents and storage of the stand-alone reagents and buffers.

- Tissue Clearing Reagent 1 and Tissue Clearing Reagent 2 should be stored at room temperature in a dry environment. Do not freeze. When stored as directed, the products are stable for 24 months from the date of receipt.
- The Tissue Clearing buffers should be stored at room temperature except for the Antibody Buffer and the Blocking Buffer which should be stored in a refrigerator (2–8°C) upon receipt and are stable for 12 months from the date of receipt.

4. Materials Required, Not Supplied

These materials are not included in the kit but will be required to successfully perform this assay.

Item
3D Cultures, organoids, spheroids, tissue, or animal organs (use positive and negative controls as needed)
Slides, coverslips, containers
Primary or secondary antibodies
PBS (phosphate buffered saline), pH 7.4 (without calcium, magnesium, or phenol red)
Ethanol (for samples containing fluorescent proteins)
Methanol (for samples without fluorescent proteins)
Hydrogen peroxide solution
DMSO, Anhydrous
4% formaldehyde, methanol-free
Sodium Azide

5. General guidelines, precautions, and troubleshooting

- Please observe safe laboratory practice and consult the safety datasheet.
- For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide: www.abcam.com/assaykitguidelines
- For typical data produced using the assay, please see the assay kit datasheet on our website.

6. Guidelines for Optimizing Antibody Concentration

– If you are using an antibody for the first time, we recommend that you validate the antibody and optimize its concentration. Antibody concentration required for the workflow can be different for thicker tissues or 3D cell culture models than for thinner sections. Thicker sections require longer incubations and make workflow times longer. Therefore, we recommend that you validate the antibody of interest using thin tissue sections first.

6.1 Fix the tissue sections with 4% paraformaldehyde overnight at 4°C. Do not over-fix the tissues.

6.2 For antibody validation and optimization, consider using tissue sections 100–250 µm thick. You will need approximately 5 tissue sections to complete the validation and optimization.

6.3 Label tissue sections using various concentrations of the primary antibody, ranging from 1:50 to 1:500 (e.g. 1:50, 1:100, 1:200, 1:300, 1:500), diluted in Tissue Clearing Antibody Buffer.

6.4 Usually, a 1:100 dilution of the secondary antibody works well. However, you might have to optimize the secondary antibody concentration if you observe low signal or high background.

6.5 You can validate antibody staining using a typical fluorescent microscope. Prepare a slide of the cleared tissue and examine for specificity of signal.

6.6 To evaluate the evenness of staining, image the tissues using a confocal microscope. Obtain a z-stack image spanning the entire thickness of the tissue section using two color channels: the channel corresponding to the fluorescent conjugate for antibody staining, and the channel used for nuclear stain. Because nuclear stains penetrate tissues rapidly and homogenously, the nuclear stain channel serves as a control for optical attenuation.

6.7 Examine the z-stacks in ImageJ program (or other image processing software). Observe the XZ and YZ planes by viewing "Orthogonal Views" and examine the evenness of staining.

Δ Note: ImageJ is a public domain image processing and analysis program available from NIH at https://imagej.nih.gov/ij/index.html.

6.8 If the staining is even, you should see relatively consistent intensity (with respect to nuclear stain) across the tissue

(Figure 1). Some dimming in the inner layers is expected, but signal should be visible across tissue.

6.9 If the concentration of the immunolabel is too high, you will see a bright ring of staining at the surface layers, with uneven staining at a lower intensity deeper into the tissue.

6.10 If the concentration of the immunolabel is too low, you will see slight staining at the surface layer, a dark interior, and uneven spots of stain.

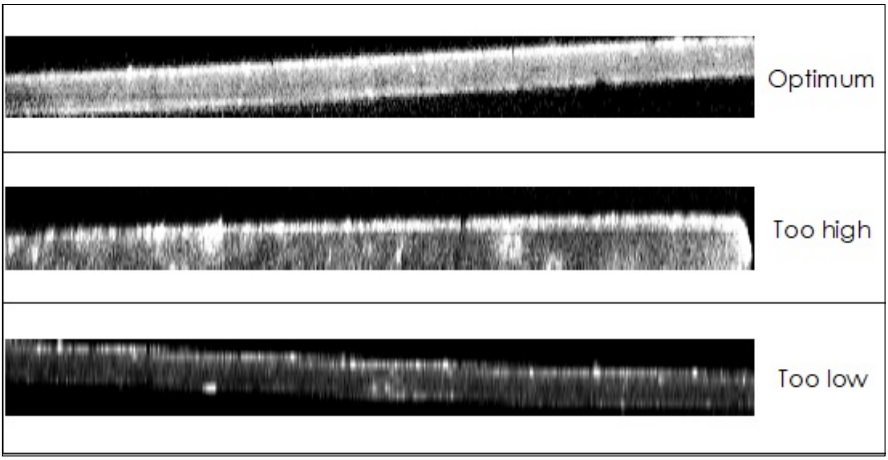


Figure 1. Evaluating the evenness of staining. Tissue sections were imaged using a confocal microscope and a z-stack spanning the entire thickness of the tissue was obtained. The XZ plane was examined for the evenness of staining. Top panel: Optimum antibody concentration. Middle panel: Antibody concentration is too high. Bottom panel: Antibody concentration is too low.

