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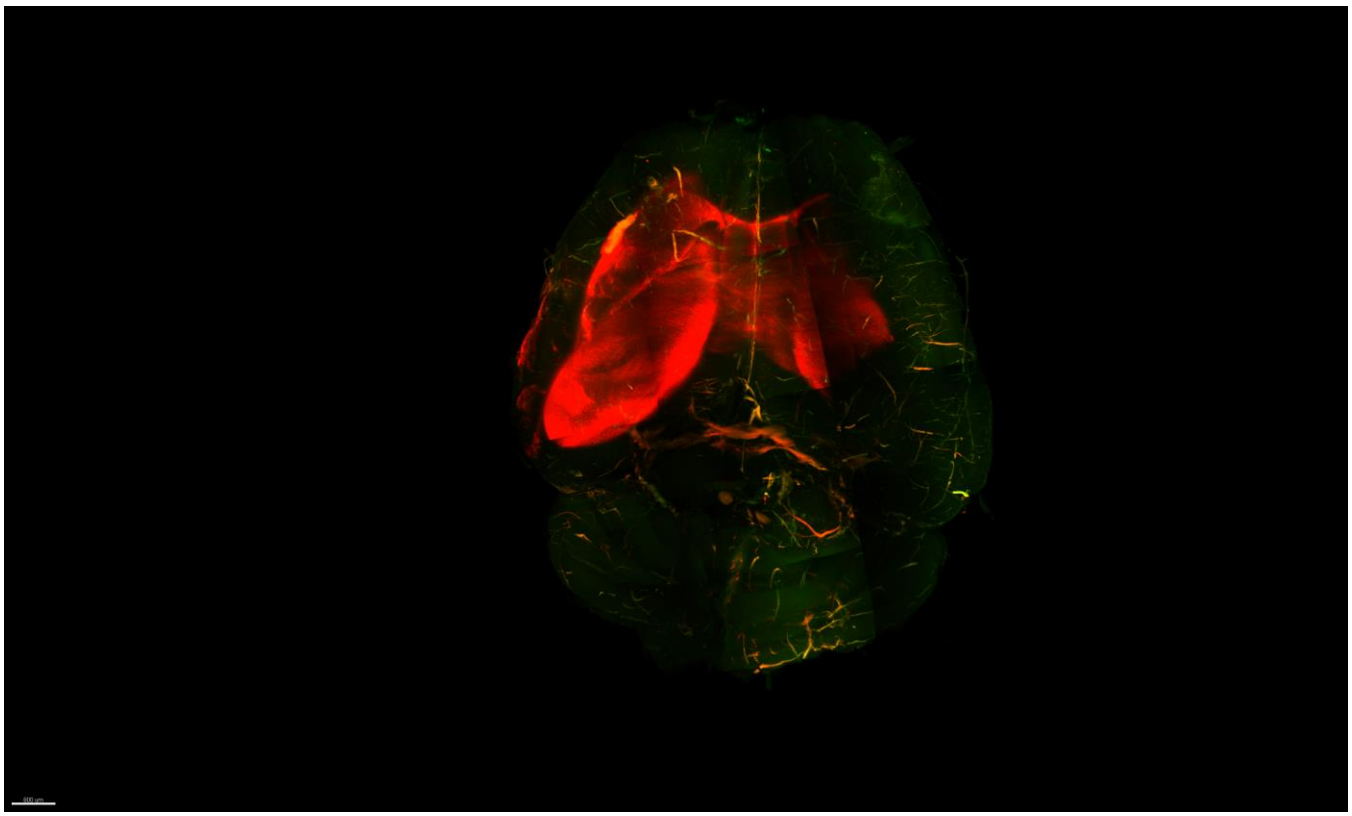
Tissue Clearing Ebook

Version 1.1



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Tissue Clearing Comparison

Overview

In the last few years there has been a significant increase in the number of researchers that want to incorporate tissue clearing and 3D histology into their bio-imaging workflows. While there are numerous tissue clearing techniques and versions of these techniques, adopting tissue clearing has been challenging for many researchers as it requires an expertise in tissue clearing and 3D imaging – we are working to make this process easier.

Class	Name	RI	Immunostainin	FP	Clearing Time*	Morphology Alterations	Preservation of Lipids	Toxic / Teratogenic
Solvent clearing techniques	BABB	1.56	Yes	No	Days	Shrinkage	No	Yes
	FluoBABB	1.56	No	Yes	Days-Weeks	Shrinkage	No	Yes
	3DISCO	1.56	Limited	Quenches	Hours-days	Shrinkage	No	Yes
	iDISCO	1.56	Yes	Quenches	Hours-days	Shrinkage	No	No
	Visikol HISTO	1.48 to 1.53	Yes	Yes	Hours-days	No	Yes	No
Aqueous hyper-hydrating clearing techniques	Sucrose	1.44	Yes	Yes	Days	Shrinkage	No	No
	FocusClear	1.47	Yes	Yes	Hours-days	No	Yes	Yes
	ClearT	1.44	Yes	No	Hours-days	No	Yes	Yes
	ClearT2	1.44	Yes	Yes	Hours-days	No	Yes	Yes
	SeeDB	1.48	No	Yes	Days	No	Yes	No
	FRUIT	1.48	No	Yes	Days	Minimal expansion	Yes	No
	TDE	1.42	Yes	Yes	Days-weeks	No	No	No
	ScaleA2	1.38	Limited	Yes	Weeks-months	Expansion	No	No
	ScaleS	1.47	Yes	Yes	Days-Weeks	Expansion, then restored	No	No
	CUBIC	1.47	Yes	Yes	Days-Weeks	Expansion	No	No
	ScaleCUBIC	1.47	No	Yes	Days-Weeks	Expansion, tissue becomes very fragile	No	No
Hydrogel embedding techniques	CLARITY	1.45	Yes	Yes	Days-Weeks	Expansion	No	Yes (acrylamide monomer)
	PACT	1.38 to 1.48	Yes	Yes	Days-Weeks	Slight expansion	No	Yes
	PARS	1.38 to 1.49	Yes	Yes	Days	No	No	Yes

Which Tissue Clearing Technique is Best for Me?

This question unfortunately does not have an easy answer and will depend entirely upon your specific research question. Each tissue clearing technique has advantages and no technique is best for all applications. However, you can narrow down the best clearing technique for your application by considering four different tissue clearing properties using the table above.

1) **Molecular Labeling Technique**

Tissue clearing techniques are compatible with either only immunolabeling or fluorescent protein (FP) or both. Each approach to tissue clearing has advantages and disadvantages whereas approaches that are compatible with both immunolabeling and FP such as FocusClear™, Sucrose, ClearT2, TDE, CUBIC, CLARITY have their own specific disadvantages such as cost, tissue shrinkage, toxicity, slow clearing, tissue swelling and complexity, respectively. While techniques that are compatible with immunolabeling can be used with FP through using anti-FP immunolabels, this might increase overall processing time when compared to techniques that are compatible with FP.

2) Processing Time

Overall tissue processing and clearing time is a major driver for which technique should be chosen for an application as this will directly impact the rate at which the technique can be adopted. The time required to render a tissue transparent will depend on several factors including; tissue type, temperature, tissue size, animal age and tissue preparation method (e.g. fixed or fresh). Additionally, each clearing technique will react differently to each one of these parameters whereas solvent based techniques can easily render fatty tissues transparent and lipid removal techniques like CLARITY cannot.

3) Ease-of-use

The use of protein expansion/denaturation clearing techniques (e.g. Scale) and solvent based techniques (e.g. Visikol HISTO, i/3/uDISCO, BABB) are relatively simple wherein tissues are placed in varying solutions until the tissues are rendered transparent and are able to be visualized on the microscope. These processes lend themselves for high-throughput applications and can be successfully executed by any laboratory technician. Conversely, hydrogel-based approaches (e.g. CLARITY) require embedding tissues in acrylamide hydrogels which is a significantly more challenging process. Though companies (Logos Biosystem, LifeCanvas Technologies) have developed CLARITY devices to automate such processing, these techniques require expensive equipment (>$15,000) and are challenging.

4) Validation

Most researchers who are using tissue clearing for basic research do not need to be concerned with validation whereas researchers and clinicians using tissue clearing for applied research or on clinical tissues do need to consider validation. For some applications researchers need to be certain that the 3D renderings acquired from tissues are indicative of the tissue's molecular and morphological properties and not the tissue clearing process. Therefore, these 3D renderings need to be correlated to the traditional histological methods that have been developed over the last century that serve as the foundation for tissue characterization. The challenge in validating these approaches is that while they can be used to acquire 3D renderings of tissues, many of the processes tend to significantly alter cellular morphology, leading to the expansion or shrinkage of tissue. Additionally, some clearing processes (e.g. CLARITY) require the removal of cellular components (e.g. lipids) from tissues to render tissues transparent. Combined, these factors lead to irreversible damage to the cellular morphology of tissues which necessitates parallel validation for these tissue clearing approaches.

Therefore, the need for validation is a significant consideration for some researchers whereas the need to validate 3D histology requires parallel 2D histological processing or a tissue clearing technique that preserves tissue morphology, does not cause damage to tissue structures, and is reversible. It has been shown that Visikol HISTO is reversible as opacity can be returned to

tissues following imaging such that 2D histology can be conducted after 3D imaging with the same tissue.

Type of Tissue and Fixation

Type of Tissue
Depending on the nature of the tissue being labeled and cleared, there are several aspects to consider. Whole tissues require longer incubation times, and tissues with highly compartmentalized structures, such as the kidney and the liver, require increased incubation times for permeabilization and staining. A rule of thumb is that doubling tissue size requires tripling of incubation times. Some of these considerations are discussed below.

Whole Organs
Whole organs are often of interest to obtain system-wide data from volume imaging. When staining, clearing, and imaging whole tissues, there are two major considerations. **First,** antibody incubation is the most time-consuming aspect of labeling whole tissues: a whole mouse brain requires approximately 96 hours of incubation in blocking solution, primary antibody, and secondary antibody each, which comes to nearly 12 days of processing, whereas brain hemispheres take less than half the time. **Second,** many confocal systems are not equipped to image whole organs, since extended working distance objectives are required when imaging whole organs. Specific considerations for common tissue types are detailed below. **Whenever possible, 0.5-1 mm sections or portions of tissues of interest should be used to greatly reduce the processing time and imaging time when using confocal microscopy.**

Brain and Spinal Tissue
Brain tissue has been the most extensively studied for tissue clearing and brain tissue reliably clears because of its overall lack of pigment, cartilage, and autofluorescence.

