Protocol
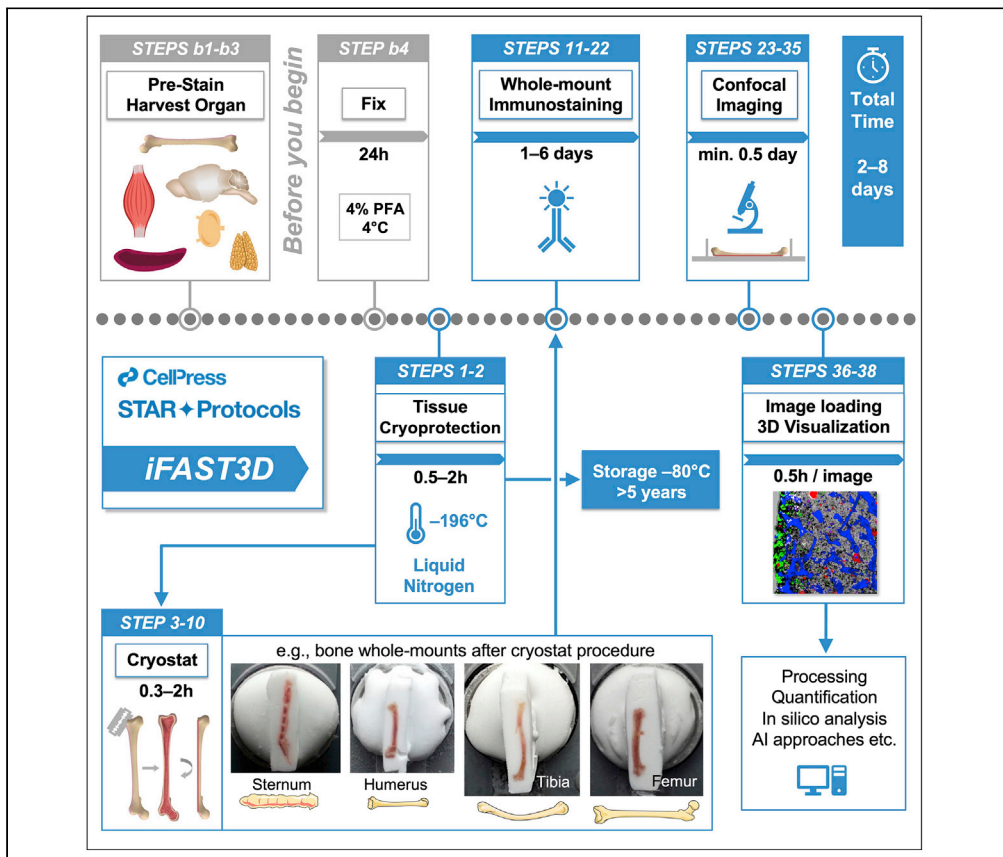
Fast and high-fidelity *in situ* 3D imaging protocol for stem cells and niche components for mouse organs and tissues

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Highlights

Whole-mount immunostaining protocol for 3D microscopy of stem cells and niche components

Fast pipeline for multiple mouse organs and tissues

High preservation of mouse tissue morphology and molecular integrity

Allows long-term (up to 5 years) storage of samples for subsequent staining and imaging

Quantitative 3D imaging of organ-wide cellular and subcellular components is central for revealing and understanding complex interactions between stem cells and their microenvironment. Here, we present a gentle but fast whole-mount immunofluorescence staining protocol for 3D confocal microscopy (iFAST3D) that preserves the 3D structure of the entire tissue and that of subcellular structures with high fidelity. The iFAST3D protocol enables reproducible and high-resolution 3D imaging of stem cells and various niche components for many mouse organs and tissues.

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

STAR Protocols

Protocol

Fast and high-fidelity *in situ* 3D imaging protocol for stem cells and niche components for mouse organs and tissues

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SUMMARY

Quantitative 3D imaging of organ-wide cellular and subcellular components is central for revealing and understanding complex interactions between stem cells and their microenvironment. Here, we present a gentle but fast whole-mount immunofluorescence staining protocol for 3D confocal microscopy (iFAST3D) that preserves the 3D structure of the entire tissue and that of subcellular structures with high fidelity. The iFAST3D protocol enables reproducible and high-resolution 3D imaging of stem cells and various niche components for many mouse organs and tissues.

For complete details on the use and execution of this protocol, please refer to Saçma et al. (2019).

BEFORE YOU BEGIN

To investigate the position of cells within a 3D space in a tissue for understanding complex cellular networks, like stem cell and stem cell niche interactions, sophisticated quantitative imaging tools are already available (Acar et al., 2015; Nombela-Arrieta et al., 2013; Coutu et al., 2018; Fra-Bido et al., 2021; Kunisaki et al., 2013; Kusumbe et al., 2014). One large drawback of these protocols is that they are technically very challenging, time consuming and often also require the use of very special equipment and tools. These restrictions might currently be in the way of a more widespread application of 3D *in situ* or *in vivo* imaging. We present here a fast while gentle methodology termed iFAST3D that enables high-fidelity 3D analysis of stem cells, endothelial and stromal structures and the determination of their spatial co-distribution directly *in situ*. iFAST3D is a straightforward protocol for 3D visualization of distinct types of cells and microstructures within a highly intact tissue architecture up to 75 μm depth and able to deliver final results already after 2 days (Figures 10–12).

The protocol below describes the specific steps for simultaneously staining of hematopoietic stem cells (HSCs) and niche components (vasculature, megakaryocytes, endosteum) using difficult to image bone (femur) and bone marrow (BM) that was initially developed in the key supporting paper (Saçma et al., 2019; Landspersky et al., 2021). With a few technical modifications (variation of antibody concentrations and incubation times), the protocol can be extended to other types of murine tissues and organs without changing the sample preparation methodology.