7. Sample Preparation

7.1 For easier-to-clear tissues like brain, lung, intestine, and skin with less than 250 µm thickness, treatment with Tissue Clearing Reagent 1 is sufficient for 3D imaging.

7.2 However, tissues thicker than 250 µm, or difficult-to-clear tissues such as kidney, liver, heart, and placenta, require an initial treatment with Tissue Clearing Reagent 1, followed by treatment with Tissue Clearing Reagent 2.

7.3 For 3D cell culture models up to 1 mm in thickness, we recommend using Tissue Clearing Reagent M (ab243299).

7.4 For hard tissues like bone, cartilage, and connective tissue, the Visikol® HISTO™ tissue clearing technology is not recommended.

7.5 For first-time users, we recommend the Tissue Clearing Kit - hydrophobic (ab243298) for whole tissues, which contain all the reagents required for the workflows described here.

7.6 All three clearing reagents, Tissue Clearing Reagent 1, Tissue Clearing Reagent 2, and Tissue Clearing Reagent M can also be used as imaging solutions during imaging on a fluorescent imaging instrument.

7.7 For first time users, we highly recommend cutting thicker tissues into 1-2 mm thick sections using a device such as the 1 (ab243315) or 2 mm (ab243312) Coronal Mouse Brain Slicer or the 1 (ab243314) or 2 mm (ab243313) Sagittal Mouse Brain Slicer.

7.8 Best results are obtained with tissues that have been fixed by perfusion with 4% paraformaldehyde. Immersion fixation in 10% neutral buffered formalin is also acceptable, but tissues larger than 6 mm (e.g. whole brains) should be perfused with ice-cold 4% paraformaldehyde.

7.9 If perfusion is not possible, slice several channels into the tissue ("bread-loafing") to allow penetration of the fixative to avoid autolysis from incomplete fixation of center portion of tissues. Place the tissues in a container containing fixative at a volume that is approximately 10X the volume of tissue. Ensure that the tissue is completely submerged in solution and at 4°C overnight, followed by incubation for 1 hour at room temperature. Finally, if long-term (>1 week) storage is required, transfer tissues to PBS with 0.05% sodium azide as a preservative. Otherwise, transfer tissues to PBS and proceed with further processing.

7.10 We recommend large tissues (e.g. whole mouse or rat brains) to be perfusion fixed, as immersion fixation of large tissues can lead to incomplete fixation, autolysis, and necrosis.

7.11 Except where otherwise stated, perform all steps in the procedure at room temperature (20°C) with gentle agitation. If background noise is a significant problem when visualizing your endogenous fluorescence, conduct all steps at 4°C and use 100% dry ethanol instead of methanol.

7.12 For liver, kidney, and lymphatic tissues, you may need to extend incubation times by 30–50%, depending on degree of fixation.

7.13 Use 100% dry ethanol for all steps involving ethanol.

7.14 Tissue Clearing Reagent 2 damages polystyrene. Use glass or polypropylene containers and tubes instead.

7.15 You can perform the tissue clearing steps (Steps 9.1.16, 9.1.17 and 9.1.18) within a chamber constructed by sticking the Tissue Imaging Chamber on a glass coverslip or slide (Silicon Imaging Chamber (1.75 mm) (ab243309); Silicon Imaging Chamber (3.5 mm) (ab243311); Silicon Imaging Chamber (7 mm) (ab243310)). The Tissue Imaging Chambers are made of silicon, which is compatible with the Tissue clearing reagents, making them ideal containers for the clearing steps and for use as imaging chambers.

8. Reagent Preparation

8.1 Tissue Clearing Washing Buffer 10X

- Dilute to 1X with PBS, pH 7.4 before use.

8.2 Ethanol solutions for samples containing fluorescent proteins

- For samples containing fluorescent proteins, prepare 30% and 50% ethanol solutions by diluting a higher concentration ethanol solution in PBS, pH 7.4.
- Prepare 70% and 90% ethanol solutions by diluting a higher concentration ethanol solution in deionized water. For best results, ensure that the 100% ethanol used in the last step of dehydration is completely dehydrated.

8.3 Methanol solutions for samples without fluorescent proteins

- For samples without fluorescent proteins, prepare 50% methanol solution by diluting a higher concentration methanol solution in PBS, pH 7.4.
- Prepare 70% and 90% methanol solution by diluting a higher concentration methanol solution in deionized water. For best results, ensure that the 100% methanol used in the last step of dehydration is completely dehydrated.

8.4 5% H_2O_2 for samples with extensive pigmentation

- For samples with extensive pigmentation (liver, kidney), prepare ice-cold 5% H_2O_2 in 20% DMSO/methanol (1 part 30% H_2O_2, 1 part 100% DMSO, 4 parts 100% methanol).

  Δ Note Bleaching with this solution is not compatible with fluorescent protein staining.

