CytoVista™ Tissue and 3D Culture Clearing for 3D Fluorescence Imaging

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product information

CytoVista™ Tissue and 3D Culture Clearing workflow optically clears tissues or 3D cultures with minimum changes to morphology and without compromising the sensitivity of detection with many fluorophores. With the easy-to-use protocol, whole mouse brain (up to 8 mm) 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 fluorescence imaging instruments such as wide-field, confocal, and light sheet microscopes, and high-content analyzers. CytoVista™ Tissue Clearing technique adequately clears tissue for 3D fluorescence imaging with minimal shrinkage or contraction of the sample. For samples that must be reused, the clearing can be reversed, and the tissue can be processed and sectioned for histology studies, such as H&E staining.

Contents and storage

Table 1 CytoVista™ kits

	CytoVista™ Tissue Clearing/Staining Kit (Cat. No. V11324)	CytoVista™ 3D Cell Culture Clearing/Staining Kit (Cat. No. V11325)	CytoVista™ Tissue Clearing Kit (Cat. No. V11304)	CytoVista™ Tissue Clearing Kit (Cat. No. V11322)	CytoVista™ Tissue Clearing Kit (Cat. No. V11323)
CytoVista™ Tissue Clearing Reagent	30 mL	—	10 mL	30 mL	100 mL
CytoVista™ Tissue Clearing Enhancer	30 mL	—	10 mL	30 mL	100 mL
CytoVista™ 3D Cell Culture Clearing Reagent	—	30 mL	—	—	—
CytoVista™ Antibody Dilution Buffer	30 mL	30 mL	—	—	—
CytoVista™ Blocking Buffer	30 mL	30 mL	—	—	—
CytoVista™ Antibody Penetration Buffer	30 mL	30 mL	—	—	—
CytoVista™ 10X Wash Buffer	70 mL	70 mL	—	—	—
CytoVista™ Tissue Permeabilization Buffer	30 mL	—	—	—	—

- CytoVista™ Tissue Clearing/Staining Kit (Cat. No. V11324) and CytoVista™ 3D Cell Culture Clearing/Staining Kit (Cat. No. V11325) are shipped and stored at multiple temperatures. Store according to product labeling.
- Store the CytoVista™ Tissue Clearing Kit (Cat. Nos. V11304, V11322, V11323) at room temperature in a dry environment. Do not freeze.
- When stored as directed, the products are stable for 6 months from the date of receipt.

Table 2 Stand-alone CytoVista™ products

Product	Cat. No.	Size	Notes	Storage
CytoVista™ Tissue Clearing Reagent	V11300	30 mL	Clearing and imaging reagent with a refractive index of 1.50	• Store at room temperature in a dry environment. • Do not freeze. • When stored as directed, the products are stable for 6 months from the date of receipt.
	V11301	100 mL		
CytoVista™ Tissue Clearing Enhancer	V11302	30 mL	Clearing and imaging reagent with a refractive index of 1.53	
	V11303	100 mL		
CytoVista™ 3D Cell Culture Clearing Reagent	V11326	10 mL	Clearing and imaging reagent a with refractive index of 1.48	
	V11315	30 mL		
	V11316	100 mL		
CytoVista™ Antibody Dilution Buffer	V11305	30 mL	PBS with 0.2% Tween™ 20, heparin, 3% donkey serum, and 5% DMSO	• Store at 2–8℃. • Follow sterile techniques while using. Sterile filtration through 0.22-µm filter is recommended. • When stored as directed, the products are stable for 3 months from the date of receipt.
	V11306	100 mL		
CytoVista™ Blocking Buffer	V11307	30 mL	PBS with 0.2% Triton™ X-100, 6% donkey serum, and 10% DMSO	
	V11308	100 mL		
CytoVista™ Antibody Penetration Buffer	V11309	30 mL	PBS with 0.2% Triton™ X-100, 0.3 M glycine, and 20% DMSO	
	V11310	100 mL		
CytoVista™ 10X Wash Buffer	V11311	70 mL	PBS with 0.2% Tween™ 20 and 10 µg/mL heparin (after the diluting the 10X stock solution to make 1X working solution)	
	V11312	200 mL		
CytoVista™ Tissue Permeabilization Buffer	V11313	30 mL	Not provided	
	V11314	100 mL		
CytoVista™ Tissue Imaging Chamber (0.75 mm deep)	V11333	Set of 6	NA	• Store at room temperature. • Do not freeze.
CytoVista™ Tissue Imaging Chamber (1.75 mm deep)	V11317	Set of 6	NA	
CytoVista™ Tissue Imaging Chamber (3.5 mm deep)	V11318	Set of 6	NA	
CytoVista™ Tissue Imaging Chamber (7 mm deep)	V11319	Set of 6	NA	
CytoVista™ 2 mm Coronal Mouse Brain Slicer	V11320	1 each	NA	
CytoVista™ 2 mm Sagittal Mouse Brain Slicer	V11321	1 each	NA	
CytoVista™ 1 mm Coronal Mouse Brain Slicer	V11335	1 each	NA	
CytoVista™ 1 mm Sagittal Mouse Brain Slicer	V11334	1 each	NA	

