Protocol

Visualization and quantification of the stromal-vascular compartment in fetal or adult mouse bones: From sampling to high-resolution 3D image analysis

Advanced techniques combining tissue clearing, deep confocal imaging, and image quantification enable researchers to characterize complex tissue architectures and understand how trauma or disease impact on their respective components. Here, we detail methodologies to prepare, immunostain, and clear thick sections of mouse bones. We describe their use to visualize and quantitatively analyze marrow blood vessels and their spatial relationship with stromal skeletal stem/progenitor cells. These approaches can help unravel the stromal-vascular interplay in the bone marrow environment in health and disease.

Nicolas Peredo, Roger Valle-Tenney, Seppe Melis, Marion Mesnieres, Elena Nefyodova, Christa Maes
christa.maes@kuleuven.be

Highlights
Deep tissue imaging of cleared bones enables 3D analysis of stromal-vascular interplay

Protocols to process, immunostain, clear, and image fetal and adult mouse bone sections

Confocal microscopy modes to visualize blood vessels and skeletal stem/progenitor cells

Procedures to quantify vascular features and stromal cell spatial relationship in 2D/3D

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Visualization and quantification of the stromal-vascular compartment in fetal or adult mouse bones: From sampling to high-resolution 3D image analysis

Nicolas Peredo,1 Roger Valle-Tenney,1 Seppe Melis,1 Marion Mesnieres,1 Elena Nefyodova,1 and Christa Maes1,2,3,*

1Laboratory of Skeletal Cell Biology and Physiology (SCEBP), Skeletal Biology and Engineering Research Center (SBE), Department of Development and Regeneration, KU Leuven, 3000 Leuven, Belgium
2Technical contact
3Lead contact
*Correspondence: christa.maes@kuleuven.be
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SUMMARY
Advanced techniques combining tissue clearing, deep confocal imaging, and image quantification enable researchers to characterize complex tissue architectures and understand how trauma or disease impact on their respective components. Here, we detail methodologies to prepare, immunostain, and clear thick sections of mouse bones. We describe their use to visualize and quantitatively analyze marrow blood vessels and their spatial relationship with stromal skeletal stem/progenitor cells. These approaches can help unravel the stromal-vascular interplay in the bone marrow environment in health and disease. For complete details on the use and execution of this protocol, please refer to Mesnieres et al. (2021).

BEFORE YOU BEGIN
This protocol describes a pipeline to dissect, embed, section, immunostain, and clear mouse bones harvested at fetal and postnatal stages for subsequent high-resolution confocal microscopy and quantification of the vascularization and its relation to dedicated cell populations, such as stromal osteoprogenitor cells. The method was recently implemented in (Mesnieres et al., 2021) to characterize the stromal-vascular bone marrow compartment in developing mouse bones, comparing control mice and mice over-expressing VEGF by osterix (Osx) osteoprogenitor cells. Increased skeletal VEGF drastically impacted the stromal-vascular architecture; the local changes concomitantly impaired the initial homing of hematopoietic stem cells (HSCs) from the fetal liver toward the developing marrow cavity and compromised the establishment of hematopoiesis inside the bone shaft (Mesnieres et al., 2021).

To function properly, the hematopoietic lineage (from HSCs to their differentiated progeny) requires specific microenvironments supported by various cell types in bone. Among these, endothelial cells, stromal cells, and osteoprogenitors create essential niches that maintain and regulate the function of hematopoietic cells throughout multiple stem/progenitor and maturation stages. Blood vessels in bone are known to regulate different cell types and to be a necessary part of multiple stem cell niches. Perivascular bone marrow stromal cells (BMSCs), for instance, are considered skeletal stem/progenitor cell (SSPC) populations with important roles in maintaining and regenerating bone tissue during life and after bone trauma, processes occurring in close interplay with the skeletal vascular network. The regulation of SSPC maintenance versus osteogenic differentiation likely involves cell-cell crosstalk between endothelial cells and SSPCs/osteoprogenitors, for instance, via
the release of cytokines or growth factors; hence their mutual spacing may be critical in the determination of cellular behavior and tissue-level outcomes.

The method detailed here outlines how to immunostain and clear thick sections of mouse bone, delivers a strategy for high-resolution image acquisition, and offers a list of quantitative features that can be used to characterize the vasculature and its spatial association with SSPCs, with or without fluorescent reporters of relevant marker gene expression.

**Institutional permissions**

All experiments on animals were approved by the Ethical Committee for Animal Experimentation of KU Leuven (ECD ethical approval licenses P117/2011, P177/2014 and P041/2017). Housing of the animals was done in the central Animal Facilities of the KU Leuven (facility accreditation of the Belgian authorities under license number LA2210393; License number of the Department: LA1210189) and conducted in compliance with the Belgian legislation (the Belgian Royal Decree of 29 May 2013) and ethical guidelines on animals’ experimentation and welfare, in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS No. 123) (European Directive, 2010/63/EU of 20 October 2010).

Before you begin methodologies as described in this protocol, it is critical that you ensure that the required permissions from the relevant institutions have been acquired. Any experiments on live vertebrates or higher invertebrates must be performed in accordance with the institutional and national guidelines and regulations.

**Preparation of fixative**

**© Timing: 2–3 h**

A necessary preceding step is to prepare fresh fixative used in the protocol. Preparing a newly dissolved solution of paraformaldehyde (PFA) is a requisite step to stabilize and preserve the morphology and ultrastructure of the biological sample and the cells and matrix constituents contained therein and to optimally retain antigenicity. This section describes the fixative solution preparation for freshly isolated fetal and adult mouse bone tissues, collected as detailed in the step-by-step method description below.

1. Freshly prepare a 4% PFA (in PBS) solution for tissue fixation. Prepare the solution on the day of intended use or at maximum one day before.

### Preparation of 4% PFA tissue fixation solution

| Reagent                  | Final concentration | Amount     |
|--------------------------|---------------------|------------|
| Paraformaldehyde (PFA)   | 4%                  | 2 g        |
| PBS                      | 1 x                 | Fill up to 50 mL |
| Total                    | n/a                 | 50 mL      |

**Note:** The preparation process can be sped up by heating the dissolving PFA solution. Therefore, place the closed recipient (e.g., a glass bottle) on a heated magnetic stirring plate and warm up to maximally 55°C–60°C. It takes ± 2 h for the PFA to be fully dissolved. Store the solution at 4°C until use and for no more than 16 h.

2. Cool down the 4% PFA solution to 4°C before use.
3. Dilute the solution to 2% PFA in PBS if needed (depending on the specific protocol that will be followed; see step-by-step method details).
4. Pre-fill the sample collection tubes with fixative and keep cold (4°C).

⚠ CRITICAL: PFA is hazardous (see safety information and chemical handling) and needs to be always handled under a fume hood and with protective measures.

### Safety information and chemical handling

| Chemical, IUPAC name | Chemical safety information | Health hazard information | Handling precautions |
|----------------------|----------------------------|---------------------------|---------------------|
| Paraformaldehyde     | Flammable                   | Causes skin irritation. May cause allergic skin reaction. Causes serious eye damage. May cause respiratory irritation. Suspected carcinogen. | Wash hands thoroughly after handling. Work in fume hood. Wear protective gloves. Wear protective clothing. |
| Triton X-100         | Irritant                    | Causes serious eye irritation. Toxic for aquatic life with long term consequences. | Wash hands thoroughly after handling. Wear safety goggles. Wear protective clothing. |
| Isopentane           | Flammable                   | May be fatal when getting into respiratory tract upon accidental swallowing. May cause dizziness and drowsiness. Toxic for aquatic life with long term consequences. | Work in fume hood. Wear protective gloves. Wear safety goggles. Wear protective clothing. |
| 2,2'-Thiodiethanol   | Irritant                    | Causes serious eye irritation. | Wash hands thoroughly after handling. Wear safety goggles. Wear protective clothing. |

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| CD140b (PDGFRβ), goat polyclonal anti-mouse (1:100) | Santa Cruz Biotechnology | Cat# SC-1627 |
| CD31, goat polyclonal anti-mouse (1:100) | R&D Systems | Cat# AF3628 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Endomucin (Emcn), rat monoclonal anti-mouse (1:100) | Santa Cruz Biotechnology | Cat# SC65495 |
| Green fluorescent protein (GFP), chicken polyclonal anti-GFP (1:100) | Abcam | Cat# ab13970 |
| PHH3 (phospho-histone H3), rabbit polyclonal anti-human IgG (1:100) | Abcam | Cat# ab47297 |
| VE-cadherin, goat polyclonal anti-mouse IgG (1:100) | R&D systems | Cat# AF1002 |
| Donkey polyclonal anti-rat IgG (H+L), DyLight 550 (1:200) | Invitrogen | Cat# SAS-10027 |
| Donkey polyclonal anti-goat IgG (H+L), Alexa Fluor 647 (1:200) | Abcam | Cat# ab150131 |
| Donkey polyclonal anti-rat IgG, Alexa Fluor 488 (1:200) | Abcam | Cat# ab150155 |
| Goat polyclonal anti-chicken IgY, Dylight 488 (1:200) | Sigma-Aldrich | Cat# A31572 |
| Donkey polyclonal anti-rat IgG, Alexa Fluor 647 (1:200) | Invitrogen | Cat# AB96951 |

**Chemicals, peptides, and recombinant proteins**

| Chemical/Peptide | Source | Identifier |
|------------------|--------|------------|
| 2,2'-Thiodiethanol | Sigma-Aldrich | Cat# 88561 |
| Donkey serum | Sigma-Aldrich | Cat# 530 |
| EDTA disodium salt dihydrate | Fisher Scientific | Cat# BP120-1 |
| Fluoroshield mounting medium (without DAPI) | Sigma-Aldrich | Cat# F6182 |
| Gelatin from porcine skin | Sigma-Aldrich | Cat# G6144 |
| Goat serum | Sigma-Aldrich | Cat# G9023 |
| Hoechst 33342 | Life Technologies | Cat# H3570 |
| Isopentane | Acros organics | Cat# 42730010 |
| KP-CryoCompound – Frozen Tissue Medium | ImmunoLogic | Cat# BVK1620-C |
| Paraformaldehyde | Sigma-Aldrich | Cat# 818715 |
| Polyvinylpyrrolidone | Sigma-Aldrich | Cat# 856568 |
| Superfrost™ Plus Adhesion Microscope Slides | Epedia | Cat# J1800AMNZ |
| Triton-X-100 | Sigma-Aldrich | Cat# 100102599 |
| Tween-20 | VWR | Cat# 2.2184.0500 |

**Deposited data**

| Test image dataset, raw images and analyzed data | BioImage Archive: www.ebi.ac.uk/bioimage-archive/BioImage Archive: S-BIAD287 |

