PRODUCT INFORMATION

The Visikol HISTO tissue clearing technique is designed for use with whole tissues (Visikol HISTO-1 and Visikol HISTO-2) and clears tissues with minimum changes to morphology and without compromising the sensitivity of detection with almost any fluorophore. With the easy-to-use protocol, whole mouse brain (8 mm thickness) can be cleared in 24 hours, or a 1 mm section in 2 hours, without using any special instrument or equipment. The clearing workflow is compatible with most fluorophores including fluorescent proteins, which can be detected with typical fluorescent imaging instruments such as wide-field, confocal, and light sheet microscopes, and high-content instruments. The Visikol HISTO tissue clearing technique is strong enough to adequately clear tissue for 3D fluorescent imaging, but not so harsh as to disrupt tissue morphology. Minimum morphological changes such as shrinkage or contraction have been observed. For precious samples, the clearing can be reversed, and tissue can be processed for histology studies such as H&E staining.

Table 1. Contents and storage of Visikol HISTO kits

	Visikol HISTO Starter Kit (Cat. No. HSK-1)	Visikol HISTO 1 & 2 Combo (Cat. No. HH-10)	Visikol ® HISTO 1+2 Combo (Cat. No. HH-30)	Visikol ® HISTO 1+2 Combo (Cat. No. HH-100)
Visikol HISTO-1	30 mL	10 mL	30 mL	100 mL
Visikol HISTO-2	30 mL	10 mL	30 mL	100 mL
Visikol Antibody Buffer	30 mL	—	—	—
Visikol Blocking Buffer	30 mL	—	—	—
Visikol Antibody Penetration Buffer	30 mL	—	—	—
Visikol HISTO Washing Buffer 10X	70 mL	—	—	—
Visikol Tissue Permeabilization Buffer	30 mL	—	—	—

- Visikol HISTO-1 and Visikol HISTO-2 should be stored tightly closed 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.
- Visikol HISTO buffers should be stored in a refrigerator (2–8°C) upon receipt and are stable for 12 months from the date of receipt.

Table 2. Contents and storage of stand-alone Visikol HISTO reagents.

Product	Cat. No.	Size	Notes	Storage
Visikol HISTO-1	H1-30	30 mL	Clearing and imaging reagent with a refractive index of 1.50	• Store at room temperature in a dry environment. [1] • Do not freeze.
	H1-100	100 mL		
Visikol HISTO-2	H2-30	30 mL	Clearing and imaging reagent with a refractive index of 1.53	
	H2-100	100 mL		
Visikol HISTO Antibody Buffer	HSK-AB-30	30 mL	PBS with 0.2% Tween™ 20, heparin, 3% donkey serum, and 5% DMSO	Store at 2–8°C. [2]
	HSK-AB-100	100 mL		
Visikol HISTO Blocking Buffer	HSK-BB-30	30 mL	PBS with 0.2% Triton™ X-100, 6% donkey serum, and 10% DMSO.	
	HSK-BB-100	100 mL		
Visikol HISTO Penetration Buffer	HSK-PB-30	30 mL	PBS with 0.2% Triton™ X-100, 0.3 M glycine, and 20% DMSO	
	HSK-PB-100	100 mL		
Visikol HISTO Washing Buffer 10X	HSK-WB-70	70 mL	10X PBS with 2% Tween™ 20 and 100 µg/mL heparin.	
	HSK-WB-200	200 mL		
Visikol HISTO Permeabilization Buffer	HSK-PMB-30	30 mL	-	
	HSK-PMB-100	100 mL		
ClearWell™ Tissue Imaging Chamber (0.75 mm deep)	CW0.75	Set of 6	-	Store at room temperature.
ClearWell™ Tissue Imaging Chamber (1.75 mm deep)	CW1.75	Set of 6	-	
ClearWell™ Tissue Imaging Chamber (3.5 mm deep)	CW3.5	Set of 6	-	
ClearWell™ Tissue Imaging Chamber (7 mm deep)	CW7	Set of 6	-	
2 mm Coronal Mouse Brain Slicer	BSLC-1	1 each	-	
2 mm Sagittal Mouse Brain Slicer	BSLS-1	1 each	-	
1 mm Coronal Mouse Brain Slicer	BSLM-1	1 each	-	
1 mm Sagittal Mouse Brain Slicer	BSLM-2	1 each	-	

[1] When stored as directed, the products are stable for 24 months from the date of receipt.