Before the staining procedure, you need to prepare the solutions (see "materials and equipment" for detailed recipe):

Fixation solution 4% PFA for the perfusion and fixation approximately 50 mL per mouse.

Washing buffer solution for washing steps and tissue covering during confocal microscopy.

Antibody diluent buffer for blocking and incubation steps.

Institutional permissions

All mouse experiments were performed in compliance with the ethical regulations according to the German Law for Welfare of Laboratory Animals and were approved by the Institutional Review Board of the local institutions, as well as by the state government of Baden-Württemberg (Protocol Number: 35/9185.81-3 / 1363). Researchers should check or obtain permissions from the relevant local institutions before undertaking this protocol.

Preparation of bones with intravital vasculature pre-staining

⏲ Timing: 1 h

1. Intravital vasculature staining.
   a. Weigh the mouse to calculate the volume of antibodies: APC-anti-CD31 0,8 μL/g and Alexa Fluor® 647-anti-CD144 0,32 μL/g.
   b. Dilute antibodies in PBS for a total of 200 μL.
   c. Inject the antibody solution intravenously into the lateral tail vein (troubleshooting 1).
   d. Leave the mouse at 18°C–25°C for 20 min.
2. Perfusion of the mouse.
   a. Anesthetize the mouse deeply: anesthesia is induced by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (16 mg/kg). Once sufficient depth of anesthesia is achieved and ensured (checked by inter-toe and tail tip, eyelid and corneal reflexes), administer repeated doses (100 μL) of anesthesia to provide deep surgical-plane anesthesia.
   b. Perfusion surgery (troubleshooting 1 and 2).
      i. Grip the skin with forceps under the chest and make a lateral and two incisions towards the collarbones with fine scissors and open the skin. (Figure 1A).
      ii. Grip the xiphisternum, make lateral incisions through the abdominal wall and carefully open the diaphragm along the rib cage with fine scissors. Subsequently, make two lateral cuts along both sides of the rib cage up to the clavicles and lift the sternum with the truncated ribs and clamp them with a hemostat to expose the chest cavity including the heart (Figure 1B).
      iii. Insert a 23-G needle or a 23-G butterfly needle through the posterior end of the left ventricle (LV) 0.3–0.5 cm deep. An incision is then made in the animal's right atrium (RA) to create the largest possible outlet without damaging the aorta (Figures 1C and 1D).
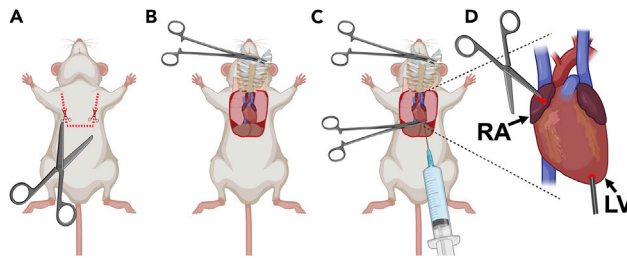
Figure 1. Perfusion surgery steps
(A) Direction and position of skin incisions.
(B) Exposed thoracic cavity.
(C) Transcardiac perfusion view.
(D) Direction and position of the needle insertion into the left ventricle (LV) and incision site on the right atrium (RA).

Note: Clamping with a hemostat can help to secure the needle from slipping and leakage (Figure 1C).

    iv. Perfuse the animal at a slow and steady rate (∼1 mL/5 s): first with ice-cold PBS (∼30 mL) followed by ice-cold 4% PFA fixation solution (∼30 mL) using a 30 mL syringe (Figure 1C).

Note: Twitching, fading liver, head and tail movement and stiffness are signs of successful perfusion.

⚠ CRITICAL: Work in a hood (PFA is harmful).

Collection and fixation of bones

⏲ Timing: 24 h

3. Bone isolation.
   a. Isolate required bones (femora, tibiae, sternum, humeri).
   b. Clean bones accurately from muscles and ligaments well (Figure 2) with fine scissors and low-lint wipes, completely dip in PBS and place in 15 mL 4% PFA fixation solution in a 15 mL centrifuge tube on ice (troubleshooting 2).
4. Fixation of bones in 15 mL 4% PFA fixation solution in 15 mL centrifuge tube for 24 h at 4°C (troubleshooting 1 and 2).

Note: We achieved similar staining results for min. 20 h to max. 30 h of fixation at 4°C for bones. We recommend 16 h fixation time for thymus, 24 h for muscle and spleen and 30 h for brain in 15 mL 4% PFA fixation solution in 15 mL centrifuge tubes at 4°C.
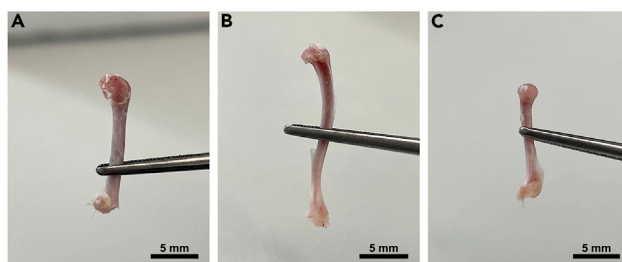

