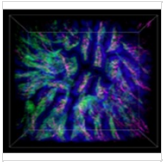

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Ce3D™ Tissue Clearing Protocol

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1 Works for me This protocol is published without a DOI.

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ABSTRACT

Biological tissues are generally composed of proteins, lipids, and water, each of which has a different refractive index (RI). Differences in refractive indices, or RI mismatch, cause the scattering of light in the tissue and result in tissue opacity. Tissue clearing reagents aim to reduce light scattering by normalizing the RI throughout the tissue, thus making the tissue transparent. Optical clearing is a valuable application that allows us gain a better understating of spatial composition, phenotypic and subtype identity, and cellular networks. BioLegend's Ce3D™ Tissue Clearing Solution pairs perfectly with an expansive portfolio of antibodies, generating mesmerizing 3D images with unparalleled depth. Read through the protocol to learn about each step in the process.

Additional Help

- To view our troubleshooting guide, visit: https://www.biolegend.com/en-us/protocols/ce3d-tissue-clearing-kit-protocol
- For additional questions on the protocol, contact us here: https://www.biolegend.com/en-us/contact-technical-service

PROTOCOL CITATION

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KEYWORDS

microscopy, 3D IHC, immunohistochemistry, multicolor, tissue clearing, Ce3D

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MATERIALS TEXT

Materials Provided

Cat. No. 427701/427702 Ce3D™ Tissue Clearing Kit, which includes:
Ce3D™ Tissue Clearing Solution
Ce3D™ Permeabilization/Blocking Buffer
Ce3D™ Antibody Diluent Buffer
Ce3D™ Wash Buffer

Materials Required (not supplied)

- HyClone™ PBS (1X) w/o calcium and magnesium
- Heparin
- Fixation buffer
- High resolution agarose
- Slides and cover glasses
- 24-well plate
- Spacer

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Biological tissues are generally composed of proteins, lipids, and water, each of which has a different refractive index (RI). Differences in refractive indices, or RI mismatch, cause the scattering of light in the tissue and result in tissue opacity. Tissue clearing reagents aim to reduce light scattering by normalizing the RI throughout the tissue, thus making the tissue transparent. Optical clearing is a valuable application that allows us gain a better understating of spatial composition, phenotypic and subtype identity, and cellular networks. BioLegend's Ce3D™ Tissue Clearing Solution pairs perfectly with an expansive portfolio of antibodies, generating mesmerizing 3D images with unparalleled depth. Read through the protocol to learn about each step in the process.

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BEFORE STARTING

The Ce3D™ (clearing-enhanced 3D) Tissue Clearing Kit works by simple immersion of tissue into the clearing reagent and does not require special equipment or containers.

This protocol is optimized for staining with samples with 500 µm thickness. For samples with a different thickness, the end user may need to perform further testing. Use 1% PFA if best clearing effect is desired, or 4% PFA for optimal epitope preservation and immunostaining. For additional support, please contact us at: biolegend.com/en-us/contact-technical-service.

Recommended Tissue Clearing Time for Different Organs

- Intestine, lymph node: 2 hours
- Spleen, thymus, lung: 4 hours
- Kidney, liver, testis, brain: >8 hours (overnight)

Whole Animal Perfusion 2h 30m

1 *Prepare fresh heparin/ PBS solution at the indicated dosage:* Add 10 units of heparin per mL of PBS. Use ≥ 50 mL per

mouse. Keep chilled at 4°C.

! It is recommended to prepare fresh heparin/ PBS solution. Steps 1-7 and step 10 must be carried out using chilled buffers.

2 *Prepare fresh PFA at the desired volume and concentration:* Use BioLegend's fixation buffer (Cat. No. 420801, 4% PFA solution). Dilute to 1% in PBS if needed. Alternatively, dilute 16% PFA in PBS to a 1% or 4% solution. Use ≥ 50 mL per mouse. Keep chilled at 4°C.

PFA is light-sensitive and should be protected from light or stored in the dark.

3 *Perform transcardial perfusion wash:* Perform perfusion in the chemical fume hood under anesthesia. Perfuse with ≥ 25 mL of chilled heparin/PBS solution at a pump speed of 0.5 mL per minute.

This step is to wash out blood containing sources of autofluorescence such as hemoglobin from organs. To avoid blood clotting and over-fixation, we recommend perfusion on a chilled pad throughout the process.

4 *Perform transcardial perfusion fixation:* Perfuse with ≥ 25 mL of 1% or 4% PFA solution at 4°C at a pump speed of 0.5 mL per minute.

Perfuse slowly on a chilled pad. Incomplete fixation or over-fixation will affect immunostaining or clearing efficiency.

5 *Harvest organ:* Place harvested organ in a 50 mL conical tube. Keep tube chilled. Wash with ≥ 25 mL heparin/PBS solution at 4°C until the solution is cleared of blood.