[2] When stored as directed, the products are stable for 12 months from the date of receipt.

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For easier-to-clear tissues like brain, lung, intestine, muscle, and skin with less than 250 µm thickness, treatment with the Visikol HISTO-1 is sufficient for 3D imaging. However, tissues thicker than 250 µm, or difficult-to-clear tissues such as kidney, liver, heart, and placenta, require an initial treatment with the Visikol HISTO-1, followed by treatment with Visikol HISTO-2.

For first-time users, we recommend the Visikol HISTO Starter Kit for whole tissues which contains all reagents required for the workflows described here.

REQUIRED MATERIALS NOT SUPPLIED

Item
Slides, coverslips, containers
Primary or secondary antibodies[1]
PBS (phosphate buffered saline), pH 7.4 (without calcium, magnesium, or phenol red)
Water-free Ethanol (for samples containing fluorescent proteins)
Methanol (for samples without fluorescent proteins)
Hydrogen peroxide solution
DMSO, Anhydrous
4% formaldehyde, methanol-free
PBS with 0.05% Sodium azide (Caution! Sodium azide is extremely toxic!)

BEFORE YOU BEGIN

PROCEDURAL GUIDELINES

- For tissues up to 250 µm thickness, treatment with Visikol HISTO-1 is sufficient for clearing, but clearing can be enhanced by incubating the sample with Visikol HISTO-2.
- All three clearing reagents, Visikol HISTO-1, Visikol HISTO-2, and Visikol HISTO-M can also be used as imaging solutions during imaging on a fluorescent imaging instrument.
- For first time users, we highly recommend cutting thicker tissues into 1-2 mm thick sections using a device such as the 1- or 2-mm Coronal Mouse Brain Slicer or the Visikol 1- or 2-mm Sagittal Mouse Brain Slicer, available at visikol.com. Alternatively, a vibratome can be used to prepare thick tissue sections
- 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.
- 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.
- 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.

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- Except where otherwise stated, perform all steps in the procedure at room temperature (20°C) with gentle agitation. If autofluorescence is a significant problem for your tissue, it may be reduced by conducting all steps at 4°C and using 100% dry ethanol instead of methanol.
- For liver, kidney, and lymphatic tissues, you may need to extend incubation times by 30–50%, depending on degree of fixation.
- Use 100% water-free ethanol for all steps involving ethanol. Reagent alcohol is a suitable choice (Fisher cat #HC-600-1GAL), containing 90% ethanol, 5% isopropanol and 5% methanol.
- **Visikol HISTO-2 is not compatible with polystyrene. Use glass or polypropylene containers and tubes instead.**
- You can perform the tissue clearing steps (Steps 2.5, 2.6 and 2.7, Steps 3.16*,* 3.17 and 3.18) within a chamber constructed by sticking the Visikol ClearWell Tissue Imaging Chamber on a glass coverslip or slide. Visikol ClearWell Tissue Imaging Chambers are made of silicon, which is compatible with the Visikol HISTO clearing reagents, making them ideal containers for the clearing steps and for use as imaging chambers.

PREPARE THE REAGENTS

1.1 Visikol HISTO 10X Washing Buffer is provided at 10X concentration. Dilute the Visikol HISTO 10X Washing Buffer to 1X with DI H20, pH 7.4 before use.

1.2 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.

1.3 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 H_2O. For best results, ensure that the 100% methanol used in the last step of dehydration is free of water. Methanol, if not sealed properly, will absorb water directly from the air, so be sure to use fresh methanol, stored with a tight seal.

1.4 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). Note that bleaching with this solution is not compatible with fluorescent protein staining.

PROTOCOL FOR FLUORESCENT PROTEIN LABELED TISSUE

The following protocol describes a general procedure for clearing a variety of tissues ranging in size from whole organs to thin sections of tissues. 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 3** for the suggested incubation times, volumes, and considerations for your particular tissue of interest.

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Table 3. Incubation times and reagent volumes required for clearing fluorescent protein or fixable fluorophore-labeled tissues

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 hours	25 mL	48 hours	10 mL
4 mm (e.g. mouse brain hemisphere)	2 hours	13 mL	36 hours	7 mL
2 mm	90 minutes	8 mL	12 hours	5 mL
1 mm	40 minutes	4 mL	4 hours	3 mL
500 µm	16 minutes	3 mL	30 minutes	2 mL
≤ 250 µm	8 minutes	2 mL	10 minutes	1 mL

[1] For liver, kidney, and lymphatic tissues, extend incubation time by 30–50%, depending on degree of fixation.