**Experimental models: Organisms/strains**

| Mouse: 'Crl:CD1(ICR)' (female young adult mice, between 8 and 12 weeks of age) | Charles River Laboratories | Strain Code Q22 (Rodda and McMahon, 2006) |
| Mouse 'Oxs-Cre-GFP': B6.Cg-Tg(Sp7-tTA, tetO-EGFP/cre)1Amc/J (used in crosses with VEGF cTg mice in Mesnieres et al., 2021) | Andrew McMahon, University of Southern California, Los Angeles, USA | (Maes et al., 2010) |
| Mouse 'VEGF cTg': Gt(ROSA)26Sor<tm1(Vegfa*)Jhai>; ROSA26-VEGF164 offspring of crosses with Oxs-Cre-GFP mice was studied between embryonic day (E)14.5 and postnatal day (P)2 in Mesnieres et al., 2021 | Andras Nagy and Jody Haigh, Mount Sinai Hospital, Samuel Lunenfield Research Institute, Toronto, Canada | |

**Software and algorithms**

| Fiji | Schindelin et al., 2012 | https://imagej.net/software/fiji/downloads |
| ImageJ 1.52a | National Institutes of Health | https://imagej.nih.gov/ij/download.html |
| NIS-elements | Nikon | https://www.microscope.healthcare.nikon.com/products/software/nis-elements |
| Prism 8 | GraphPad Software | www.graphpad.com |
| Imares 9.6 | Oxford instruments | https://imaris.oxinst.com/ |
MATERIALS AND EQUIPMENT

Equipment
The following equipment was used for the protocols explained in this manuscript:

- Nikon TiE inverted C2 confocal microscope with the following specifications:
  - C2-DU3 detector
  - Plan Apo 10× objective
  - Plan Apo VC 20× objective
  - Diode lasers (measured downstream, in the focal plane of a Nikon Plan Apo VC 20× objective):
    - 405 nm (LU-N4 – 1.25 mW)
    - 488 nm (LU-N4 – 3.06 mW)
    - 561 nm (LU-N4 – 3.75 mW)
    - 640 nm (LU-N4 – 3.94 mW)
  - Emission filters:
    - Blue: 425–475 nm and 465–500 nm
    - Green: 500–550 nm, 515–550 nm, 525–555 nm, 500–530 nm
    - Red: 570–620 nm, 560–618 nm
    - Far-red: 663–738 nm

- Nikon NiE upright spinning disk confocal microscope with the following specifications:
  - Camera (Teledyne Photometrics Prime 95B sCMOS):
    - 95% QE
    - 11 × 11 μm pixel size
    - 40 fps (16-bit) / 80 fps (12-bit)
    - 1.6e – read-noise
  - Stage (Luigs & Neumann 380FM-U shifting table + bridge 500):
    - 25 mm travel in XY
    - 2.5 nm motor resolution
    - Reproducibility less than 1 μm
    - Weight: 23 kg
    - Dimensions: 622 × 505 70 mm
    - Spindle lead 0.5 mm
  - Lasers (Agilent MLC400) (measured in the focal plane of a Nikon Plan Apo VC 20× objective):
    - 405 nm (diode – violet cube 0.23 mW)
    - 488 nm (blue diode – sapphire 1.83 mW)
    - 561 nm (green diode – sapphire 2.27 mW)
    - 640 nm (red diode – sapphire 1.76 mW)
  - Filters:
    - Quad cube: 432/515/595/730-25 nm
    - Blue 405 nm single: 450/50 nm
    - Green 488 single: 525/50 nm
    - Red 561 single: 595/50 nm
    - Far-red 640 nm single: 700/75 nm

- Analysis PC (HP Z840)
  - Operating system: Windows 10
  - RAM: 192 GB
  - Intel(R) CPU ES-2623 v3 @ 3.00 GHz 2.99 GHz (2 processors) #16
  - NVIDIA Quadro RTX 4000

- Analysis PC (Dell precision tower 5810)
  - Operating system: Windows 10
  - RAM: 64 GB
  - Intel(R) Xeon(R) CPU E5-1660 v3 @ 3.00 GHz - #16
  - NVIDIA Quadro K4200
Preparation of 0.5 M ethylenediaminetetraacetate (EDTA) bone decalcification buffer

| Reagent                             | Final concentration | Amount         |
|-------------------------------------|---------------------|----------------|
| Na₂EDTA - 2H₂O (MW = 372.24)       | 0.5 M               | 186.12 g       |
| NaOH                                | ± 20 g              |                |
| PBS                                 | 1 x                 | 800 mL (dissolve first) |
| PBS                                 | 1 x                 | Fill up to 1 L total solution |
| Total                               | n/a                 | 1 L            |

Note: Store the solution at 4°C until use for no more than 3 months.

- Dissolve the Na₂EDTA - 2H₂O in 800 mL AD in a 1 L bottle on a magnetic stirring plate;
- Add half of the NaOH pellets from the start and let dissolve;
- When dissolved, gradually add the remaining NaOH pellets and continue the stirring;
- Measure pH and adjust with HCl or NaOH if necessary to reach pH=7.4;
- Complete to 1 L with PBS;
- Store the EDTA solution at 4°C.

Cryoprotection medium

| Reagent              | Concentration   |
|----------------------|-----------------|
| D(+) Sucrose         | 20% (w/v)       |
| Polyvinylpyrrolidone | 2% (w/v)        |
| Distilled water      | n/a             |

Note: Prepare at least 5 mL per sample (2.5 mL for pre-conditioning the sample in cryoprotection medium (step 3b of the step-by-step method details), and 2.5 mL as basis for preparing the gelatin embedding medium, see step 3c and the ‘Gelatin embedding medium’ Table below). Store the solution at 4°C until use and for no more than 2 months.

Gelatin embedding medium

| Reagent              | Concentration   |
|----------------------|-----------------|
| Cryoprotection medium| n/a             |
| Gelatin 100          | 8% (w/v)        |

Note: This solution is not suitable for storage and should be used the same day it is made (step 3c).

Preparation of blocking solution

| Component    | Final concentration | Amount | Component function          |
|--------------|---------------------|--------|----------------------------|
| Tween-20     | 0.1%                | 1 µL   | Washing and permeabilization|
| Triton-X-100*| 0.2%                | 2 µL   | Permeabilization            |

(Continued on next page)
Note: Additional solutions required during the embedding and staining procedures, are outlined in the relevant sections of the step-by-step method details.

Mouse lines
This protocol was originally implemented (Mesnieres et al., 2021) to monitor the stromal-vascular bone marrow compartment in developing mouse bones, in a study designed to assess the impact of genetically induced VEGF over-expression in the skeleton on hematopoietic stem cell (HSC) homing to the newly forming marrow cavity inside the bone shaft. Therefore, the protocol was applied to mice carrying a conditional VEGF164 transgene (Maes et al., 2010), recombined under the control of the osteoprogenitor cell (OPC)-directed Osx-Cre:GFP driver (Rodda and McMahon, 2006), generating (Osx-Cre:GFP+;VEGF(Tg/+)) mutant embryos and their control (Osx-Cre:GFP+;VEGF(+/+)) littermates.

These techniques, or modules thereof, can be further applied to any other mouse strain and likely to any other tissue as long as it can be consistently immunostained and cleared.

Data management
Imaging tiled Z-stacks for subsequent 2D and 3D spatial analysis yields large images of 5–10 GB depending on the size of the Region of Interest (ROI). The image files are initially stored in the format of the microscope system software; in this protocol NIS-Elements for Nikon microscopes using the “.nd2” file extension. “.nd2” files were processed as TIF files to analyze the images using FIJI. Finally, images shown in the figures were compressed to JPEG format. Note that jpg should not be used for analysis. Regarding data storage, as an example, a 1 mm² tiled scan containing 4 channels and 52 single optical sections requires ~5 GB of storage space. In addition, different image processing and analysis steps generate multiple copies of the image to use for quantification purposes, further increasing the amount of storage space needed in correlation with the generated number of copies. To analyze these datasets, we used either HP or Dell workstations running with Windows 10,
equipped with 64–192 GB of RAM, dual processors, 16 cores, and either an NVIDIA Quadro RTX4000 or K4200 graphics card.

In our experiments, acquired images were first temporally stored in the local drive of the microscope’s computer. Then, following each imaging session, raw images were transferred into secured internal servers provided by KU Leuven IT Service and stored. For processing and analysis, copies of the images were transferred to local drives of image processing workstations for efficient computation. All images and analysis data were finally transferred back to a KU Leuven maintained and backed-up storage server for long-term data preservation and archiving.

**STEP-BY-STEP METHOD DETAILS**

Figure 1 provides a schematic overview of the overall workflow including the steps and options in the procedures.

### Dissection and processing of mouse limbs

**Timing:** 2–16 days (2 days for dissection and fixation of all sample types; add an additional 2 days for decalcification of fetal/newborn samples, and 14 days for decalcification of adult bones).

1. **Embryonic and newborn tissue sampling, fixation, and decalcification**
   a. The day or hours before the dissection, prepare fresh 2% PFA in PBS for tissue fixation and store the solution at 4°C (see ‘before you begin’ section).
   b. Dissection of embryonic and newborn mouse limbs
      i. For embryo isolation, euthanize the pregnant dam at embryonic day E18.5 or the desired fetal age, determined from the day of conception, with E0.5 being the morning the copulation plug was observed, by CO2 inhalation in a glass desiccator designated for this purpose, filling the lower chamber with CO2 ice.
      ii. Position the pregnant female mouse belly upwards and spray the surface of the abdomen region with 70% ethanol. Open the abdomen using scissors and tweezers to make an incision in the fur and skin initially, and next reach the peritoneal cavity by cutting the peritoneal membrane. Pull out the uterus using forceps, lifting the uterine horn at the ovarian side out of the abdominal cavity, and cutting at the level of the cervix. Place the uterus into a pre-filled petri dish with PBS solution and clean the uterus from the mesometrium membrane.
      iii. Isolate the embryos by cutting the uterine horns and liberating the embryos from the embryonic sac using forceps, removing the extraembryonic tissues and organs. Cut the umbilical blood vessels to remove the placenta and euthanize the embryos according to the local guidelines, adhering to the legislation and ethical regulations provided by the relevant authorities at your institution.
      iv. Immerse the isolated embryos in clean PBS in a second petri dish.
      v. For newborn bone harvesting, sacrifice the pups by the appropriate prescribed method, rinse with PBS, and immerse in clean PBS in a second petri dish.

   2. **Dissect embryonic and newborn mouse limbs in a clean petri dish with PBS using a stereo-sopic microscope, small scissors, and a pair of fine tweezers; delicately separate the limbs from the mouse embryo, avoiding putting pressure or damaging the sample.**

   *Note:* Useful resources and videos detailing these steps can be found on Jove.com (e.g., see https://doi.org/10.3791/59509-v and https://doi.org/10.3791/54978) and on the internet / Youtube.)
skin and excess soft tissue / muscles to facilitate optimal penetration of the fixating solution into the bones.

c. Fixation

Figure 1. Outline of the overall procedure and the respective steps and options

Depending on the final purpose and analysis goals, fetal/newborn and juvenile/adult samples can be processed for cryo- versus gelatin-embedding, and sectioned into thin slices versus thick specimens. See text for details on these options. Bottom right, confocal image examples, reprinted with permission from (Mesnieres et al., 2021). Scale bars, 100 µm.
i. Collect limbs and fix for 12–16 h at 4°C in 2% PFA (prepared in PBS) solution.