Multiple organs can be placed in a 50 mL conical tube.

Organ Fixation and Washing 1d 3h

6 *Perform organ fixation:* Fix organ with ≥ 25 mL of 1% or 4% PFA at 4°C for 24 hours. Use the same percentage of PFA as in step 4. 1d

Replace PFA with freshly prepared solution if the solution becomes cloudy or pink to ensure complete removal of blood and efficient fixation.

7 *Perform PBS wash:* Wash organ in PBS at 4°C for 1 hour. Repeat wash two additional times. 3h

Residual PFA may lead to over-fixation and affect immunostaining. The fixed organ can be store at 4°C in a buffer containing 0.05% sodium azide in PBS for 2 weeks.

Agarose Embedding and Tissue Sectioning 4h

8 *Prepare 2% agarose solution in PBS:* Measure the appropriate amount of solid agarose and PBS. Melt the agarose in a beaker containing PBS in the microwave with occasional stirring until the agarose is completely dissolved. Keep the solution on a 65°C hot plate to stop agarose from solidifying.

Volume per organ depends on the container used for embedding.

9 *Embed fixed organ in 2% agarose:* Stir the agarose before pouring into a small plastic weight boat. The recommended temperature for agarose is 40-42°C. Transfer the organ using a spatula. Use a Kimwipe and gently wipe around the organ to remove excess PBS. If a specific orientation is required, use a small amount of 2% agarose solution to first make an agarose bed. Then, place the tissue on the agarose bed and fill the container until the organ is completely immersed in agarose. Wait until the agarose has solidified before tissue sectioning.

Do not embed in hot agarose. This may cause tissue autofluorescence. To accelerate agarose solidification, the organ-agarose block can be transferred to a humidity chamber to keep the block hydrated. Cover the container and place at 4°C for 30-60 minutes. The tissue block can be stored at 4°C overnight in a well-hydrated container.

10 Prepare tissue sections using a vibratome: Cut 500 µm sections at 4°C. The suggested parameters for PFA-fixed mouse tissue are speed at 0.3 mm/s and amplitude at 1.0 mm. Transfer tissue sections to a 24-well plate. 4h

Tissue sections can be stored at 4°C in a buffer containing 0.05% sodium azide in PBS for up to two weeks.

Immunostaining and Tissue Clearing Protocol 5d 4h

11 *Permeabilize and block tissue section:* Move tissue section to a 24-well plate. Handle with care to avoid tissue damage. Use 500 µL of Ce3D™ Permeabilization/Blocking Buffer per well per tissue section. Incubate at RT with gentle shaking for 2 days. 2d

Replace with fresh Ce3D™ Permeabilization/ Blocking Buffer if the solution becomes cloudy or pink. Tissue section can be stored in this buffer at 4°C for up to one week.

12 *Prepare antibody dilution at the desired concentration:* Discard the Permeabilization/Blocking Buffer and replace with antibody cocktail prepared in Ce3D™ Antibody Diluent Buffer. Use 500 µL per well per tissue section. Incubate at RT with gentle shaking for 2 days. 2d

Centrifuge the diluted antibody solution at 13000 rpm for 10 minutes to remove antibody aggregates. Tissue sections can be stored at 4°C in this buffer for up to 1 week. For BioLegend- validated antibodies, we recommend the concentration range listed for 2D IHC.

13 *Wash tissue section:* Discard the antibody cocktail solution. Add 500 µL of Ce3D™ Wash Buffer per well per tissue section. Incubate at RT with gentle shaking for ~8 hours. Discard the wash buffer and repeat this step twice more. At the second washing step, a counterstaining dye, such as DAPI, can be added to the wash buffer. 1d

Tissue sections can be stored at 4°C in Ce3D™ Wash Buffer for up to one week. Tissues can be washed up to ten times if extra washes are desired.

14 *Clear tissue section:* Remove wash buffer completely as excess buffer may interfere with efficient tissue clearing. Add 500 µL of Ce3D™ Tissue Clearing Solution per well per tissue section. Incubate at RT with gentle shaking for ~2 -12 hours. Refer to the "Recommended Tissue Clearing Time for Different Organs" section of the abstract. 2h

Ce3D™ Tissue Clearing Solution is viscous. Gently mix before use.

15 *Prepare sample chamber:* Assemble the sample chamber by attaching a spacer onto a slide. Fill the space with Ce3D™ Tissue Clearing Solution. Carefully transfer and place the tissue section in the solution and cover with a coverslip. Avoid air bubbles. Use nail polish to seal the edges of the coverslip. Air-dry for 2 hours. Slides can be stored in the dark at 4°C for 2 weeks. 2h

Choose appropriate spacer thickness.

16 *(Optional) Reverse tissue clearing:* If 2D IHC or other downstream application is desired, remove the tissue section from the imaging chamber and wash in PBS at RT for 30 minutes to reverse the clearing effect.