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Cell lines for 3D cultures, organoids, spheroids, tissue, or animal organs (use positive and negative controls as needed)	MLS
Slides, coverslips, containers	MLS
Primary or secondary antibodies[1]	MLS
PBS (phosphate buffered saline), pH 7.4 (without calcium, magnesium, or phenol red)	10010031
Ethanol (for samples containing fluorescent proteins)	MLS
Methanol (for samples without fluorescent proteins)	MLS
Hydrogen peroxide solution	MLS
DMSO, Anhydrous	D12345
4% formaldehyde, methanol-free	FB002

[1] To search through the vast Thermo Fisher Scientific primary antibody collection, use our Antibody Search tool at **thermofisher.com/us/en/home/life-science/antibodies.html**.

Recommended workflows

- For first-time users, we recommend the CytoVista™ Tissue Clearing/Staining Kit (Cat. No. V11324) or the CytoVista™ 3D Cell Culture Clearing/Staining Kit (Cat. No. V11325), which contain all of the reagents that are required for the workflows described here.
- For easier-to-clear tissues, such as brain, lung, intestine, and skin, with less than 250-µm thickness, treatment with the CytoVista™ Tissue Clearing Reagent is sufficient for 3D imaging. Clearing can be enhanced, however, by incubating the sample with the CytoVista™ Tissue Clearing Enhancer.
- For tissues thicker than 250 µm, or difficult-to-clear tissues, such as kidney, liver, heart, and placenta, we recommend a first treatment with the CytoVista™ Tissue Clearing Reagent, followed by a treatment with the CytoVista™ Tissue Clearing Enhancer.
- For organoids and spheroids up to 1,000-µm thickness, use the CytoVista™ 3D Cell Culture Clearing Reagent in the clearing step.
- For plant tissue clearing and mounting, we recommend using Invitrogen™ Image-iT™ Plant Tissue Clearing Reagent (Cat. No. V11328) and Image-iT™ Plant Tissue Hard-set Mountant (Cat. No. V11331).

Procedural guidelines

- CytoVista™ Tissue Clearing technique is not recommended for hard tissues, such as bone, cartilage, and connective tissue.
- CytoVista™ reagents are not compatible with most 3D cell culture matrices. We recommend harvesting the cells from the matrix to enhance the penetration of antibodies and other staining reagents.
- All three clearing reagents, the CytoVista™ 3D Cell Culture Clearing Reagent, the CytoVista™ Tissue Clearing Reagent, and the CytoVista™ Tissue Clearing Enhancer, can also be used as imaging solutions during imaging on a fluorescence imaging instrument.
- For first-time users, we highly recommend cutting thicker tissues into 2-mm thick sections using a device such as the CytoVista™ 2 mm Coronal Mouse Brain Slicer or the CytoVista™ 2 mm Sagittal Mouse Brain Slicer.
- Except where otherwise stated, perform all steps in the procedure at room temperature (20°C) with gentle agitation. If background noise is a significant problem when visualizing your endogenous autofluorescence, conduct all steps at 4°C.
- For liver, kidney, and lymphatic tissues, you may need to extend incubation times by 30–50%, depending on the degree of fixation.
- For 3D cell culture models, tissue dehydration is not required, but it can enhance tissue clearing speed for dense 3D cell culture models (for example, neuronal models).
- Use 100% dry ethanol for all steps involving ethanol.
- Use glass or polypropylene containers and tubes with CytoVista™ Tissue Clearing Enhancer. Do not use polystyrene.
- You can perform the tissue clearing steps in a chamber that is constructed by pasting the CytoVista™ Tissue Imaging Chamber on a glass coverslip or slide. CytoVista™ Tissue Imaging Chambers are made of silicon, which is compatible with CytoVista™ clearing reagents, making them ideal containers for the clearing steps and for use as imaging chambers.

