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🌐 Transparent Bones: An Improved BABB Protocol for Clearing and 3D Imaging of Intact Calcified Tissues

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We use this protocol and it's working

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Abstract

Tissue-clearing techniques have become indispensable in biomedical research, especially for three-dimensional (3D) imaging. These techniques enable the visualization of complex biological structures by rendering tissues transparent while preserving their structural integrity. Calcified tissues, such as bone and cartilage, pose unique challenges in imaging studies due to their inherent opacity and rigidity. In this work, we introduce a novel modification to the Benzyl Alcohol and Benzyl Benzoate (BABB) clearing protocol specifically designed to enhance clearing efficacy for calcified tissues. Our enhanced tissue-clearing method addresses the specific challenges associated with calcified tissues, significantly improving tissue transparency while maintaining structural preservation. This advancement has the potential to facilitate more precise and comprehensive studies in fields such as developmental biology, orthopedics, and regenerative medicine.

Guidelines

Introduction

The ability to examine intact biological specimens in three dimensions has been transformed by the advent of tissue-clearing protocols, which now represent a cornerstone technology in contemporary biomedical science [1–4]. By removing light-scattering elements while maintaining cellular architecture, these methodologies allow researchers to peer deep within specimens, revealing spatial relationships and morphological features previously obscured in traditional sectioning approaches. A classic method employed in tissue clearing is the Benzyl Alcohol and Benzyl Benzoate (BABB) technique, which has been widely utilized due to its simplicity and effectiveness [2,5,6]. However, despite its broad applicability, the BABB method faces significant challenges when applied to calcified tissues, notorious for their opacity and density.

In calcified tissues, traditional clearing techniques often fall short, limiting the resolution and depth of imaging [7]. Therefore, optimizing tissue-clearing protocols for calcified tissues is critical for advancing our understanding of bone and cartilage biology, pathology, and regeneration.

In this work, we introduce a novel modification to the BABB clearing protocol aimed at enhancing the clearing efficacy specifically for calcified tissues. Our modified protocol leverages adjustments in the composition and application of clearing agents to improve the transparency and preservation of calcified tissues. By systematically optimizing each step of the process, we aim to achieve superior clearing while maintaining the structural and molecular integrity of the tissues.

This paper details our modified BABB clearing protocol. We present a comprehensive analysis of its efficacy compared to the traditional BABB method and current state-of-the-art clearing technique, the polyethylene glycol (PEG)-associated solvent system (PEGASOS). Furthermore, we demonstrate the applicability of our protocol using light-sheet microscopy to capture images of delicate nerve structure in whole bone tissue. Our results show significant improvements in the visualization of calcified tissues, opening new avenues for detailed anatomical and functional studies relevant to animal and human health.

Discussion

Here, we report a novel approach for clearing calcified tissues using a modified BABB method. Through adjustments in the composition and application of clearing agents, our approach provides enhanced transparency and faster clearing while maintaining the structural integrity of calcified tissues. We achieved high image resolution and imaging depth through intact mouse long bones. To demonstrate the capabilities of this approach, we have provided sample images of nerves in the mouse third metatarsal labeled with immunofluorescence. Notably, we achieved robust labeling of fine nerve fibers throughout the intact bone tissue.

Our cleared tissue samples can be employed with multiple imaging modalities. In this paper, we highlight the robust image quality achieved by light-sheet microscopy. However, with appropriate configurations, tissues cleared with our approach can also be imaged using multi-photon, confocal, or other fluorescent imaging techniques. Users are encouraged to further optimize this approach to better fit their specific applications. For

example, bones from larger species (e.g., rats, rabbits) may require longer periods for decalcification or clearing. Researchers should be aware of this and perform validation studies before applying our clearing approach to experimental data.

There are notable challenges and limitations to the clearing approach reported here. First, imaging datasets of intact bones with light-sheet microscopy produces very large files. Users should be aware that high-performance computing workstations are required for analyzing these data. As mentioned above, decalcification, clearing, and immunolabeling steps may require longer incubation periods than specified, depending on bone size. Researchers should consider their sample requirements and validate all protocol timing details. A major limitation is that this approach relies on a non-aqueous refractive index matching solution for achieving clarity. Placing samples into aqueous solutions will reverse the tissue clearing and render samples opaque. This presents a challenge for many microscopes that rely on water immersion objectives. Consequently, long-working distance objectives are required for imaging these tissues, which can limit the overall achievable magnification. Clearing in general offers the benefit of high-resolution imaging of thick samples at incredible speed, obviating the need for laborious thin section preparation. A final limitation of note is that the imaging resolution of cleared samples does not fully match those of thin sections on a slide. As such, users should decide on methodologies that suit their needs.

