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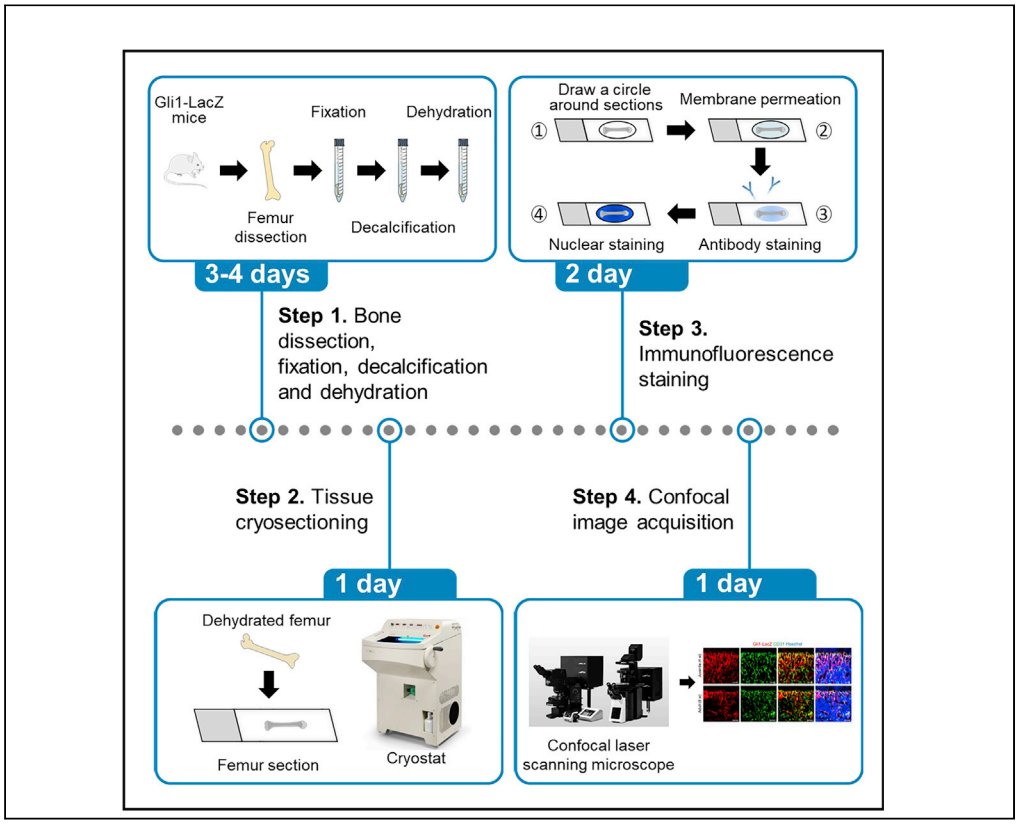
Protocol

Optimized immunofluorescence staining protocol for identifying resident mesenchymal stem cells in bone using LacZ transgenic mice



Glioma-associated oncogene homolog 1 (Gli1) marks a subpopulation of endogenous mesenchymal stem cells (MSCs) characterized by perivascular location. Here, we present an optimized immunofluorescence staining protocol to identify resident Gli1$^+$ MSCs in fixed/frozen bone sections from LacZ transgenic mice. This protocol describes the preparation of fixed/frozen tissue sections and the use of LacZ immunofluorescent staining for the *in vivo* characterization of endogenous MSCs, regarding their specific identity and specialized niches, and is applicable to LacZ-expressing cells of diverse organs.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Sample preparation of mouse skeletal tissue for confocal imaging

Immunofluorescence staining protocol of fixed/frozen bone sections

Identifying resident Gli1$^+$ MSCs in bone sections from LacZ transgenic mice

Protocol is applicable to LacZ-expressing cells of diverse organs

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Optimized immunofluorescence staining protocol for identifying resident mesenchymal stem cells in bone using LacZ transgenic mice

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SUMMARY

Glioma-associated oncogene homolog 1 (Gli1) marks a subpopulation of endogenous mesenchymal stem cells (MSCs) characterized by perivascular location. Here, we present an optimized immunofluorescence staining protocol to identify resident Gli1$^+$ MSCs in fixed/frozen bone sections from LacZ transgenic mice. This protocol describes the preparation of fixed/frozen tissue sections and the use of LacZ immunofluorescent staining for the *in vivo* characterization of endogenous MSCs, regarding their specific identity and specialized niches, and is applicable to LacZ-expressing cells of diverse organs.

For complete details on the use and execution of this protocol, please refer to Chen et al. (2020).