Figure 2. Isolated and cleaned long bones
Isolated and cleaned (A) femur, (B) tibia and (C) humerus.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-B220 (clone RA3-6B2) (1:100)	Thermo Fisher Scientific	Cat# 13-0452-85; RRID: AB_466450
anti-CD11b (clone M1/70) (1:100)	Thermo Fisher Scientific	Cat# 13-0112-85; RRID: AB_466360
anti-CD8a (Clone 53-6.7) (1:100)	Thermo Fisher Scientific	Cat# 13-0081-85; RRID: AB_466347
anti-Gr-1 (clone RB6-8C5) (1:100)	Thermo Fisher Scientific	Cat# 13-5931-85; RRID: AB_466801
anti-Ter119 (1:100)	Thermo Fisher Scientific	Cat# 13-5921-85; RRID: AB_466798
PE anti-CD150 (clone TC15-12F12.2) (4 μg/mL), critical reagent	BioLegend	Cat# 115904; RRID: AB_313683
anti-CD41 (clone MWReg30) (1:2500)	Thermo Fisher Scientific	Cat# 13-0411-85; RRID: AB_763489
anti-CD48 (clone HM48-1) (1:100)	Thermo Fisher Scientific	Cat# 13-0481-82; RRID: AB_466470
APC anti- CD31 (clone MEC13.3) (0,8 μL/g)	BioLegend	Cat# 102510; RRID: AB_312917
Alexa Fluor® 647 anti-CD144 (clone BV13) (0,32 μL/g)	BioLegend	Cat# 138006; RRID: AB_10569114
Streptavidin-eFluor®450 (1:60)	Thermo Fisher Scientific	Cat# 48-4317-82; RRID: AB_10359737
FITC anti-Ki-67 (clone SolA15) (1:100)	Thermo Fisher Scientific	Cat# 11-5698-82; RRID: AB_11151330
anti-FABP4/A-FABP (1:100)	R&D Systems	Cat# AF1443; RRID: AB_2102444
anti-goat Alexa Fluor® 647-conjugated (1:100)	Jackson ImmunoResearch	Cat# 705-605-003; RRID: AB_2340436
anti-LepR (1:100)	R&D Systems	Cat# AF497; RID: AB_2281270
anti-goat Cy3-conjugated (1:100)	Jackson ImmunoResearch	Cat# 705-165-003; RRID: AB_2340411
anti-H4K16ac (1:200)	Millipore	Cat# 07-329; RRID: AB_310525
anti-rabbit Alexa Fluor® 488-conjugated (1:200)	Jackson ImmunoResearch	Cat# 711-545-152; RRID: AB_2313584
PE anti-Ki-67 (clone SolA15) (1:100)	Thermo Fisher Scientific	Cat# 12-5698-80; RRID: AB_11149672
Streptavidin-PE (1:60)	Thermo Fisher Scientific	Cat# 12-4317-87
Streptavidin-APC (1:60)	Thermo Fisher Scientific	Cat# 17-4317-82
Chemicals, peptides, and recombinant proteins		
DAPI	Sigma-Aldrich	Cat# D9542-1MG
Donkey normal serum	Jackson ImmunoResearch	Cat# 017-000-121; RRID: AB_2337258
Goat normal serum	Dako	Cat# X090710-8
DPBS, Dulbecco's PBS without Ca^{2+} Mg^{2+}	PAN-Biotech	Cat# P04-36500
Formaldehyde solution ROTIPURAN® 37%	Carl Roth	Cat# 4979.1
Ketanest S 25 mg/mL	Pfizer	Cat# 37087.00.00
Penicillin/Streptomycin, P/S	PAN-Biotech	Cat# P06-07100
Xylazine, Rompun 2%	Bayer Animal Health	Cat# 14840
Triton™ X-100	Sigma-Aldrich	Cat# T9284-100ml
Experimental models: Organisms/strains		
Mouse C57BL/6J, 2–26 months old, either gender	Janvier Labs, internal divisional stock	Cat# C57BL/6JRj
Mouse Nestin-GFP, 8–16 weeks old, either gender	Jackson Laboratory, internal divisional stock	Cat# 033927
Mouse Rag2$^{-/-}$gc$^{-/-}$Kit$^{W/W}$, 8–16 weeks old, either gender	internal divisional stock	N/A
Mice AcRFP, AcYFP, AcCFP, 2–26 months old, either gender	internal divisional stock	N/A
Mouse SCL-tTAxH2B-GFP, 8–16 weeks old, either gender	DKFZ Heidelberg	N/A
Software and algorithms		
Volocity	Quorum Technologies, PerkinElmer	www.volocity4d.com; www.perkinelmer.com
ImageJ	National Institutes of Health	https://imagej.nih.gov/ij/
LAS-X acquisition software	Leica	LAS-X-3.1.5.16308
Zen acquisition software	Zeiss	Zen-2.3-SP1
Other		
Leica TCS SP8 confocal microscope	Leica	SP8
Zeiss LSM-710 confocal microscope	Zeiss	LSM-710
Objective HC-PL-APO-CS2 20×/0.75	Leica	Cat# 11506343

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Objective HC-PL-FLUOTAR 10×/0.30	Leica	Cat# 11506505
Objective Plan-APO 20×/0.8 M27	Zeiss	Cat# 420651-9911000
Objective Plan-APO 10× 0.3 M27	Zeiss	Cat# 420640-9800000
Cryostat CryoStar NX70	Thermo Scientific	Cat# 957000
Microtome blade A35	Feather	Cat# 207500011
4-well-μ-slide with glass bottom	ibidi	Cat# 80427
24-well culture plates, suspension	Sarstedt	Cat# 83.3922
OCT, Embedding medium Tissue-Tek™ O.C.T.	Sakura	Cat# 4583
Cryomold Tissue-Tek™ Cryomold® (25 × 20 × 5 mm)	Sakura	Cat# 4557
Wipes, low-lint	Kimberly-Clark	Cat# 7552
15 mL centrifuge tube, 120 × 17 mm	Sarstedt	Cat# 62.554.002
Fine scissors	Fine Science Tools	Cat# 14058-11
Hemostat	Fine Science Tools	Cat# 13008-12
Forceps	Fine Science Tools	Cat# 11008-13
Long-armed tweezers	Carl Roth	Cat# 0951.1
23-G needle	Henke-Sass, Wolf	Cat# 4710006025
30 mL syringe	B. Braun	Cat# 4616308F
23-G butterfly needle	B. Braun	Cat# 4056353

MATERIALS AND EQUIPMENT

4% PFA fixation solution

Reagent	Final concentration	Amount
37% Formaldehyde	37%	60.61 mL
DPBS	n/a	500 mL
NaOH 1 M	n/a	adjust pH=7.4, ~1080 μL
HCl 1 M	n/a	adjust pH=7.4
Total	4%	n/a

Note: 4% PFA fixation solution need to be prepared weekly and can be stored at +4°C.