**Note:** Ensure the width of the tube and the volume of fixative is sufficient to fully immerse and expose the sample to the fixative. Always use freshly prepared PFA for this protocol.

ii. After fixation, take off the PFA solution and wash the sample with PBS 3 × 5 min.

d. Decalcification
   i. Decalcify perinatal bones (E18.5 to P2 samples) in 0.5 M EDTA for 2 days at 4°C with mild agitation and daily renewed EDTA solution.

**Note:** Decalcification is normally not necessary for fetal mouse bones before the stage of E18.5, given the limited mineralization of the developing skeleton by then.

ii. Wash samples 3 × 5 min in PBS at 20°C–22°C (room temperature) with mild agitation.

2. Adult mouse bone dissection, fixation, and decalcification
   a. Euthanize adult mice by CO2 inhalation in a glass desiccator designated for this purpose, filling the low chamber with CO2 ice.
   b. Spray limbs with 70% ethanol, cut and retract the skin from the legs, remove muscles using scissors and tweezers, and collect the bones of interest. For tibia and femur: remove the foot by cutting at the ankle using scissors, and separate the tibia and femur from each other by carefully cutting the soft tissues / ligaments in between the bones at the level of the knee.
   c. Tissue fixation: Fix samples 12–16 h using freshly prepared 4% PFA in PBS at 4°C.

**Note:** Ensure the width of the tube and the volume of fixative is sufficient to fully immerse and expose the sample. Always use freshly prepared PFA for this protocol.

d. Wash samples 3 × 20 min with PBS at 20°C–22°C with constant agitation.
   e. Decalcification: Decalcify juvenile and adult samples in 0.5 M EDTA for 2 weeks in constant agitation at 4°C, changing the EDTA solution weekly.

**Note:** Shorter decalcification periods may be sufficient to obtain high-quality sections; our recommendation is to test and adjust it on a sample batch-specific basis.

f. Wash samples 3 × 20 min with PBS at 20°C–22°C with constant agitation and proceed with embedding protocol.

**Preparation of histological specimens: Tissue embedding**

© **Timing:** 2 days

We describe two embedding protocols, gelatin-embedding and cryo-embedding, which are each optimal for specific final imaging purposes and analysis goals. Briefly, for 3D analysis it is better to use gelatin-embedding, because the section thickness limit is larger. To perform 2D analyses, cryo-embedding is better. More specifically: Gelatin-embedding, based on (Caire et al., 2019), allows for the bone samples to be sectioned (using a regular cryostat, see step 5 on ‘Preparation of histological specimens: tissue sectioning’ below) at a thickness of the resulting specimens ranging from 50 up to 300 μm. This choice of processing is ideal when the main aim is to perform deep tissue imaging for 3D analysis, requiring thick sections. Of note, thin sections, if desired, can also be generated from the gelatin blocks (for instance, generating some thick and some thin sections from the same block), but the quality may not be optimal and the lower limit for the sections’ thickness in our hands was around 10–12 μm.
If thinner sections of high quality are the priority (e.g., 5 μm), for instance, when high-resolution 2D image analysis is aimed for, we recommend performing cryo-embedding, because thin cryosections of bone can be generated with optimal preservation of the tissue integrity. Thick sectioning is also possible from cryo-embedded bone samples, but in our hands the maximum thickness achieved with this method, without jeopardizing tissue integrity, was 50 μm. This still allows for 2D analysis of confocal images.

For the purpose of deep tissue imaging and 3D analysis, the final option is to cut half-trimmed bone specimens from cryo-embedded blocks.

For an overview of the various options and downstream analysis possibilities, see Figure 1.

Figure 2 provides information on the gelatin and cryo-embedding procedures.

3. Gelatin embedding
   a. On the first day of the gelatin embedding protocol, prepare cryoprotection medium and store at 4°C until use.
   b. Pre-condition samples in cryoprotection medium, using ~2.5 mL per sample to fully cover the tissue, and incubate 24 h at 4°C.
   c. Prepare gelatin embedding medium by adding 8% (w/v) of gelatin to cryoprotection medium. Stir gently (to avoid air bubbles) at 65°C until the gelatin is dissolved completely (this can take ~4 h or can be performed for 12–16 h overnight).

Figure 2. Gelatin- and cryo-embedding set-up and examples
(A) Pouring warm gelatin embedding solution in molds for tissue embedding.
(B) Optimal orientation for an adult femur.
(C) Material set-up for cryo-embedding.
(D) Freezing samples in OCT cryo-embedding medium using liquid N2-chilled 2-methylbutane.
(E) Cryo-embedded tissue example.
d. On the second day, embed the tissues in gelatin blocks as follows:
   i. Label molds and fill them completely with warm (~65°C) gelatin embedding medium. Pour the medium gently into the molds, avoiding bubbles (Figure 2A).
   ii. Using tweezers, submerge the bone sample in the gelatin, positioned in the center of the mold and orientated in such a way that the growth plate is perpendicular to the vertical axis of the mold, and the bone shaft is leveled horizontally (to facilitate later trimming and sectioning). Figure 2B shows the optimal orientation for an adult femur.
   iii. Let the samples cool down on the bench at 20°C–22°C for 15–30 min until the gelatin solidifies, and store the blocks at −80°C.

4. Cryo-embedding
   a. Pre-condition samples in a solution of 30% sucrose in PBS (see ‘Recipes for general bone sample preparation solutions’ section under the ‘Materials and Equipment’ header) for 24 h at 4°C.
   b. Transfer the tissue samples to labeled plastic molds and cover them with Optimal Cutting Temperature (OCT) compound.
   Note: Submerge the bones gently to avoid bubble formation.

   c. Prepare the setup for freezing the tissues: place a borosilicate glass recipient (wide tube or beaker) containing 2-methylbutane at a volume that fills ~8–10 cm of the tube, hanging inside a wide insulated container with liquid nitrogen (N₂), as shown in Figure 2C. Allow the 2-methylbutane solution to cool down adequately, i.e., until the bottom part (~1 cm) of the 2-methylbutane solution solidifies and turns opaque white, while the upper part of the solution remains fluidic.
   Note: An equivalent set-up with other materials can be used to generate a solution of liquid N₂-chilled 2-methylbutane.

d. Using long forceps, submerge the mold containing the tissue sample into the cold 2-methylbutane solution, until it quickly becomes completely frozen (takes around 10 s) (Figures 2D and 2E). Allow the tissue matrix to solidify completely and remove block from 2-methylbutane.
   Note: Do not overcool the sample by submerging it longer than necessary in 2-methylbutane, as this may cause the block to crack.

e. Collect samples on dry ice until finishing the set of samples; then store them at −80°C.

Preparation of histological specimens: Tissue sectioning

@ Timing: 1–2 h per bone, dependent on experience and number of sections per sample

5. Prepare thin or thick sections, as desired according to the selected embedding procedure and output goals (see Figure 1 and Table 1).
   a. Retrieve gelatin- or cryo-embedded blocks from −80°C and transfer them to the cryostat at −20°C for temperature conditioning.
   b. Trim the excess of gelatin or OCT compound, respectively, using a blade or a scalpel.
   c. Mount the block into the cryostat chuck (specimen disk) using OCT compound and place it into the fast-freezing station inside the cryostat.
   d. Place the chuck into the moving arm and trim the sample cutting ~50 μm slices until the bone surface of the sample appears.

△ CRITICAL: Beware of the sample orientation, and delicately redirect the sample holder in X, Y and Z to obtain even, longitudinal sections across the bone.
e. Cut tissue sections at the desired thickness.

f. Collect the bone sections on coated adhesion microscope slides (e.g., Superfrost Plus).
   i. For gelatin sections: Let the sections condition at 20°C–22°C (room temperature) for ~30 min to ensure adhesion to the slide.
   ii. For cryo-sections: It is important that the slides are at 20°C–22°C (room temperature) for the section to stick to the slide.

   **Note:** In case problems arise during sectioning, please refer to troubleshooting 1, 2, and 3.

g. Store the slides with the bone sections at 0°C for short-term or at –80°C for long-term preservation.

### Immunostaining of bone sections

© Timing: 2–4 days (depending on the type of immunostaining and type of sections; see Figure 1 and Table 2)

Bones are immunostained to detect blood vessels via antigens typical for endothelial cells, to identify skeletal stem/progenitor cells within the tissue stroma, and/or to visualize GFP-expressing cells or EdU-containing proliferating cells, with the selected options depending on the specific application.

6. Blocking and permeabilization

   a. Let the slides come to 20°C–22°C and dry for 1 h to improve adherence of the section to the slide.

   **Note:** If the samples contain an endogenous fluorescence signal that needs to be preserved, e.g., fluorescent reporter expression, ensure to protect them from light for the entirety of the protocol.

   **Note:** If the sections fail to adhere well to the slide, please refer to troubleshooting 4.

   b. Incubate with PBS-T for 10 min at 20°C–22°C (room temperature) to rehydrate the tissue.

| Table 1. Section thickness |
|----------------------------|
| **Embedding** | **Thickness** | **Comments** |
| Cryo (OCT) | 5–10 µm | Obtaining very thin sections may be difficult and require practice (also dependent on the equipment specifics). |
| | 50 µm | No comments. |
| | Half bone | Trim the block until the middle of the bone and collect the rest as half bone sample. |
| Gelatin | 10–12 µm | Collect the section by putting the empty slide upside down over the section. |
| | 50–300 µm | Above 50 µm thickness, the sections can be taken with tweezers to transfer them to the slide. |

| Table 2. Blocking and antibody incubation times |
|-----------------------------------------------|
| **Type of section** | **Imaging purpose** | **Blocking solution** | **Primary antibody incubation** | **Secondary antibody incubation** |
| Thin | 2D | 45 min | 12–16 h | 75 min |
| Thick | 2D | 45 min | 12–16 h | 75 min |
| Thick/Half bone | 3D | 12–16 h | 24 h | 24 h |

**Note:** For gelatin sections thicker than 200 µm, the primary antibody incubation time may be prolonged up to 48–72 h if required to obtain good penetration into the tissue.
CRITICAL: Apply every solution in the entire procedure gently, as the bone sections are very delicate; particularly the bone marrow is easily washed off the slide if not handled carefully.

c. Clean the excess cryo-embedding medium or gelatin remnants from the surroundings of the bone section using a wipe or blade, and draw a circle around the section using a hydrophobic pen.

Note: The hydrophobic pen facilitates the creation of a delimited region of the slide containing the tissue section, reducing the volumes required to incubate the tissue with precious/costly reagents (e.g., antibodies).

Note: Be careful not to spill any hydrophobic ink on the section.

CRITICAL: Throughout the entire staining procedure, ensure to keep the rehydrated tissue sections immersed in water-based solutions or at the minimum sufficiently moist to avoid that they would dry out. Perform the incubations in a humidified, closed chamber, and keep the sections protected from light when fluorescent signals are involved. Performing the blocking and antibody incubation steps on a very gently rocking platform can help ensure homogeneous staining of the tissues; a risk could be to lose sections if the movements result in detaching of the sections from the slides.

d. Block and permeabilize samples by adding 100 μL of Blocking Solution (see ‘Recipes for general bone sample preparation solutions’) per section for 45 min at 20°C–22°C (sections) or 12–16 h at 4°C (very thick sections for 3D imaging and trimmed half bones) in a humid/moisture chamber as detailed in Table 2.