Guidelines for tissue fixation

- Best results are obtained with tissues that are fixed by perfusion with 4% paraformaldehyde. Immersion fixation in 10% neutral buffered formalin is also acceptable, but tissues larger than 6 mm (for example, 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. This procedure helps to prevent autolysis due to incomplete fixation of the center portion of the tissues. Place the tissues in a container containing fixative at a volume that is approximately 10X the volume of the tissue. Ensure that the tissue is submerged in the fixative. Incubate the tissue in the fixative at 4°C overnight, then incubate for 1 hour at room temperature. Transfer the tissues to PBS, then proceed with further processing. If long-term storage (>1 week) is required, transfer the tissues to PBS with 0.05% sodium azide as a preservative.
- We recommend perfusion fixation for large tissues (for example, whole mouse or rat brains). Immersion fixation of large tissues can lead to incomplete fixation, autolysis, and necrosis.

Before you begin

Prepare the reagents

1. CytoVista™ 10X Wash Buffer is provided at 10X concentration. Dilute the CytoVista™ 10X Wash Buffer to 1X with PBS, pH 7.4 before use.

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 that is used in the last step of dehydration is completely dehydrated.

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

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.

Clear fluorescent protein- or fixable fluorophore-labeled tissue (without antibody detection)

The following protocol describes a general procedure for clearing various tissues ranging in size from whole rat brains to 3D cell culture models (for example, organoids, microtissues, spheroids). The procedure is effective at clearing unfixed tissues, tissues that are fixed with various fixatives, and tissues that have been stored in formalin for years. See Table 3 for the suggested incubation times, volumes, and considerations for your particular tissue of interest.

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 CytoVista™ reagents[1]	Volume of CytoVista™ 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
3D cell culture models	10 minutes	0.2 mL	4 minutes	0.2 mL

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

[2] CytoVista™ Tissue Clearing Reagent only, or CytoVista™ Tissue Clearing Reagent and CytoVista™ Tissue Clearing Enhancer, depending on the tissue thickness. For 3D cell culture models, use only the CytoVista™ 3D Cell Culture Clearing Reagent.

Clear tissues labeled with fluorescent proteins

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

Note: You can perform the tissue clearing steps (step 6 and step 7) in a chamber that is constructed by pasting the CytoVista™ Tissue Imaging Chamber on a glass coverslip or slide. CytoVista™ Tissue Imaging Chambers are made of silicon, which is compatible with CytoVista™ clearing reagents, making them ideal containers for the clearing steps and for use as imaging chambers.

1. Obtain tissues of interest. See "Guidelines for tissue fixation" on page 4.

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

3. (*Optional for most tissues*) Incubate tissues that are difficult to clear due to the presence of pigment, collagen, or blood (for example, liver tissue, whole kidney, over-fixed human tissues) in CytoVista™ Tissue Permeabilization Buffer overnight with gentle shaking before proceeding with dehydration.

   IMPORTANT! Do not incubate 3D cell culture models in CytoVista™ Tissue Permeabilization Buffer.

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

4. 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.

   Note: For 3D cell culture models, tissue dehydration is not required, but it can enhance tissue clearing speed for dense 3D cell culture models (for example, neuronal models).

   a. Treat tissues with 30% ethanol in PBS with gentle shaking.
   b. Treat tissues with 50% ethanol in PBS with gentle shaking.
   c. Treat tissues with 70% ethanol in deionized water with gentle shaking.
   d. Treat tissues with 90% ethanol in deionized water with gentle shaking.
   e. Treat tissues with 100% dry ethanol with gentle shaking.

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

5. Remove the tissues from ethanol. Ensure that all excess ethanol is absorbed with Kimwipes™ laboratory tissue or a paper towel, then removed from the sample.
   - For tissue samples, proceed to step 6.
   - For 3D cultures, proceed to step 8.

6. For tissues, add CytoVista™ Tissue Clearing Reagent 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). 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 CytoVista™ Tissue Clearing Enhancer (step 7) requires the use of glass or polypropylene containers. Other plastic containers are not compatible with the enhancer and can ruin the samples.

7. Transfer larger or thicker tissues (>200 µm) to CytoVista™ Tissue Clearing Enhancer to finish the clearing process at 4°C with gentle agitation, then proceed to step 9. Otherwise, directly proceed to step 9.