⚠ CRITICAL: Paraformaldehyde is a flammable solid and is harmful if swallowed or inhaled, may causes skin irritation, skin allergic, serious eye damage, respiratory irritation, even genetic defects and cancer. Follow all safety guidelines.

Washing and storage buffer solution

Reagent	Final concentration	Amount
DPBS	1×	495 mL
Penicillin/Streptomycin P/S	1%	5 mL
Total	n/a	500 mL

Note: The washing buffer solution can be stored at 4°C up to several weeks.

Antibody diluent buffer

Reagent	Final concentration	Amount
Washing buffer solution	n/a	49.75 mL
Triton™ X-100	0.5%	0.25 mL
Total	n/a	50 mL

Note: The antibody diluent buffer solution can be stored at 4°C up to several weeks.

⚠ CRITICAL: Triton™ X-100 is harmful if swallowed and causes skin irritation and serious eye damage. It is very toxic to aquatic life with long lasting effects. Follow all safety guidelines.

Blocking and permeabilization solution

Reagent	Final concentration	Amount
Antibody diluent buffer	n/a	0.8 volume
Normal goat serum or donkey serum*	20%	0.2 volume
Total	n/a	1 volume

Note: *if a used antibody is raised from goat (host species), we use normal donkey serum to prevent unspecific binding. The blocking and permeabilization solution can be stored at 4°C for up to 2 days.

Cryostat settings: The temperature of the specimen's head and the blade should be adjusted to the manufacturer's manual and the tissues. For bones, we recommend the specimen holder between −18°C to −22°C and for the blade between −15°C to −19°C. Set the trimming thickness to 40 μm and the fine section thickness to 5 μm. The recommended cutting angle is 10°.

Alternatives: Theoretically, the reagents and operating materials listed in the key resources can be replaced by equivalent items from other vendors. However, the impact of alternative reagents on performance has not been tested.

Microscope settings: Acquisition software of the Leica-SP8 was LAS-X-3.1.5.16308 and the used objectives were HC-PL-APO-CS2 20×/0.75 and HC-PL-FLUOTAR 10×/0.30. The detectors were 3 photomultiplier (PMT) and two internal hybrid detectors (HyDs). The acquisition software of the ZeissLSM-710 was Zen-2.3-SP1 and the used objectives were Plan-APO 10× 0.3 and 20×/0.8 M27. The detectors were 4 PMTs. The laser sources were 405-Diode, Argon, DPSS-561 and HeNe-633 with the laser lines 405 nm, 458 nm, 476 nm, 488 nm, 496 nm, 514 nm, 561 nm or 633 nm.

STEP-BY-STEP METHOD DETAILS
Tissue cryoprotection

⏱ Timing: 30 min–2 h

In this step, embedding in water-soluble embedding medium and snap freezing in liquid nitrogen for tissue preservation is described.

1. Embedding in optimal cutting temperature compound (OCT).
    a. Rinse the bones 2× with 10 mL PBS in 15 mL tubes to get rid of PFA solution.
    b. Dry the bones with tissue; make sure that they are clean; if necessary, remove redundant tissue.
    c. Fill the cryomolds with OCT until the upper limit.
    d. Positioning of the tissue in OCT (Figure 3).
        i. Place the bones longitudinally close to the left side of the OCT containing cryomold.
        ii. Make sure the bones are fully immersed and parallel to the left edge of the cryomold (Figure 3), to achieve the maximal frontal tissue surface after cryo-shaving.
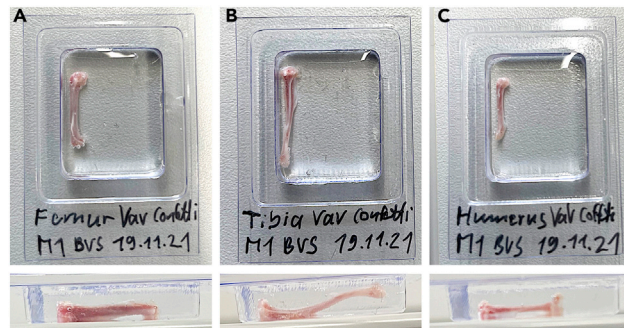   iii. Move bubbles out if they are formed.

Figure 3. Embedding position of long bones
Embedding position of (A) femur, (B) tibia and (C) humerus in OCT.

2. Snap freezing in liquid nitrogen (LN2).
   a. For dealing with LN2, wear protective equipment in appropriate rooms.
   b. Grab the filled cryomold with long-armed tweezers with the help of a table surface (Figures 4A and 4B).
   c. Touch gently with the bottom of the cryomold the surface of the LN2 in an appropriate open LN2 Dewar flask. Attachment is ensured when a bubbling sound begins (Figure 4C).

   ⚠ CRITICAL: Too quick plunging of the sample into LN2 can cause formation of bubbles.

   d. Let the OCT get freeze (white) from bottom to top (Figure 4D).
   e. When the OCT is totally frozen (Figure 4E, drop it into the LN2 (Figure 4F).

   ⚠ CRITICAL: Fast snap freezing is essential to prevent formation of ice crystals which induce cellular and architectural tissue damage.

   ⚠ CRITICAL: For long-term preservation (up to 5 years, see Figure 14) frozen and embedded tissue should be stored subsequently at −80°C.

Cryostat shaving procedure

⏱ Timing: 20 min/tissue block

This section describes the cryostat shaving procedure of the specimen for obtaining optimal bisected bone whole-mounts for 3D confocal imaging.