9. Protocol for Immunolabelling Tissues

- The following protocol describes a general procedure for immunolabeling and clearing a variety of tissues.
- If you are analyzing samples and combining imaging of fluorescent proteins with immunolabeling, then use this protocol. If you are only imaging fluorescent proteins, then use the fluorescent proteins protocol in the Appendix (Section 13.1).
- Refer to Table 3 for the suggested incubation times and Table 4 for the required reagent volumes to immunolabel and clear your tissue of interest.
- Except where otherwise stated (samples with fluorescent protein), perform all steps in the procedure at room temperature (20°C) with gentle agitation.
- Stopping points are noted in the protocol and detailed in subsection 9.2.
- The protocol is written for samples that do not contain fluorescent protein. Please refer to subsection 9.3 for modifications to the protocol when working with samples with fluorescent protein.

Thickness	8 mm (e.g. whole mouse brain)	4 mm (e.g. mouse brain hemi-sphere)	2 mm	1 mm	500 µm	≤ 250 µm
Permeabilization and dehydration	2 h	2 h	90 min	40 min	16 min	8 min
Penetration/ Permeabilization	8 h	6 h	4 h	2 h	1 h	30 min
Blocking [1]	120 h	80 h	28 h	10 h	3 h	1 h
Antibody incubation [1]	240 h	80 h	28 h	10 h	3 h	90 min
Washing steps	4 h + overnight for last wash	2 h + overnight for last wash	90 min	1 h	40 min	20 min
Incubation in Tissue Clearing reagents [1] [2]	48 h	36 h	12 h	4 h	30 min	10 min
[1] For liver, kidney, and lymphatic tissues, extend incubation time by 30–50%, depending on degree of fixation. [2] Tissue Clearing Reagent 1 only, or Tissue Clearing Reagent 2 and Tissue Clearing Reagent 2, depending on the tissue thickness.						

Table 3: Suggested incubation times for immunolabeling and clearing tissues.

Thickness	8 mm (e.g. whole mouse brain)	4 mm (e.g. mouse brain hemi-sphere)	2 mm	1 mm	500 µm	≤ 250 µm
Permeabilization and dehydration	25 mL	13 mL	8 mL	4 mL	3 mL	2 mL
Penetration/ Permeabilization /Washing	20 mL	10 mL	6 mL	4 mL	2 mL	1.6 mL
Blocking/ Antibody incubation	10 mL	5 mL	3 mL	2 mL	1 mL	0.8 mL
Clearing	10 mL	7 mL	5 mL	3 mL	2 mL	1 mL

Table 4: Reagent volumes required for immunolabeling and clearing tissues.

9.1.1 Obtain tissues of interest and fix them, if needed. See Section 7 for guidelines on fixation.

9.1.2 Wash tissues twice in PBS, pH 7.4 (without calcium, magnesium, or phenol red) for at least 1 hour.

9.1.3 (Optional for most tissues) Incubate tissues that are particularly difficult to clear due to the presence of pigment, collagen, or blood (e.g. liver tissue, whole kidney, over-fixed human tissues) in Tissue Clearing Permeabilization Buffer overnight with gentle shaking before proceeding with permeabilization.

Δ Note: See section 9.3.1 for note on tissues that contain fluorescent protein.

9.1.4 Permeabilize tissues by washing them in increasing concentrations of methanol at 4°C with gentle agitation. See Tables 3 and 4 for required volumes and incubation times.

9.1.4.1 Wash tissues twice in PBS, once in 50% methanol in PBS, 80% methanol in deionized water, and finally in 100% dry methanol.

Δ Note: See section 9.3.2 for modification for tissues that contain fluorescent protein.

Δ Note: Stopping point 1 (see 9.2.1)

9.1.5 (Optional) Bleach tissues containing substantial quantities of blood or pigment (such as non-perfused heart, lung, kidney, or liver tissue) by submerging them in ice-cold 5% H_2O_2 in 20% DMSO/methanol (1 part 30% H_2O_2, 1 part 100% DMSO, 4 parts 100% methanol) and incubating at 4°C overnight. This step significantly reduces background fluorescence caused by hemoglobin.

Δ Note: See section 9.3.3 for note on tissues that contain fluorescent protein.

9.1.6 Wash samples before proceeding with further staining:

9.1.7 Wash the tissues in 20% DMSO/methanol, in 80% methanol in deionized water, in 50% methanol in PBS, in 100% PBS, and finally in PBS with 0.2% Triton X-100.

Δ Note: See section 9.3.4 for modification for tissues that contain fluorescent protein.

Δ Note: Stopping point 2 (see 9.2.2)

9.1.8 Incubate the samples in Tissue Clearing Penetration Buffer with gentle shaking.

9.1.9 Block the samples in Tissue Clearing Blocking Buffer with gentle shaking at 37°C.

Δ Note: Stopping point 3 (see 9.2.3)

9.1.10 Transfer the samples to primary antibody dilutions prepared in Tissue Clearing Antibody Buffer and incubate at 37°C with gentle shaking.