Note: For thin sections, use Blocking Solution without Triton-X-100.

7. Primary and secondary antibody incubations
   a. Incubate samples with 100 μL of primary antibodies diluted in Blocking Solution for 12–16 h (thin or relatively thick sections) or for 24 h (very thick sections and trimmed half bones) at 4°C as listed in Tables 2 and 3.
   b. Rinse briefly once and then wash samples at least 3–4 times for 10 min each, using PBS-T at RT.

Pause point: Here, samples can be stored in the fridge for up to one day. They should always be kept humid.

c. Incubate samples with 100 μL of secondary antibodies diluted at 1:200 in PBS-T for 75 min at 20°C–22°C (for 2D imaging) or 24 h at 4°C (for 3D imaging). See Tables 2, 3, and 4.

8. Washes and Hoechst staining
   a. Rinse briefly once and then wash in PBS-T for 10 min at least 3–4 times at 20°C–22°C.
   b. Incubate samples with 100 μL of Hoechst 1:500 (final concentration 20 μg/mL) for 10 min for thin sections and 30 min for thick sections and half bones at 20°C–22°C.
   c. Rinse briefly and then wash the samples in PBS-T for 10 min once at 20°C–22°C.

Table 3. Antibody concentrations used for immunostaining

| Primary antibody  | Concentration | Secondary antibody         | Concentration |
|-------------------|---------------|----------------------------|---------------|
| Emcn (rat)        | 1:100         | Donkey anti-rat (DyLight 550) | 1:200         |
| CD31 (goat)       | 1:100         | Donkey anti-goat (Alexa Fluor 647) | 1:200         |
| PDGFRb (goat)     | 1:100         | Donkey anti-goat (Alexa Fluor 647) | 1:200         |
| GFP (chicken)     | 1:100         | Goat anti-chicken (Dylight 488) | 1:200         |
| PHH3 (rabbit)     | 1:100         | Donkey anti-rabbit (Alexa Fluor 555) | 1:200         |
| VE-cadherin (goat)| 1:100         | Donkey anti-goat (Alexa Fluor 647) | 1:200         |
Pause point: Here, samples can be stored in the fridge for up to a day. They should always be kept humid.

Note: For problems related to uneven staining or high background signal of the tissue sample, please refer to troubleshooting 5, 6, and 7.

### Clearing the bone tissue

**Timing:** 4 h

This step applies to thick sections and trimmed half bones aimed for 3D deep-tissue imaging. For samples that do not require clearing, skip this section and proceed to step 11.

**Note:** 2-2' thiodiethanol (TDE) may quench or change the excitation/emission properties of some dyes when used at high concentrations (>50%). If you are using a GFP reporter or a green fluorescent dye (e.g., Alexa Fluor 488) do not exceed 50% TDE.

For more information on clearing using TDE, see (Aoyagi et al., 2015; Staudt et al., 2007).

#### 9. Clearing the bone tissue

a. Clear samples using TDE series of 10%, then 25%, then 50% and finally 100% (or 95% if higher purity reagent was not possible to acquire) in PBS. Each incubation step should last > 1 h at 20°C–22°C.

i. Rinse samples and incubate in 10% TDE in PBS for at least 1 h at 20°C–22°C.

ii. Rinse samples and incubate in 25% TDE in PBS for at least 1 h at 20°C–22°C.

iii. Rinse samples and incubate in 50% TDE in PBS for at least 1 h at 20°C–22°C.

iv. Rinse samples and incubate in 95%–100% TDE for at least 1 h at 20°C–22°C (*).

**Note:** (*) do not perform this last step when using green fluorescent signals.

**Note:** For problems related to Hoechst signal becoming diffuse following clearing, please refer to troubleshooting 8.

### Mounting the samples

**Timing:** 5 min per slide for sections and 15–20 min for half bone

This procedure differs between cleared thick sections (follow step 10), non-cleared sections (follow step 11), and half bone samples (follow step 12).

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**Table 4. Considerations for the choice of fluorophore**

| Laser wavelength | Suggested fluorophore | Advantages | Disadvantages |
|------------------|-----------------------|------------|---------------|
| 488 nm (Green fluorophores) | Alexa Fluor 488, Dylight 488 | Higher resolution possible than with longer wavelengths. | High tissue autofluorescence. Very sensitive to TDE clearing (next step). |
| 561 nm (Red fluorophores) | DyLight 550, Alexa Fluor 555 | Low tissue autofluorescence: deep imaging possible. | Can blend into the green channel. |
| 647 nm (Far red fluorophores) | Alexa Fluor 647 | No tissue autofluorescence: deep imaging possible. Most recommended channel for imaging. | |

---
10. Mounting cleared thick sections.
   a. Mount the slides in 200 μL of TDE at the final concentration used in the clearing steps.

   **Note:** If the samples are half-bones, omit this step and proceed to step 12 ‘Preparing half bones for image acquisition’.

   △ **CRITICAL:** Mounting slides in TDE is delicate as it does not harden like other mounting mediums. Take this into account when holding the coverslip on top of the slide. Avoid bubble formation by slowly placing the coverslip. If a bubble is generated, tilt the slide with its long edge orienting vertically until air fuses into a big bubble on top, and re-fill the area under the coverslip with TDE using a micropipette.

   b. Remove the excess TDE by slightly pressing with a finger on top of the coverslip.
   c. Seal the slides with nail polish and keep them at 4°C. Image in the next few days.

   ![Pause point: Samples are stable in the fridge for a few weeks. Keep them protected from light.](https://www.star-protocols.org)

   **Note:** When sections are maintained longer than a few days, there is an increasing risk of air infiltration between the slide and the coverslip, hampering subsequent imaging. This risk is higher for sections of increasing thickness.

11. Mounting immunostained, non-cleared sections on the slide
   a. Apply 3–4 drops of Fluoroshield mounting medium on top of the section.
   b. Place the edge of the coverslip on a corner of the slide and move it towards the section until touching the mounting medium with the coverslip.
   c. Carefully lower the coverslip into place and gently press using blunt-end tweezers to remove excess mounting medium.

   **Optional:** after drying, coverslips can be sealed on the slides with nail polish.

   d. Keep the slides at 4°C and protected from light. Image in the next few days.

12. Preparing half bones for image acquisition

   **Note:** Imaging of half bones must be performed with the lens pointing at the flat, trimmed side. Therefore, the specimen must be stabilized at the side of the half bone that is not trimmed. The following steps describe how to make a mold that will hold the half bone in position for imaging.

   a. Use a steel spatula to place a small amount of Vaseline in a petri dish and mold it into a rectangle that is large and deep enough to hold the half bone.
   b. Cut a square of parafilm as large as the Vaseline square and carefully stretch it until it becomes thin and malleable.
   c. Cover the Vaseline with the stretched parafilm carefully to avoid tearing it.
   d. Place the half bone on top of the parafilm/Vaseline mold and put a Menzel-Gläser coverslip on top of it.
   e. Apply pressure on top of the coverslip delicately until the trimmed half bone side is horizontal and stable.
   f. Proceed to image the half bone.

   **Note:** In case blurry spots appear during visualization, please refer to troubleshooting 9.
**Image acquisition**

© Timing: Variable (The image acquisition time can range from e.g., 10 min to 2 h per image, depending on the microscope, the number of stacks per image, and the user’s experience)

Images in the work to which this protocol applies (Mesnieres et al., 2021) were acquired using laser-scanning confocal microscopy and spinning disk confocal microscopy. Both are very useful imaging tools, each having its advantages and disadvantages. Laser-scanning confocal microscopy is very powerful at capturing details that demand high resolution and contrast, but it requires relatively long imaging times, and as a result, it can have relatively high photo-toxicity and long microscope usage time. Spinning disk confocal microscopy works at high speed and has increased sensitivity of fluorescence detection but reveals fewer details than laser-scanning confocal microscopy. Therefore, for applications as described in this work, the use of laser-scanning confocal microscopy is recommended for imaging structures that require high levels of detail and resolution (e.g., cell dendrites), and spinning disk confocal microscopy for organ-scale images.

The second choice to make relates to the objective lens to use. Objective lenses have the following main features to consider: numerical aperture (NA), working distance (WD), field of view (FoV), and refractive index (RI). The higher the NA, the better the resolution and sensitivity. The longer the WD, the deeper the imaging can go. The bigger the field of view, the more tissue area can be imaged per image taken. The closer the RI the lens is corrected for and the RI of the sample match, the fewer the optical aberrations, the higher the sensitivity and the deeper volume that can be imaged.

Finally, the desired pixel resolution, thickness, and Z-step size to use for acquisition should be defined prior to imaging. To determine the fitting acquisition settings, Tables 5 and 6 provide common starting points.

### 13. Imaging using laser-scanning confocal microscopy

**Note:** The following steps apply when using a Nikon TiE inverted C2 confocal microscope equipped with NIS-Elements software. A detailed description of the microscope can be found in the ‘Materials and Equipment’ section.

a. Turn on the microscope, the detection system, the computer, and the lasers.

| Table 5. Example settings for laser scanning confocal image acquisition |
|---------------------------------------------------|
| **Parameter** | **Microscope settings** |
| Modality | Laser Scanning Confocal |
| Software | NIS-Elements 5.21.00 |
| Detection system | C2-DU3 detector |
| Lens | Plan Fluor 20x MImm DIC N2 |
| Calibration | 0.62 μm/px |
| Refractive index | 1.51 |
| Numerical aperture | 0.75 |
| Working distance | 0.51–0.33 mm |
| Pinhole size | 20–30 μm |
| Scan size | 512 px |
| Scanning speed | 0.5–1 fps |
| 405 nm laser (1.25 mW) power / digital gain | 5% / 80–100 |
| 488 nm laser (3.06 mW) power / digital gain | 5% / 80–100 |
| 561 nm laser (3.75 mW) power / digital gain | 5% / 80–100 |
| 640 nm laser (3.94 mW) power / digital gain | 10% / 80–100 |
| Z-volume / step sizea | 100 μm / 2 μm |

aOnly necessary for 3D analysis. For 2D analysis, the Z-dimension is not used.
b. Place the sample on the microscope slide holder with the coverslip facing down.

**Note:** Always place the sample so that the coverslip is facing the lens.

**Note:** In case of using an immersion lens, apply the proper immersion medium (e.g., oil) onto the lens prior to placing the slide on the holder.

**Note:** If a multi-immersion lens with dynamic RI is used, ensure to adjust the RI ring position to the medium used for imaging.

**Optional:** If a movable slide holder is available in the microscope stage, place the sample such that the long bone axis is parallel to either the X- or the Y-axis of the microscopic field of view. This will facilitate defining the region of interest for image analysis later in the procedure.

c. Visualization:
   i. Open the microscopy acquisition software and use a wide-field optical configuration that relies on LEDs or a mercury lamp to find and orient the sample.
   ii. Open the shutter and select the color of the fluorophore that will be used to locate the sample through the microscope’s eyepiece and bring it into focus.

**Note:** You should have the option between three colors: red, green, and blue. If Hoechst was used to label cell nuclei blue, it is recommended to use this channel to localize your sample.

d. Acquisition:
   i. Once the sample is in the right position and focused, the next steps involve determining the laser power, scanning speed, or exposure time (depending on the detection system), the pinhole size (if it is dynamic), and the dimensional parameters.