   Note: Image larger tissue samples in CytoVista™ Tissue Clearing Enhancer.

8. For 3D cultures such as organoids and spheroids up to 1,000 µm thick, add CytoVista™ 3D Cell Culture Clearing Reagent, incubate for 5 minutes, then proceed to step 9.

   Note: For 3D cell culture models, use only CytoVista™ 3D Cell Culture Clearing Reagent for tissue clearing.

   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.

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

Immunolabel and clear tissues

The following protocol describes a general procedure for immunolabeling and clearing various tissues ranging in size from whole rat brains to 3D cell culture models (for example, organoids, microtissues, spheroids). See Table 4 for the suggested incubation times and Table 5 for the required reagent volumes to immunolabel and clear your particular tissue of interest.

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 CytoVista™ 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 hours	6 hours	80 hours	80 hours	2 hours + 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
3D cell culture models	10 minutes	30 minutes	30 minutes	1 hour	10 minutes	10 minutes

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

[2] CytoVista™ Tissue Clearing Reagent only, or CytoVista™ Tissue Clearing Reagent and CytoVista™ Tissue Clearing Enhancer, depending on the tissue thickness. For 3D cell culture models, use only the CytoVista™ 3D Cell Culture Clearing Reagent.

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
3D cell culture models	0.2 mL	0.2 mL	0.2 mL	0.2 mL

Immunolabel and clear tissues

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

Note: You can perform the tissue clearing steps (step 16 and step 17) in a chamber that is constructed by pasting the CytoVista™ Tissue Imaging Chamber on a glass coverslip or slide. CytoVista™ Tissue Imaging Chambers are made of silicon, which is compatible with CytoVista™ clearing reagents, making them ideal containers for the clearing steps and for use as imaging chambers.

1. Obtain tissues of interest, then fix them, if needed. See "Guidelines for tissue fixation" on page 4.

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

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

   IMPORTANT! Do not incubate 3D cell culture models in CytoVista™ Tissue Permeabilization Buffer.

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 Table 4 and Table 5 for required volumes and incubation times.
   a. **Samples without fluorescent protein:** Wash tissues twice in PBS, then once in 50% methanol in PBS, 80% methanol in deionized water, and finally in 100% methanol.
   b. **Samples with fluorescent protein:** Wash tissues twice in PBS, then once in 50% ethanol in PBS, 80% ethanol in deionized water, and finally in 100% dry ethanol.

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.

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), then incubating at 4°C overnight. This step significantly reduces background fluorescence that is caused by hemoglobin among others.

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

6. Wash samples before proceeding with further staining:
   a. **Samples without fluorescent protein:** Wash the tissues in 20% DMSO/methanol, then 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, then in 80% ethanol in deionized water, in 50% ethanol in PBS, in 100% PBS, and finally in PBS with 2% Triton™ X-100.

STOPPING POINT (*Optional*) You can store the tissues at 4℃ in the dark for up to 3 days without detrimental effects. For 3D cell culture models, transfer the samples to 100% PBS (without Triton™ X-100) before storing.

7. Incubate the samples in CytoVista™ Antibody Penetration Buffer with gentle shaking.

8. Block the samples in CytoVista™ 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. For 3D cell culture models, transfer the samples to 100% PBS before storing.

9. Transfer the samples to primary antibody dilutions prepared in CytoVista™ Antibody Dilution Buffer, then incubate at 37°C with gentle shaking.

   Note: For most broadly expressing epitopes, a dilution of 1:50 to 1:500 is typically required, but antibody concentration should be optimized for tissues according to the guidelines described in Appendix B, "Guidelines for validating antibodies and optimizing antibody concentration".

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

10. Wash the samples 5 times in CytoVista™ Wash Buffer (diluted to 1X in PBS; see "Prepare the reagents" on page 4) with gentle shaking.

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

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 CytoVista™ Antibody Dilution 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.

12. (*Optional*) Add nuclear stain (for example, DAPI) to a dilution of 1:1000 to 1:5000 (depending on the stain). You can perform this step concurrently with antibody labeling steps (recommended), or separately in CytoVista™ Wash Buffer.

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

    Note: Samples that 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.

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 Table 4 and Table 5 for required volumes and incubation times. Using an excess volume in the dehydration steps ensures proper clearing.

    Note: For 3D cell culture models, tissue dehydration is not required, but it can increase tissue clearing speed for dense 3D cell culture models (for example, neuronal models).