Δ Note: For most broadly expressing epitopes, a dilution of 1:50 to 1:500 is typically required, but antibody concentration should be optimized for tissues according to the guidelines described in Section 6.

Δ Note: Stopping point 4 (see 9.2.4)

9.1.11 Wash the samples 5 times in Tissue Clearing Washing Buffer 10X (diluted to 1X in PBS; see Step 8.1) with gentle shaking.

Δ Note: Stopping point 5 (see 9.2.5)

9.1.12 If using secondary antibody detection, incubate the samples in secondary antibody dilutions (1:50 to 1:500, depending on the dilution of the primary antibody) in Tissue Clearing Antibody Buffer at 37°C with gentle shaking.

Δ Note: Stopping point 6 (see 9.2.4)

9.1.13 (Optional) Add nuclear stain (e.g. DAPI) to a dilution of 1:1,000 to 1:5,000 (depending on the stain). You can perform this step concurrently with antibody labeling steps, or separately in Tissue Clearing Washing Buffer.

9.1.14 Wash the samples 10 times in Tissue Clearing Washing Buffer, 5 – 90 minutes each time, at 37°C, with gentle shaking. You can keep the samples in Tissue Clearing Washing Buffer indefinitely before proceeding with the subsequent steps.

Δ Note: Samples which have not been stained with antibodies normally require only 3 washes. If excess background staining still occurs, increase the number of washes.

Δ Note: Stopping point 7 (see 9.2.2)

9.1.15 Dehydrate the tissues with increasing concentrations of methanol at 4°C with gentle shaking. See Tables 4 and 5 for required volumes and incubation times. Using an excess volume in the dehydration steps ensures proper clearing.

9.1.15.2 Treat tissues with 50% methanol in PBS, then with 80% methanol in deionized water, and finally in 100% methanol with gentle shaking.

Δ Note: See section 9.3.5 for modification for tissues that contain fluorescent protein.

Δ Note: Stopping point 8 (see 9.2.5)

9.1.16 Remove the tissues from methanol. Ensure that all excess methanol is absorbed with a paper towel and removed from the sample.

Δ Note: See section 9.3.6 for modification for tissues that contain fluorescent protein.

9.1.17 Add Tissue Clearing Reagent 1 to completely cover the sample with gentle shaking (conduct at 4°C for samples with fluorescent protein).

Δ Note: Required reagent volume and clearing time vary with tissue sample size (see Table 3 and 4). However, tissue clearing can be accelerated substantially at 37°C with gentle shaking without damage to tissue, at the compromise of increased autofluorescence.

Δ Note: Incubation in Tissue Clearing Reagent 2 (Step 9.17) requires the use of glass or polypropylene containers. Other plastic vessels are not compatible with the enhancer and will ruin the samples.

9.1.18 Transfer larger or thicker tissues (>200 µm) to Tissue Clearing Reagent 2 to finish the clearing process with gentle agitation. Otherwise, directly proceed to Step 9.1.19 (conduct at 4°C for samples with fluorescent protein).

Δ **Note**: Larger tissue samples should be imaged in Tissue Clearing Reagent 2.

Δ **Note**: You can perform the tissue clearing steps within a chamber. See 7.15.

Δ **Note**: Stopping point 9 (see 9.2.6)

9.1.19 Image the cleared samples using confocal, light sheet, or single or multi-photon microscopy.

Δ **Note:** Samples should be mounted for imaging in Tissue Clearing Reagent 1 or 2.

9.2 Stopping Points

– These are optional.

9.2.1 You can store the tissues in methanol (samples without fluorescent protein) or ethanol (samples with fluorescent protein) at 4°C for up to 2 weeks without detrimental effects.

9.2.2 You can store the tissues at 4°C in the dark for up to 3 days without detrimental effects.

9.2.3 You can store the tissues at 4°C for up to 1 month without detrimental effects.

9.2.4 You can store the tissues at 4°C for up to 2 weeks without detrimental effects.

9.2.5 You can store the tissues at 4°C for up to 3 days without detrimental effects.

9.2.6 You can seal and store the cleared samples at 4°C in the dark indefinitely without detrimental effects. Depending on the sample type and the fluorophore, mounted samples can be imaged weeks to months after mounting.

9.3 Modifications for when using sections containing Fluorescent Proteins

9.3.1 Tissue Clearing Permeabilization Buffer cannot be used if immunolabeling tissues that contain fluorescent protein.

9.3.2 Permeabilize tissues by washing them in increasing concentrations of ethanol at 4°C with gentle agitation. See Tables 3 and 4 for required volumes and incubation times.

9.3.2.3 Wash tissues twice in PBS, once in 50% ethanol in PBS, 80% ethanol in deionized water, and finally in 100% dry ethanol (conduct at 4°C).

9.3.3 Bleaching samples with 5% H_2O_2 in 20% DMSO and methanol is not compatible with imaging fluorescent proteins.

9.3.4 Wash samples before proceeding with further staining:

9.3.4.4 Wash the tissues in 20% DMSO/ethanol, in 80% ethanol in deionized water, in 50% ethanol in PBS, in 100% PBS, and finally in PBS with 0.2% Triton X-100 (conduct at 4°C).