3. Set up the cryostat settings according to the "materials and equipment" section.
4. Transfer the samples to dry ice to prevent OCT melting.
5. Freeze the frozen block on a specimen chuck on the long side using OCT and with the tissue upside down. If available, use a quick freeze chamber.

   ⚠ CRITICAL: The chuck should be at 18°C–25°C prior to use to ensure optimal adhesion.

6. Allow time (5–10 min) for the temperatures within the cryostat to stabilize.
7. Start to shave the block with the trimming function until the bone marrow is maximally exposed. Also see Methods video S1.

   ⚠ CRITICAL: To prevent severe damage, ensure that the blade does not crash into the sample.
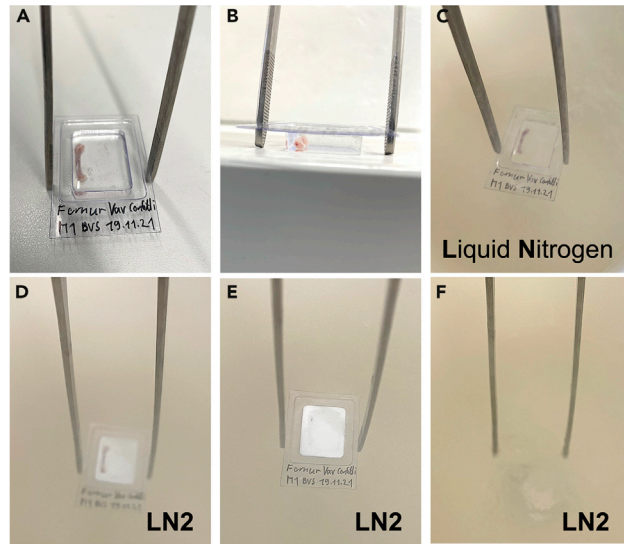
Figure 4. Snap freezing in liquid nitrogen
(A) Grabbing the filled cryomold with long-armed tweezers.
(B) Side view of the cryomold with a table surface.
(C) Touching the bottom of the cryomold with the surface of the LN2.
(D) Freezing of the OCT (white) from bottom to top.
(E) Totally frozen OCT.
(F) Dropping the specimen into LN2.

8. Switch to the sectioning thickness and shave the specimen 10–20×. Also see Methods video S1.

   ⚠ CRITICAL: To ensure a planar shaved surface, check during the shaving steps the blade for notches caused by the bone matrix. If notches occur, shift the blade to an undamaged part or use a new one (troubleshooting 2). Be cautious during the shaving procedure as the blades are very sharp.

   Note: Use a brush to remove debris from the sample.

9. Cut the frozen tissue block with a razor blade from the specimen chuck.
10. The shaved samples can be stored 3–4 weeks at −80°C prior to immunofluorescence staining.

Whole-mount immunostaining

   ⏲ Timing: 1–7 days

This section describes the immunostaining procedure to image 3D information of hematopoietic stem cells and niche components (vasculature, endosteum, megakaryocytes) throughout the whole femoral bone marrow section with optimized antibodies, which, in total, need 5 days. The incubation times and concentrations of the used antibodies in this protocol are the same for other appropriate tissues. Novel antibodies should be validated before use.

11. Purification from melting OCT.
    a. Place the shaved and frozen samples into a box with separating chambers which are filled with ∼150 mL cold (4°C) washing buffer solution to cover the samples (Figures 5A and 5B).

   ⚠ CRITICAL: Throughout the remaining parts of the protocol, avoid touching the exposed bone marrow to prevent losing cells and causing tissue artifacts (troubleshooting 2).
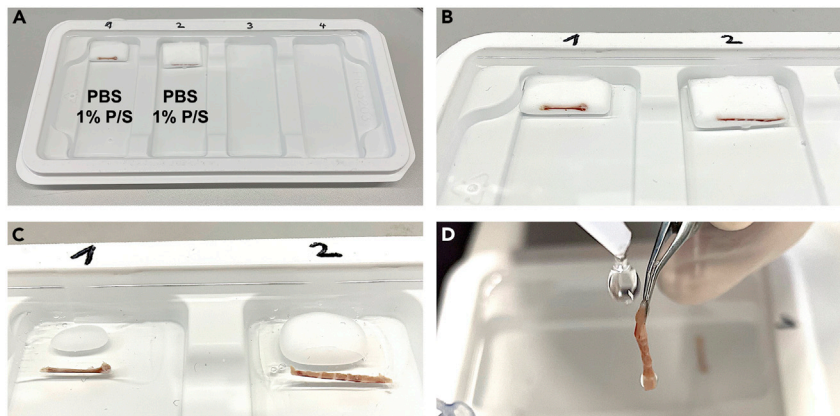
Figure 5. Purification from melting OCT
(A and B) Frozen samples in PBS.
(C) Melting samples.
(D) Washing samples with washing buffer solution.

   b. Wait until the OCT around the sample is melted in the cold (4°C) washing buffer solution (Figure 5C).

   c. After the OCT is melted (Figure 5C), take out the exposed samples cautiously with tweezers and wash the bones with washing buffer solution with a plastic bottle to get rid of sticky OCT (Figure 5D).

   ⚠ CRITICAL: Don't pour the washing solution directly to the exposed bone marrow to avoid causing artifacts.

   Note: Use a see-saw rocker with ca. 30 rpm and cover the samples with a box from light for every step from now on.

12. Prepare 24-well plates (wp) for fixation and washing (Figure 6A) and 0.2 mL PCR tubes for blocking and antibody incubation (Figure 6B).

   Note: Solution amounts in 0.2 mL PCR tubes for blocking and antibody incubation for femur is 260 μL, for tibia 250 μL, for humerus and sternum 200 μL. In general, the processed tissue should be fully covered by the solution in the tube.