    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.

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

15. Remove the tissues from methanol or ethanol. Ensure that all excess methanol or ethanol is absorbed with Kimwipes™ laboratory tissue or a paper towel, then removed from the sample.
    - For tissue samples, proceed to step 16.
    - For 3D cultures, proceed to step 18.

16. Add CytoVista™ Tissue Clearing Reagent 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). 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 CytoVista™ Tissue Clearing Enhancer (step 17) requires the use of glass or polypropylene containers. Other plastic containers are not compatible with the enhancer and can damage the samples.

17. Transfer larger or thicker tissues (>200 µm) to CytoVista™ Tissue Clearing Enhancer to finish the clearing process at 4°C with gentle agitation, then proceed to step 19. Otherwise, directly proceed to step 19.

    Note: Image larger tissue samples in CytoVista™ Tissue Clearing Enhancer.

18. For 3D cultures such as organoids and spheorids up to 1,000 µm thick, add CytoVista™ 3D Cell Culture Clearing Reagent, incubate for 5 minutes, then proceed to step 19.

    Note: For 3D cell culture models, use only CytoVista™ 3D Cell Culture Clearing Reagent for tissue clearing.

    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.

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

    Note: Mount samples for imaging in CytoVista™ Tissue Clearing Enhancer or CytoVista™ 3D Cell Culture Clearing Reagent.

Appendix A Troubleshooting

Observation	Possible cause	Recommended action
Cannot image past 500–1,000 µm The labeling appears uneven and drops off significantly when imaged past 500–1,000 µm.	Antibody concentration was too high: ring of intense staining near the surface, which then drops off significantly.	Reduce antibody concentration. If the signal is too weak, use a lower antibody concentration for half of the time, then re-incubate with antibodies at a higher concentration.
	Antibody concentration was too low: signal drops off in the middle of the tissue.	Increase the antibody concentration.
	Optical attenuation occurred due to absorption of photons by the upper tissue layers that "shadows" the tissue below, even with perfect staining.	Increase the laser power and gain with increasing depth. Some microscopes can automate laser power and gain corrections. **IMPORTANT!** Higher laser power increases the rate of photobleaching.
		Ensure that the samples do not contain 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.

Observation	Possible cause	Recommended action
Intense band of labeled tissue at the surface, then a significant drop-off afterwards	The antibody concentration was too high.	Reduce the antibody concentration by increasing the dilution factor.
The tissue did not clear	Incompatible plastic was used.	CytoVista™ Tissue Clearing Enhancer degrades polystyrene. Use polypropylene and glass in your workflow, where possible. Plastic leaching into your sample can affect the clearing ability of CytoVista™ reagents.
	The dehydration/clearing was incomplete.	Ensure that you are using pure, dehydrated ethanol or methanol for drying. Impure methanol or ethanol that contains water will not remove all of the water from the tissue, resulting in cloudiness.
		Ensure that the sample vessel is sealed properly. CytoVista™ Tissue Clearing Enhancer is hygroscopic and will draw water.
		Ensure that you are using a sufficient volume of CytoVista™ Tissue Clearing Reagent, CytoVista™ Tissue Clearing Enhancer, or CytoVista™ 3D Cell Culture Clearing Reagent for your tissue size. Using an insufficient volume of CytoVista™ reagents can cause inadequate clearing.
		Add a 100% (dehydrated) ethanol/methanol dehydration step before clearing.
		Increase the incubation time for methanol/ethanol dehydration and clearing steps.
Fluorescent protein looks quenched	The sample containing fluorescent protein was dehydrated using methanol.	To visualize fluorescent proteins, dehydrated samples using ethanol at 4°C instead of methanol.
	The sample was bleached.	Keep cleared samples in the dark and cover them with aluminum foil. 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.
	The background fluorescence was too high.	Shift all steps in the protocol to 4°C and increase their duration by 50%.
The antibody did not label the tissue	The antibody was 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 an IHC-validated antibody is not available, an IF/ICC validated antibody might also work.
The center of the tissue looks dark	The antibody concentration was too low.	Increase the antibody concentration. Explore a range of antibody concentrations on a small section of the tissue before scaling to large tissues.
	Optical attenuation occurred. Optical attenuation leads to diminished signal at increasing depths. Optical attenuation depends 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 the laser power and gain according to the 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.