9.3.5 Dehydrate the tissues with increasing concentrations of ethanol at 4°C with gentle shaking. See Tables 3 and 4 for required volumes and incubation times. Using an excess volume in the dehydration steps ensures proper clearing.

9.3.5.5 Treat tissues with 50% ethanol in PBS, then with 80% ethanol in deionized water, and finally in 100% ethanol with gentle shaking (conduct at 4°C).

9.3.6 Remove the tissues from ethanol. Ensure that all excess ethanol is absorbed with paper towel and removed from the sample.

10. Imaging

- To achieve 3D volume imaging, you need to use a confocal microscope, a light sheet microscope, or a single/multiphoton microscope.
- Imaging a whole mouse brain in 3D requires a light sheet microscope with long working distance Refractive index-matched objectives (>6 mm).
- Imaging a 200 micron thick section of tissue requires an inverted or upright confocal microscope with a wide range of objectives.
- Imaging tissue of 1 mm thick or less can be done with almost every imaging set-up.
- RI mismatch is the main problem encountered when imaging cleared tissues. Cleared specimens have a RI of 1.50 or 1.53 (Tissue Clearing Reagent 1 or Tissue Clearing Reagent 1), very equivalent to glass.

10.1 Confocal microscopy

- The most important consideration to make prior to imaging cleared tissue specimens is the microscope objective available on the confocal instrument. The two most important aspects to consider are:
- 1) whether the objective is an air, water, glycerol, or oil objective;
- 2) the working distance and numerical aperture of the objective lens.

10.1.1 Imaging with air objectives

- While air objectives are not ideal for visualizing whole organs, excellent results can and have been obtained for tissues up to 1-2 mm thick. This is accomplished by visualizing tissues 500-1,000 µm thick and then flipping the tissue over and repeating.
- One can compensate somewhat for attenuation of signal by adjusting the gain setting as the scan proceeds through the cleared tissue. There may be some spherical aberration as depth approaches the maximum, and this may result in "smearing" across the z-axis (this can easily be seen in xz or yz projections of image stacks in ImageJ by going to command Image>Stacks>Orthogonal Views).

- Most air objectives have a working distance of several millimeters, and so the only limit to imaging is related to the attenuation of signal from aberration. A numerical aperture (NA) of less than 0.3 is not appropriate for volume imaging, as the depth of field at 500 nm wavelength light is >40 microns, which is insufficient z-resolution for volume imaging, since adjacent optical sections will bleed through to the focal plane with such a wide depth of field.

10.1.1.1 Mounting Specimens for imaging with an air objective

- An imaging chamber can be constructed by sticking a Tissue Imaging Chamber on a glass coverslip or slide (Silicon Imaging Chamber (1.75 mm) (ab243309); Silicon Imaging Chamber (3.5 mm) (ab243311); Silicon Imaging Chamber (7 mm) (ab243310)). The Tissue Imaging Chambers are made of silicon, which is compatible with the Tissue clearing reagents.

10.1.2 Imaging with immersion objectives

- Immersion objectives were developed to reduce scattering and noise caused from refractive aberrations.
- Immersion objectives are well-suited to deep tissue imaging due to the resultant reduction of RI mismatch between sample and objective, as the lens is immersed directly in the medium, eliminating mismatch between the liquid medium and air, typically encountered for air lenses.

10.1.2.1 Oil immersion lenses

- The working distance on oil immersion lenses is typically 250 microns or less, which presents a 500 micron limit on the thickness of tissue to image.
- Since some objectives may be corroded or damaged from immersion in Tissue Clearing Reagent 2, you should utilize a double chambered cuvette. The cuvette allows the sample to be mounted in Tissue Clearing Reagent 2 in the inner cuvette, and fill the outer cuvette with water/glycerol mixture to match RI of the objective.

10.1.2.2 Water immersion lenses

- Allow for 2-3 mm depth of imaging, however some RI mismatch will still occur, since water has a RI (1.33) significantly different than Tissue Clearing Reagent 2 (1.53).

10.1.2.3 Glycerol immersion lenses

- Improvement in imaging depth can be obtained by using glycerol immersion objectives. Some RI mismatch will still occur, however substantially less aberration will result due to the closer match in RI between glycerol (1.47) and Tissue Clearing Reagent 2 (1.53). Double chambered cuvettes should be used.

10.1.2.4 Multi-immersion objectives

- When imaging very thick tissues (> 1 mm) or whole organs, one should use multi-immersion objectives containing a correction collar. However, a correction collar isn't required for immersion objectives if proper optical design has been done to allow imaging over a range of RI.
- The objectives (shown in Table 5) offer the deepest tissue imaging, allowing for imaging of complete, intact organ systems. The only limitation to depth with these objectives is the effective working distance, since a sample approximately twice the thickness of the working distance length can be imaged.