BEFORE YOU BEGIN

Endogenous mesenchymal stem cells (MSCs) play a vital role in the development, homeostasis maintenance and regeneration of a variety of organs, including bone, which is a representative organ for studying the *in vivo* characteristics of MSCs (Mendez-Ferrer et al., 2010; Zheng et al., 2020). Specifically, it has been found that Glioma-associated oncogene homolog 1 (Gli1) marks a subpopulation of MSCs characterized by perivascular location, which also localize in the bone (Schneider et al., 2017; Shi et al., 2017). Notably, Gli1-LacZ mouse line in which the expression pattern of inserted LacZ gene product, β-galactosidase, is identical to that of Gli1 provides a useful tool for labeling endogenous Gli1$^+$ cells (Zhao et al., 2014). Moreover, since many studies have proved that CD31 is a specific marker for blood vessels, the perivascular localization of Gil1$^+$ MSCs can be observed by staining blood vessels with CD31. Here we describe the immunofluorescence staining protocol of identifying resident Gli1$^+$ MSCs in long bone sections from Gli1-LacZ mice (Chen et al., 2020). In principle, this protocol can be applied to virtually any bone of LacZ transgenic mice models. Moreover, other organs can also be imaged using this protocol, with relevant modifications.

Animal breeding

Gli1-LacZ mice (JAX#008211) (4-week-old and 8-week-old) as well as wild-type (WT) mice of the same age are obtained from the Jackson Laboratory. For genotyping, genomic DNA is isolated from tail samples and undergoes PCR, using the primer sequences and the protocol provided by the Jackson Laboratory. Mice are maintained under specific pathogen-free (SPF) conditions (24°C, 12 h light/dark cycles and 50% humidity), and are kept feeding and drinking ad libitum.

Institutional permissions

All procedures are performed in accordance with the Guidelines of Institutional Animal Care and Use Committee of the Fourth Military Medical University from China, and the ARRIVE guidelines. The acquirement of permissions from the relevant institutions is essential for experiments on liver vertebrates or higher invertebrates.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chicken polyclonal anti-β-galactosidase (1:200)	Abcam	Cat#ab9361; RRID: AB_307210
Alexa Fluor® 488 Goat polyclonal anti-CD31/PECAM-1 (1:100)	R&D	Cat#FAB3628G; RRID: AB_10972784
Alexa Fluor® 647 Goat Anti-chicken IgG (1:200)	Jackson Immuno Research Labs	Cat#103-605-155; RRID: AB_2337392
Chemicals, peptides, and recombinant proteins		
Paraformaldehyde (PFA)	Sigma-Aldrich	Cat#158127; CAS: 30525-89-4
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	Cat#E5134; CAS: 6381-92-6
Sucrose	Sigma-Aldrich	Cat#S7903; CAS: 57-50-1
Optimal cutting temperature (OCT) tissue freezing medium	Leica	N/A
Goat serum	Boster Biological Technology	Cat#AR0009
Hoechst 33342	Invitrogen	Cat#H1399
Triton® X-100 10%	Thermo Fisher Scientific	Cat#85111
SlowFade Antifade Reagents	Thermo Fisher Scientific	Cat#S36937
Experimental models: Organisms/strains		
Mouse: Gli1-LacZ, 4 weeks and 8 weeks old males	The Jackson Laboratory	JAX#008211
Software and algorithms		
Olympus FV10-ASW Viewer	Olympus	https://www.olympus-lifescience.com/en/
Image-Pro Plus	Media Cybernetics	https://www.mediacy.com/imageproplus
Other		
Leica CM1950 Clinical Cryostat	Leica	N/A
MX35 UltraTM Low-Profile Blade	Epredia	Cat#3053835
Microscope Slides	CITOTEST Scientific	Cat#80312-3161-16
Microscope Cover Glass	CITOTEST Scientific	Cat#80340-2810
PAP pen	Biosharp	Cat#BC004
FV1000 Confocal Laser Scanning Microscope	Olympus	N/A
Sharp dissection scissors	Thermo Fisher Scientific	Cat#08-935
Forceps	Thermo Fisher Scientific	Cat#12-000-127

MATERIALS AND EQUIPMENT

4% PFA solution, pH 7.4		
Reagent	Final concentration	Amount
PFA	4%	4 g
PBS	N/A	To 100 mL
Total	**N/A**	**100 mL**

Note: After adding PFA, heat to 60°C, and continue to heat and stir until PFA is completely dissolved.

Note: 4% PFA solution needs to be freshly prepared. It can be stored at 4°C for up to 2 weeks or −20°C for up to 1 year.

⚠ CRITICAL: PFA is highly hazardous, and needs to be handled in the fume hood. Wear personal protective equipment (PPE).