**Note:** Table 5 provides a starting guide for defining fitting acquisition settings.

   ii. Change to the confocal imaging optical configuration and start with a laser power between 5%–10%, a gain of 80–100, and a pinhole size as close as possible to 1 AU, to find the top layer of the sample by turning the coarse focus or the fine one.

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**Table 6. Example settings for spinning disk confocal image acquisition**

| Parameter                           | Microscope settings |
|-------------------------------------|---------------------|
| Modality                            | Spinning Disk Confocal |
| Software                            | NIS-Elements 5.21.00 |
| Camera type                         | Andor DU-885         |
| Lens                                | Plan Fluor 20× MImm DIC N2 |
| Calibration                         | 0.33 μm/px           |
| Refractive index                    | 1.51                 |
| Numerical aperture                  | 0.75                 |
| Working distance                    | 0.51–0.33 mm         |
| Pinhole size                        | 30 μm                |
| 405 nm laser (0.23 mW) exposure time/power | 100 ms/100% |
| 488 nm laser (1.83 mW) exposure time/power | 100 ms/100% |
| 561 nm laser (2.27 mW) exposure time/power | 100 ms/100% |
| 640 nm laser (1.76 mW) exposure time/power | 100 ms/100% |
| Z-volume / step size*               | 100 μm /2 μm         |

*Only necessary for 3D analysis. For 2D analysis, the Z-dimension is not necessary.*
Note: The pinhole size is critical for the resolution in the Z-axis. The smaller it is, the higher the resolution, but the lower the amount of light that is captured.

Note: For determining the settings, click the “fast scan icon” in NIS-Elements or select a 512 x 512 px resolution and a scanning speed of 0.5–1 frames per second (fps).

iii. Once the sample is in focus, define the dimensions in X and Y (i.e., the size of the final tiled image) by determining the top left and bottom right borders and adding these locations to the system parameters.

Note: For problems regarding signal heterogeneity in the XY axis, please refer to troubleshooting 10.

Optional: Define the desired acquisition range in the Z direction (i.e., the depth of the tissue to be confocally imaged; this is especially relevant when working with a thick section or half bone). Add the locations of the Z-range’s top and bottom layers to the software window’s acquisition settings.

Optional: If imaging in Z, define the separation between the individual optical sections. The ideal Z-spacing depends on the NA of the lens (the Z-resolution of the microscope) and the intended analyses. If the purpose is 3D visualization and 2D blood vessel analysis on middle single optical sections or maximum intensity projections (MIPs), the Z-spacing does not require to be extremely narrow (e.g., in our hands, up to 10 μm or even higher has worked well). For 3D nearest neighbor distance calculations though, a Z-step at least 2 times smaller than the smallest object to quantify (within the resolution power of the lens) is needed (e.g., a 4 μm cell nucleus requires a Z-step of maximally 2 μm between the optical sections). The Nyquist ratio can be used to avoid under- or over-sampling, especially if the image will follow a deconvolution step.

iv. When the XY tile-scan area and the Z range and separation have been defined, find the proper laser power, gain, and resolution for the desired image.

Note: Too much laser power can bleach the sample. Too much gain will result in increased Poisson noise. Too much resolution will result in a longer imaging duration. Find the right balance to get the required resolution and best signal-to-noise ratio.

Note: When the final aim is to compare samples from different experimental groups (e.g., quantitative or qualitative image analysis between conditions or genotypes), it is important to use the same imaging settings for all the samples.

v. When the image is fully acquired, save it in the microscope’s original software format (e.g., “.nd2” for images generated using a Nikon microscope).

Note: For problems with stitching, please refer to troubleshooting 11.

14. Imaging using spinning disk confocal microscopy

Note: The following steps apply when using a Nikon NiE upright confocal microscope equipped with NIS-Elements software. The microscope’s full set-up is detailed in the ‘Materials and Equipment’ section.

Note: The upright configuration of this microscopy system offers advantages for imaging thick sections and half bones, as they can be held upward and immersed in TDE clearing medium during imaging.
a. Start the microscopy system
   i. Turn on the microscope, the detection system, the computer, and the lasers.
   ii. For acquiring large-scale images using the spinning disk microscope, the use of a multi-immersion objective is recommended (e.g., Nikon CFI Plan Apo 10XC Glyc, NA 0.5, WD 5.5 mm).

   **Note:** after installing the objective, ensure to change the RI ring position to the one corresponding to the medium used for imaging (e.g., RI 1.51 for 95%–100% TDE or oil; RI 1.42 for 50% TDE).

   **Note:** It is also possible to use an air lens if multi-immersion objectives are not available. However, the resolution that can be reached will be lower.

b. Position the sample on the stage
   i. Either place the slide directly into the slide holder position of the stage or use a 100 mm petri dish holding the slide (thick section) or sample (half bone). The dish can be filled with TDE, such that the specimen can be imaged using an immersion lens even when the working distance (WD) is relatively large.

   **Note:** For positioning a slide with a section in a TDE-filled dish, gently place the slide on the bottom of the dish avoiding bubbles formation. Carefully press the slide towards the bottom of the dish by the corners, avoiding touching the coverslip. This will displace the TDE solution underneath such that the slide is not floating.

   ii. Place the petri dish on the microscope stage and slowly move the objective to the TDE solution avoiding bubbles formation under the objective lens.

   **Optional:** Orient the bone along the X- or Y-axis of the field of view to reduce the number of fields that need to be scanned and to facilitate image presentation and analysis.

c. Visualization and acquisition.
   i. Open the shutter and select the color of the fluorophore used to find the sample through the microscope’s eyepiece. Next, locate the tissue area of interest and bring it into focus.

   ii. Change to the confocal imaging optical configuration for each color and find the proper laser power, gain, and resolution for the desired image.

   **Note:** Table 6 can be used as a starting guide for defining fitting acquisition settings.

   **Note:** For problems related to sample visualization, please refer to troubleshooting 12.

   iii. For large image acquisition, place the objective to the secondary spongiosa region and define a ROI that encloses the whole bone by setting these coordinates as the center point (a ROI of 3 x 8 fields using a 10X objective with a 15% overlap would work for an adult femur); find the top left corner and low right corner to check if the bone is inside the ROI.

   iv. Determine the desired Z-range and add the coordinates of the top and bottom positions to the acquisition window.

   **Note:** When imaging a large specimen (e.g., the full bone) it is recommended to explore the sample in Z at multiple distant regions of the sample (e.g., close to the growth plate as well as further down in the diaphysis) to ensure the desired Z-range is completely included in the imaged stack for all the ROIs / the full bone.
v. Set steps of 10 μm in Z if the aim is to perform 2D blood vessel analysis (this resolution is sufficient for blood vessel network visualization).

**Note:** For 3D spatial quantifications, it is recommended to take the input image using a 20X lens, and the Z-spacing should be set to maximally 2 μm.

**Note:** For problems with stitching, please refer to troubleshooting 11.

### Quantitative analysis of the bone vasculature architecture in 2D images

**Timing:** 1–3 h per image, highly dependent on the user’s experience

The following section describes in detail how to segment and quantify different features of the blood vessel network morphometry and topology in 2D, starting from single optical sections generated through confocal imaging as described above.

15. Optional image processing steps: deconvolving, denoising, and Z-intensity equalization

Stacked tiles used for 3D visualization and/or quantification of different parameters always have reduced Z-axis resolution and quality compared to the XY-axis due to convolution of the underlying sample structure with the point spread function of the microscope. One way to increase the resolution and thus the quality of the image and subsequent analysis is to perform deconvolution on the image, followed by denoising and a Z-intensity equalization step to reduce the pixel intensity differences between Z-planes from different depths.

These image pre-processing steps can be performed using NIS-Elements software (AR 5.30.01 64-bit or later versions), as described here, or using another software with comparable image processing tools (e.g., Huygens, Arivis).

a. Open the raw “.nd2” image in NIS-Elements.

b. Select the deconvolution tool followed by the type of algorithm you want to use. In our hands, the “Blind” and the “Richardson-Lucy” algorithms (with a computer-estimated point spread function (PSF)) worked best for this protocol, as determined qualitatively by evaluating the improvement of the signal to noise ratio, and the increased sharpening of existing structures without artifact introduction along the XY, XZ and YZ axes (for examples, see Figure 3).

**Note:** For problems related to deconvolution, please refer to troubleshooting 13.

c. Use the denoise.ai tool in NIS-Elements and apply it to all the channels of the image to reduce the amount of Poisson noise introduced by the detection system.

**Note:** It is normal for images to look slightly smoothened following this step (Figures 3D and 3H).

d. Select the brightest slice from your image stack that will be used for equalizing the less intense slices to that intensity. Click on Image>ND processing>Equalize intensity in Z and apply the histogram stretching option to the whole image.

16. Tissue area segmentation (selecting the ROI)

As a first step, the region of interest (ROI) for the analysis needs to be determined, in a consistent manner for all samples that are intended to be comparatively analyzed. Several of the blood vessel features to be quantified need to be normalized to the size of the ROI, i.e., the total tissue area under consideration, to express them as a fraction, frequency, or density relative to the tissue surface area comprising the ROI. To select the ROI, follow these steps, illustrated in Figures 4A–4C:
a. Open the processed or raw `.nd2` image containing at least one channel with the stained nuclei and a second one with labeled blood vessels.

b. Use the "Polygon" or "Freehand" selection tools in FIJI to outline the bone marrow ROI, as illustrated in Figure 4C. Alternatively, select an ROI of predetermined shape and size (e.g., a rectangular area of a fixed size positioned in the metaphysis).

Note: The growth plate and the endosteal surfaces can be used as delimiters for the ROI selection.

c. Add the selected area to the ROI Manager through the shortcut “Ctrl + T”.

d. Create a new black 8-bit image of the same dimensions as the image in which the ROI was drawn by clicking on “File>New>Image”.

Note: The “slices” box should be set to 1 in order to have a 2D image.

e. Select the drawn ROI from the ROI Manager, and click on "More>Fill" within the ROI Manager window.

Note: This will be used later in the protocol to obtain the ROI’s tissue area and filter out the blood vessels located outside of the ROI. This image will be referred to as the “tissue mask image” for the rest of the protocol.

17. Blood vessel segmentation in 2D.
Antibodies directed against blood vessel markers such as endomucin (Emcn) and CD31 label endothelial cell membrane proteins, thereby staining the linings of the blood vessels specifically, but not the lumens inside the blood vessel walls (Figure 4A). To perform the analyses described in the following section, binary images must first be created that contain the vessel linings and lumens altogether. Therefore, using single optical sections (Figure 4B) and within the selected ROI (Figure 4C), the segmented immunostained blood vessel outlines (Figure 4D)
Figure 4. 2D blood vessel segmentation and analysis pipeline for describing the architecture of the bone vasculature

(A) Representative MIP of an E18.5 tibia stained against CD31 for blood vessels and Hoechst for cell nuclei.
(B) Example single optical section used as a starting point for the pipeline.
(C) ROI comprising the bone marrow area inside the bone shaft, shown as a binary mask.
(D) Binary CD31 signal within the ROI.
(E) Binary blood vessels, closed manually and filled to include both the vessel walls and lumens.
(F) Filtered segmented blood vessel image used for analysis.
(G–J) Process of skeletonization. (G) Segmented blood vessels prior to skeletonization. (H) Example of an inadequately (over-) skeletonized image, resulting from insufficient smoothing. (I) Example of a properly smoothened and skeletonized image. (J) Example of an inadequately (under-) skeletonized image, resulting from smoothing too much.
have to be closed manually whenever the lining is incomplete and then filled using an image processing software (Figures 4E and 4F).