Kidney Tissue
Kidney tissue can be somewhat difficult to clear due to the structural complexity of the organ and its various membranes for controlling diffusion and osmosis. As such, kidney tissue holds on to water stronger than other tissues, and requires increased incubation in alcoholic solutions to dehydrate the tissue prior to clearing. Clearing can also be substantially accelerated (2-3x) by bisecting the kidney. Due to the large quantity of blood retained by kidneys, these tissues should be perfused whenever possible. Bleaching with 5% H_2O_2 in Methanol/DMSO (1 part 30% H_2O_2, 4 parts methanol, 1 part 100% DMSO) at 4°C is effective to reduce pigment in kidney tissue. For best results, treat whole kidney tissues with the **Tissue Permeabilization Buffer** prior to further steps.

Liver Tissue
The major difficulty encountered in clearing and imaging liver tissue is due to the elevated level of autofluorescence caused by the high content of collagen. It is recommended to use perfused liver tissue whenever possible. Bleaching with 5% H_2O_2 in Methanol/DMSO (1 part 30% H_2O_2, 4 parts methanol, 1 part 100% DMSO) at 4°C overnight is effective to reduce pigment in liver tissue. For best results, large segments of liver tissue should be treated with **Tissue Permeabilization Buffer** prior to further steps.

Heart and Lung Tissue
When using perfused tissue specimens, clearing heart and lung tissue is simple, and there are no special considerations. When perfused tissue is not available, the level of blood that remains in the heart and lung tissue can significantly impair optical clearing due to the high concentration

of heme pigment. These tissues can be effectively bleached using 5% H_2O_2 in Methanol/DMSO (1 part 30% H_2O_2, 4 parts methanol, 1 part 100% DMSO) at 4°C overnight prior to staining and clearing steps.

Muscle Tissue
Muscle tissue clears rapidly in most cases. To increase clearing efficiency in muscular tissues that contain significant quantities of blood, tissues may be bleached using 5% H_2O_2 in Methanol/DMSO (1 part 30% H_2O_2, 4 parts methanol, 1 part 100% DMSO) at 4°C overnight prior to staining and clearing steps.

Archived Human Tissues
Due to the excessive crosslinking and higher lipid content of archived human tissues, we recommend treatment with **Tissue Permeabilization Buffer** prior to staining and clearing steps. Furthermore, archived tissues may require prolonged treatment in antigen retrieval and dehydration steps to ensure accessibility of stain and clearing agent to the deeper layers of tissue. Especially difficult tissues should be incubated in 20 mM tris buffer at pH 9 at elevated temperatures to reverse formalin cross-linking.

3D Tissue Cultures and Scaffold Supported Tissue Cultures
Staining and clearing 3D tissue cultures, organoids, spheroids, etc. has the same considerations as whole tissues, where the major difference comes to incubation times. Incubation times (and volumes of solutions) can be significantly decreased for smaller tissues. Furthermore, for small micro-tissues (< 500 μm), the dehydration steps can be skipped. Many types of micro-tissues, both scaffold-supported (on Matrigel or similar matrix) as well as unsupported, have been successfully stained, cleared and imaged using Visikol HISTO-M.

Maximum thickness of tissues possible for 3D imaging
Due to limits in the working distance of modern microscope objectives, the **maximum theoretical thickness possible to image is limited to ~34 mm**, 2x the highest working distance achieved in modern objectives (since a tissue can be imaged from each side at a radius of the working distance). The resolution at this thickness is limited to 2-10x; higher magnification lenses have significantly shorter working distances.

Considerations about Fixation

Optimum Results: Perfusion with Paraformaldehyde
For optimum results, specimens should be perfused with cold freshly prepared 4% PFA solution upon sacrifice, and harvested tissues should be immediately immersed in 4% PFA solution at 4°C overnight, followed by at least 24 hours at 4°C; followed by at least 1 hour at room temperature to ensure complete fixation. To avoid over-fixation for tissues that require long term storage, they can be transferred to PBS with 0.05% sodium azide as a preservative and can stored at 4°C indefinitely.

If Perfusion is Not Possible
Tissues of interest should be removed as quickly as possible following sacrifice. Large tissues (> 6 mm thickness) should be either cut into small pieces, or "bread-loafed," i.e. several channels should be sliced into tissue to ensure the uniform penetration of fixative into tissue. To remove excess blood, prior to fixation, tissues can be washed with 10 mM EDTA solution, which prevents blood clotting and greatly reduces the quantity of blood in tissue.

For optimum results, tissues should be fixed for 24 hours at 4°C, no longer than 72 hours, then for 1 hour at room temperature, and finally transferred to PBS with 0.05% sodium azide as a preservative for storage at 4°C. **Overfixation can lead to poor antibody penetration, slow tissue clearing, and increased background fluorescence.**

Formalin Fixed Tissue
10% Neutral Buffered Formalin (NBF) has been used successfully as a fixative with Visikol HISTO, and tissues stored for several years in 10% NBF have been successfully labeled and imaged. However, over-fixation can lead to increased background fluorescence (autofluorescence), as well as the need to extend incubation times for penetration buffers and fluorescent labels to achieve uniform penetration at large depths. Considerations for managing autofluorescence are discussed in the *Controlling Autofluorescence* section.

Problems with Formalin Fixation
Overfixation leads to two major issues: increased autofluorescence, and a decrease in the permeability of the tissue to antibody labeling. Overfixation can be partially mitigated by incubation of tissue in 20 mM tris buffer, pH 9, for 30 minutes at 37°C.

Other fixatives: HoPE, HistoChoice, etc.
Proprietary fixatives that are designed specifically for use with immunohistochemistry are an excellent choice for tissues where immunolabeling and clearing is desired. Follow the manufacturers protocol for processing tissues with these fixatives.

Methanol / Alcohol / Carnoy's Fixed Tissue
Fixation with alcohols can greatly increase penetration of antibodies into tissue for 3D immunolabeling, and as such can be used when labeling labile epitopes or low-expression biomarkers. However, some protein loss is possible when fixing with alcohol, and for quantitative proteomic applications where loss of protein signal is unacceptable, a cross-linking fixative should be employed.

Fresh-Frozen Tissues
Fresh-frozen tissues can be treated just as formalin fixed tissues, following dehydration with methanol/ethanol. Unfixed, frozen tissues tend to exhibit superior antibody penetration and low autofluorescence compared to formalin fixed tissues, at the compromise of potential loss of unbound proteins (and thus epitopes). Frozen formalin fixed tissues, once thawed, can be processed just as other formalin fixed tissues, described above.

Formalin Fixed Paraffin Embedded Tissue Blocks
Paraffinized tissue has been successfully cleared using Visikol HISTO. Paraffinized tissues must first be washed with xylenes (or equivalent nonpolar solvent) at elevated temperature (40°C) 3-5 times to remove excess paraffin. The tissue is then washed through a gradient of ethanol to restore and rehydrate tissues for further processing. Since they tend to be highly cross-linked, paraffinized tissues may require extensive pre-treatment in **Tissue Permeabilization Buffer** to achieve consistent staining. Especially difficult tissues should be incubated in 20 mM tris buffer at pH 9 at elevated temperatures to reverse formalin cross-linking.

Controlling Autofluorescence

The best strategy for combatting autofluorescence is to prevent it as much as possible from the start. There are a few approaches that can be employed to prevent autofluorescence during tissue collection, and if these are not possible, there are several strategies for reducing autofluorescence.

A chart of common endogenous fluorophores is found following this section.

Autofluorescence has several causes:

1. Fixation Induced Autofluorescence

Cross-linking of tyrosine and tryptophan residues in proteins with formaldehyde generates increased autofluorescence due to formation of fluorescent formaldehyde adducts. Glutaraldehyde exacerbates this issue even further.

The best way to avoid autofluorescence due to fixation is to be sure to fix tissues for the minimum amount of time required for the size and type of tissue. Generally, tissues should be fixed in freshly prepared 4% PFA (paraformaldehyde) or 10% NBF (neutral buffered formalin) overnight at 4°C and then at room temperature for 1-2 hours. Larger tissues may require longer incubation times, but in general, immersion fixed tissues should be less than 6 mm thick. After fixation, tissues should be transferred to PBS containing 0.05% sodium azide or similar preservative, and stored at 4°C.

While fixation induced autofluorescence can occur to some degree in most tissues, it can be avoided by using non-crosslinking fixatives. Non-crosslinking fixatives will also increase the ability of antibodies and stains to penetrate the tissues. However, the use of non-crosslinking fixatives may lead to loss of some epitopes if the protein of interest is not bound to the structure of the tissues.

2. Heat and Dehydration induced Autofluorescence

Treatment of tissues at elevated temperatures can increase autofluorescence in fixed tissues. To minimize autofluorescence, incubate tissues at room temperature for dehydration, staining, and clearing steps. Dehydration of tissues is a necessary step in clearing tissues with Visikol HISTO. Alcohol dehydration increases background autofluorescence by shifting equilibrium of formalin-adducts in fixed tissues with the effect of increasing the content intensity of autofluorescence. This effect is much greater in the red (530-600 nm) region than in the green or blue.

3. Endogenous Pigments: Heme Autofluorescence

The heme group is the primary pigment in blood cells. This porphyrin ring structure exhibits broad autofluorescence in tissues and can complicate analysis. The best way to control heme autofluorescence is simply to perfuse tissues with PBS prior to fixation at the time of sacrifice. This procedure will remove blood cells from tissues and eliminate this problem at the source.

If it is not possible to perfuse the tissue (i.e. archived specimens), there are techniques that can be applied to bleach the tissue and reduce autofluorescence of heme. The procedure involves incubating tissues in 5% H_2O_2 in Methanol/DMSO (1 part 30% H_2O_2, 4 parts methanol, 1 part 100% DMSO) at 4°C overnight prior to staining and clearing steps.

4. Endogenous pigments: Lipofuscin, Collagen, and others

Lipofuscin is a lipophilic pigment that accumulates through normal aging processes in animal tissue and is significantly autofluorescent. Lipofuscin often appears as small yellow granules in fluorescent imaging. Collagen is a structural protein which occurs in high concentrations in many tissues (e.g. liver, muscle) and is strongly autofluorescent in the blue region (350-450 nm), and lesser so in the green region (475-550 nm).

There are also several other biomolecules that are innate to animal tissue that can exhibit autofluorescence. These include nicotinamides (NADP), retinols and carotenoids, bile acids and downstream products like bilirubin.

The fluorescent spectra of many endogenous pigments are shown on page 9 following this section.