[2] Visikol HISTO-1 only, or Visikol HISTO-1 and Visikol HISTO-2, depending on the tissue thickness.

Except where otherwise stated, perform all steps in the procedure at (4ºC) with gentle agitation.

2.1 Obtain tissues of interest. See "Procedural guidelines" on page 3 for guidelines on fixation.

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

STOPPING POINT. (Optional) You can store the tissues at 4°C in the dark for up to 3 days without detrimental effects.

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

2.3.1 Treat tissues with 30% ethanol in PBS with gentle shaking.

2.3.2 Treat tissues with 50% ethanol in PBS with gentle shaking.

2.3.3 Treat tissues with 70% ethanol in deionized water with gentle shaking.

2.3.4 Treat tissues with 90% ethanol in deionized water with gentle shaking.

2.3.5 Treat tissues with 100% dry ethanol with gentle shaking.

STOPPING POINT. (Optional) You can store the tissues at 4°C in the dark for up to 5 days without detrimental effects.

2.4 Remove the tissues from ethanol. Ensure that all excess ethanol is removed by dabbing tissue with a Kimwipe, laboratory tissue, or a paper towel.

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2.5 For tissues, add Visikol HISTO-1 to completely cover the sample, then incubate at 4°C with gentle shaking.

Note: Required reagent volume and clearing time vary with tissue sample size (see Table 3, page 5). However, tissue clearing can be accelerated substantially at 37°C with gentle shaking without damage to tissue, at the compromise of increased autofluorescence in green and red channels.

IMPORTANT! Incubation in Visikol HISTO-2 (Step 2.7) requires the use of glass or polypropolyne containers. Other plastic vessels are not compatible the enhancer and will ruin the samples.

2.6 Transfer larger or thicker tissues (>200 µm) to Visikol HISTO-2 to finish the clearing process at 4°C with gentle agitation, then proceed to Step 2.8. Otherwise, directly proceed to Step 2.8.

Note: Larger tissue samples should be imaged in Visikol HISTO-2.

Note: You can perform the tissue clearing steps (Steps 2.5 and 2.6) within a chamber constructed by sticking the Visikol ClearWell Imaging Chamber on a glass coverslip or slide. Visikol ClearWells are made of silicon, which is compatible with the Visikol HISTO clearing reagents, making them ideal containers for the clearing steps and for use as imaging chambers. Be sure the slide is clean and dust-free, or the ClearWell may not seal properly, and it may leak. Use a glass coverslip to close the ClearWell.

Note: DAPI gives much better results with cleared samples and is preferable to Hoechst 33342.

STOPPING POINT. 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.

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

PROTOCOL FOR IMMUNOLABELING TISSUES

The following protocol describes a general procedure for immunolabeling and clearing a variety of tissues ranging in size from whole rat brains to 250 µm thick sections. Refer to **Table 4** for the suggested incubation times and **Table 5** for the required reagent volumes to immunolabel and clear your tissue of interest.

Except where otherwise stated, perform all steps in the procedure at room temperature (20°C) with gentle agitation.

3.1 Obtain tissues of interest and fix them, if needed. See "Procedural guidelines" on page 3 for guidelines on fixation.

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

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3.3 (Optional for most tissues) Incubate tissues that are particularly difficult clear due to the presence of pigments, durable extracellular matrix (e.g. collagenous tissues), or blood (e.g. liver tissue, whole kidney, over-fixed human tissues) in Visikol HISTO Permeabilization Buffer overnight with gentle shaking before proceeding with permeabilization.

Table 4. Suggested incubation times for immunolabeling and clearing tissues

Thickness	Permeabilization and dehydration	Penetration/ Permeabilization	Blocking[1]	Antibody incubation[1]	Washing steps	Incubation in Visikol HISTO tissue clearing reagents[1] [2]
8 mm (e.g. whole mouse brain)	2 hours	8 hours	120 hours	240 hours	4 hours + overnight for last wash	48 hours
4 mm (e.g. mouse brain hemisphere)	2 hour	6 hours	80 hours	80 hours	2 hour + overnight for last wash	36 hours
2 mm	90 minutes	4 hours	28 hours	28 hours	90 minutes	12 hours
1 mm	40 minutes	2 hours	10 hours	10 hours	1 hour	4 hours
500 μm	16 minutes	1 hour	3 hours	3 hours	40 minutes	30 minutes
≤ 250 μm	8 minutes	30 minutes	1 hour	90 minutes	20 minutes	10 minutes

[1] For liver, kidney, and lymphatic tissues, extend incubation time by 30–50%, depending on degree of fixation.