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 antibody and optimize the antibody concentration. The required antibody concentration for thicker tissues or 3D cell culture models can be different from the concentration required for thinner sections. Thicker sections require longer incubations and take a longer time for the entire workflow. Therefore, we recommend that you validate the antibody of interest using thin tissue sections first.

Guidelines for validation and optimization

- 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 that are 100–250 µm thick. You 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 (for example, 1:50, 1:100, 1:200, 1:300, 1:500), diluted in CytoVista™ Antibody Dilution Buffer.
- Usually, a 1:100 dilution of the secondary antibody works well. If you observe a low signal or high background, it may be necessary to optimize the secondary antibody concentration.

Guidelines for imaging

- 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 uniformity 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 Celleste™ Image Analysis Software (Cat. No. AMEP4816) or 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 public domain image processing and analysis program available from NIH at **imagej.nih.gov/ig**.
- If the staining is even, you should see relatively constant intensity (with respect to nuclear stain) across the tissue (Figure 1). Some dimming in the inner layers is expected, but signal should be visible across tissue.
- 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.

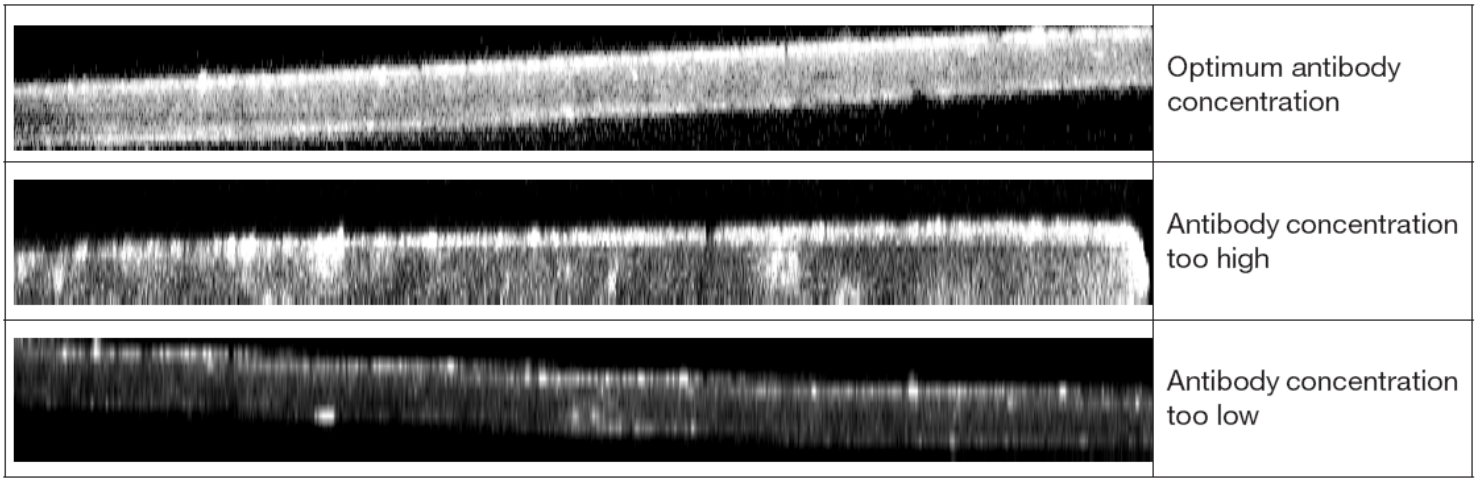

Figure 1 Evaluating the uniformity of staining. Tissue sections were imaged using a confocal microscope and a z-stack spanning the entire thickness of the tissue was obtained. The XZ plane was examined for the uniformity of staining.

Appendix C Reverse cleared tissue

CytoVista™ 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 CytoVista™ Tissue Clearing method can be integrated into any bio-imaging process without disrupting other assays or histological processing that is used in traditional workflows.

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

2. Larger and more vascular tissues (for example, whole kidney) can require 2–3 washes of alcohol over the course of several hours or a gradient down from 100% ethanol or methanol.