Model (Manufacturer)	Working Distance	Numerical Aperature	Refractive Index Range
Leica - 25X FLUOTAR	6 mm	1.0	1.45
Zeiss - 20X LSFM Clearing	5.6 mm	1.0	1.45
Olympus - 10X UIS2-XLPN10XSVMP	8 mm	0.6	1.33 – 1.52
Olympus - 25X UIS2-XLSPLN25XGMP	8 mm	1.0	1.41 – 1.52
ASI – Immersion Objective for Cleared Tissues	12 mm	0.37 – 0.43	1.33 – 1.56

Table 5: Objectives optimized for deep tissue imaging.

10.2 Light Sheet Microscopy

- Many light sheet microscopes such as the ZEISS Z.$_1$ and the OpenSPIM will have water immersion objectives that are matched to a RI of 1.33. As mentioned in 10.1.2.2 these objectives have disadvantages for imaging tissues that have been cleared with Tissue Clearing Reagent 2:

  1) The mismatch in RI will limit imaging depth to approx. 3 mm due to spherical aberrations, and overall sample thickness to 6 mm if you image from both sides.

  2) The sample will need to be mounted in a cuvette that is placed within the imaging chamber (the imaging chamber cannot be filled with Tissue Clearing Reagent 2 when using water immersion objectives as it is possible that the objective can be damaged by Tissue Clearing Reagent 2).

- A Solvent compatible light sheet imaging system like the LaVision BioTec Ultramicroscope allows imaging directly in Tissue Clearing Reagent 2. It has been designed to work with almost every clearing solution (1.33 to 1.56) and will not get damaged by solvents.

10.2.1 Mounting specimens

- For mounting specimens for imaging, we suggest using thick microscope slides with deep wells. An imaging chamber can be constructed by sticking a Tissue Imaging Chamber on a glass coverslip or slide (Silicon Imaging Chamber (1.75 mm) (ab243309); Silicon Imaging Chamber (3.5 mm) (ab243311); Silicon Imaging Chamber (7 mm) (ab243310)). The Tissue Imaging Chambers are made of silicon, which is compatible with the Tissue clearing reagents.
- The sample is placed in the well, covered with Tissue Clearing Reagent 2 and then a cover slip is placed on top of the slide, allowing usage with water or glycerol immersion objectives.

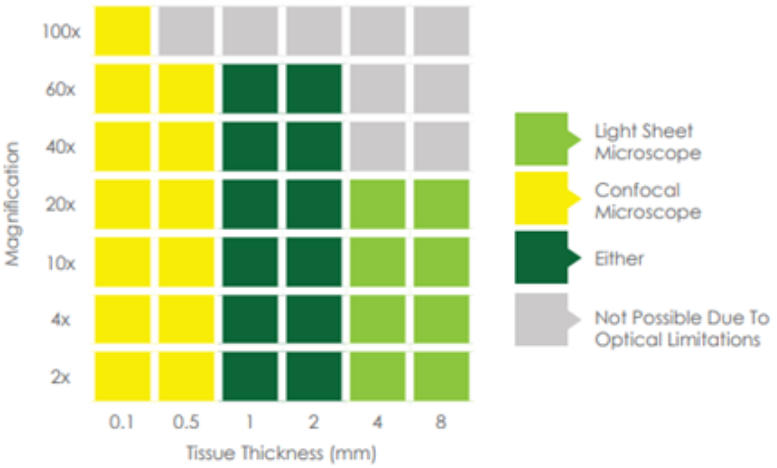


Figure 2. Imaging guidelines.

11. Reverse Tissue Clearing

– The tissue clearing process is non-destructive and reversible, allowing traditional 2D histology to be conducted after 3D imaging. Because of the reversible nature of this approach, the tissue clearing method can be integrated into the many bio-imaging processes without disrupting the other assays or histological processing or traditional workflows.

11.1 Place cleared tissue directly into a large volume (at least 10–20 times tissue volume) of absolute or histological grade ethanol or methanol. Leave tissue at room temperature until opacity has been restored.

11.2 Larger and more vascular tissues (e.g. whole kidney) may require 2–3 washes of alcohol over the course of several hours.

11.3 After reversal, samples can be processed directly for paraffin-embedding histological preparations.

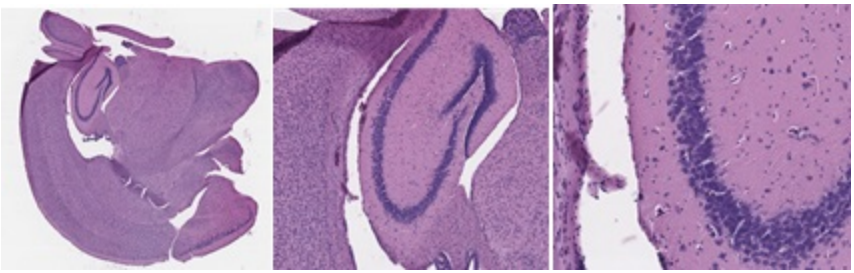


Figure 3A. Untreated mouse brain tissue section was formalin-fixed and paraffin-embedded, then stained with H&E, depicting the hippocampus.