[2] Visikol HISTO-1 only, or Visikol HISTO-1 and Visikol HISTO-2, depending on the tissue thickness.

Table 5. Reagent volumes required for immunolabeling and clearing tissues

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

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Note: Visikol HISTO Permeabilization Buffer may quench signal from fluorescent proteins. If immunolabeling tissues containing fluorescent protein, you may want to consider using an antibody for the fluorescent protein used in your tissues.

3.4 Permeabilize tissues by washing them in increasing concentrations of methanol (samples without fluorescent protein) or ethanol (samples with fluorescent protein) **at 4°C** with gentle agitation. See **Tables 4 and 5** for required volumes and incubation times.

a. **Samples without fluorescent protein:** Wash tissues twice in PBS, once in 50% methanol in PBS, 80% methanol in deionized water, and finally in 100% dry methanol.

b. **Samples with fluorescent protein:** 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).

STOPPING POINT. (Optional) 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.

3.5 (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.

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

3.6 Wash samples before proceeding with further staining:

a. **Samples without fluorescent protein:** 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 .2% Triton™ X-100.

b. **Samples with fluorescent protein:** 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 .2% Triton™ X-100 (conduct at 4°C).

STOPPING POINT. (Optional) You can store the tissues at 4°C in the dark for up to 3 days without detrimental effects.

3.7 Incubate the samples in Visikol HISTO Penetration Buffer with gentle shaking.

3.8 Block the samples in Visikol HISTO Blocking Buffer with gentle shaking at 37°C.

STOPPING POINT. (Optional) You can store the tissues at 4°C for up to 1 month without detrimental effects.

3.9 Transfer the samples to primary antibody dilutions prepared in Visikol HISTO Antibody Buffer and incubate at 37°C with gentle shaking.

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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 on page 13.

STOPPING POINT. (Optional) You can store the tissues at 4°C for up to 2 weeks without detrimental effects.

3.10 Wash the samples 5 times in Visikol HISTO Washing Buffer (diluted to 1X in PBS; see Step 1.1, page 4) with gentle shaking.

STOPPING POINT. (Optional) You can store the tissues at 4°C for up to 3 days without detrimental effects.

3.11 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 Visikol HISTO Antibody Buffer at 37°C with gentle shaking.

STOPPING POINT. (Optional) You can store the tissues at 4°C for up to 2 weeks without detrimental effects.

3.12 (Optional) Add nuclear stain (e.g. DAPI) to a dilution of 1:1000 to 1:5000 (depending on the stain). You can perform this step concurrently with antibody labeling steps, or separately in Visikol HISTO Wash Buffer. DAPI should be used as a nuclear stain instead of Hoechst.

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

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.

STOPPING POINT. (Optional) You can store the tissues at 4°C in the dark for up to 3 days without detrimental effects.

3.14 Dehydrate the tissues with increasing concentrations of methanol samples without fluorescent protein) or ethanol (samples with fluorescent protein) **at 4°C** with gentle shaking. See Tables 4 and 5 (page 7/8) for required volumes and incubation times. Using an excess volume in the dehydration steps ensures proper clearing.

a. Samples without fluorescent protein: Treat tissues with 50% methanol in PBS, then with 80% methanol in deionized water, and finally in 100% methanol with gentle shaking.

b. Samples with fluorescent protein: 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).

STOPPING POINT. (Optional) You can store the tissues at 4°C for up to 3 days without detrimental effects.

3.15 Remove the tissues from methanol or ethanol. Ensure that all excess methanol or ethanol is absorbed with Kimwipe™ laboratory tissue or a paper towel and removed from the sample.

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3.16 Add Visikol HISTO-1 to completely cover the sample with gentle shaking (conduct at 4°C for samples with fluorescent protein).