Methods to manage autofluorescence

1. Incubate tissues at room temperature or 4°C for antigen retrieval, labeling and clearing steps. Incubation times should be increased by 1.5-2x at 4°C due to slow diffusion.

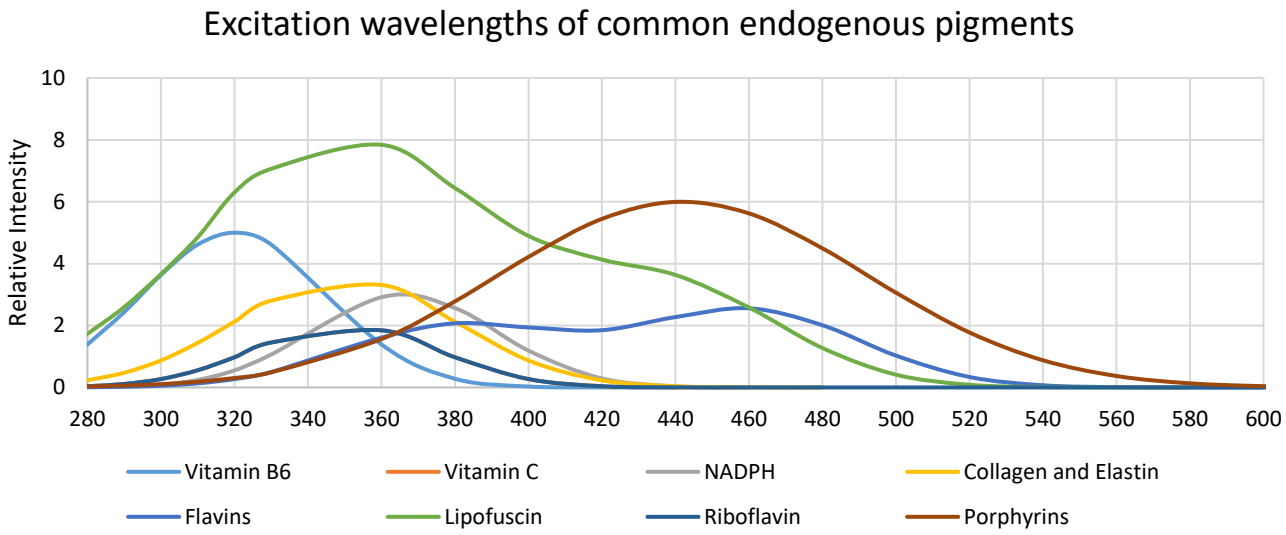
2. Perfuse tissues with PBS to remove blood and other soluble pigments prior to fixation.

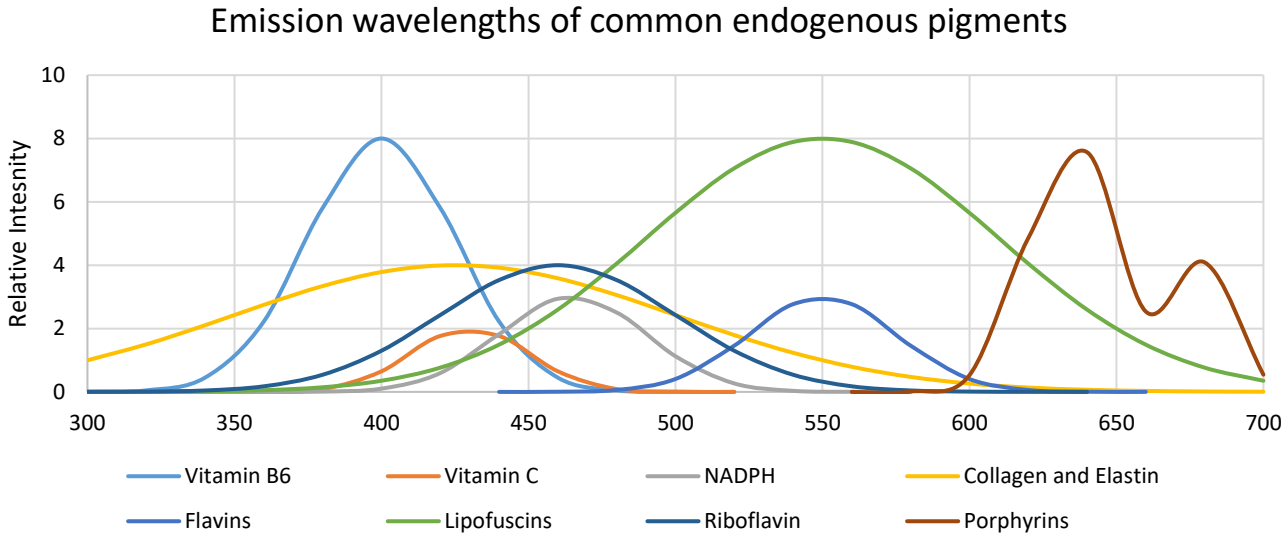
3. Bleach tissues with 5% H_2O_2 during antigen retrieval step (not compatible with fluorescent protein).

   To address autofluorescence derived from these sources, bleaching tissues with peroxide can be effective. The procedure involves incubating tissues in 5% H_2O_2 in Methanol/DMSO (1 part 30% H_2O_2, 4 parts methanol, 1 part 100% DMSO) at 4°C overnight prior to staining and clearing steps.

4. Autofluorescence can be subtracted during image processing to reveal signals otherwise difficult to detect. Autofluorescence is highest in the red channel (TRITC, 530-600 nm), so select green, or far red for important labels, and use blue for nuclear staining. During imaging, capture your signal channel, and a channel of autofluorescence, and subtract the autofluorescent image stack from the signal channels to decouple autofluorescence from signal.

Diagram: Common Endogenous Fluorophores





Fluorescence excitation and emission maxima of common endogenous fluorophores

Pigment	*Excitation (nm)*	*Emission (nm)*	*Pigment*	*Excitation (nm)*	*Emission (nm)*
Vitamin B6	320	400	Collagen and Elastin	350	350
Vitamin C	350	430	Flavins	380, 460	380, 460
NADPH	366	465	Lipofuscin	350, 440	350, 440
Vitamin D	390	470	Riboflavin	350	350
Lignin	530	488	Porphyrin	442	442

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Tissue Labeling Techniques

There are three major ways that tissues are labeled for 3D visualization: 1) Fluorescent protein transgenes; 2) Immunolabeling; 3) Small-molecule fluorescent probes. Considerations for each of these techniques are discussed in this section.

Considerations for Imaging Fluorescent Proteins: GFP, YFP, td-Tomato, etc.

Imaging fluorescent proteins does not require antigen retrieval steps. Furthermore, imaging fluorescent proteins requires the use of ethanol at 4°C instead of methanol for dehydration steps, since methanol quenches fluorescent protein.

Considerations for Immunolabeling

When conducting immunolabeling techniques on whole tissues, large sections, or pieces of tissue, there are several considerations that apply to whichever technique is selected for labeling.

Validation and Optimization of Antibody

Before immunolabeling large pieces of tissue (>3 mm) or whole organs, please read the following considerations and validate your antibody and dilution using the procedure described below and detailed in the "Getting Started" page included in the kit. Optimizing staining conditions for 100 µm tissue sections prior to large tissue labeling can save weeks of wasted effort. It is easy to extrapolate from small tissue sections, as doubling tissue thickness requires tripling incubation times with antibodies. See page 15 for a table of incubation times. 100-300 µm sections can be labeled and cleared in a single workday, allowing for rapid optimization and validation.

Method for Validation and Optimization of Antibody with Visikol HISTO Approach

1. Obtain tissue of interest. Using a vibratome (or razor-blade) cut five 100-200 µm thick sections of the tissue of interest.
2. Incubate solutions in antigen retrieval steps.

Replace Methanol with ethanol and process tissues at 4°C if using fluorescently labeled tissues

   a. Incubate in methanol for 15 minutes
   b. Incubate twice in 20% DMSO/80% methanol (v/v) for 15 minutes
   c. Incubate in methanol for 15 minutes
   d. Incubate tissues for 15 minutes in **Penetration Buffer**
   e. Block tissues by incubation for 15 minutes in **Blocking Buffer**

3. Incubate each tissue section at 1:500, 1:250, 1:150, 1:100, 1:50 dilutions of antibody in minimum **Antibody Buffer** for 30 minutes. Wash tissues 5x with PTwH for 5 mins. Repeat for secondary if using indirect labeling and wash 5x with PTwH for 5 mins.
4. Dehydrate tissues in 100% methanol for 15 minutes. Clear with **Visikol HISTO-1** and **Visikol HISTO-2** for 30 minutes each
5. Image tissue sections using confocal laser scanning microscope (or multiphoton or light sheet instrument). Acquire stack at ~10µm z-step on 10x objective. Examine stacks for evenness of staining across increasing depth.
6. Examine orthogonal view of image stack to look for evenness of stain on the XZ and YZ planes.

Not all antibodies are created equal: some antibodies stubbornly refuse to work in 3D. We have found that successful labeling of certain targets (e.g. GFAP) is highly dependent on what particular antigen was used to generate the antibody utilized. For example, in our hands, GFAP was difficult to label using common rabbit IgG anti-GFAP, but was highly successful using

chicken IgY anti-GFAP (Encor Bio, CPCA-GFAP). Other epitope targets, for example NeuN, label successfully with almost every antibody we have screened.

Choose antibodies from a host species differing from the species of your tissue of interest. For example, if studying mouse tissue, use antibodies from rabbit, donkey, etc.

How to avoid uneven labeling

There are several factors at play to which affect uniformity of antibody labeling in tissues.

1) Antibody concentration

Since antibodies are rather large with respect to tissue pore size, and the exhibit high binding constants to their targets, at concentrations too high, antibodies bind to the tissue and block off available diffusion channels in the tissue pores. This is analogous to a disabled vehicle blocking the lane of a road: other cars (unbound antibodies) are slowed, increasing the local concentration of cars, as they must find a different path around the disabled vehicle (bound antibody). Take careful note of the concentration of the antibody stock solution from the supplier: the typical concentration for primary antibody is 1 mg/mL, however can vary depending on supplier.

2) Incubation time

Insufficient incubation time will lead to uneven staining of sample, since not enough time has passed for antibody to diffuse evenly throughout the tissue. Incubation time is directly tied to tissue thickness.