13. Fixation in 24-wp.
   a. Fill one well for each sample with 2 mL 4% PFA fixation solution.
   b. Place the bone samples into the fixation solution with the shaved surface, so that the BM doesn't touch the bottom and incubate for 30 min at 18°C–25°C.
14. Wash the samples 3× 20 min at 18°C–25°C by relocating from well to well with each well containing 2 mL of washing buffer solution.
15. Place the samples in 0.2 mL PCR tubes with blocking and permeabilization buffer; incubate at 18°C–25°C for 1 h.
16. Primary hematopoietic lineage antibody staining (troubleshooting 3 and 4):
   a. Prepare the lineage antibody cocktail solution with biotinylated anti-CD41 (1:2500), anti-CD48 (1:100), anti-CD11b (1:100), anti-B220 (1:100), anti-CD5 (1:100), anti-Gr-1 (1:100), anti-Ter119 (1:100), anti-CD8a (1:100) in antibody diluent buffer.
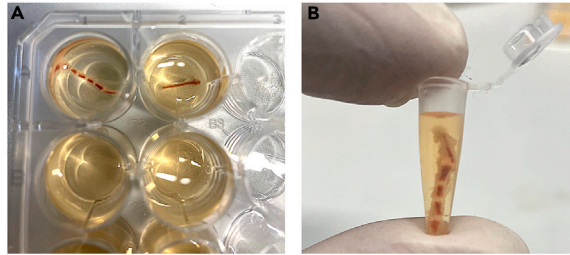   b. Incubate at 4°C for 2 days.

Figure 6. Washing and incubation equipment
(A) 24-wp for fixation and washing.
(B) 0.2 mL PCR tubes for blocking and antibody incubation.

⚠ CRITICAL: Spin down the antibody vials to remove aggregates and precipitates to avoid the occurrence of non-specific fluorescent spots.

17. Wash the samples 3× 20 min at 18°C–25°C in washing buffer solution (troubleshooting 4).
18. Secondary antibody staining (troubleshooting 3 and 4):
    a. Prepare secondary antibody solution with streptavidin (eFluor®450- or FITC- conjugated, 1:60) in antibody diluent buffer.
    b. Incubate at 18°C–25°C for 2 h.
19. Wash the samples 3× 20 min at 18°C–25°C in washing buffer solution (troubleshooting 4).
20. SLAM antibody staining (troubleshooting 3 and 4):
    a. Prepare antibody solution with anti-CD150-PE (4 μg/mL) in antibody diluent buffer.
    b. Incubate at 4°C for 3 days.
21. Wash the samples 3× 20 min at 18°C–25°C in washing buffer solution.
22. Place the bone samples in 24-wp in storage buffer solution (2 mL per well) with the shaved surface and store at 4°C in the dark until microscopy.

⏸ Pause point: Samples can be imaged 1–3 days after the staining procedure.

Confocal microscopy

⏱ Timing: 0.5 days

This section describes the sample mounting and confocal 3D microscopy. Every immunostaining experiment has special settings and here we give the basic settings for 3D imaging we are using for the iFAST3D protocol.

Note: With many confocal microscope acquisition software you can retrieve settings from previous experiments.

23. Drop 30 μL–80 μL washing and storage buffer solution to the bottom glass of a 4-well-μ-slide (Figure 7A) to prevent tissue desiccation.
24. Place the fluorescently labeled tissue cut-face down onto the drop in the 4-well-μ-slide (Figures 7B and 7C) and check from the bottom side that the sample surface is completely touching the glass planar (Figure 7D).

⚠ CRITICAL: Avoid too much pressure which can press out BM and prevent formation of air bubbles (troubleshooting 2 and 5).

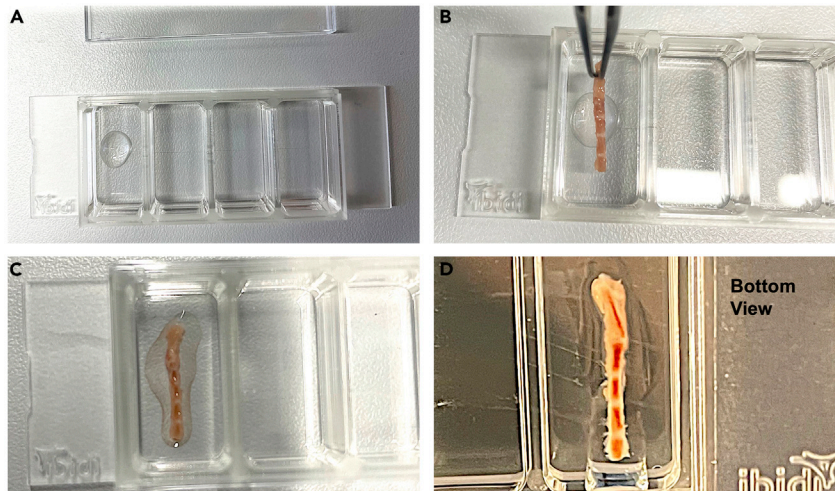25. Cover the microscope glass chamber with the associated lid.

Figure 7. Microscope glass chamber
(A) 4-well-μ-slide chamber with 60 μL washing and storage buffer solution.
(B and C) Placing of a whole-mounted sternum.
(D) View from the bottom side of the sample.

26. Switch on the confocal microscope system.
27. Place the glass chamber on the stage of the microscope.
28. Use a 10× objective for preview and choose a region for setting up in the widefield mode.
29. Set up the acquisition settings for confocal microscopy:
    a. Set up the channel configurations based on the excision/emission parameters of the used fluorochromes and dyes in the staining.

    Note: If available, use the auto-set-up mode with the ideal option.

    b. Use transmitted light detection (DIC) for trabecular and cortical bone matrix detection and for structural details.
    c. Use an image size resolution of 1024 × 1024 with bidirectional scanning (600 Hz–800 Hz) and 3–5× averaging.
30. Switch to a 20× objective and confocal live mode.
31. Optimize the detector parameters gain, laser power etc. by negative controls and/or by a software over-saturation option (troubleshooting 3 and 4).