Here, we describe how to segment blood vessels using FIJI, but technically similar other software packages can be used alternatively (e.g., CellProfiler, Icy).

**Note:** FIJI (Fiji is just ImageJ) is an image processing package distribution of the open-source software ImageJ. To process and analyze confocal images using the method described here, the following plugin should be installed in FIJI: IJPB-plugins, [https://sites.imagej.net/IJPB-plugins/](https://sites.imagej.net/IJPB-plugins/)

a. Import the processed or raw image into FIJI by dragging and dropping the file onto FIJI’s window.

b. Duplicate the channel containing the blood vessel signal by clicking on “Image>Duplicate…”

**Note:** If it is a stacked image, select the slice with the most complete blood vessel network and write the selected slice number in the “Range” box of the duplication window such that a single optical section is used from here on.

**Note:** Be sure that the “Duplicate Hyperstack” box is unchecked and that the proper channel number is selected.

c. Perform a “Default” or “Huang” global threshold.

**Note:** The blood vessel marker signal should be relatively homogenous in the XY axes and sufficiently bright for these algorithms to work. If this is not the case, refer to the ‘troubleshooting’ section.

**Optional:** To ensure the ImageJ software uses white as the “Foreground” color and black as the “Background” color, use “Process>Binary>Convert to Mask” and check the box “Black background”.

⚠️ **CRITICAL:** The same threshold algorithm should be used for all images analyzed within a batch of samples that are to be compared afterwards.

d. Select the binary image generated in the previous step and use the paintbrush tool to draw a line where needed to close every incompletely closed blood vessel outline within the image.

**Note:** The drawing color should be white. If it is not, double-click on the paintbrush icon and select the white color before starting. If the problem persists, please refer to troubleshooting 14.

e. Once every vessel is properly closed, apply a manual threshold of 1–255 to convert the image into a binary image.

f. Use the “Analyze particles…” tool and click on the “Add to Manager” option.
g. Verify that all the blood vessels in the original image were detected and select them all. Use the "More>fill" tool within the ROI Manager window to fill all the detected blood vessels (Figure 4E).

*Note:* This image will further be referred to as “filled blood vessels image”.

*Note:* For problems related to incompletely filled blood vessels, please refer to troubleshooting 15.

h. To filter out contaminating signal particles and unspecific background signal, use the “Analyze>Analyze Particles...” tool; set the “size” parameter to an appropriate value (e.g., fill out 50 to eliminate particles smaller than 50 μm²) and select the “Show>Masks” option (Figure 4F).

*Note:* Depending on the image, this parameter can be increased to 100 μm² to eliminate larger particles, but it is important to ensure that no meaningful signal is lost in the process.

18. Selecting blood vessels specifically within the ROI

To only quantify those blood vessels that localize within the ROI, external blood vessels are omitted from the analysis image prior to performing the quantitative analysis.

a. Use the “Process>Image calculator...” tool from FIJi’s main window and apply an “AND” operation using tissue mask image as “Image 1” and the filled blood vessels image as “Image 2”.

*Note:* This step should generate an image lacking any signal outside the borders of the selected ROI.

*Note:* The resulting image should show the segmented blood vessels in white and the background in black.

19. Aligning the bone and blood vessel orientation

To determine the blood vessel directionality, the image needs to be rotated so that all samples are aligned in the same manner with respect to a reference point or structure. We commonly employ a vertical orientation for the bone shaft, with the growth plate positioned horizontally (as in Figure 4).

a. Rotate the image in steps of 90° until a smaller angle is required using the “Image>Transform>Rotate 90 degrees to the Right/Left” tools.

b. Rotate the image with respect to the growth plate or another reference within the tissue using “Image>Transform>Rotate...”. Find the proper angle; ensure to check the “Enlarge image” and “Preview” boxes and tick off the “Grid lines” box.

*Note:* To better preserve the image’s original appearance while rotating, a bi-linear (for 2D) interpolation can be considered. Bear in mind that this may result in changes in pixel intensity, thus precluding measuring pixel intensity if that would be additionally desired (this does not apply to the quantifications presented here).

*Note:* This image will be further referred to as “Processed blood vessels image”.

*Note:* For problems related to loss of information following image rotation, please refer to troubleshooting 16.

20. Determine the blood vessel area and perimeter fraction (see Figure 4K)

a. Select the “Processed blood vessels image” and measure the segmented blood vessel area and perimeter using the “Plugins>MorphoLibJ>Analyze>Analyze Regions” tool.
b. Select the “tissue mask image” and measure the tissue area using the “Plugins>MorphoLib-J>Analyze>Analyze Regions” tool.
c. Store all the generated values by transferring them to an excel table or save them as “.csv” files.

21. Determine the blood vessels width (see Figure 4L)
a. Select the “Processed blood vessels image” and measure local blood vessel width using Analyze>Local Thickness>Local Thickness (masked, calibrated, silent)”.

Note: The generated image will be further referred to as “local thickness image”.
b. Measure the frequency distribution of blood vessel width using “Analyze>Histogram” on the generated image. Select the number of desired bins and the range of values to plot in microns (e.g., 0–200).

Note: The number of bins can be calculated by dividing the maximum value of the range by the desired step number (e.g., maximum value of 200 μm in 10 μm steps will be equivalent to 20 bins).
c. Select the histogram window and click on the “list” icon. Save this list as “.csv” or copy the data into an excel table for future analysis.
d. To generate blood vessel pseudo-coloration according to vessel width intervals, duplicate the local thickness image using the “Image>Duplicate…” tool. The image should be duplicated once for every interval desired to become color-labeled. For 4 intervals, duplicate the image four times and binarize each one of them using “Image>Adjust>Threshold” by setting interval ranges manually.

Note: Ranges should not overlap; they can be equal in size or variable, at the user’s discretion. For equal intervals, e.g., spanning 200 μm width, the 4 intervals would range 1–50, 51–100, 101–150, and 151–200.
e. To generate the pseudo-colored combined image, use the “Image>Color>Merge Channels…” tool and assign the individual binary images (corresponding to the different width ranges) each to a channel in the order of size (e.g., Channel 1 containing the 0–50 μm width range, Channel 2 the 51–100 μm range, etc.).
f. Store the image as “.tiff”, which will preserve the different channels.

Note: Saving the image as “.jpg” flattened image may enable more easy handling later, but not all the information is retained.

22. Determine the blood vessel topology
To calculate the topology of the vascular network, the blood vessels need to be skeletonized (Figures 4G–4J). This is a necessary step to obtain information on the number of blood vessel branches, their connectivity (total junctions), the number of triple and quadruple junctions, and the blood vessel directionality.
a. Blood vessel centerline definition (skeletonization of the vasculature):
   i. Use the “Process>Filters>Gaussian Blur” filter with a sigma of 8 to smoothen the blood vessels outlines to facilitate accurate skeletonization.

Note: Skeletonization (i.e., centerline generation) is very sensitive to rough outlines as it uses cycles of erosion until generating the centerline of one pixel in width (Figure 4I). When the outline of the structure that will be skeletonized is not smooth enough, artificial branches might appear (Figure 4H). If the structure is smoothened too much, real blood
vessels might be left out of the analysis (Figure 4J). In any of these cases, change the sigma accordingly.

   ii. Select the “Processed blood vessel image” and obtain its centerline using Plugins>Skeleton>Skeletonize (2D/3D).

**Note:** This image will be referred to as “Skeletonized image”.

b. Analyze the skeleton using the “Analyze>Skeleton>Analysis Skeleton” tool. Check the “Elimination of End-points: Prune ends” and the “Results and Output: Show detailed info” boxes (example images are shown in Figures 4M and 4N).

**Note:** For problems arising during skeletonization, please refer to troubleshooting 17.

c. Save the resulting two tables in an excel file or as “.csv” files. The “Results” table data can be used to calculate the total number of branches, and the total, triple and quadruple junctions. The “Branch information” table data is used as input to generate the frequency distribution graph of branch lengths.

23. Determine the blood vessel directionality

Blood vessel directionality is a useful parameter to gain quantitative knowledge on the vascular organization in vivo in various conditions. For this calculation, the skeletonized image is used. Since the centerline also functions as a projection of the blood vessel direction at that given position, it is the proper input to use to obtain the frequency distribution of blood vessel angles.

a. Select the “Skeletonized image” and use the “Analyze>Directionality” tool applying the “Local gradient orientation” method and tick the “Build orientation map”, “Display color wheel”, “Display table”.

**Note:** The color wheel contains the color map assigned to the different calculated angles.

b. Copy the data of the table into an excel file or save it as “.csv” file for later plotting and analysis.

24. Quantitative analysis of endothelial cell proliferation

This quantification requires an image containing nuclear, endothelial, and proliferation markers as an input. For this protocol, we used images containing Hoechst-labeled nuclei, Emcn-labeled endothelial cells, and PHH3-stained proliferating cells (Figure 5).

a. Open the processed “.nd2” image by dragging and dropping it into FIJI’s window.

b. Make a composite image with the 3 channels clicking on “Image>Color>Make Composite” to facilitate the detection of the proliferating endothelial cells.

**Note:** Endothelial cells can be distinguished based on Emcn-positive signal around a Hoechst-positive nucleus. If this nucleus co-localizes with a PHH3 signal, then the endothelial cell is considered a proliferating cell (Figures 5B–5D).

c. To use the same quantification surface area, draw a rectangle using the “Rectangle” selection tool and add it to FIJI’s ROI Manager using “Ctrl + T”. Once the rectangle is drawn, use “Image>Duplicate” to generate a new image containing only the selected ROI.

**Note:** You can now use the same rectangle on other images, as long as the ROI Manager stays open.

d. To count and save the location of the proliferating and non-proliferating endothelial cells, use the “Multi-point” selection tool in FIJI.
Optional: You can save multi-point selections within the ROI Manager window by selecting them all and saving them as ".roi" files.

△ CRITICAL: To avoid bias, always perform quantifications in a blinded fashion.

### 3D quantitative analysis of the spatial relationship between osteoprogenitors and blood vessels

Timing: 1–3 h per 3D image, highly dependent on the researcher’s experience

The following steps describe how to measure the distance of cells belonging to a specified cell population (e.g., osteoprogenitors marked by Osx-GFP expression) to the nearest blood vessel surface (e.g., detected using immunostaining against CD31 or Emcn) in 3D (Figure 6). Therefrom, a frequency distribution can be generated, outlining the cells’ nearest distance to blood vessels (nearest neighbor distance (NND)), and infer if there are changes in the way cells distribute in space with respect to blood vessels in certain conditions or settings. This can be applied to bone but also to any other vascularized tissue.