Decalcification solution, pH 7.4–7.6		
Reagent	Final concentration	Amount
EDTA	0.5 M	186.1 g
ddH_2O	N/A	To 1,000 mL
Total	**N/A**	**1,000 mL**

Note: It is important to store decalcification solution away from light, which can be stored at 4°C for up to 6 months.

⚠ CRITICAL: EDTA is slightly hazardous in case of skin contact, eye contact, ingestion, and inhalation. Wear PPE.

30% Sucrose solution		
Reagent	Final concentration	Amount
Sucrose	30%	30 g
PBS	N/A	To 100 mL
Total	**N/A**	**100 mL**

Note: 30% Sucrose solution needs to be freshly prepared. It can be stored at 4°C for up to 2 weeks or −20°C for up to 6 months.

Permeabilization solution		
Reagent	Final concentration	Amount
Triton™X-100 10%	0.3%	150 µL
PBS	N/A	To 5 mL
Total	**N/A**	**5 mL**

Note: Permeabilization solution needs to be prepared just before use.

Blocking solution		
Reagent	Final concentration	Amount
Goat serum	5%	250 μL
PBS	N/A	To 5 mL
Total	N/A	5 mL

Note: Blocking solution needs to be prepared just before use.

Hoechst solution: Add 100 mg Hoechst 33342 to 10 mL deionized water to create a 10 mg/mL (16.23 mM) stock solution, which can be stored at −20°C for up to 1 year. At the time of staining, dilute the Hoechst stock solution 1:2,000 in PBS to obtain a working solution.

Note: Hoechst solution needs to be prepared just before use and protected from light.

Note: The liquids used for fixation and decalcification need to be pre-cooled in advance.

Alternatives: In principle, researchers can replace the reagents and equipment listed in the "key resources table" with comparable items from other suppliers.

STEP-BY-STEP METHOD DETAILS

Tissue harvest, fixation, decalcification, and dehydration: Day 1–4

Timing: 30 min for tissue harvest of one mouse; 4 h for fixation; 24–48 h for decalcification; 12–16 h for dehydration

This major step describes dissection of mouse femurs, which undergo fixation, decalcification, and dehydration (Figure 1), yielding intact femora that are ready for embedding and cryosectioning.

1. Add 10 mL pre-cooled 4% PFA solution to a 15 mL centrifuge tube and place the tube on ice.
2. Cull the mouse and surface-sterilize the skin using 75% ethanol.
3. Place the mouse on a dissection board and use sterile forceps and scissors to cut through the lower extremity skin.
4. Carefully remove the surrounding muscle and expose the femur.

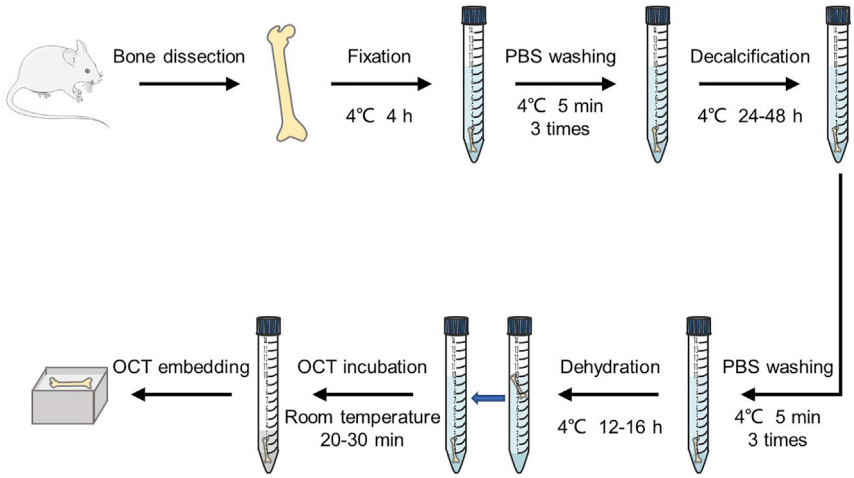

Figure 1. Schematic illustration of the tissue preparation steps before cryosectioning