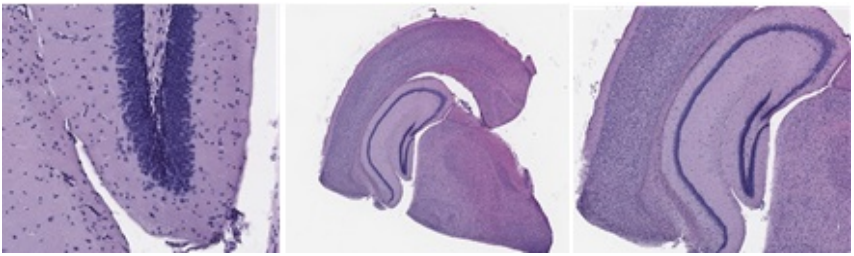


Figure 3B. Mouse brain tissue was cleared using the tissue clearing technique. Cleared tissue was then reversed, embedded in paraffin, sectioned, and stained with H&E, depicting hippocampus. Tissue Clearing workflow does not appreciably affect tissue histology.

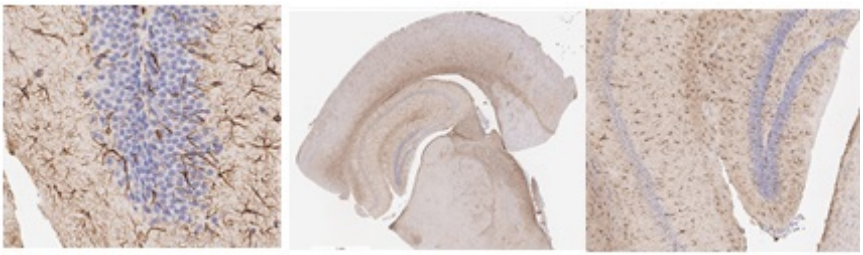


Figure 3C. Mouse brain tissue was cleared using the tissue clearing technique. Cleared tissue was then reversed, embedded in paraffin, sectioned, and immunostained for GFP, labeling astrocytes. The tissue clearing workflow does not affect antigenicity of tissues.

12. Troubleshooting

Problem	Reason	Solution
Cannot image past 500-1,000 µm. Labelling appears uneven and drops off significantly at this depth	Antibody concentration is too high: ring of intense staining near the surface, drops off significantly after that.	Reduce antibody concentration. If the signal is too weak, use a lower antibody concentration for half of the time, then re-incubate with antibodies at a higher concentration.
	Antibody concentration too low: signal drops off in the middle of the tissue.	Increase antibody concentration
	Optical attenuation due to absorption of photons by the upper tissue layers "shadows" the tissues below even with perfect staining.	- Increase laser power and gain with increasing depth. Some microscopes can automate laser power and gain corrections. Δ Note: Higher laser power increases the rate of photobleaching. - Ensure that the samples contain no air bubbles. - Compare intensity loss to nuclear stain intensity. Because nuclear stain diffuses very fast into tissues, you can use this signal to correct for signal loss in image processing.
There is an intense band of labeled tissue at the surface, then a significant drop-off afterwards.	Antibody concentration too high.	Reduce antibody concentration by increasing the dilution factor.
Tissue did not clear.	Plastic incompatibility.	Tissue Clearing Reagent 2 degrades polystyrene. Use polypropylene and glass in your workflow, where possible. Plastic leaching into your sample can affect the clearing ability of reagents.
		- Ensure that you are using pure,

	Incomplete dehydration /clearing.	dehydrated ethanol or methanol for drying. Impure methanol or ethanol that contains water will not remove all the water from the tissue, resulting in cloudiness. - Ensure that the sample vessel is sealed properly. Tissue Clearing Reagent 2 is hygroscopic and will draw water. - Ensure that you are using sufficient volume of Tissue Clearing Reagent 1 or 2 for your tissue size. Using insufficient volume of can cause inadequate clearing.
Fluorescent protein looks quenched.	Sample containing fluorescent protein is dehydrated using methanol.	To visualize fluorescent proteins, samples must be dehydrated using ethanol at 4°C instead of methanol.
	Sample is bleached.	- Keep cleared samples in the dark and cover them with aluminum foil, because fluorescent proteins photobleach rapidly when exposed to ambient light. - Do not treat fluorescent protein-labeled samples with H_2O_2. This step oxidizes fluorescent proteins, resulting in loss of signal. - Do not treat fluorescent protein-labeled samples with permeabilization buffers.
	Background fluorescence too high.	Shift all steps in the protocol to 4°C and increase their duration by 50%.
Antibody did not label the tissue.	Antibody is not compatible with 3D immunolabeling.	- Validate the specificity of your antibody on small tissue sections before proceeding to larger tissues. Contact Technical Support, if you have any questions about your specific antibody. - Only use antibodies that have been validated for use in IHC. If IHC validated antibody is not available, then IF/ICC validated antibody might also work.
Center of Tissue	Antibody	Increase the antibody

Looks dark.	concentration too low.	concentration. Explore a range of antibody concentrations on a small section of the tissue before scaling to large tissues.
	Optical attenuation.	- Optical attenuation leads to diminished signal at increasing depths depending on several factors, such as concentration of label bound in upper layers of the tissue, level of autofluorescence, type of objective, and laser power. - Modify laser power and gain according to tissue depth to account for optical attenuation. This can be automated in systems such as the Leica SP5 and SP8. - Histogram matching during image processing can account for optical attenuation at the cost of increased noise at greater depths.