The approach reported here enables precise and comprehensive studies of musculoskeletal tissues in fields such as developmental biology, orthopedics, and regenerative medicine. We provide protocols for both decalcified and undecalcified samples, with cleared undecalcified specimens offering the significant advantage of preserving fine cellular structures and interactions while allowing 3-dimensional imaging of immunolabeled features. Future advancements should focus on improving image quality for cleared samples, particularly through the development of improved aqueous refractive index matching solutions and innovations in long-working distance objective design. Our method represents an important step forward for calcified tissue clearing by reducing processing time, enhancing clarity, establishing effective bulk immunolabeling of intact mouse tissues, and providing distinct approaches for both decalcified and undecalcified samples.



Materials

Reagents:

- Z-FIX/Zinc Formalin: NC9937162 (Fisher Scientific)
- EDTA: E5134-1KG (Sigma-Aldrich)
- 30% Hydrogen Peroxide: 470301-282 (VWR)
- Acetonitrile: A998-1 (Fisher Scientific)
- Ethylene Glycol Monoethyl Ether/2-Ethoxyethanol: E180-1 (Fisher Scientific)
- Benzyl Alcohol: 402834-1L (Sigma Aldrich)
- Benzyl Benzoate: B6630 (Sigma-Aldrich)
- PBS with Tween 20 (PBST; Sigma-Aldrich)

Equipment

- 15 mL conical tubes
- 50 mL conical tubes
- Eppendorf tubes
- Weighing boats
- Electronic scale
- Disposable plastic spatulas
- Hot plate with stirrer
- Shaker with temperature control
- pH meter
- 10 µL micropipette
- 100 µL micropipette
- 1000 µL micropipette
- 5 mL electronic pipette
- 50 mL electronic pipette

 Anatech Ltd Z-FIX/4/GALLON **Fisher Scientific Catalog #NC9937162**

 Ethylenediaminetetraacetic acid disodium salt dihydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #E5134-1KG**

 Hydrogen Peroxide **Avantor Sciences Catalog #470301-282**

 Acetonitrile (HPLC) **Fisher Scientific Catalog #A998-1**

 Ethylene Glycol Monoethyl Ether (Laboratory) **Fisher Scientific Catalog #E180-1**

 Benzyl alcohol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #402834-1L**

 Benzyl Benzoate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #B6630**



Decalcified Protocol

20m

1

Note

All procedures and reagents are approved by the Institutional Animal Care and Use Committee of Cornell University.

BioRxiv Link: <https://www.biorxiv.org/content/10.1101/2024.07.23.604611v2>

Anesthetize mice with 3-5% aerosolized isoflurane in an induction chamber. Maintain anesthesia with 2% isoflurane delivered via nosecone.

1.1 Prior to starting perfusion, perform a hind paw pinch to assure full anesthesia is established. Perfuse mice with 10% Z-FIX.

1.2 After perfusion is completed, perform cervical dislocation as a secondary means of sacrifice.

1.3 Dissect bones and place them in Z-FIX  Overnight at  4 °C .



Note

Technical note: If perfusion is not possible, fixation time should be increased accordingly.

2 Decalcify bones in 20% EDTA, pH 7-7.5 at  37 °C for 5 days. Change solutions every day.

2.1 Rinse samples well with DI water before the next step.

Note

Technical note: Other decalcification reagents may be used. If using EDTA, decalcification can take longer depending on the reagent source and sample dimensions. As such, decalcification should be verified using Micro-CT analysis. Zinc-based fixatives may cause salt artifacts in Micro-CT images and need to be thresholded out.

3 ■ Dilute the 30% stock hydrogen peroxide in DI water by a factor of 2.

20m



- Incubate samples in 15% hydrogen peroxide for 00:20:00 or until marrow becomes opaque, then rinse well with DI water.

- 4
 - Whole-mount immunofluorescent staining of samples takes place at Room temperature with constant shaking.
 - Use either 15 mL conical tubes or Eppendorf tubes for these steps.
 - Starting at the secondary antibody, cover all your tubes with aluminum foil for the rest of the process to avoid photobleaching of fluorescent antibodies.

- 4.1 Block using 10% BSA in PBST Overnight .

Note

If you know the species of the primary antibodies, you can instead use 3% BSA with 10% species serum.

- 4.2 Dilute 1:100 primary antibody in blocking buffer. Incubate samples Overnight at Room temperature or for 24:00:00 at 4 °C .