Note: The 3D image visualization and quantification as described here uses the software package Imaris. Imaris enables to generate meshed surfaces on 3D images to perform different types of quantifications and has algorithms to model labeled nuclei (like Osx-GFP+ osteoprogenitors) in 3D.

Use the “Imaris Converter” software to transform either raw or processed images into “.ims” images to be able to visualize them in Imaris.
25. Image quality inspection prior to 3D analysis

This section requires image sets that include stacks of multiple slices that can be rendered in 3D. The following steps describe how to inspect the image for key quality markers and the potential presence of exclusion criteria.

a. Open the generated “.ims” image in Imaris. Explore the image using the “Slice” or “Section” views.
b. If the bone marrow presents holes, cracks or ruptures of considerable size (e.g., compromising >10% of the surface of a single optical section or observable in Z-axial views), exclude the sample from the analysis and refer to the troubleshooting section on how to avoid this problem.

c. Check that the different used stainings penetrated the tissue homogeneously by looking at multiple slices along the Z-axis using the “Section” view. If any of the markers shows scattered penetration lines instead of a uniform horizontal pattern, exclude the image from the analysis.

**Note:** For problems concerning uneven staining or high background signal of the tissue sample, please refer to troubleshooting 5, 6, and 7.

d. Check the homogeneity of the stainings and signals in the XY fields of view, i.e., per section. If a marker shows considerable heterogeneity, i.e., large differences in signal intensity observed on similar structures/cells, exclude the image from the analysis.

26. Blood vessel segmentation in 3D

The following steps delineate how to model 3D blood vessels as a surface mesh using Imaris.

a. Set the viewer in 3D view mode.

b. Click on “3D view/Surfaces” to model blood vessel signals as a surface mesh.

**Note:** If the image is too large to be handled smoothly (e.g., >20 GB), crop it into multiple smaller images using “Edit/Crop 3D…”

**Note:** If using Imaris 9.6 or newer versions, tick the “Object-Object Statistics” box to open the software’s spatial statistics tool.

c. Select the channel with the blood vessel marker as “Source channel” and determine the ideal level of smoothing for the image.

**Optional:** Imaris “Smooth” uses a 3D Gaussian filter that can help eliminate noise (e.g., coming from the detection system) and unwanted small particles.

**Note:** It is important not to apply too much smoothing as this bears the risk of impacting the vascular signal. This risk applies especially to relatively moderate or low resolution; in such cases, the “Smooth” function is not recommended. If this is case, refer to troubleshooting 18.

d. Threshold the vasculature using the “Absolute intensity” option.

**Note:** If the threshold value proposed by Imaris does not succeed in including all blood vessels in the selection, adapt the value manually to obtain adequate segmentation of the vascular signal as judged by the original image.

**△ CRITICAL:** The same threshold should be applied to all conditions that are compared.

e. Filter out the remaining particles or unwanted blobs using the “Number of voxels” filter.

f. Finalize the surface creation by clicking the multiple arrows icon.

27. Osteoprogenitor detection

Osx-Cre:GFP-expressing osteoprogenitor cells (SSPCs and osteolineage cells) exhibit nuclear localization of GFP, allowing for them to be modeled as particles using algorithms embedded in Imaris that look for bright intensity centers (3D maxima) of a certain size. The software places a sphere for every detected GFP” cell (Figure 6F). Similar pipelines can be used to other nuclear markers, either transgenic or immunostained.

a. Click on “3D view/Spots” to start the particle detection process.
b. Select the channel with the nuclear signal of interest (e.g., Osx-GFP).
c. To estimate the proper XY diameter of the spots (cell nuclei) to be detected, use the “Slice” view to draw the diameter of a few nuclei in 2D; this is done by left-clicking on two opposite borders within the longer axis of the selected cell nuclei. Use the average diameter as the “Estimated XY Diameter” in the spot modeling tool.

Optional: If the nuclear signal is highly elongated in Z due to anisotropic resolution, tick the “Model PSF-elongation along Z-axis” box. Also see troubleshooting 19.

d. Select 3–5 different small ROIs within your image to evaluate the quality of the automatic spot detection.
e. Manually count the spots in each of these ROIs and deduct therefrom the true versus false positive and negative detections made by the software. Find the “Quality” value threshold for which the automatically detected spots most accurately represent the real signal.

Note: Imaris “Spots” object creation tool window has a filtering step incorporated into the creation process that is automatically set in the “Quality” feature. This feature is the intensity at the center of the spot of the channel used for spot detection. Additional filters can be applied depending on the user’s needs (e.g., intensity mean in another channel).

28. NND between detected osteoprogenitor cells and blood vessels
The following steps only need to be applied if using an older version than Imaris 9.6. In newer versions of the software, the “spots” object creation tool automatically provides the spatial statistics data.

a. Select the generated blood vessel surface from the Imaris scene.
b. Click on “Image Processing>Surfaces Functions>Distance Transformation”.

Note: This will generate a new channel containing the distance to the nearest blood vessel surface as image intensity values per voxel.

c. Select the modeled spots from the Imaris scene.
d. Click on the statistics tab and export the “Detailed>Specific Values>Intensity Center” using the channel number of the distance transformed image.

EXPECTED OUTCOMES
This protocol details all the steps that need to be performed to dissect pre- and postnatal mouse long bones, and process them for high-end 3D imaging, with a focus on the stromal-vascular bone marrow compartment. The procedures as outlined can guide the user in generating different types of sections, thereby making well-mediated decisions on the choice of embedding material and the section thickness, depending on the ultimate analysis goals. For outcomes and applications that range from basic 2D image visualization to more complex 3D spatial quantifications, the various required steps are given, including detailed protocols for immunostaining and clearing the bone samples, acquiring high-quality images, and performing quantitative morphometric analyses on them. We describe several features that we used recently to characterize the skeletal vasculature and its relation to osteoprogenitors (including perivascular and stromal SSPCs) in a fetal VEGF overexpression model; expected outcomes can be appreciated in the original publication linked to this STAR Protocol (Mesnieres et al., 2021).

Beyond this exemplifying implementation, the protocol can undoubtedly be useful to study any condition (mutation, treatment, or any other specific setting) for a potential impact on the skeletal vascular architecture and organization. Moreover, on the condition that a nuclear marker is used to detect a cell population of interest, the method described here can also be used to quantify
the spatial proximity of any cell type or cell subset of interest to the tissue’s blood vessels. Finally, this method details the generation of 2D or 3D images of bone tissue labeled with different fluorescent markers and their subsequent blood vessel quantifications including morphometrical features, branch and junction numbers, length, width and directionality; with tissue-specific optimization and adaptations in the protocol, it is expectedly applicable to any other tissue of interest in the biomedical research community.

QUANTIFICATION AND STATISTICAL ANALYSIS

This method generates data of mainly two types: feature averages and frequency distributions. As detailed previously in the quantification steps, the blood vessel surface and perimeter fractions, density, junction number and proliferation are graphed as averages per mouse sample. Blood vessel width, directionality, branch length and spatial proximity to osteoprogenitors (NND) can be displayed according to frequency distribution.

Graphing feature averages

1. Open the tables containing the data obtained on blood vessel surface and perimeter fractions, blood vessel density, number of branches, junction number, triple/quadruple junctions, and number of proliferating endothelial cells.
2. Open GraphPad Prism (or a similar data graphing software) and create a new column table.
3. Copy and paste the averaged data from excel to the Prism sheet for each condition and feature.
4. When comparing two conditions with similar variance and a normal distribution, apply an unpaired Student’s t-test for statistical testing.

Note: Using a $P \leq 0.05$ threshold is common for considering data to be statistically significant.

Graphing frequency distributions

Frequency distributions are a useful way to perform population statistics. These data are displayed as histograms where the X-axis represents the feature that is being quantified (e.g., blood vessel width) and the Y-axis represents the number of counts or the frequency/percentage obtained for each feature value.

5. Open GraphPad Prism (or a similar data graphing software) and create a new XY table.
6. Copy and paste the excel data per sample and apply a frequency distribution analysis.

Note: Use relative distributions when normalizing to the total population is useful. Use absolute distributions when comparing the absolute difference between conditions.

Note: Bin size might vary depending on the data distribution. The bigger the bin size the more data points will be combined together in a single bin.

7. For statistical testing, apply a multiple comparison 2-way ANOVA test and compare the mean between rows.

LIMITATIONS

For this method to be precise in characterizing tissue parameters in different experimental conditions, the images used as input must be of high quality from multiple perspectives.

As a first prerequisite, the tissue sections should be intact, with optimal preservation of the tissue integrity, and be attached firmly to the slide. Tissue sections that became damaged or (partially) washed off during the staining and clearing procedures, cannot provide reliable data. Secondly, the immunostaining signals within each of the applied color channels must display homogeneously
distributed pixel intensity along the XY axes and no significant loss of intensity in the Z-axis. Thirdly, the image processing for blood vessel segmentation requires precision, dedication, and time investment, to correctly close and fill the blood vessels within the ROI prior to the quantifications. These requirements can become limitations if challenged by technical difficulties; investing time and efforts in fine-tuning the procedure to achieve optimal performance on one’s own on-hand samples may possibly be needed. Lastly, there are limitations to the 3D analysis detailed here: (i) the described NND calculation pipeline applies to nuclear signals to detect the cells of interest; (ii) the cells of interest for which the distance to the tissue vasculature is to be quantified should not reside inside blood vessels; and (iii) since the lumens of the blood vessels are not filled in this procedure, most morphometrical measurements cannot be performed using the segmented blood vessel walls as inputs.

TROUBLESHOOTING

A wide range of problems may arise during tissue embedding and sectioning. Below we report some of the troubleshooting we have faced during our optimizing of this protocol for staining and imaging of mouse bones. For more general troubleshooting on histology please refer to other resources and available online guides.

Problem 1
The bone marrow becomes detached from the bone cortex during sectioning (associated with: “Preparation of histological specimens: tissue sectioning” section, step 5f). This problem may be attributed to inadequate or insufficient tissue fixation.

Potential solution
To avoid fixation problems, always prepare fresh PFA fixative solution and keep it at 4°C before use. Fixation duration can be further optimized and adjusted on a sample batch basis to solve this problem.

Problem 2
The OCT-embedded cryo-block is cracked (associated with: “Preparation of histological specimens: tissue sectioning” section, step 5f). This may result in the frozen OCT medium providing insufficient support to the sample and generating poor-quality sections with substantial loss of tissue integrity. This problem can occur when the sample is being held in the liquid nitrogen-cooled 2-methylbutane solution for too long.

Potential solution
Test and adjust the adequate freezing time in the cryo-embedding procedure for the specific samples in your experiment.

Problem 3
The sections are broken (diagonally or horizontally), insufficiently flat (jeopardizing optimal orientation and adherence to the slide), or borders are cracked/folded (associated with: “Preparation of histological specimens: tissue sectioning” section, step 5f). These problems can arise due to insufficient decalcification, leading to stiff bones, which are difficult to section properly.

Potential solution
Ensure that the bone samples have been decalcified adequately before embedding and sectioning.