13. Appendix

13.1 PROTOCOL FOR CLEARING TISSUES WITH FLUORESCENT PROTEINS WHERE YOU ARE NOT ALSO IMMUNOLABELING

- The following protocol describes a general procedure for clearing a variety of tissues ranging in size from whole rat brains to 3D cell culture models (e.g. organoids, microtissues, spheroids).
- The procedure is effective at clearing unfixed tissues, tissues fixed with a variety of fixatives, as well as tissues that have been stored in formalin for years.
- Refer to Table 6 for the suggested incubation times, volumes, and considerations for your particular tissue of interest.
- Except where otherwise stated, perform all steps in the procedure at room temperature (20°C) with gentle agitation.

Thickness	Ethanol dehydration	Volume of ethanol for each step	Incubation in Visikol HISTO clearing reagents [1]	Volume of Visikol tissue clearing reagents [2]
8 mM (e.g. whole mouse brain)	4 h	25 mL	48 h	10 mL
4 mm (e.g. mouse brain hemisphere)	2 h	13 mL	36 h	7 mL
2 mm	90 min	8 mL	12 h	5 mL
1 mm	40 min	4 mL	4 h	3 mL
500 µm	16 min	3 mL	30 min	2 mL
≤ 250 µm	8 min	2 mL	10 min	1 mL
[1] For liver, kidney, and lymphatic tissues, extend incubation time by 30 – 50%, depending on degree of fixation. [2] Tissue Clearing Reagent 1 only, or Tissue Clearing Reagent 1 and Tissue Clearing Reagent 2, depending on the tissue thickness.				

Table 6: Incubation times and reagent volumes required for clearing fluorescent protein or fixable fluorophore-labeled tissues.

13.1.1 Obtain tissues of interest. See Section 7 for guidelines on fixation.

13.1.2 Wash tissues twice in PBS, pH 7.4 (without calcium, magnesium, or phenol red) for at least 1 hour.

Δ Note: Stopping point 1 (see 13.2.1).

13.1.3 Dehydrate the tissues with increasing concentrations of ethanol at 4°C. See Table 6 for required volumes and incubation times. Using an excess volume in the dehydration steps ensures proper clearing.

1. Treat tissues with 30% ethanol in PBS with gentle shaking.
2. Treat tissues with 50% ethanol in PBS with gentle shaking.
3. Treat tissues with 70% ethanol in deionized water with gentle shaking.
4. Treat tissues with 90% ethanol in deionized water with gentle shaking.
5. Treat tissues with 100% dry ethanol with gentle shaking.

Δ Note: Stopping point 2 (see 13.2.1).

13.1.4 Remove the tissues from ethanol. Ensure that all excess ethanol is absorbed with a paper towel and removed from the sample.

13.1.5 For tissues, add Tissue Clearing Reagent 1 to completely cover the sample, then incubate at 4°C with gentle shaking.

Δ Note: Required reagent volume and clearing time vary with tissue sample size (see Table 6). However, tissue clearing can be accelerated substantially at 37°C with gentle shaking without damage to tissue, at the compromise of increased autofluorescence.

Δ Note: Incubation in Tissue Clearing Reagent 2 (Step 13.1.6) requires the use of glass or polypropylene containers. Other plastic vessels are not compatible the enhancer and will ruin the samples.

13.1.6 Transfer larger or thicker tissues (>200 µm) to Tissue Clearing Reagent 2, to finish the clearing process, at 4°C with gentle agitation, and then proceed to step 13.2.7. Otherwise, proceed directly to step 13.2.7.

Δ Note: Larger tissue samples should be imaged in Tissue Clearing Reagent 2.

Δ **Note**: You can perform the tissue clearing steps (Steps 13.1.5 and 13.1.6) within a chamber. See Step 7.15.

Δ **Note**: Stopping point 3 (see 13.2.2).

13.1.7 Image the cleared samples using any fluorescent imaging analyzer such as widefield microscope, confocal, light sheet or single/multi-photon microscope, or high content analyzer. You can image the samples in any appropriate container, such as mounted slides, 96-well plates, light sheet microscope chambers, etc.

13.2 Stopping Points

– These are optional.

13.2.1 You can store the tissues at 4°C in the dark for up to 3 days without detrimental effects.

13.2.2 You can seal and store the cleared samples at 4°C in the dark indefinitely without detrimental effects. Depending on the sample type and the fluorophore, mounted samples can be imaged weeks to months after mounting. You might need to re-stain with a nuclear stain depending on how long the same has been stored for.

Technical Support

For all technical or commercial enquiries please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)