- 4.3 Wash in PBST.



- 4.4 Dilute 1:200 secondary antibody in PBST. Incubate samples Overnight at Room temperature or for 24:00:00 at 4 °C .



- 4.5 Wash in PBST.



- 5 Incubate samples in 2-ethoxyethanol for 04:00:00 to Overnight .



- 6 Pour off 2-ethoxyethanol and replace with acetonitrile with three changes over 04:00:00 to Overnight .

- 7 Pour off acetonitrile and replace with benzyl alcohol for 2-4 hours.



- 8 Pour off benzyl alcohol and replace it with benzyl benzoate. Incubate  Overnight until samples are fully transparent. 

Note

Samples may be stored in benzyl benzoate until ready for histology processing or imaging. If imaging, be aware that benzyl benzoate is an organic solvent that can damage many objectives.

Undecalcified Protocol

1h 30m

- 9 Anesthetize mice with 3-5% aerosolized isoflurane in an induction chamber. Maintain anesthesia with 2% isoflurane delivered via nosecone.
- 9.1 Prior to starting perfusion, perform a hind paw pinch to assure full anesthesia is established. Perfuse mice with 10% Z-FIX.
- 9.2 After perfusion is completed, perform cervical dislocation as a secondary means of sacrifice. Dissect bones and place them in Z-FIX  Overnight at  4 °C . 

Note

Technical note: If perfusion is not possible, fixation time should be increased accordingly.

- 10 Post-fixation: rinse samples well with DI water. 
- 11 Incubate samples in 30% hydrogen peroxide for  01:30:00 or until marrow becomes opaque, then rinse well with DI water.

1h 30m

 
- 12
- Whole-mount immunofluorescent staining of samples takes place at  Room temperature with constant shaking.
 - Use either 15 mL conical tubes or Eppendorf tubes for these steps.
 - Starting at the secondary antibody, cover all your tubes with aluminum foil for the rest of the process to avoid photobleaching of fluorescent antibodies.



12.1 Block using 10% BSA in PBST Overnight .



Note

If you know the species of the primary antibodies, you can instead use 3% BSA with 10% species serum.

12.2 Dilute 1:100 primary antibody in blocking buffer. Incubate samples Overnight at Room temperature or for 24:00:00 at 4 °C .



12.3 Wash in PBST.



12.4 Dilute 1:200 secondary antibody in PBST. Incubate samples Overnight at Room temperature or for 24:00:00 at 4 °C .



12.5 Wash in PBST.



13 Incubate samples in 2-ethoxyethanol for 08:00:00 to Overnight .



14 Pour off 2-ethoxyethanol and replace with acetonitrile with three changes over 24:00:00 .

15 Pour off acetonitrile and replace with benzyl alcohol for 08:00:00 to Overnight .

16 Pour off benzyl alcohol and replace with benzyl benzoate. Incubate until samples are fully transparent (time may vary depending on bone size).



17 Samples may be stored in benzyl benzoate until ready for histology processing.

18

Expected result

Results:

Our approach provided successful clearing of bone tissue. To assess the ability to perform bulk immunohistochemistry and light sheet imaging with our clearing method, we processed our samples through a protocol for labeling nerves in bone tissue. We labeled tyrosine hydroxylase (TH) and vesicular acetylcholine transferase (VACHT) which represent adrenergic and cholinergic nerves, respectively. Compared to the current state-of-the-art protocol for clearing mineralized tissues, PEGASOS, our bone was clearer (Fig 1 A-B). The availability of binding epitopes for antibodies was confirmed via chromogenic stain of plastic-embedded sections of cleared bone tissue (Fig 1 C-F).