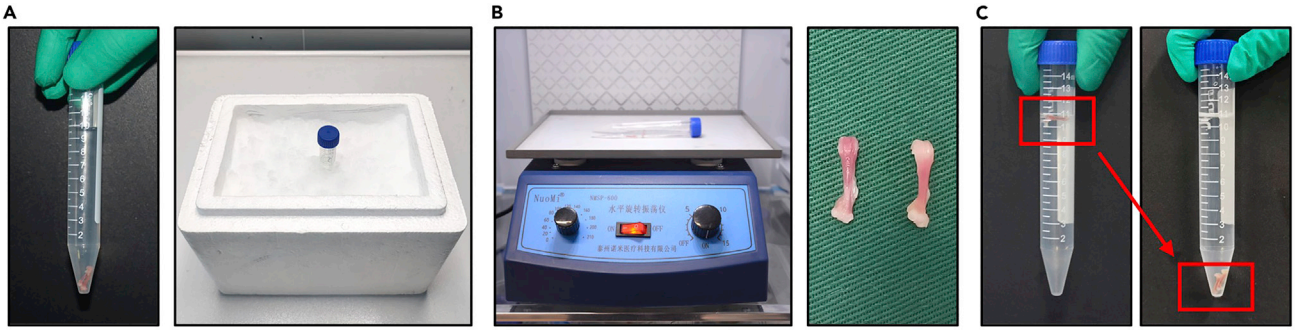

Figure 2. Representative images showing the steps of tissue dissection, fixation, decalcification and dehydration

(A) Fixation of femora dissected from 4-week-old Gli1-LacZ mice in 4% PFA in an ice box.

(B) Decalcification of femora on a shaker at 4°C, and side by side images of freshly harvested (left) and decalcified (right) bones.

(C) Dehydration of femora by 30% sucrose solution, during which the bones will first float for a few minutes and then descend through the solution to the bottom of the tube.

5. Dissect the femur by proximally separating the femoral head from the acetabulum and distally separating the lower end of the femur from the upper end of the tibia.

   Note: When the femur is separated from the upper and lower joints, it is recommended that the articular surfaces of the two adjacent bones be accurately separated in a visual environment so as to avoid damage to the femur.

6. Further remove the attached soft tissue on the femur.

   ⚠ CRITICAL: It is important to completely remove all the residual muscle and connective tissue on the bone surface, the remaining of which will interfere with the process of fixation and decalcification.

7. Immediately transfer the separated femur to the prepared fixation solution (4% PFA solution) and fix for 4 h on ice (Figure 2A).

   Note: It is best not to put more than four long bones in each tube, so as not to lower the fixation efficiency.

8. After fixation, discard the fixative completely and add 10 mL pre-cooled PBS to wash the bone on a shaker (100–150 rpm) at 4°C for 5 min. Repeat this step another two times and replace PBS after each wash.

   Note: It is important to wash the bone samples thoroughly so as to remove all of the PFA.

   Note: For the femur, 4 h is sufficient, and over-fixation will inactivate the antigen, leading to decreased signal in imaging.

9. Discard the PBS completely and add 10 mL pre-cooled decalcification solution (Harms et al., 2002). Incubate the mixture on a shaker (100–150 rpm) at 4°C for 24–48 h (Figure 2B).

   ⚠ CRITICAL: In this protocol, rapid decalcification method is applied, as indicated by previous study (Kusumbe et al., 2015), which uses a sufficient amount of decalcification solution and maintains constant shaking for 24–48 h, without changing the decalcification solution, thus helping preserve antigenic activity.

Note: In principle, the time needed for decalcification depends on the calcification degree of bones. For example, due to insufficient bone calcification, the femur of juvenile mice (2–4 weeks old) can be decalcified in 24 h. Nevertheless, the decalcification of bones from aged mice (> 50 weeks old) requires incubation for at least 48 h.

10. After decalcification, discard the decalcification solution completely and add 10 mL pre-cooled PBS to wash the bone on a shaker (100–150 rpm) at 4°C for 5 min. Repeat this step another two times and replace PBS after each wash.
11. Discard the PBS completely and add 10 mL 30% sucrose solution. Incubate the mixture at 4°C for 12–16 h with the tube being upright (Figure 2C, Methods video S1).

Note: When adding sucrose, the degree of decalcification can be determined. In specific, if the decalcification is complete, the bones will first float for about 20 min (Figure 2C) and then descend through the solution to the bottom of the tube (Figure 2C, Methods video S1). If not, repeat the above decalcification process.

Embedding and cryosectioning: Day 4

Timing: 1 h for tissue embedding; 30 min for cryosectioning of one sample

This major step describes embedding (Figure 1) and cryosectioning of mouse femur, resulting in tissue sections that are ready for immunofluorescence staining.

12. Set the temperature of the cryostat to −23°C in advance for pre-cooling.
13. Choose an appropriate embedding mold according to the shape and size of the bone sample, or use tin foil to make a personalized mold.

Note: The size of the mold should be appropriately larger than that of the tissue sample, so that when slicing the tissue sample, there is sufficient margin left.