3. After reversal, samples can be processed directly for paraffin-embedded histological preparations.

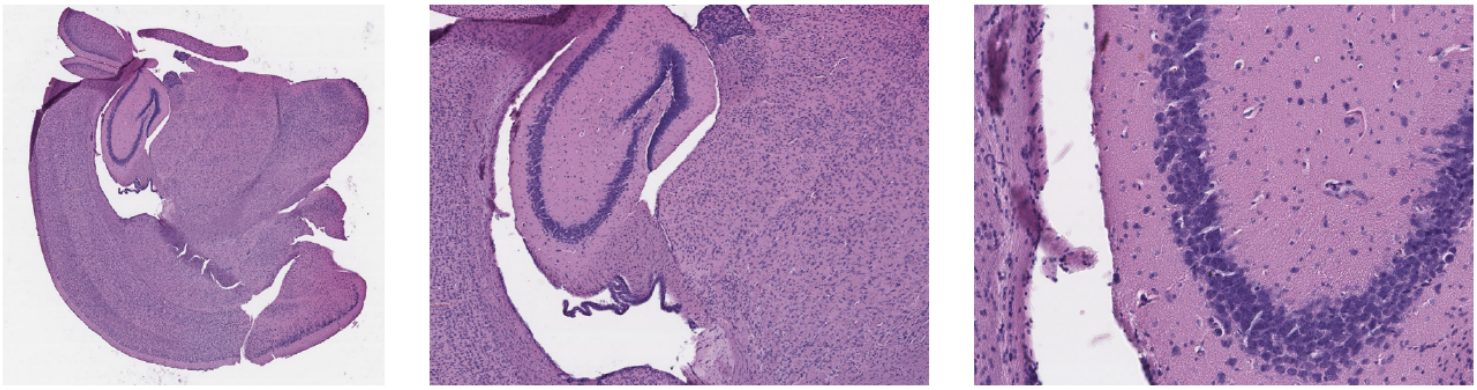

Figure 2 Untreated mouse brain tissue section was formalin-fixed and paraffin-embedded, then stained with H&E, depicting the hippocampus.

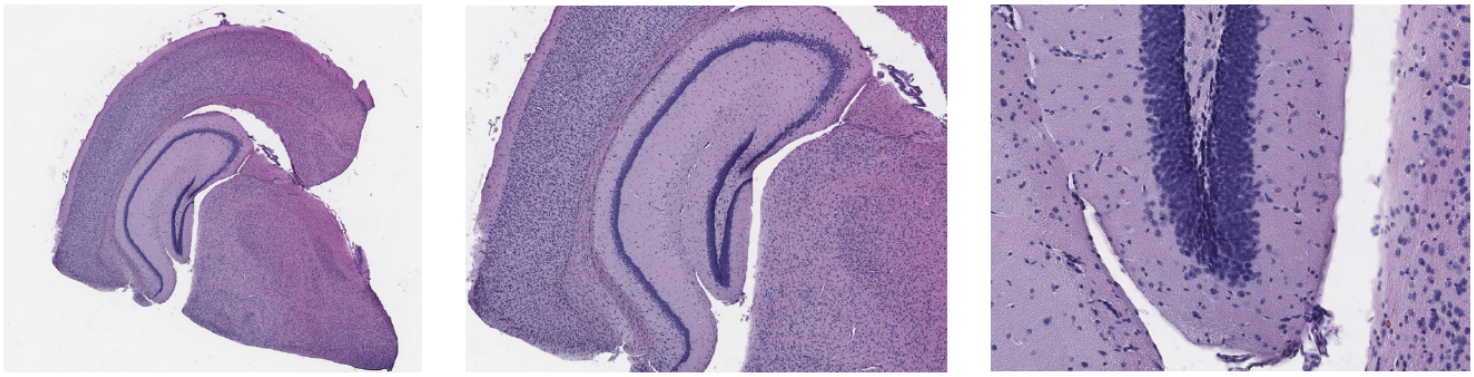

Figure 3 Mouse brain tissue was cleared using the CytoVista™ Tissue Clearing workflow. The cleared tissue was then reversed, embedded in paraffin, sectioned, and stained with H&E, depicting hippocampus. The CytoVista™ Tissue Clearing workflow does not significantly affect tissue histology.