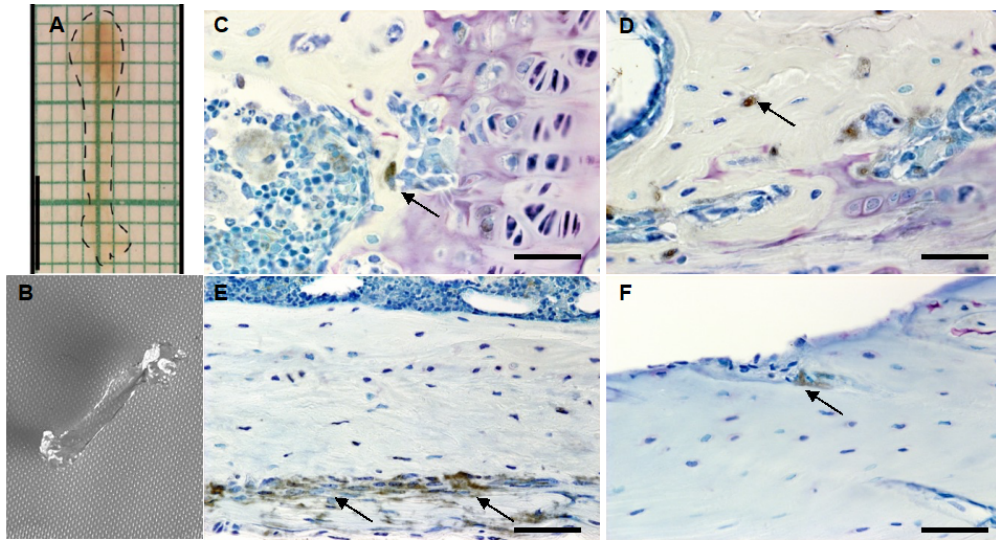


Figure 1: **A)** PEGASOS cleared bone and **B)** bone cleared by our modified approach. Both samples are adult mouse femurs. Note that our new approach removes more of the marrow space pigment, offering the potential for higher quality resolution of fluorescent markers via 3D imaging of intact samples. **C-F**, Plastic embedded sections of clear bone. Chromogenic stain was used to label VACHT for cholinergic fibers with a toluidine blue counterstain (scale bar 100 μ m). Cholinergic fibers are shown to permeate trabecular (panel **A**) and cortical bone (panels **B-D**), as indicated by the black arrows. These images confirm that our clearing method does not damage binding epitopes and the presence of cholinergic fibers in bone matrix near embedded osteocytes. (PEGASOS image sourced from D. Jing et al, Cell Research 2018)

Bulk immunohistochemistry for TH (red) and VACHT (green) was successful. In the example images here, we show a mouse third metatarsal (MT3) that has been whole-mount labeled and imaged using light sheet microscopy (Fig 2). Note that nerves of both types can be seen to track together (indicated by a yellow color) and individually, and that the patterns of their anatomy differ depending on location along the length of the bone. Moreover, extensively labeled features can be seen in the marrow space. These results exhibit the utility of our approach and the opportunity for analyses of biological/anatomical features in intact calcified tissues.

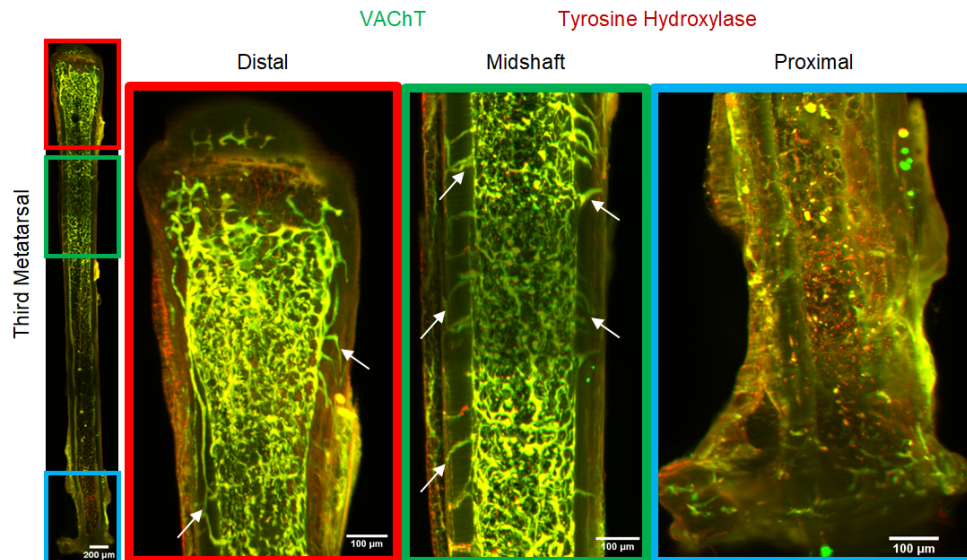


Figure 2: 3D projection light sheet microscopy images of a cleared bones bulk immunohistochemistry-stained mouse third metatarsal (MT3) with VACHT (Green) and tyrosine hydroxylase (Red) representing cholinergic and adrenergic nerve fibers, respectively. At the distal and proximal ends of the bone clear distinct nerve labeling can be seen. At the midshaft, transcortical innervation can be observed, with apparent extensive branching throughout the distal half of the bone. White arrows indicate transcortical cholinergic fibers. These preliminary results validate our approach and showcase the ability to map nerves in 3-dimensions.



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