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

IMPORTANT! Incubation in Visikol HISTO-2 (Step 3.17) requires the use of glass or polypropylene containers. Other plastic vessels are not compatible with the enhancer and will ruin the samples.

3.17 Transfer larger or thicker tissues (>200 μm) to Visikol HISTO-2 to finish the clearing process with gentle agitation, then proceed to Step 3.19. Otherwise, directly proceed to Step 3.19 (conduct at 4°C for samples with fluorescent protein)

Note: Larger tissue samples should be imaged in Visikol HISTO-2.

Note: You can perform the tissue clearing steps (Steps 3.16 and 3.17) within a chamber constructed by sticking the Visikol ClearWell Tissue Imaging Chamber on a glass coverslip or slide. Visikol ClearWell Tissue Imaging Chambers are made of silicon, which is compatible with Visikol clearing reagents, making them ideal containers for the clearing steps and for use as imaging chambers.

STOPPING POINT. 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.

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

Note: Samples should be mounted for imaging in Visikol HISTO-1 or Visikol HISTO-2.

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APPENDIX A: TROUBLESHOOTING

<table>
<tr><th>Observation</th><th>Possible cause</th><th>Recommended action</th></tr>
<tr><td rowspan="4">Cannot image past 500–1000 µm. Labeling appears uneven and drops off significantly at this depth.</td><td>Antibody concentration too high: ring of intense staining near the surface, drops off significantly after that.</td><td>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.</td></tr>
<tr><td>Antibody concentration too low: signal drops off in the middle of the tissue.</td><td>Increase antibody concentration</td></tr>
<tr><td>Using LED illumination on confocal microscope</td><td>Use laser based illumination system</td></tr>
<tr><td>Optical attenuation due to absorption of photons by the upper tissue layers “shadows” the tissue below, even with perfect staining.</td><td>Increase laser power and gain with increasing depth. Some microscopes can automate laser power and gain corrections. Caution! 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.</td></tr>
<tr><td>There is an intense band of labeled tissue at the surface, then a significant drop-off afterwards.</td><td>Antibody concentration too high</td><td>Reduce antibody concentration by increasing the dilution factor.</td></tr>
<tr><td rowspan="2">Tissue did not clear</td><td>Plastic incompatibility</td><td>Visikol HISTO-2 degrades polystyrene. Check to see if plastic looks deformed and/or melted.

Use polypropylene and glass in your workflow, where possible. Plastic leaching into your sample can affect the clearing ability of Visikol HISTO reagents.</td></tr>
<tr><td>Incomplete dehydration/clearing</td><td>Ensure that you are using fresh, water-free ethanol or methanol for dehydration. If not stored properly, methanol/ethanol will absorb water from the air. Methanol or ethanol that contains water will not remove all water from the tissue, resulting in cloudiness.
Ensure that the sample vessel is sealed properly. Visikol HISTO-2 is hygroscopic and will draw water.
Ensure that you are using sufficient volume of Visikol HISTO-1, Visikol HISTO-2, or Visikol HSITO-M for your tissue size. Using insufficient volume of Visikol HISTO reagents can cause inadequate clearing.</td></tr>
</table>

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Observation	Possible cause	Recommended action
Fluorescent protein is 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.
Fluorescent protein is quenched	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 Looks dark	Antibody concentration too low.	• Increase the antibody concentration. Explore a range of antibody concentrations on a small section of the tissue before scaling to large tissues.
Center of Tissue Looks dark	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

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APPENDIX B: GUIDELINES FOR VALIDATING ANTIBODIES AND OPTIMIZING ANTIBODY CONCENTRATION

If you are using an antibody for the first time, we recommend that you validate the anti-body and optimize its concentration. Antibody concentration required for the workflow can be different for thicker tissues 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.

- Fix the tissue sections with 4% paraformaldehyde overnight at 4°C. Do not over-fix the tissues.
- 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.
- 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 Visikol HISTO Antibody Buffer.
- 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.
- You can validate antibody staining using a typical fluorescent microscope. Prepare a slide of the cleared tissue and examine for specificity of signal.
- 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.
- 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.

  Note: ImageJ is a free, open-sourced, public domain image processing and analysis program available from NIH at **https://imagej.nih.gov/ij/index.html**. Fiji is a release of ImageJ which comes pre-installed with many useful plugins, available at **https://imagej.net/Fiji/Downloads**

- If the staining is even, you should see relatively consistent intensity (with respect to nuclear stain) across the tissue (see **Figure 1**, below). Some dimming in the inner layers is expected, but signal should be visible across tissue.
- 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.
- 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.