    ⚠ CRITICAL: Avoid long-lasting laser exposure of the region of interest (ROI) to prevent photobleaching of sensitive types of labeled antibodies.

32. Scan for a ROI using 10× objective in the widefield mode.
33. Switch back to a 20× objective and confocal live mode.
34. Set up the z-stack configurations:
    a. Set up the lower and upper focal plane scanning limits. A limit is reached when the image is out of focus. Z-thickness size can vary between 20 μm–75 μm.
    b. For optimal and fast scanning, use a step size between 1.0 μm to 3.5 μm (z-step size).

    Optional: Set start and stop points for z-stack tile scanning.

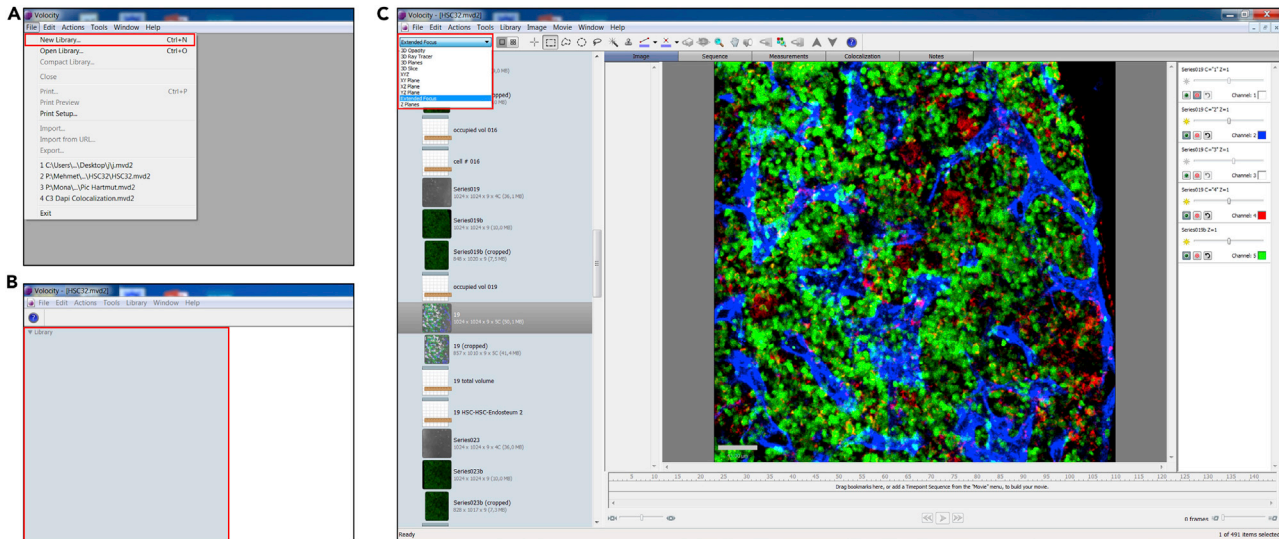35. Start the scanning procedure for 3D imaging and save your z-stack data subsequently.

Figure 8. Key steps for importing and loading raw images in Volocity
(A) "New Library…" selection in the "File" menu.
(B) "Library" view section.
(C) Image view modes.

Processing and 3D visualization of raw data using Volocity analysis software

⏱ Timing: 30 min per image

This section describes how to import raw data, how to process images to visualize in 3D and how to export images by using Volocity software as an example. For 3D reconstruction and subsequent quantification and analysis of structural, cellular, and subcellular properties, also other image analysis platforms (e.g., Imaris, ImageJ, FIJI) can be used.

Note: Common file formats with metadata, generated by conventional confocal microscope systems, are supported and can be added to Volocity by drag and drop. The software saves every step automatically.

36. Import of raw images:
    a. Go to the "File" menu and select "New Library…" (Figure 8A).
    b. Name the library and choose the destination for storage.
    c. Drag the raw image, or folder with images, into the "Library" view on the left side of the screen (Figure 8B).
    d. Image sequences with the appropriate XYZ dimensions, channels and timepoints will be generated automatically.
    e. Imported images can be viewed in different modes, like "3D Opacity", "XYZ" and "Extended Focus" (Figure 8C).

Note: We use the "Extended Focus" mode for fast screening of the images and enhancement of the signal intensity and brightness for individual channels.