To improve this step:

• The incubation in 0.5 M EDTA can be prolonged (e.g., to 3 or 5 days for fetal/newborn samples, and to 3 weeks for postnatal bones);
• The volume and frequency of renewing the EDTA solution can be increased to avoid saturation of the calcium chelation (e.g., daily changes of EDTA).
A combination of these mitigation options may provide the best results. The decalcification procedure may need to be optimized for the respective type of bone samples under investigation, as the degree of mineralization is dependent on the age of the mice, the genetic background, the specific bone analyzed, etc.

**Problem 4**
Sections are not well adhered to the slide, which may lead to the full section or its bone marrow content washing off the slide during the staining procedure (associated with: “Immunostaining of bone sections” section, step 6a).

**Potential solution**
Ensure to use coated adhesion microscope slides (e.g., Superfrost Plus) for collecting bone sections. Ensure to dry the sample for ~1 h at 20°C–22°C before starting the rehydration incubation in the immunostaining procedure to enable proper adhesion of the sample to the slide. Increase the drying time when problems persist. Inevitably, sectioned bone samples are very delicate; therefore, during the protocol, ensure to apply every solution gently and to handle the samples carefully.

**Problem 5**
Uneven staining of the tissue sample (associated with: “Immunostaining of bone sections” section, step 6-8). This problem may be attributed to dehydration of (parts of the) sample during long antibody incubation steps or to leakage of the antibody solution out of the hydrophobic circle surrounding the sample.

**Potential solution**
Ensure proper closure of the humid chamber to avoid evaporation. In case of longer antibody incubation steps, re-check the status of the immunostaining on a daily basis to avoid dehydration. Ensure proper closure of the hydrophobic circle surrounding the sample to avoid leakage of incubation solutions. Avoid putting the solutions in excessive volume in order to prevent leakage out of the hydrophobic circle.

**Problem 6**
The stained samples show high background signal (associated with: “Immunostaining of bone sections” section, step 6-8). This problem may be attributed to insufficient blocking of the sample or inadequate washing. However, the high background signal is often related to improper (excessive) antibody concentration.

**Potential solution**
Extend the time of blocking solution incubation and/or increase the frequency of washing steps after each antibody incubation step. Ensure to titer the antibody concentration upon first use, as the concentration may vary among different brands, batches (for polyclonal antibodies) and nature of the specimen.

**Problem 7**
Upon imaging, there is a loss of signal when moving in Z (associated with: “Immunostaining of bone sections” section, step 6-8). This problem may arise due to insufficient antibody penetration and particularly occurs in very thick tissue sections.

**Potential solution**
When the goal is to image gelatin sections thicker than 200 μm, the primary antibody incubation time may be prolonged up to 48 h or 72 h, and the Triton-X-100 concentration can be increased to 0.5% to ensure proper antibody penetration for deep tissue imaging.

**Problem 8**
Cleared samples show diffuse Hoechst signal, not specific to cell nuclei, and/or poor signal or loss of signal in the green channel (associated with: “Clearing the bone samples” section, step 9a). This problem may relate to the clearing procedure.
Potential solutions

Problem 9
Blurry spots appear on the image when visualizing half bone sections (associated with: “Mounting the samples” section, step 12). These spots may be due to the presence of Vaseline remnants on the sample or coverslip.

Potential solution
To avoid this problem, be careful not to tear the parafilm that is placed on top of the Vaseline. In case some of the Vaseline sticks to the sample, avoid imaging these parts that touched the Vaseline, as it is difficult to clean and leads to blurry images. Another solution could be to develop an in-house alternative method to mount the half bone in a stabilized manner, for instance, without the use of Vaseline.

Potential solution
Verify that the slide is perfectly positioned on the slide holder of the microscope. Ensure to perform all the immunostaining and clearing steps inside a humidified chamber, and use a stirring platform (see materials and equipment) to facilitate homogeneous spreading and penetration of the solutions into the sample. Enough solution should be applied to each section such that no part of the section is devoid of solution; on the other hand, avoid using excess volume as this may lead to spillover and leakage of the solutions out of the hydrophobic area.

Problem 11
The acquired image shows poor stitching between the tiles making up the full mosaic image, recognized as displaced frames or unmatching assembly of the individual tile image (associated with: “Image acquisition” section, step 13d).

Possible reasons may include:

• Displacement of the microscope’s stage or movement of the slide holding the specimen during image acquisition;
• Too low local drive memory space to perform the calculations at the moment of acquisition, for instance because of insufficient available free memory or because of performing parallel tasks (e.g., examining other images) while the image acquisition is running.
Potential solutions
During image acquisition, avoid touching the microscope to prevent movement of the stage. Avoid installing the microscope in rooms subjected to any kind of vibrations. If the microscope enables it, save the tile-stacked image in an image sequence format and perform the stitching manually afterward. Regularly clean up the local drive for the software to run smoothly. Do not use the microscope’s computer to run other tasks while the image acquisition is ongoing.

Problem 12
When visualizing the sample, it appears dark, or it is impossible to properly get in focus (associated with: “Image acquisition” section, step 14c).

Possible reasons include:
- The presence of an air bubble between the lens and the sample (in case of an immersion lens);
- Not using oil for a lens that requires it (or using too little oil);
- Misplacement of the slide (coverslip on the wrong side with respect to the lens);
- Improper adjustment of the refractive index ring (in case of a multi-immersion lens);
- Immersing the lens into nonhomogeneous TDE solution.

Potential solution
Carefully look for the presence of bubbles between the lens and the sample. In case of bubbles, take the immersion lens out of the oil or TDE solution. If bubbles remain in the liquid, remove them using a 1 mL tip. If bubbles persist on the lens, use a lens wipe to clean it and then re-immers it very slowly into the solution. If the bubbles persist, repeat this process. Ensure to always use the proper medium intended for each lens. When using an air lens, pay attention not to use oil or TDE. When using an oil or multi-immersion lens, ensure to use enough solution to cover the entire lens. Verify if the slide was placed such that the coverslip is oriented properly (facing the lens), and if not, correct it. When using a multi-immersion lens, verify that the refractive index ring is in a position according to the imaging solution (e.g., oil or TDE). When using a lens immersed in a petri dish containing TDE, ensure to always mix the TDE gently in order to homogenize the solution after introducing a slide for image acquisition.

Problem 13
When acquiring images for 3D analysis, no gain in resolution is obtained upon deconvolution (associated with: “Quantitative analysis of the bone vasculature architecture in 2D images” section, step 15).

Possible reasons for this include:
- The Z-resolution of the image is too low;
- Wrong or incomplete information was given as input for the deconvolution algorithm.

Potential solution
Use a smaller Z-separation. If the cells of interest are densely packed together, a Z-separation of half the smallest cell size will not be sufficient to split them up properly. Hence a Z-separation of up to four times smaller might be needed (i.e., for a cell of interest of 4 μm in thickness, use a 1 μm Z-step). Ensure to check that the deconvolution algorithm tool takes the proper information, especially regarding the sample refractive index and the fluorophores excitation and emission wavelengths.

Problem 14
When using the drawing tool in ImageJ, it appears too small or is of a color different than white. (associated with: “Quantitative analysis of the bone vasculature architecture in 2D images” section, step 17d).
Possible reasons include:

- The size of the brush was not properly adjusted;
- The color was not set to white at the beginning of the step.

**Potential solution**
Double click on the paintbrush tool in ImageJ and adjust the size as desired. Ensure selection of the color white.

**Problem 15**
After the blood vessel closing procedure during image analysis, some blood vessels remain unfilled (associated with: “Quantitative analysis of the bone vasculature architecture in 2D images” section, step 17g). This issue could be due to improper closure of remaining blood vessels.

**Potential solution**
Assess the remaining unfilled blood vessels for the presence of missing gap closures. When finished, repeat the procedure starting from the closing blood vessels step 17d.

**Problem 16**
The image was cropped and important areas of interest were lost following rotation (associated with: “Quantitative analysis of the bone vasculature architecture in 2D images” section, step 19a-b).

Possible reasons include:

- The field of view being too tight, with some of the signal of interest locating close to the image borders;
- The rotation was performed without interpolation.

**Potential solution**
Take the image using a sufficiently large field of view. When rotating the image post-acquisition, ensure to check on the ‘enlarge image’ box and select ‘bilinear interpolation’.

**Problem 17**
The skeletonized image has too many or too few branches, not properly fitting the actual stained vessel pattern in the raw image (associated with: “Quantitative analysis of the bone vasculature architecture in 2D images” section, step 22a-b). A possible reason could be improper smoothening of the segmented blood vessel image.

**Potential solution**
In case there are too many branches with respect to the original image, use a bigger Gaussian filter. In case some blood vessels are lost during the procedure, use a smaller Gaussian filter, as shown in Figures 4H–4J.

**Problem 18**
When segmenting blood vessels in 3D using the surface tool in Imaris, the segmented image appears to lack important details of the original image (associated with: “3D Quantitative analysis of the spatial relationship between osteoprogenitors and blood vessels” section, step 26c).

Possible reasons include:

- Usage of a threshold that was set too high;
- Usage of a smoothing sigma that is too big to capture the signal of interest adequately.
Potential solution
Adjust the threshold to include all of the meaningful signals in the selection. Consider reducing or checking off the smooth box.

Problem 19
The cells of interest detected using a nuclear label appear very elongated in Z or appear fused instead of showing as individual entities (associated with: “3D Quantitative analysis of the spatial relationship between osteoprogenitors and blood vessels” section, step 27c).

Possible reasons include:

- Insufficient Z-resolution of the image;
- The cells of interest are small and strongly clustered together.

Potential solutions
Increase Z-resolution by taking smaller Z-steps. Use a higher resolution lens in case cells are clustered together in such a way that identifying them as separate entities is challenging.

RESOURCES AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Christa Maes (christa.maes@kuleuven.be).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The original/source dataset employed for illustrating the processing and analysis pipeline described here is accessible within the BioImage Archive repository. The accession number for the image dataset reported in this paper is BioImage Archive: S-BIAD287. These data relate to the published article by (Mesnieres et al., 2021) including image datasets for fetal bone generated or analyzed during this study, with permission for reuse. The dataset contains: 1) a single optical section of an E18.5 mouse bone containing Hoechst, Emcn, CD31, and Osx-GFP signals (Figure 4B); 2) a binary mask delimitating the embryonic bone (Figure 4C); 3) the binary segmented CD31+ blood vessels; 4) the blood vessel centerlines; 5) the segmented CD31+ blood vessels color labeled with respect to their local width; 6) a 3D image of an E18.5 mouse bone containing Hoechst, Emcn, CD31, and Osx-GFP signals (Figure 6), with the Emcn and CD31 signals modeled as surfaces and the detected Osx-GFP signals as 3D spots, in Imaris format. The dataset also contains excel tables with example data obtained by applying the 2D and 3D image analysis pipelines with the given images as input, and an excel file containing a detailed description of each file in the dataset and the corresponding step in the present protocol it relates to.

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AUTHOR CONTRIBUTIONS
Conceptualization, N.P. and C.M.; investigation, N.P., R.V., S.M., M.M., and E.N.; writing – original draft, N.P., R.V., S.M., and CM.; writing – review & editing, N.P., R.V., S.M., M.M., E.N., and C.M.; funding acquisition, C.M.; supervision, C.M.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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