3) Thickness of tissue

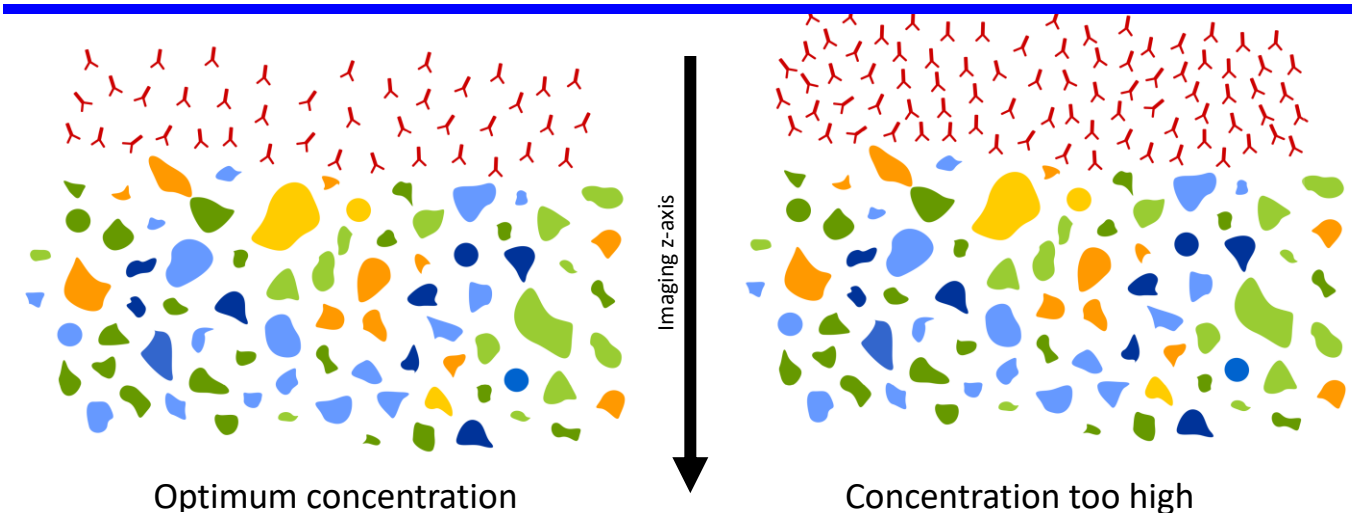
In general, doubling the thickness of tissue requires tripling the incubation time. As such, mouse brain hemispheres require ~1.5 days incubation, whereas whole mouse brains require ~5 days. Smaller tissues can be labeled in just hours: 1 mm tissue sections require a 5 hour incubation.

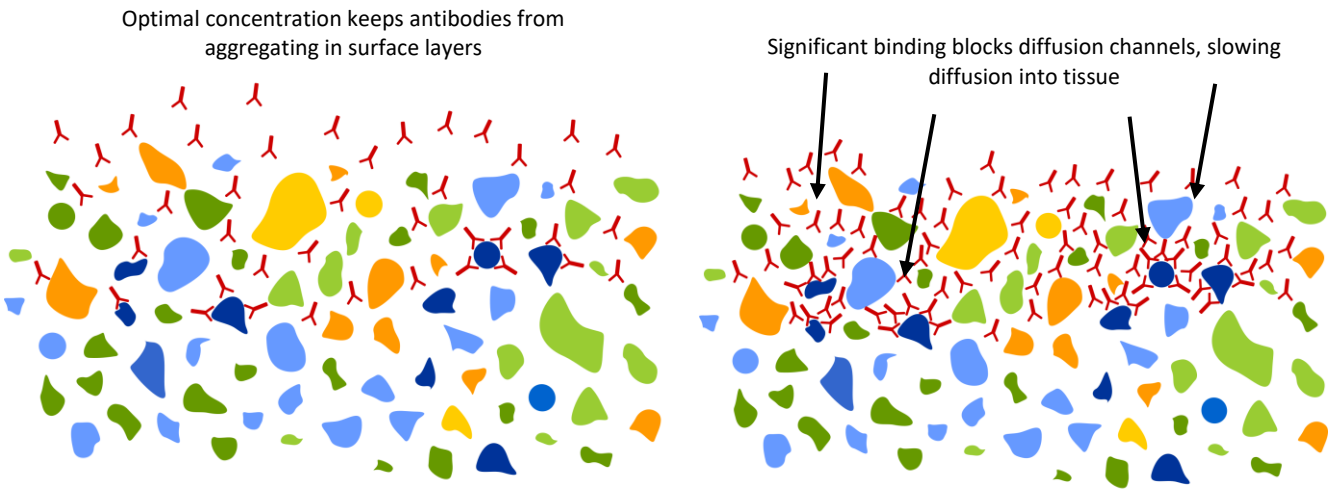
4) Tissue porosity

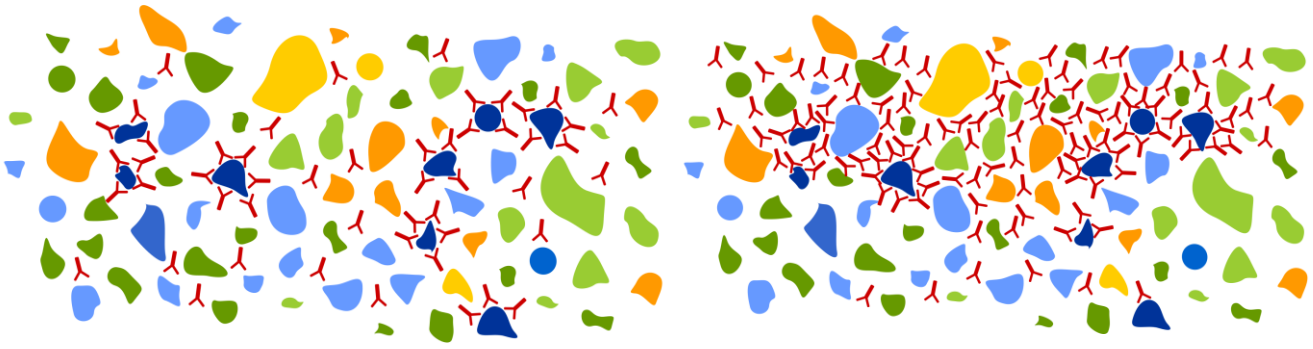
The denser the tissue, the slower an antibody will permeate into tissue. The antigen retrieval, penetration, and permeabilization steps discussed in this section are designed to increase the size of pores in the tissue, increasing antibody penetration.

*The **two main factors** to control for uniform antibody labeling are **incubation time** and **antibody concentration**. Antibody concentration can be optimized on small sections of tissue rapidly before moving to large tissue. The general rule is to increase incubation times 3x for each doubling of tissue thickness.*

Diagram: Effect of Antibody Concentration on Labeling







Optimum concentration
Even staining along z axis

Concentration too high
Uneven staining at equilibrium

As can be seen by this diagram, increasing concentration too much will lead to increased binding in the upper layers of the tissue which in turn blocks available diffusion channels, preventing diffusion to deeper parts of tissue, and increasing the signal intensity at the upper layers.

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Optical Factors

There are also optical factors that can contribute to what appears to be uneven labeling, as seen by an attenuation of signal intensity on evenly labeled tissue. This can be observed by imaging nuclear-stained tissues: since nuclear stains are very small and stain very evenly, the attenuation due to a specific optical system can be observed.

The main factor influencing optical attenuation of signal in immunolabeling is the final concentration of fluorophores bound to the tissue during imaging. Labeling at too high a concentration causes the upper layers of the tissue to absorb significant quantities of incident laser, causing a "shadowing" of the deeper layers. As concentration of stain approaches the "optimum level" (determined experimentally as discussed above) attenuation from absorption becomes a less significant factor.

How to check for uneven labeling: Bisect the tissue and examine the staining by comparison of the center of the image to the periphery. Compare the intensity of staining at the surface at the cut to the outer surface exposed during staining.

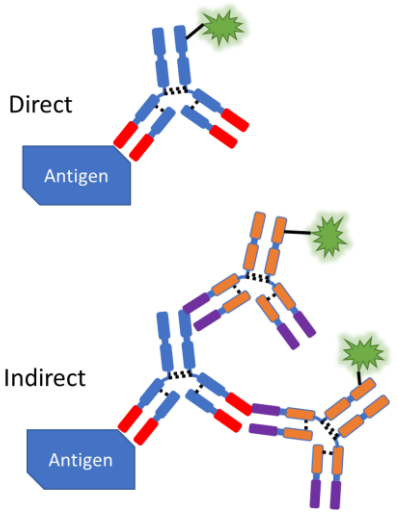
Direct vs. Indirect labeling

Direct labeling

- *Antibody conjugated to fluorescent marker*
- *No amplification of signal—sometimes not sufficient for larger tissues or tissues with high background*
- *Faster (only requires incubation with primary)*

Indirect labeling

- *Labeling with primary antibody followed by labeling primary with secondary antibody conjugated to fluorescent marker*
- *Signal amplified since more than 1 antibody per target – gives brighter signal*
- *Slower (requires two incubations and washings)*



Antigen Retrieval

Since tissue labeling is conducted prior to clearing, the Visikol HISTO process uses a special series[1] of treatments intended to permeabilize the tissue to stain, allowing for complete penetration and even labeling of the tissue. This is commonly known as antigen retrieval. Antigen retrieval helps to open pores in the cell membranes, exposing epitopes and increasing permeability of the tissue to antibodies into the tissue. The technique involves washing tissues through a methanol or ethanol (at 4°C) gradient, washing with DMSO, and returning to PBS through the reverse gradient. For larger, more collagenous, and difficult to label and/or clear tissues (e.g. liver tissue, archived human tissue, etc.), incubation overnight in the **Tissue Permeabilization Buffer** is recommended.

Washing Steps

Successful tissue labeling is dependent on the washing steps, which are conducted to remove excess and unbound stain from tissue. Heparin is included in the **Washing Buffer** to inhibit non-specific protein binding, reducing background staining due to non-specific binding of stains. Issues with high background staining can often be resolved by increasing the length of time the tissues are washed following staining steps.

Immunolabeling in Fluorescent Protein Transgenic Tissue

Immunolabeling fluorescent protein expressing tissue is simple. Follow the same steps described for immunolabeling, however omit the H_2O_2 bleaching step, and use ethanol at 4°C instead of methanol in antigen retrieval and dehydration steps.

[1] Adapted from Renier, N., Wu, Z., Simon, D. J., Yang, J., Ariel, P., & Tessier-Lavigne, M. (2014). iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. *Cell*, *159*(4), 896-910.

Visikol HISTO Immunolabeling Buffers

The buffers required to use Visikol HISTO contain common reagents that can be made in almost every lab. These buffers can be easily prepared, and substitutions with other buffer salts (e.g. tris buffered saline instead of PBS) have no overall effect on the labeling procedure. These buffers can be prepared from their components, or ordered directly on our website at https://visikol.com/products/store/. The recipes for the buffer components are found below.