14. Discard the sucrose solution and add an appropriate amount of embedding medium (OCT) to the tube that can completely cover the tissue sample. Incubate at 25°C for 20–30 min.
15. Add a small amount of OCT into the mold and put the mold in the pre-cooled cryostat for solidification. The purpose of this step is to form a base for the sample.
16. Add an appropriate amount of OCT into the mold and embed the bone sample into the OCT. Adjust the position and angle of the bone so that the long axis of the bone is parallel to the base of the mold, the posterior side of the bone faces the blade and the anterior side faces the base of the mold (Figure 3A, Methods video S2), and put the mold in the pre-cooled cryostat for at least 30 min to allow complete solidification.

Note: Take care to avoid bubbles during the embedding process.

Pause point: The embedded bone samples can be stored at −80°C for up to 6 months.

17. Set the slice thickness to 30 μm and install the blade in the holder for pre-cooling. Also, place tweezers and a camel-hair brush in the cryostat for pre-cooling.
18. Take out the sample from the mold and glue it to a specimen disc using OCT. If samples are kept at −80°C, place them in the cryostat for 60 min before isolating them from the mold.

Note: It is important to ensure strong adhesion between sample and disc so as to avoid movements or detachments when sectioning.

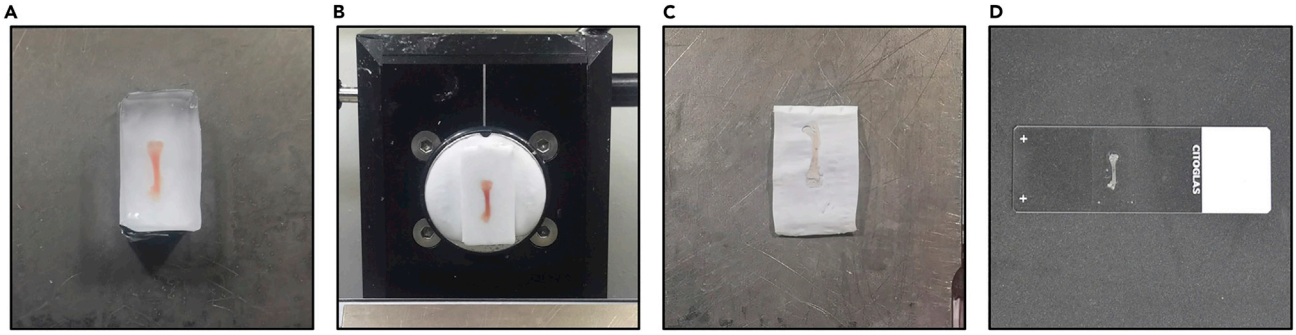

Figure 3. Representative images showing the steps of tissue embedding and cryosectioning
(A) Embedding of mouse femur in OCT.
(B) Cryosectioning of mouse femur.
(C) Flattened tissue section before collection.
(D) Collected tissue section on slide.

19. Fix the disc on the specimen head, and slice the solidified embedding agent on top of the sample. During this step, the position of the sample as well as of the anti-roll glass plate is adjusted.
20. When the bone tissue is revealed, further adjust the position of the sample to ensure sectioning of the bone as a whole (Figure 3B). Then, slice the bone sample according to the set thickness (Methods video S3).

⚠ CRITICAL: Be careful with the blades since they are very sharp.

21. For collection of bone tissue sections, open the anti-roll glass plate, quickly use tweezers to hold one end of the slice and gently flatten the slice with a camel-hair brush (Figure 3C, Methods video S3).
22. Take out the slide placed at 25°C and press down quickly towards the section to allow adherence of the section to the slide as a whole (Figure 3D, Methods video S3).

⚠ CRITICAL: Try to transfer the tissue section to the slide within 1 min of slicing to avoid freeze-drying of the tissue. Besides, do not use the same slide to collect consecutive sections so as to keep the temperature of slide at 25°C.

23. Air-dry the slides for 30 min at 25°C to avoid section detachment caused by insufficient drying.

Pause point: The slides can be stored at −20°C for up to 1 year.

Immunofluorescence staining: Day 5–6

Timing: 24 h

This major step describes the immunofluorescence staining of sections, which includes membrane permeation, blocking, primary antibody labeling, secondary antibody labeling, and nuclear staining.

24. Take out the frozen slides from the refrigerator and thaw them at 25°C for at least 20 min.
25. Use a PAP pen to draw a circle or square around the tissue section to avoid the spillage of liquid during the staining process (Figure 4A).

Note: When drawing the hydrophobic barrier, be careful not to touch or get too close to the sample.