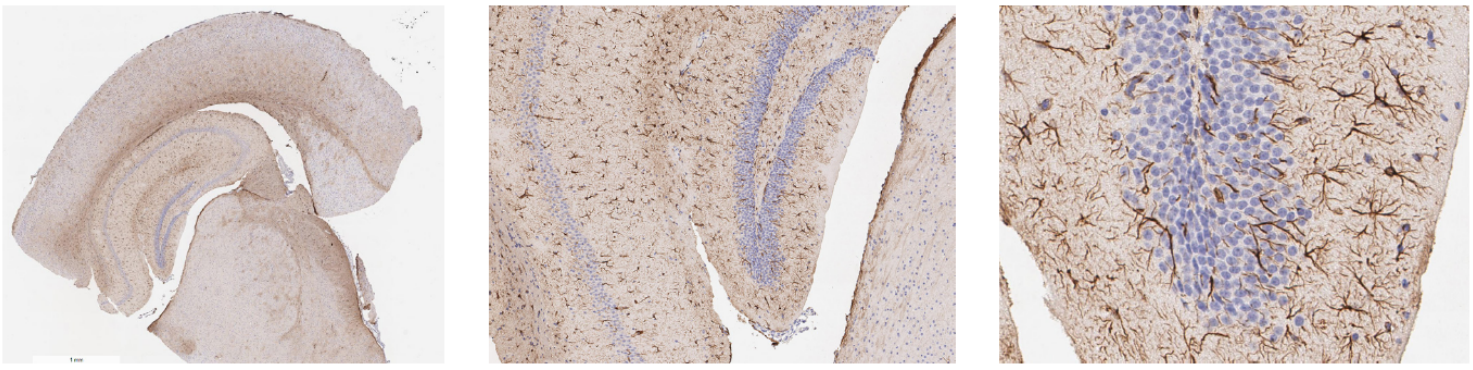

Figure 4 Mouse brain tissue was cleared using the CytoVista™ Tissue Clearing workflow. The cleared tissue was then reversed, embedded in paraffin, sectioned, and immunostained for GFAP, labeling astrocytes. The CytoVista™ Tissue Clearing workflow does not affect antigenicity of tissues.

Appendix D Ordering information

Cat. No.	Product name	Size
V11324	CytoVista™ Tissue Clearing / Staining Kit	1 kit
V11300	CytoVista™ Tissue Clearing Reagent	30 mL
V11301	CytoVista™ Tissue Clearing Reagent	100 mL
V11302	CytoVista™ Tissue Clearing Enhancer	30 mL
V11303	CytoVista™ Tissue Clearing Enhancer	100 mL
V11304	CytoVista™ Tissue Clearing Kit	10 mL set
V11322	CytoVista™ Tissue Clearing Kit	30 mL set
V11323	CytoVista™ Tissue Clearing Kit	100 mL set
V11305	CytoVista™ Antibody Dilution Buffer	30 mL
V11306	CytoVista™ Antibody Dilution Buffer	100 mL
V11307	CytoVista™ Blocking Buffer	30 mL
V11308	CytoVista™ Blocking Buffer	100 mL
V11309	CytoVista™ Antibody Penetration Buffer	30 mL
V11310	CytoVista™ Antibody Penetration Buffer	100 mL
V11311	CytoVista™ 10X Wash Buffer	70 mL
V11312	CytoVista™ 10X Wash Buffer	200 mL
V11313	CytoVista™ Permeabilization Buffer	30 mL
V11314	CytoVista™ Permeabilization Buffer	100 mL
V11333	CytoVista™ Tissue Imaging Chamber (0.75 mm Deep)	Set of 6
V11317	CytoVista™ Tissue Imaging Chamber (1.75 mm Deep)	Set of 6
V11318	CytoVista™ Tissue Imaging Chamber (3.5 mm Deep)	Set of 6
V11319	CytoVista™ Tissue Imaging Chamber (7 mm Deep)	Set of 6
V11334	CytoVista™ 1 mm Sagittal Mouse Brain Slicer	1 each
V11335	CytoVista™ 1 mm Coronal Mouse Brain Slicer	1 each
V11320	CytoVista™ 2 mm Coronal Mouse Brain Slicer	1 each
V11321	CytoVista™ 2 mm Sagittal Mouse Brain Slicer	1 each
V11326	CytoVista™ 3D Cell Culture Clearing Reagent	10 mL
V11315	CytoVista™ 3D Cell Culture Clearing Reagent	30 mL
V11316	CytoVista™ 3D Cell Culture Clearing Reagent	100 mL
V11325	CytoVista™ 3D Cell Culture Clearing / Staining Kit	1 kit

Appendix E Documentation and support

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs

  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

    Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

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Revision	Date	Description
B.0	23 August 2019	• Added new CytoVista™ products to "Contents and storage" on page 1. • Added recommended products for plant tissue clearing and mounting. • Reorganized guidelines sections for clarity. • Minor edits for style and consistency.
A.0	14 June 2018	New user guide.