Recipes for Buffer Solutions used in Immunolabeling

Recipes for 100 mL – prepare in volumetric flask

Buffer Name	Composition	Recipe for 100 mL	Storage
PBS-TX100	• PBS (1X) • Triton™ X-100[1] (0.2% wt.)	• 200 mg Triton™ X-100 X-100 • Dilute to 100 mL with 1X PBS	RT
PBST	• PBS(1X) • Tween® 20[2] (0.2% wt.)	• 200 mg Tween® 20 • Dilute to 100 mL with 1X PBS	RT
Penetration Buffer	• PBS • 0.2% Triton™ X-100[1] • 0.3 M Glycine[3] • 20% DMSO[4]	• 2.25 g Glycine • Add 20 mL 1X PBS-TX100 • 20 mL DMSO • Dilute to 100mL with ~60 mL PBS-TX100	RT
10X PTwH (10X washing buffer)*	• 10X PBS • 2% Tween® 20[2] • 100 ug/mL Heparin[6]	• 10 mg Heparin • Add 20 mL 10X PBS • 2 g Tween 20 • Dilute to 100 mL with 10X PBS	4° C, 6 months. Add 0.05% sodium azide for long term storage‡
Blocking Buffer	• PBS • 0.2% Triton™ X-100[1] • 6% Donkey Serum[6] • 10% DMSO[4]	• 6 mL Donkey Serum • Add 10 mL PBS-TX100 • Add 10 mL DMSO • Dilute to 100 mL with PBS-TX100	4° C Add 0.05% sodium azide for long term storage‡
Antibody Buffer	• PBS • 0.2% Tween 20[2] • Heparin[5] • 3% Donkey Serum[6] • 5% DMSO[4]	• 3 mL Donkey Serum • Add 20 mL 1X Washing Buffer • Add 5 mL DMSO • Dilute to 100 mL with 1X Washing Buffer	4° C Add 0.05% sodium azide for long term storage‡

* To make 100 mL of 1X washing buffer, dilute 10 mL 10X buffer with 90 mL of DI H_2O

‡ Sodium azide will interfere with horseradish peroxidase-based protocols, but has no effect on immunofluorescent labeling

Catalog numbers

1. Triton™ X-100 – Fisher Scientific – Catalog # BP151
2. Tween® 20[i] – Fisher Scientific – Catalog # BP337
3. Glycine – Fisher Scientific – Catalog # G46
4. DMSO – Fisher Scientific – Catalog # BP231
5. Heparin – Alfa Aesar – Catalog # A16198
6. Normal Donkey Serum – Jackson Immunoresearch – Catalog #: 017-000-121

i. Triton is a trademark of The Dow Chemical Company or an affiliated company of Dow.

ii. Tween is a registered trademark of Croda International PLC

Nuclear stains, cell viability dyes, ClickItEdU, mitochondrial probes

Cell-permeant nuclear stains and cell-viability stains can be used without antigen retrieval permeabilization steps. Cell impermeant (e.g. SYTOX, Propidium iodide) stains will only stain cells with compromised cell membrane, however, treating tissue with cell impermeant stains after performing antigen retrieval steps or permeabilization steps will result in ubiquitous staining of nuclei for all cells.

Cell viability dyes and mitochondrial probes which rely on enzymatic reaction to release fluorescent substrates (e.g. Calcein AM) should be used in vivo prior to fixation of cells for further processing. There are many fixable cell viability and mitochondrial probes available from ThermoFisher and BioLegend, and they are compatible with Visikol HISTO.

The ClickIt!EdU cell-proliferation kit should be performed as described by the manufacturers specifications, and should be performed in vivo prior to fixation and tissue processing.

Nuclear staining as a counterstain for immunolabeling should be conducted at the same time as incubation with antibodies. Dilution of nuclear stains is typically 1:50 to 1:250, but due to the wide variety of nuclear labels, should be evaluated for each stain and tissue combination.

Nuclear stains can be added even after clearing with Visikol HISTO. Simply remove the tissue from the clearing solution and place onto a glass dish and pipet the nuclear stain directly onto the cleared tissue. Re-incubate the tissue in a minimum volume of Visikol HISTO-2 to allow the nuclear stain to diffuse, and then re-mount in Visikol HISTO-2 for imaging.

Mounting and Storage of Labeled Samples

Fluorescently labeled samples can be stored for > 6 months in Visikol HISTO-2 without any apparent degradation of signal. Some nuclear stains fade quickly (Hoescht 33342, DAPI, Propidium iodide) but nuclear stain can be reapplied to a sample that has been stored in Visikol HISTO-2. Simply remove the sample, and directly pipet a small volume of nuclear stain onto the tissue, and allow to incubate for 30 minutes to ensure even staining. Fluorescent protein will maintain fluorescence over long periods as long as extra care to avoid ambient light is taken.

Incubation Times for Various Tissues

In general, the antibody incubation time triples for a doubling of given tissue thickness. Thus, incubating small sections (0.5-1 mm) for various times and comparing results will lead directly to extrapolation of time required for larger tissues. While the table below is useful as a general guideline, optimum incubation times should be determined experimentally by optimizing on small tissue sections, and extrapolating to larger thicknesses.

Thickness	Antigen retrieval and dehydration	Penetration / Permeabilization	Blocking *	Antibody incubation*	Washing steps	HISTO-1and HISTO-2 *
8 mm (e.g. whole mouse brain)	2 hr	4 hr	2.5 days	120 hrs	2 hr + overnight for last wash	24 hr
4 mm (e.g. mouse brain hemisphere)	1 hr	3 hr	40 hr	40 hrs	1 hr + overnight for last wash	18 hr
2 mm	45 min	2 hr	13.5 hr	13.5 hrs	45 min	12 hr
1 mm	30 min	1 hr	4.5 hr	4.5 hrs	30 min	8 hr
500 um	20 min	30 min	90 min	90 min	20 min	4 hr
250 um	15 min	15 min	30 min	45 min	10 min	2 hr
125 um	10 min	15 min	15 min	30 min	5 min	30 min

* for liver, kidney, and lymphatic tissues, extend incubation time by 30-50% depending on degree of fixation

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Table of Antibodies Validated with Visikol HISTO

ANTIGEN	HOST SPECIES	TARGET SPECIES	SUPPLIER	CAT #
GLIAL FIBRILARAL ACIDIC PROTEIN (GFAP)	Chicken	Ms,Rt, Hu	EnCor Bio	CPCA-GFAP
CALBINDIN	Chicken	Ms,Rt, Hu	EnCor Bio	CPCA-Calb
CALRETNIN	Chicken	Ms,Rt, Hu	EnCor Bio	CPCA-Calretinin
MYELIN BASIC PROTEIN	Chicken	Ms,Rt, Hu	EnCor Bio	CPCA-MBP
NEUROFILAMNET-HEAVY CHAIN	Chicken	Ms,Rt, Hu	EnCor Bio	CPCA-NFH
VIMENTIN	Chicken	Ms,Rt, Hu	EnCor Bio	CPCA-Vime
FOX3/NEUN	Mouse (monoclonal)	Ms, Rt, Hu, all vertebrates	EnCor Bio	MCA-1B7
GLIAL FIBRILARAL ACIDIC PROTEIN (GFAP)	Mouse (monoclonal)	Ms, Rt, Hu, all mammals	EnCor Bio	MCA-5C10
MYELIN BASIC PROTEIN	Mouse (monoclonal)	Ms, Rt, Hu, all mammals	EnCor Bio	MCA-7G7
NEUROFILAMENT-HEAVY CHAIN	Mouse (monoclonal)	Ms, Rt, Hu, all mammals	EnCor Bio	MCA-AH1
VIMENTIN	Mouse (monoclonal)	Ms, Rt, Hu, all mammals	EnCor Bio	MCA-2D1
ADENYLATE CYCLASE III	Rabbit	Ms, Rt	EnCor Bio	RPCA-ACIII
CFOS	Rabbit	Ms, Rt, Hu	EnCor Bio	RPCA-c-Fos-AP
FOX3/NEUN	Rabbit	Ms, Hu, vertebrates	EnCor Bio	RPCA-Fox3
METHY CPG-BINDING PROTEIN 2	Rabbit	Ms, Hu	EnCor Bio	RPCA-MeCP2
NEUROFILAMENT-MEDIUM CHAIN	Rabbit	Ms	EnCor Bio	RCPA-NF-M
E-CADHERIN	Rabbit (monoclonal)	Ms, Hu	AbCam	ab40772
BETA CATENIN	Rabbit (monoclonal)	Ms, Hu	AbCam	ab32572
BETA INTEGRIN	Rabbit (monoclonal)	Ms, Hu	AbCam	ab52971
KI67	Rabbit	Ms, Hu	AbCam	ab15580
KI67	Rabbit (monoclonal)	Ms, Hu	AbCam	av16667
NEUN-ALEXAFLUOR488 CONJUGATE	Rabbit	Ms, Rt	Millipore	ABN-90
EGFR	Rabbit	Hu, Ms, Rt, Nhp	Invitrogen	PA1-1110
PARVALBUMIN	Mouse (monoclonal)	Hu, Ms, Rt	Swant	PV-235

Ms = mouse, Rt = rat, Hu = human, Nhp = non-human primate

Please see our website https://visikol.com/products/visikol-histo/visikol-histo-validated-antibodies/ for a continually updated list of antibodies known to work (and not work) with Visikol HISTO.

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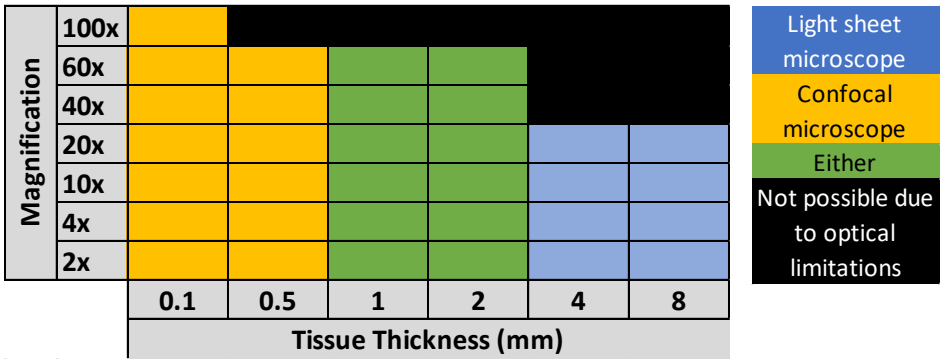
3D Imaging Guidance

3D Imaging is as much art as it is science, and a good relationship with the fundamentals of fluorescence and advanced microscopy (confocal, single/multiphoton, or light sheet microscopy).