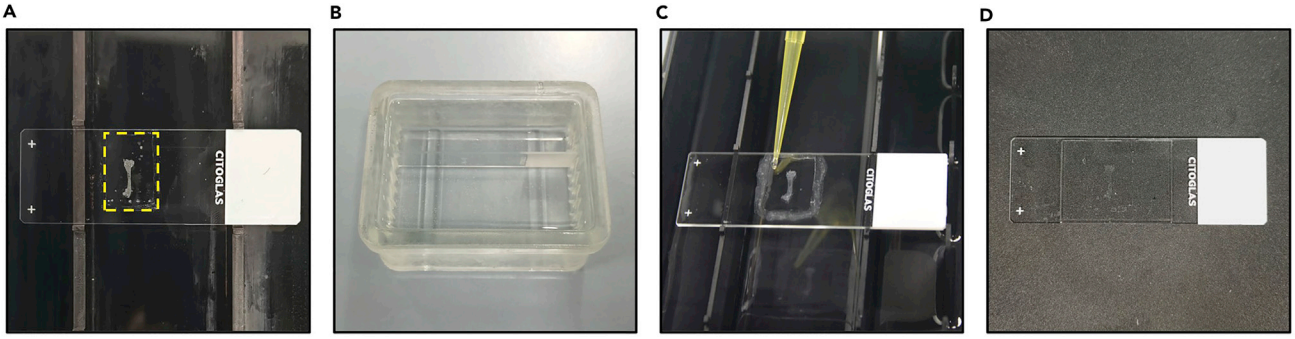

Figure 4. Representative images showing the steps of immunofluorescence staining
(A) Drawing of hydrophobic barrier around femur section, marked with dotted line.
(B) Rehydration in a staining jar.
(C) Example of adding solution to section.
(D) Mounting with coverslip.

26. Rehydrate the sections by putting the slides inside a staining jar with PBS at 25°C for 5 min (Figure 4B). Repeat this step another two times and carefully replace PBS after each wash.

⚠ CRITICAL: It is important to keep the tissue sections rehydrated from this point forward.

27. Place the slides in a humidified chamber and add 200 μL freshly prepared permeabilization solution to each section (Figure 4C). Incubate at 25°C for 20 min.

Note: Instead of being added directly onto the tissue sections, the solution should be pipetted adjacent to the tissue sections so as to prevent damage to the sample.

Note: In principle, permeabilization solution is used for thicker sections (> 30 μm) or staining of intracellular targets.

28. Discard permeabilization solution *via* pipetting and wash the slides in a staining jar with PBS at 25°C for 5 min. Repeat this step another two times and carefully replace PBS after each wash.
29. Place the slides in a humidified chamber and add 200 μL freshly prepared blocking solution to each section. Incubate at 25°C for 30 min.

⚠ CRITICAL: Blocking solution will vary with host background of the used primary antibodies. In this protocol, goat serum is used since the primary antibodies are generated in goats.

30. Discard blocking solution *via* pipetting and add 100 μL freshly prepared primary antibody dilutions to each sample. Incubate at 4°C for 12–16 h.
    a. The primary antibody dilutions: anti-β-galactosidase (1:200) and anti-CD31 (1:100) in blocking solution.

⚠ CRITICAL: In this protocol, the anti-β-galactosidase requires a secondary antibody step, while the anti-CD31 is directly conjugated and does not require a secondary antibody step.

Note: For primary antibody coupled with fluorescent dyes, perform this step and subsequent experimental steps away from light to avoid fluorescence quenching.

Note: Use secondary antibody alone as the negative control for background signal.

31. Take out the humidified chamber and allow the tissue sections to return to 25°C for 20 min.
32. Discard the primary antibody *via* pipetting and wash the slides in a staining jar with PBS at 25°C for 5 min. Repeat this step another two times and carefully replace PBS after each wash.
33. Place the slides in a humidified chamber and add 100 μL freshly prepared secondary antibody dilutions to each sample. Incubate at 25°C for 1.5 h.

Note: For steps 33–39, be careful to protect sections from light.

34. Discard the secondary antibody *via* pipetting and wash the slides in a staining jar with PBS at 25°C for 5 min. Repeat this step another two times and carefully replace PBS after each wash.
35. Place the slides in a humidified chamber and add 100 μL freshly prepared Hoechst solution to each sample. Incubate at 25°C for 30 min.
36. Discard the Hoechst solution *via* pipetting and wash the slides in a staining jar with PBS at 25°C for 5 min. Repeat this step another two times and carefully replace PBS after each wash.
37. Carefully wipe off excess PBS and add a drop of mounting medium around the tissue which gradually flows to cover the tissue.
38. Gently use forceps to lay coverslips on top of the sections (Figure 4D).

Note: Too much mounting medium will cause the coverslip to easily slip while too little will cause the generation of air bubbles. According to our experimental experience, it is recommended that the amount of mounting medium for small coverslips (24 mm × 32 mm) be about 50 μL, and that for large coverslips (24 mm × 50 mm) be about 100 μL.

39. Dry slides at 25°C for 30 min and store dried slides at 4°C for imaging.

Pause point: The slides can be stored at 4°C for a few days, protected from light.