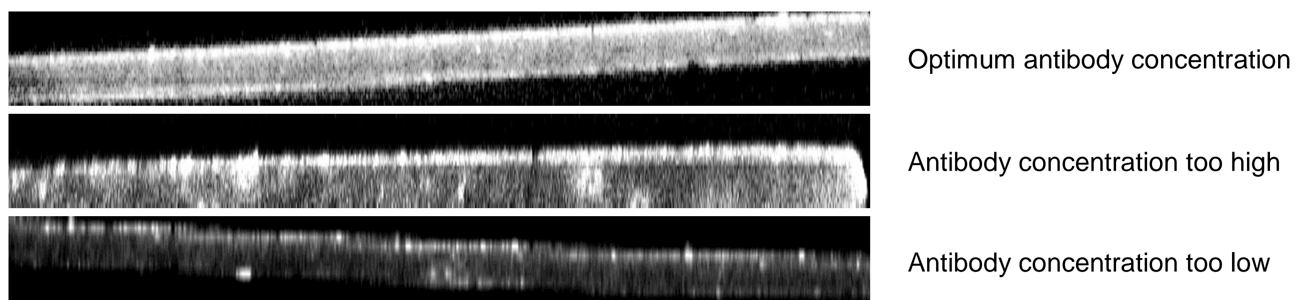

Figure 1. Evaluating the evenness of staining. Tissue sections were imaged using a confocal microscope and a z-stack spanning the entire thickness of the tissue was obtained. The XZ plane was examined for the evenness of staining.

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APPENDIX C: REVERSE TISSUE CLEARING

The Visikol HISTO 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 Visikol HISTO tissue clearing method can be integrated into the many bio-imaging process without disrupting the other assays or histological processing or of traditional workflows.

- 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.
- Larger and more vascular tissues (e.g. whole kidney) may require 2–3 washes of alcohol over the course of several hours.
- After reversal, samples can be processed directly for paraffin-embedding histological preparations.

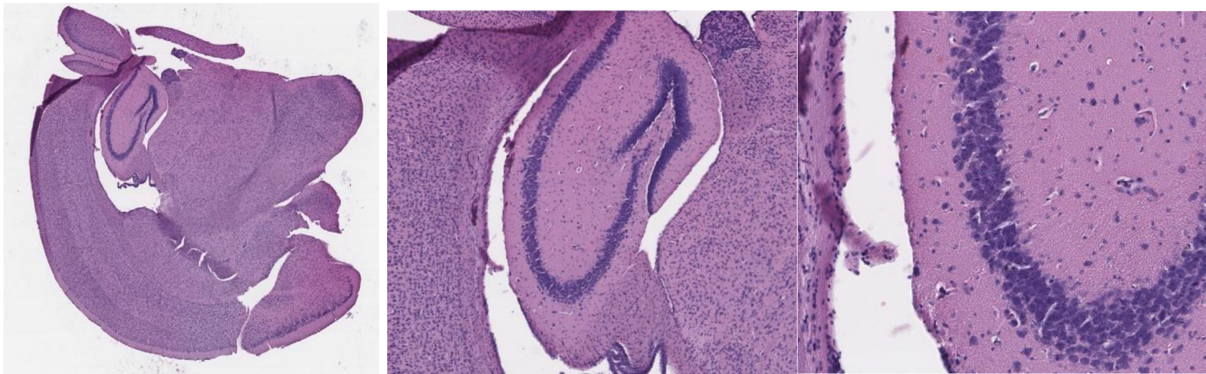

Figure 2A. Untreated mouse brain tissue section was formalin-fixed and paraffin-embedded, then stained with H&E, depicting the hippocampus.