Choice of Microscope

After a tissue has been labeled and rendered transparent, the last step in acquiring 3D information from your tissue is to image the tissue using a 3D imaging modality. There are three primary imaging modalities (e.g. confocal, light sheet, single/multiphoton) that can be used for this purpose and the one that you use will depend entirely upon your research question as each imaging modality has a specific set of advantages and disadvantages. More specifically, the imaging modality you chose will be based upon your desired magnification and the thickness of the tissue you are imaging. In general, confocal microscopy is best used for high resolution imaging of small volume tissues (≤ 2 mm thickness) and light sheet microscopy is best used for lower resolution imaging of large volume tissues (> 2 mm thickness).



Confocal Microscopy

Confocal microscopy is the most prevalent 3D microscopy imaging modality as it has numerous applications and allows for ultra-high-resolution imaging of tissues. However, confocal microscopy is slower than light sheet microscopy and can photo-bleach tissues as areas of tissue outside of the focal plane are illuminated during imaging. Confocal microscopy is best at high resolution imaging of small volumes where light sheet microscopy is best at low resolution imaging of large volumes. However, the specific imaging capabilities (e.g. depth, resolution) of a confocal microscope will be primarily dictated by the instruments objectives. See below for a more detailed discussion of objectives.

Light Sheet Microscopy

The concept of light sheet microscopy has been around for almost a century and was only applied recently to the 3D visualization of tissues through the combination of tissue clearing with fluorescent labeling. Light sheet imaging can generate large volume 3D renderings of whole tissues while causing minimal photo-bleaching, but is limited in resolution compared to confocal microscopy. A light sheet microscope is very simple in nature and operates by passing an ultra-thin orthogonal laser light sheet through a cleared tissue in the same plane as the imaging objective's focal plane. By moving the tissue up and down through the light sheet, a stack of Z projections can be generated.

Commercially Available Light Sheet Microscopes

While less prevalent than confocal microscopes, there are several commercially available light sheet microscopes: Zeiss – Z1, ASI – DISPIM and ISPIM, LaVision BioTec – Ultramicroscope II, PhaseView – Alpha3, and the Luxendo – MuVi-SPIM. Of all of these light sheet microscopes, we suggest using the Ultramicroscope II as it is the only light sheet microscope directly compatible with solvents like Visikol HISTO.

All commercially available light sheet microscopes except the Ultramicroscope II are not directly compatible with Visikol HISTO. The Ultramicroscope has a solvent compatible dipping objective and can be used without modification with Visikol HISTO. With other light sheet microscopes, you will need to mount your sample in Visikol HISTO within a double chambered cuvette, with the outer chamber filled with thiodiethanol (TDE). Please see our website where we have compiled the locations of light sheet microscopes and imaging cores across the US and EU (https://visikol.com/products/visikol-histo/whole-tissue-imaging-guidance/).

Objectives for 3D Imaging

Important: For the depth required to image tissues thicker than 1 mm with a confocal microscope, the use of high refractive index matched, high numerical aperture immersion lenses (e.g. BABB immersion objective, glycerol immersion objective, CLARITY optimized objective, water immersion objective), or an objective with a refractive index adjusting collar is required. The maximum depth of imaging obtainable with a 10x air objectives is approximately 500-800 µm due to attenuation caused by spherical aberration. While immersion objectives can be used with inverted confocal microscopes, it is suggested that for imaging deeper than 500-800 µm that an upright confocal instrument is used.

Mounting specimens

For imaging using an air objective, we recommend using the Silicone ClearWells™ included in the Visikol HISTO starter kit or available through https://visikol.com/products/store/ . These solvent resistant silicon cut-outs stick to glass slides and are the perfect size for standard 0.15 mm coverslips. Simply stack the ClearWells until the desired depth is achieved for your tissue. Stick the ClearWells to a dry microscope slide, and press firmly to ensure a tight seal. Place the tissue in the well and fill with Visikol HISTO-2 solution. Be careful not to bump the sides of the well, as breaking the silicone seal will cause leakage.

Immunolabeled samples can be stored for > 6 months in Visikol HISTO-2 without any apparent degradation of signal. Some nuclear stains fade quickly (Hoescht 33342, DAPI, Propidium iodide) but nuclear stain can be reapplied to a sample that has been stored in Visikol HISTO-2. Simply remove the sample, and directly pipet a small volume of nuclear stain on the tissue, and allow to incubate for 30 minutes to ensure even staining.

Anti-Fade Media

Anti-fading media are typically aqueous, and will disrupt Visikol HISTO-cleared samples if transferred. The use of an anti-fade medium is not required as Visikol HISTO includes anti-fade agents to stabilize fluorescence. Tissues have been stored in the dark at room temperature > 6 months without apparent decrease in signal.

Attenuation of Signal from Optical Factors

Absorption of Incident Light from Fluorophores

Tissues stained evenly with a fluorescent probe will exhibit a natural decrease in signal intensity as the depth of imaging increases due to absorption of incident light by the upper layers of tissue. This relationship is governed by Beer's Law:

$$A = log_{10} \frac{I_0}{I_T} = \varepsilon[c]l$$

Where:

- A = absorbance in absorbance units
- I_0 = Intensity of incident light
- I_T = Intensity of transmitted light
- ε = extinction coefficient – rate of light absorption per concentration and length - unique to each fluorophore
- $[c]$ = concentration of fluorophore
- l = path length (depth traveled by incident light)

Absorbance is defined as the base 10 log of the ratio of the incident light intensity to the transmitted light intensity, thus gives an absorbance of 0 when the intensity of the incident light is equivalent to the intensity of the transmitted light. As the ratio of I_0/I_T increases, the absorbance increases proportionate to the log of the ratio. As can be seen, the absorption increases proportionately to concentration and path length, which is equivalent to depth of tissue. Therefore, the intensity of transmitted light is inversely proportional to imaging depth into tissue. An easy way to think about this is by thinking about how tinted windows block certain wavelengths, and the thicker the glass, the darker the tint. See the diagram on page 21 to see the impact of increasing concentration of fluorescent label by depth.

This issue is mitigated substantially in light sheet microscopy, where the incident light is perpendicular to the imaging axis, and thus the effect of attenuation is much less affected by depth of imaging.

Quantum Yield

The quantum yield is the ratio of photons fluoresced to the photons absorbed by the fluorophore. The quantum yield is an intrinsic value to each fluorophore and can be found in the vendor's documentation, or a quick Google search. Modern fluorophores (AlexaFluor series, Cy series, SYTOX and SYTO nuclear stains) have superior quantum yield and are less prone to photobleaching compared to the first generation fluorophores (DAPI, propidium iodide).

Refractive index mismatch and numerical aperture

Each objective is designed for a specific refractive index. Air objectives are designed to observe through air and a 0.15 mm thick coverslip. As you increase depth of imaging in cleared tissue, the refractive index mismatch with the air causes a reduction of the effective numerical aperture (NA), meaning that less signal is getting through the sample and out to the lens. This causes attenuation of the signal as the signal misses the lens. In air objectives, this limits the depth attainable in confocal microscopy to approximately 500-800 μm. See the diagram on page 22.

Ideally, an immersion objective with a long working distance that is RI matched to glycerol or BABB should be used for imaging with Visikol HISTO to reduce attenuation.

Light scattering and Autofluorescence

While optical clearing of tissue drastically reduces autofluorescence, due to the complex optical properties of proteins, lipids, and carbohydrates, many molecules exhibit birefringence, and the refractive index differs based on orientation of the molecule. As such, even transparent tissues scatter some light due to subtle variations in refraction in the tissue. These effects increase with thickness of the tissue.

Another contributor to attenuation due to the tissue is from autofluorescent pigments native to the tissue. Due to absorbance effects described in the text above dictated by Beer's law, intense autofluorescence in the tissue will absorb incident light and reduce the available photons to be absorbed by labels, thereby reducing the signal as depth increases.

Confocal Pinhole

The purpose of the confocal pinhole is to achieve the narrow depth of field required for the effectiveness of the confocal system in obtaining clear images through thicker tissues. Decreasing the pinhole size increases resolution achievable in the z-axis because the depth of field is narrowed with decreasing pinhole size. However, the pinhole excludes much of the light relevant to creating a signal at deeper levels, and thus reduces intensity of tissue. This is not an issue in light sheet microscopy.

Laser Power, Gain, and Pixel Exposure Time

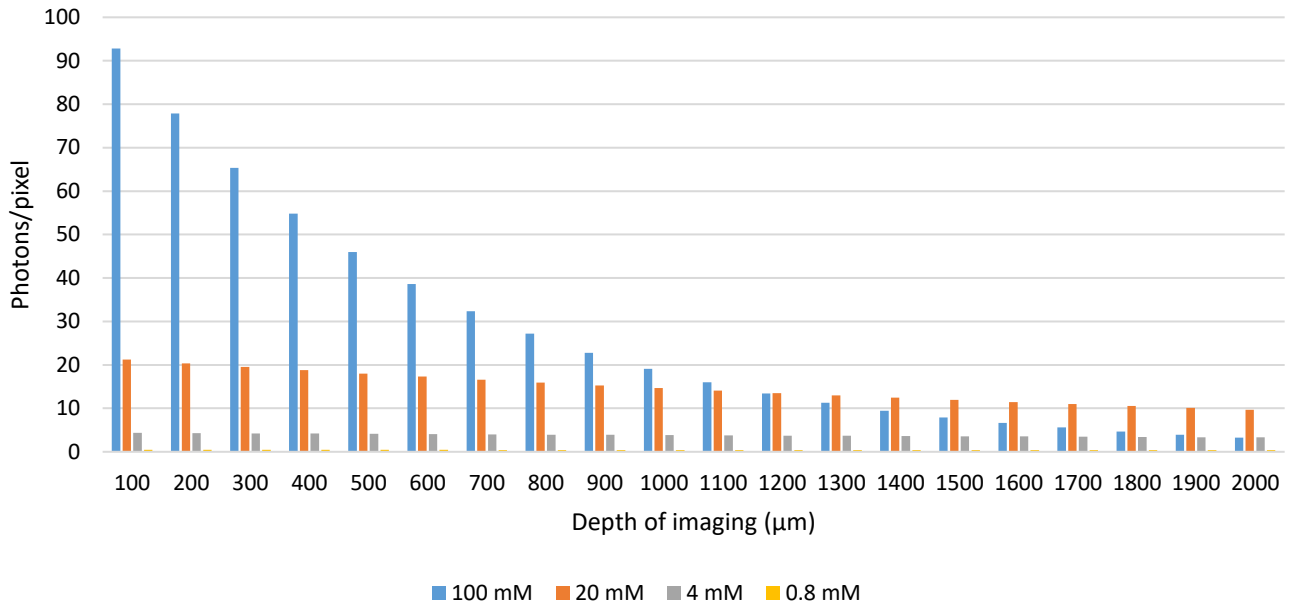
Based on the cumulative effect of the factors discussed above, the laser power reaching a given fluorophore is attenuated through the sample as depth into tissue increases. As such, at a given laser power and constant gain, signal will appear to drop off as depth of tissue increases, regardless of homogeneity of labeling.