Note: Imaging within 72 h and avoiding repeated imaging are recommended as the immunofluorescence signal diminishes with time or repeated imaging.

Confocal image acquisition: Day 7

Timing: 4–6 h

This major step describes how to set the confocal laser scanning microscope, scan the sections, and acquire images.

40. Turn on the computer, the lasers (405 nm light, 488 nm light and 633 nm light), and the mercury lamps.

Note: The purpose of this step is to allow warm-up of the equipment.

41. Wipe off extra liquid on the prepared slide and put it on the stage.
42. Open the FV10-ASW Viewer software, and choose the Hoechst fluorescent channel (i.e., DAPI).

⚠ CRITICAL: Be careful to avoid direct eye contact with the high-power lasers.

43. Observe the sample through microscope eyepiece, and move the region of interest (ROI) to the center of the visual field.
44. Choose the desired fluorochrome channels that match the used secondary antibodies and preview the chosen ROI. Here, we will introduce the steps for Z-stack scanning that can realize sequential depth imaging of the sections and obtain 3D-reconstructed images.
    a. Choose the desired magnification.

b. Configure the parameters of laser powers and channel detectors, including HV, Gain and Offset.
c. Set the scanning speed, the upper and lower limits of focal planes, and the StepSize. The number of slices will be automatically calculated by the software.

⚠ CRITICAL: When adjusting the parameters, try to make the brightness of the images moderate. If the brightness is too high, it will cause overexposure and increase the background, which may lead to false positives; if the brightness is too low, it may lead to false negatives.

45. Scan the sections with a resolution of 1,024 × 1,024.

Note: During scanning, it is important to keep the space protected from light and the operating desk stable.

46. Acquire the confocal images and overlay the Z-stack images by the composite button. Save the files and make further quantification analysis by appropriate softwares, such as ImageJ and Image Pro-Plus.

EXPECTED OUTCOMES

This manuscript has described an optimized immunofluorescence staining protocol to identify resident Gli1$^+$ MSCs in fixed/frozen bone sections from LacZ transgenic mice. Compared with LacZ staining assay, this protocol can produce 3D-reconstructed images of thick bone tissue, enabling the visualization of spatial organization and cellular networks (Kusumbe et al., 2015). Moreover, the application of immunofluorescence staining allows Gli1$^+$ cells to be quantitatively and qualitatively analyzed with flexible combination of antibodies labeling various targets (Kusumbe et al., 2014, 2015).

Consistent with our previous study (Chen et al., 2020), Figure 5 shows the distribution of Gli1$^+$ cells in the femoral metaphysis of Gli1-LacZ mice, which are located adjacent to CD31$^+$ endothelial cell (ECs) (Figure 5), confirming the identity of Gli1$^+$ cells as a specific subpopulation of MSCs residing in the perivascular niche. As to the age-related changes of Gli1$^+$ cells, Figure 5 shows that there are more Gli1$^+$ cells in juvenile (4-week-old) individuals compared with adult (8-week-old) compartments.

LIMITATIONS

Poor morphology is a major limitation regarding this protocol. On the one hand, compared with paraffin sections, the tissue architecture and cytomorphology of frozen tissues is relatively poorly preserved. On the other hand, given that the fixing time is short, the tissue morphology of bone samples is prone to be damaged. For example, the bone cortex is easily separated from cancellous bone during sectioning.

Some steps are hard to master. For example, the decalcification degree needs to be determined before dehydration, which is important for acquiring complete bone sections. Using syringe to puncture the bone can help determine the degree of decalcification, in which the syringe can puncture through the decalcified bone while not through undecalcified bone. But this method may damage the bone marrow tissue and affect the results. Besides, during the cytosectioning process, frozen slices, especially thick slices, are prone to curl after sectioning, which is difficult to be flattened. Moreover, the transfer of sections to slides requires the whole section to be adhered to the slides synchronously; otherwise, the sections will be twisted or folded on the slides, which will lead to detachment of the sections.