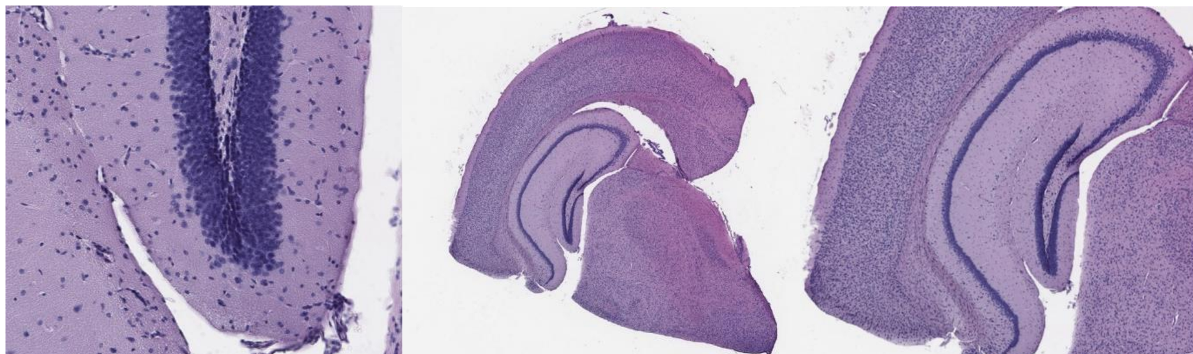

Figure 2B. Mouse brain tissue was cleared using the Visikol HISTO tissue clearing technique. Cleared tissue was then reversed, embedded in paraffin, sectioned, and stained with H&E, depicting hippocampus. Visikol Tissue Clearing workflow does not appreciably affect tissue histology.

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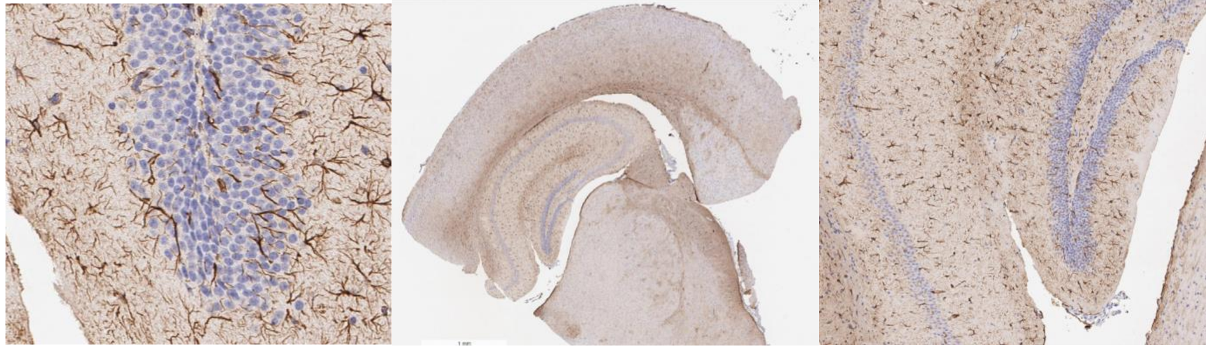

Figure 2C. Mouse brain tissue was cleared using the Visikol HISTO tissue clearing technique. Cleared tissue was then reversed, embedded in paraffin, sectioned, and immunostained for GFP, labeling astrocytes. The Visikol HISTO tissue clearing workflow does not affect antigenicity of tissues.

ORDERING INFORMATION

Product	Cat. No.	Size
Visikol HISTO-1	H1-30	30 mL
	H1-100	100 mL
Visikol HISTO-2	H2-30	30 mL
	H2-100	100 mL
Visikol HISTO Antibody Buffer	HSK-AB-30	30 mL
	HSK-AB-100	100 mL
Visikol HISTO Blocking Buffer	HSK-BB-30	30 mL
	HSK-BB-100	100 mL
Visikol HISTO Penetration Buffer	HSK-PB-30	30 mL
	HSK-PB-100	100 mL
Visikol HISTO Washing Buffer 10X	HSK-WB-70	70 mL
	HSK-WB-200	200 mL
Visikol HISTO Permeabilization Buffer	HSK-PMB-30	30 mL
ClearWell™ Tissue Imaging Chamber (1.75 mm deep)	CW1.75	Set of 6
ClearWell™ Tissue Imaging Chamber (3.5 mm deep)	CW3.5	Set of 6
ClearWell™ Tissue Imaging Chamber (7 mm deep)	CW7	Set of 6
2 mm Coronal Mouse Brain Slicer	BSLC-1	1 each
2 mm Sagittal Mouse Brain Slicer	BSLS-1	1 each
1 mm Coronal Mouse Brain Slicer	BSLM-1	1 each
1 mm Sagittal Mouse Brain Slicer	BSLM-2	1 each

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