One practical way to mitigate optical attenuation and extend the depth achievable in imaging is to vary the laser power and gain according to depth of imaging. This is automatable in the Leica SP5 and SP8 confocal microscopes.

Pixel exposure time directly affects the intensity of the signal that reaches each pixel on the detector per scan. Pixel exposure time is related to the number of pixels per scan line, and the scanning frequency. The slower the scanning frequency, the more time each pixel is exposed to the light emitted from the sample, increasing the total photons collected per pixel. The trade-off is that slower scanning frequencies result in increased overall image acquisition time.

Diagram: Effect of Fluorophore Concentration on Signal

Concentration is Critical

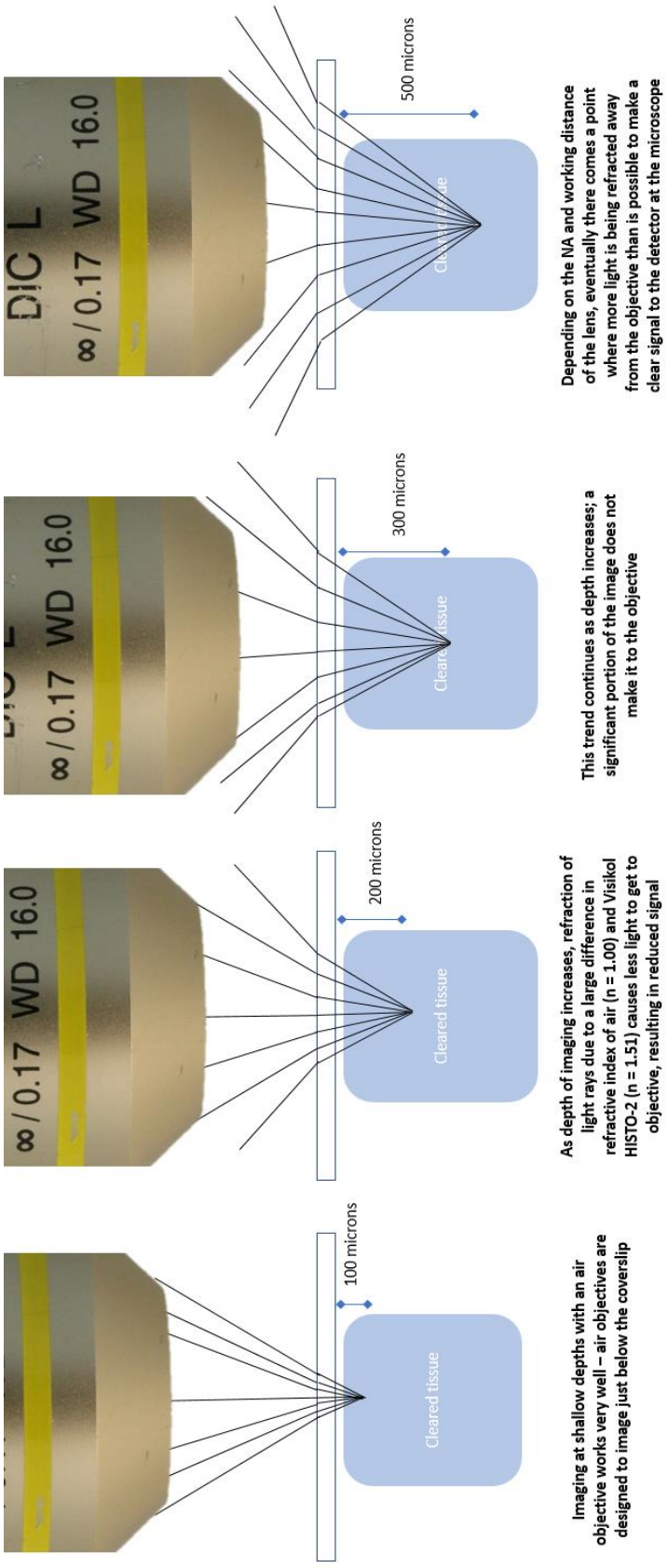


Note: concentrations in figure represent the hypothetical concentration of fluorophore bound to the tissue, **these do not represent incubation concentrations**. This diagram is used to explain the relationship between overstaining and loss of signal and is based on theoretical predictions.

Data calculated for AlexaFluor488, using a confocal microscope with a pinhole, equipped with an air objective with a 0.3 NA and a 16 mm working distance. Assumes 512x512 pixels, total scan time of 1 s / image. Assumes homogenous fluorophore distribution.

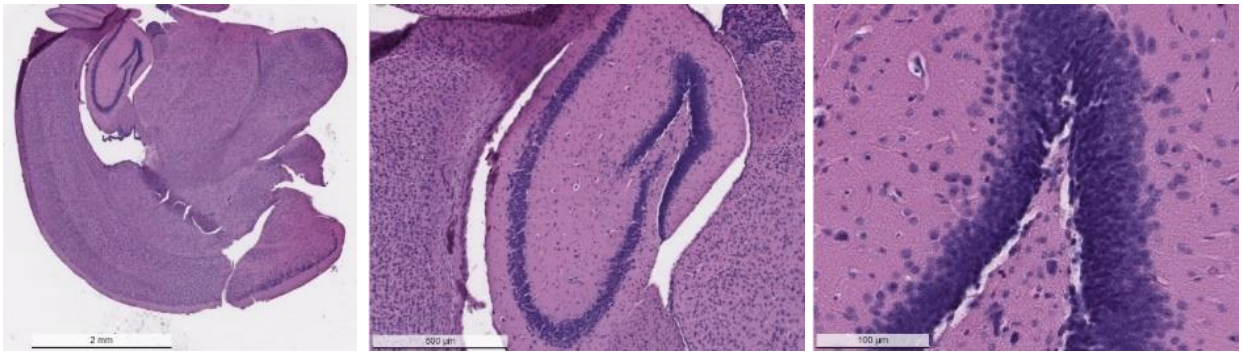
- At very high concentration of fluorophore in the tissue (100 mM), there is an intense signal at the surface, but it attenuates quickly as depth increases due to absorption of incident photons at higher layers (think tinted windows) and so deeper layers have fewer photons available for fluorescence.
- At optimum concentration (20 mM), while the signal is decreased with respect to the over-stained concentration (100 mM), the relative intensity decreases very slowly through the sample, and is still detectable at the deepest layers.
- At low concentrations (4 mM), the signal is quite low, but does not readily attenuate across tissue. This signal may not be detectable above background fluorescence.
- At sub-threshold concentrations (0.8 mM), the signal is not detectable because too few photons are available to detect at each pixel.

Diagram: Limitation of Imaging Depth from RI Mismatch

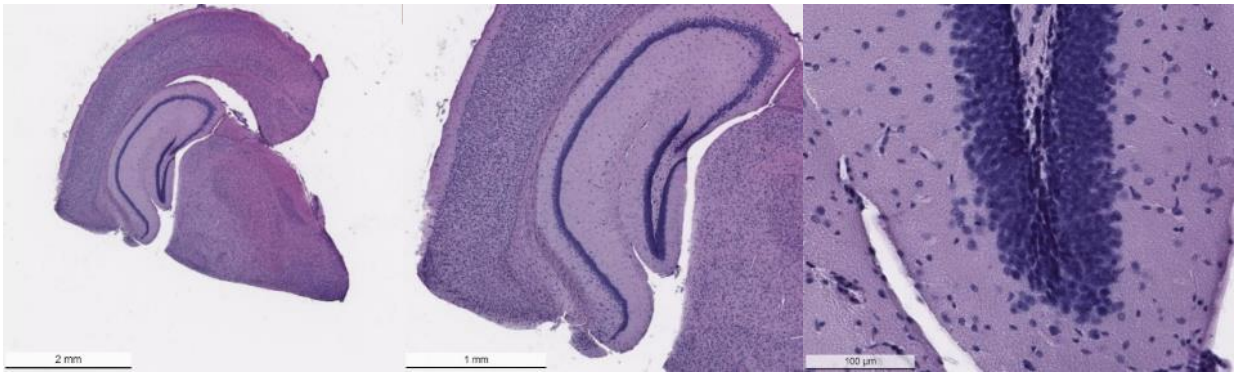


Reversal of clearing and 2D Histology

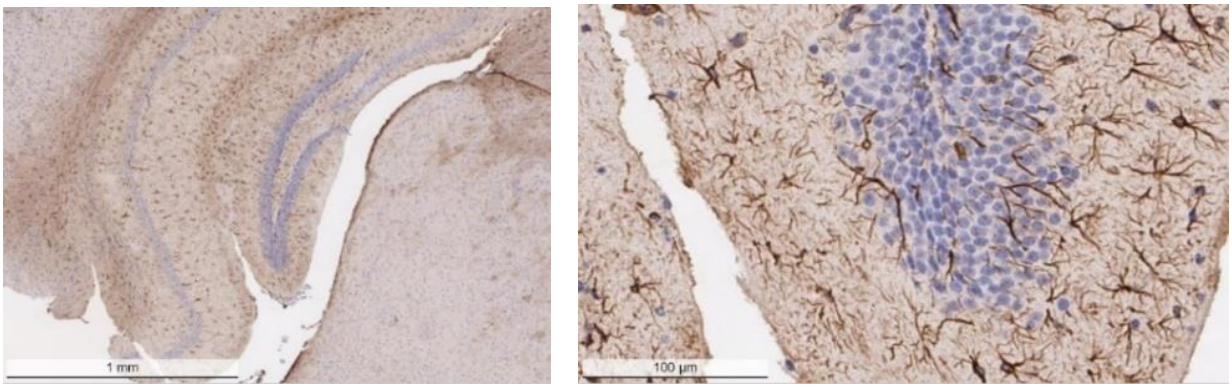
The reversal of cleared tissues to restore them for further techniques such as histological sectioning and staining, SDS-PAGE, or MALDI is a very simple process. Tissues are washed several times with large volumes of ethanol until opacity is restored. This process usually takes no longer than 24 hours. This protocol is described in detail on page **Error! Bookmark not defined.** of this booklet.