Compared with LacZ staining assay with commercial kit or reagents, the incidence of false positive is relatively high when detecting with anti-β-galactosidase antibody. Therefore, we recommend

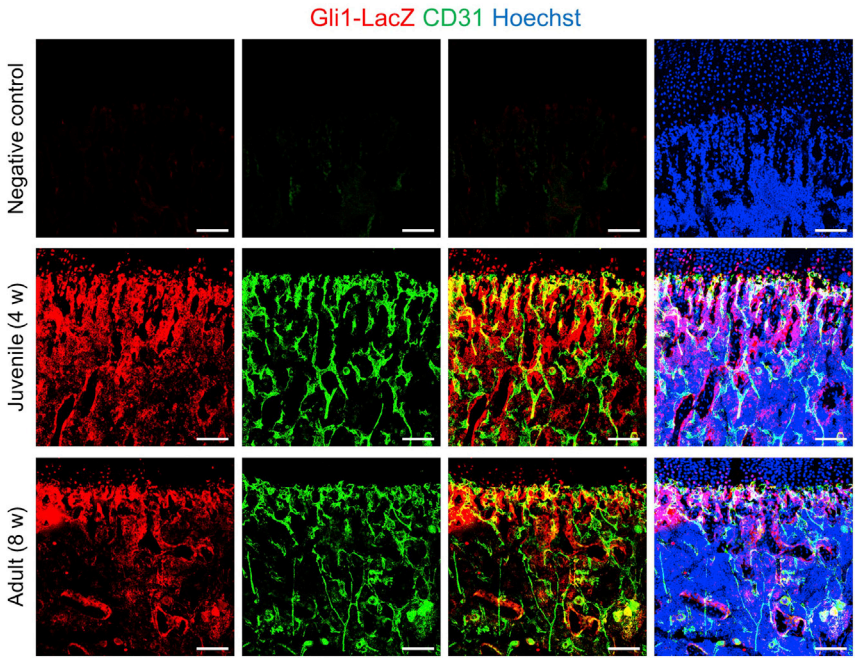

Figure 5. Representative immunofluorescence staining images

β-galactosidase immunostaining showing Gli1-LacZ^{+} cells (red) and CD31 (green) co-immunostaining in the femoral metaphysis of juvenile (4-week-old) and adult (8-week-old) Gli1-LacZ mice, counterstained by Hoechst (blue). Secondary antibody alone is used as the negative control. Scale bars, 50 μm.

application of both methods for quantitative analysis of LacZ-positive cells. Besides, unlike reporter mice expressing fluorescent proteins that can be used for cell isolation *via* fluorescence-activated cell sorting, the analysis methods are relatively limited for LacZ transgenic mice (Degirmenci et al., 2018).

TROUBLESHOOTING

Problem 1

Breaking of tissue architecture.

Potential solution

A major cause for this problem is insufficient decalcification. To solve this, it is important to completely remove all the adherent muscle and connective tissue on the bone surface (step 6). Besides, for bone samples with complex structure, such as the spine, researchers can separate them into multiple vertebrae for decalcification. In addition, provide sufficient amount of decalcification solution and do not put too many bones in one tube (step 9). Also, constant shaking is necessary for efficient decalcification (step 9). Moreover, it should be determined whether the bones have been completely decalcified before proceeding to cytosectioning.

Other solutions include preventing the existence of air bubbles when embedding the bone samples (step 16), using sharp blade without breaks and placing the anti-roll glass plate at appropriate position when sectioning (steps 17 and 19), and quickly transferring the slices to the slides as a whole (step 22).

Problem 2

Determination of the extent of decalcification.

Potential solution

It should be determined whether the bones have been completely decalcified after decalcification and washing (step 10). During the dehydration process, if the bones can float for about 20 min first and then settle to the bottom of the tube, this proves that the decalcification is complete (step 11). However, if the bones do not float or float for a very short time, it means that the decalcification is not complete so it is necessary to repeat the decalcification process (step 9).

Problem 3

Detachment of sections from the slides.

Potential solution

During the cryosectioning process, try to acquire flat slices when cutting sections and transferring them to slides, which will decrease the incidence of detachment (steps 21 and 22). Also, ensure adequate drying of the sections after cryosectioning (i.e., A minimum of 30 min) (step 23). The drying time can be appropriately extended when the room temperature is relatively low. Furthermore, the washes of slides during immunostaining should be performed gently.

Problem 4

Weak or no staining signal.

Potential solution

It is important to keep the samples at low temperature (4°C) during the fixation and decalcification processes (steps 7–10). Besides, since prolonged exposure to PFA can mask the epitope, the fixation time needs to be restricted (step 7). Moreover, the application of fresh antibodies and the increase of primary antibody concentrations as well as incubation time can help enhance the positive signals (step 30).

Problem 5

High background staining.

Potential solution

During the staining process, it is important to prevent tissue sections from drying out (steps 27–39). In addition, incubation with appropriate blocking solution can help lower the background (step 29). Moreover, the concentration of antibodies can be lowered (steps 30 and 33).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, bingdong@fmmu.edu.cn (B.D.S.).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze any dataset or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101674.

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AUTHOR CONTRIBUTIONS

C.X.Z., J.C., and J.Y.T. performed the experiments and wrote the manuscript. X.Y.H. performed the experiments. Y.J. and B.D.S. designed and supervised the study, and revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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