Untreated mouse brain tissue, formalin fixed paraffin embedded sections, stained with H&E depicting hippocampus



Mouse brain tissue, cleared with Visikol HISTO, and reversed, then embedded in paraffin, sectioned, and stained with H&E depicting hippocampus. Visikol HISTO does not appreciably affect tissue histology.



Mouse brain tissue, cleared with Visikol HISTO, and reversed, then embedded in paraffin, sectioned, and immunostained for GFAP, labeling astrocytes. Visikol HISTO does not affect antigenicity of tissues.

Protocol for Validating Antibody and Optimizing Concentration

Obtain and Fix tissue of interest

The general process for fixing tissues is to fix tissues in 4% PFA overnight at 4°C followed by one hour of clearing at room temperature. The tissue is then placed in PBS with azide (0.04%).

It is best to use a 24-well plate for treatment steps for small sections. However trim tissue to whatever containers are available, Eppendorf tubes are an excellent choice as well.

Tissue Preparation and Antigen Retrieval

Replace methanol with ethanol at 4°C in all steps if using fluorescently labeled tissues

1. Cut 100-250 µm sections of the tissue using a vibratome. Cut 5 sections for each antibody intended for validation and optimization
2. Incubate tissues in PBS for 5 minutes
3. Pipet off PBS, add 1 mL methanol, incubate tissues for 15 minutes.
4. (Optional step for highly pigmented tissues e.g. non-perfused tissues, kidney, liver.) Prepare (using refrigerated reagents stored at 4°C) 5% H_2O2 in 20% DMSO/methanol by adding 1 mL 30% H_2O_2 (careful! Strong oxidizer!) to 4 mL methanol. Add 1 mL DMSO. This solution should be kept at 4°C until use. Incubate tissue in 1 mL of this solution at 4°C for 30 minutes.
5. Remove solution from tissue and add 1 mL of 20% DMSO in methanol, incubate for 15 minutes.
6. Incubate tissues in 1 mL 100% methanol for 15 minutes.
7. Incubate tissues in 1 mL PBS with 1% Triton X-100 for 15 minutes.

Immunolabeling

8. Incubate tissues in ~400 µL (increase volume as necessary to completely cover tissue) incubation buffer for 15 minutes.
9. Incubate tissue in ~400 µL (increase volume as necessary to completely cover tissue) blocking buffer for 15 minutes.
10. Incubate tissue in ~400 µL (increase volume as necessary to completely cover tissue) antibody buffer containing varying dilutions of antibody ranging from 1:50 to 1:500 (e.g. 1:50, 1:100, 1:200, 1:300, 1:500. Incubate for 30 minutes. Add nuclear label at 1:500 dilution.
11. Wash tissues by exchanging buffer with washing buffer 5x with 5 minutes incubation time between exchanges.
12. (Optional step if using indirect labeling with secondary antibody) Incubate tissue in ~400 µL (increase volume as necessary to completely cover tissue) antibody buffer containing 1:100 dilution of secondary antibody (concentration may also need optimization using this method of varying dilution, depending on distribution of fluorophore).
13. (Optional step if using indirect labeling with secondary antibody) Wash tissues by exchanging buffer with washing buffer 5x with 5 minutes incubation time between exchanges.

Clearing

14. Transfer to 1 mL 100% methanol for 30 minutes.
15. Transfer tissues to 400-500 µL Visikol HISTO-1 for 10-30 minutes.
16. Remove from polystyrene well plate and transfer to slide. Mount in 400-500 µL Visikol HISTO-2.

Imaging and interpretation of results is discussed on the following page.

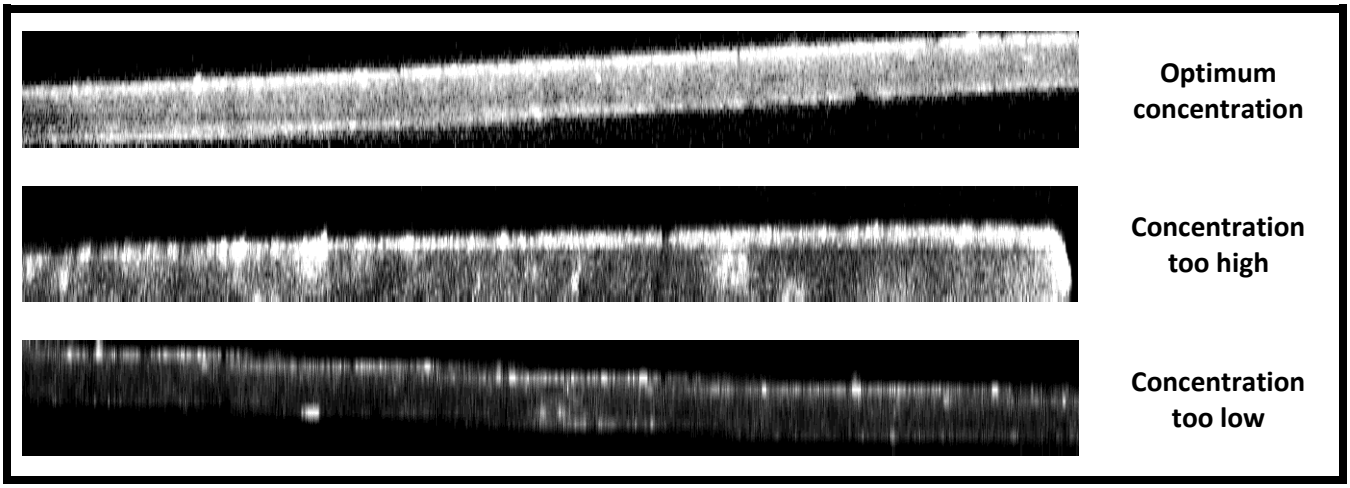
Imaging

Validation of staining of antibodies can be accomplished simply by using a typical fluorescent microscope. Prepare a slide of the cleared tissue and quickly examine for specificity of signal.
To examine evenness of staining, image tissues using a confocal microscope. Obtain a z-stack spanning the entire thickness of the tissue section with two color channels: the channel corresponding to the fluorescent conjugate for antibody staining, and the channel used for nuclear stain. Since nuclear stains penetrate tissues rapidly and homogenously, the nuclear stain channel serves as a control for optical attenuation.

Examine the z-stacks in ImageJ (or other image processing software). Observe the XZ and YZ plane by viewing "Orthogonal Views" to examine for evenness of staining. If staining is even, you should see relatively constant intensity (with respect to nuclear stain) across the tissue (see table below). Some dimming in 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 into the tissue (see table below). If the concentration of the immunolabel is too low, you will see slight staining at the surface layer, with a dark interior and uneven spots of stain.

Visualization of staining with XZ planes of image stacks of tissue sections



Troubleshooting

1. I can't image past 500-800 µm. Labeling appears uneven, and drops off significantly at this depth.
   - Antibody concentration too high: ring of intense staining near surface, drops off significantly after that
     > **Solution:** Reduce antibody concentration, if signal is too weak, incubate in lower concentration for half of time, and then re-incubate in higher concentration.
   - Antibody concentration too low: signal drops off into middle of tissue
     > **Solution: Increase antibody concentration**
   - Optical attenuation due to absorption of photons by upper layers of tissue causes "shadow" to tissues below, even with perfect staining
     > **Solution:** Increase laser power and gain as depth increases
       > Caution: increased laser power increases rate of photobleaching, be sure samples contain no air bubbles.
       > Leica SP5 and SP8 can automate laser power and gain corrections
       > Compare intensity loss to nuclear stain intensity, since nuclear stain diffuses very fast into tissue. Can use this signal to correct for signal loss in image processing

2. Intense band of labeled tissue at surface, then significant drop-off afterwards
   - Antibody concentration too high.
     > **Solution:** reduce concentration by increasing dilution factor. See page 12 for more details.

3. My tissue didn't clear!

   Plastic Incompatibility
   Visikol HISTO-2 will degrade polystyrene. For processing tissues with the Visikol HISTO approach we suggest moving away from polystyrene and towards polypropylene and glass where possible. Plastic leaching into your sample may affect the clearing ability of Visikol HISTO.

   Incomplete Dehydration/Clearing
   Most of the time a lack of tissue transparency is simply due to not completely dehydrating a sample. If you use methanol or ethanol for dehydration that has water in it and is not pure, you will not remove all the water from your tissue, resulting in tissue cloudiness. This can also be caused by not sealing the vessels containing your sample when clearing, as Visikol HISTO-2 is hygroscopic. Additionally, not using enough volume of Visikol HISTO-1 and Visikol HISTO-2 for your tissue size can cause inadequate clearing.

   Solution:
   For a mouse brain that is not completely clear, we suggest placing the brain back into 7-10 mL of Visikol HISTO-1 for 24 hours, followed by transfer to 25 mL methanol for 2 hours. Then transfer back to 7-10 mL Visikol HISTO-1 for 24 hours, followed by 7-10 mL of fresh Visikol HISTO-2 for 24 hours.

4. Fluorescent protein quenched

- To visualize fluorescent protein, samples must be dehydrated using ethanol at 4°C instead of methanol.
- Keep cleared samples in the dark, and protect your specimens with aluminum foil as fluorescent proteins photobleach rapidly when exposed to ambient light.
- Do not treat fluorescent protein labeled samples with H_2O_2 bleaching step; this step will oxidize fluorescent protein and signal will be lost.

5. My antibody didn't label the tissue
   - Some antibodies are not compatible with 3D immunolabeling. Validate the specificity of your antibody on small tissue sections before proceeding to larger tissues. Contact us if you have any questions about your specific antibody.
   - Only purchase antibodies that have been validated for use in IHC.
6. Center of tissue appears dark
   - Insufficient antibody concentration
     > Increase antibody concentration. A range of concentrations should be explored on a small section of tissue prior to scaling to large tissues.
   - Optical attenuation leads to diminished signal at increasing depths depending on several factors, such as concentration of label bound in upper layers of tissue, level of autofluorescence, type of objective, and laser power. Please read the section about optical attenuation on page 19.
     > Laser power and gain can be modified according to depth in tissue 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 increase noise at greater depths.

Contact Info

The Visikol team is here to help and will provide feedback and guidance on tissue clearing and 3D tissue imaging. We enjoy working with our customers and service clients and look forward to your feedback and questions. If you do not want to try tissue clearing yourself but still want to acquire 3D images from your tissues, ask us about our 3D tissue imaging services.

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