37. 3D visualization:
    a. Select the image from the "Library" view for 3D visualization.
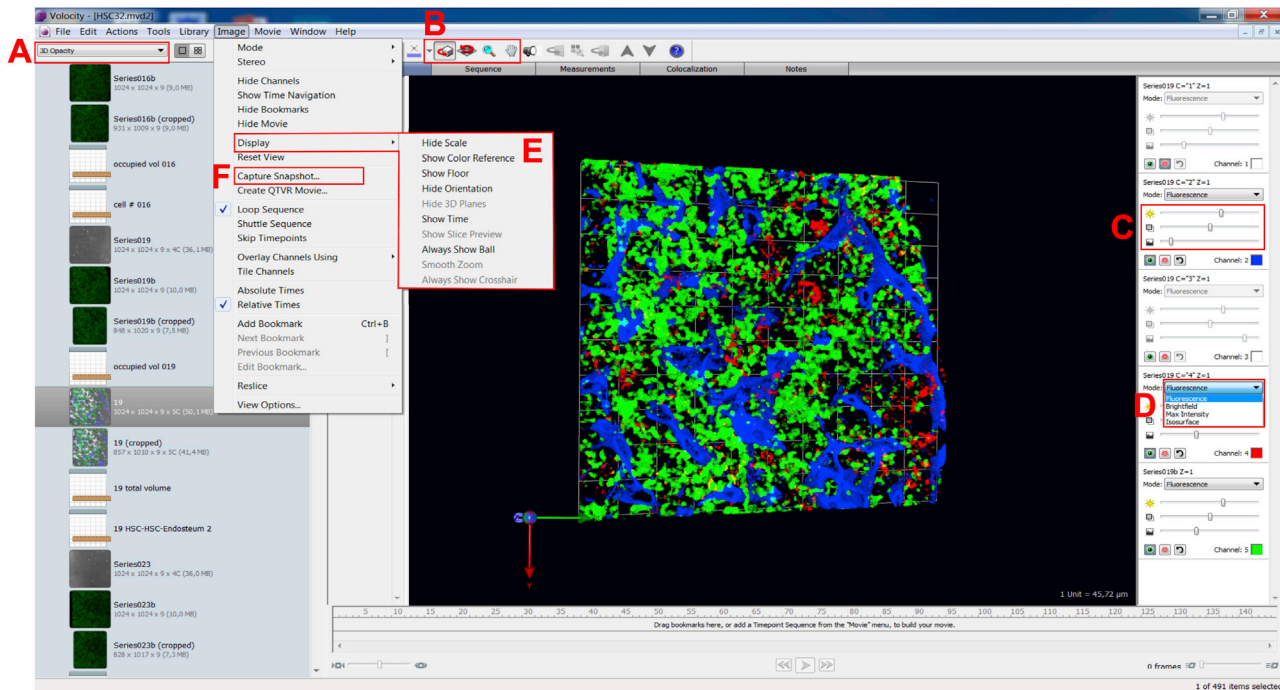   b. In the drop-down menu at the top of the screen, change the display option to "3D Opacity" (Figure 9A).

Figure 9. Key steps for processing and 3D visualization and exporting images in Volocity
(A) Display option "3D Opacity".
(B) Tools for change actions.
(C) Channel control slider.
(D) Rendering mode selection.
(E) "Image" menu with the "Display" submenu.
(F) "Capture Snapshot…" selection.

    c. Use the tools at the top of the window to change actions for rotating and zooming for a required 3D view (Figure 9B).

    d. Optimize the brightness (recommended 1×–4×), density (recommended 50%) and the black level (recommended 2%–10%) for individual channels on the channel control view on the right side of the screen (Figure 9C).

    e. Choose from the channel control view the proper rendering "Mode" (Fluorescence, Isosurface, Max Intensity) for the best presentation of your data and observations (Figure 9D).

    f. To show a scale bar and the XYZ-orientation on the image, go to the "Image" menu, the "Display" menu and select "Show Scale" and "Show Orientation" (Figure 9E).

38. Export of 3D visualized images:

    a. To create a single image illustration of the current 3D view, go to the "Image" menu and select "Capture Snapshot…" (Figure 9F).

    b. A snapshot image is added at the bottom of the library list.

    c. Select the snapshot image from the library list and in the "File" menu choose "Export…".

    d. The snapshot of the 3D visualization can now be exported in any supported file format (e.g., TIFF, BMP, JPEG).

EXPECTED OUTCOMES

The protocol for iFAST3D is straightforward, fast and gentle to allow for subsequent confocal whole-mount immunofluorescence staining and analysis. This enables comprehensive 3D analyses of different mouse organs and tissues from large field down to subcellular levels at high resolution and quantitative analysis of *in situ* 3D stem cell and niche component distribution. In the bone samples typically, you can see HSCs (CD150$^+$CD41$^-$CD48$^-$LIN$^-$, marked by yellow dots), non-HSC
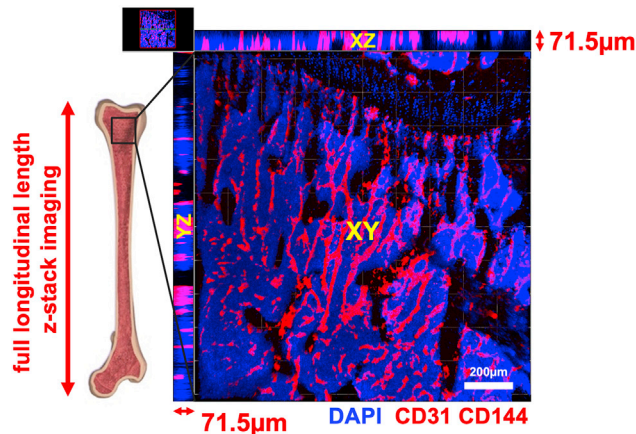
Figure 10. iFAST3D imaging depth for 3D information
Representative 3D reconstruction of confocal images of epiphysis/metaphysis with schematic longitudinal surface of a bisected bone and BM and affiliated xy-, xz- and yz-projections. Vasculature (red) and nuclei (in blue, DAPI).

hematopoietic cells (white, CD150$^-$CD41$^+$CD48$^+$LIN$^+$), big-shaped megakaryocytes (big red cells, CD150$^+$CD41$^+$), endothelial cells forming the BM vascular network (blue) and bone (gray) (Methods video S2).

iFAST3D requires minimal histology equipment for sample collection and staining and only conventional confocal microscopy for deep imaging up to 75 μm (Figure 10).

The protocol minimizes the use of chemical solvents, incubation time and mechanical stress to preserve tissue morphology, and as well as molecular integrity. Thus, there is no necessity for frequently artifact-causing and time-consuming processes of decalcification, clearing, dehydration or sectioning.

The pipeline allows very fast high resolution 3D analysis of tissue and endothelial and stromal structures and the determination of spatial distribution of a large number of single cells and structures and their interrelationships directly *in situ* within 2 days already (Figure 11).

The protocol is extremely versatile and can be applied to different tissues and organs without changing the sample preparation methodology. Moreover, the staining can be customized without altering critical steps in the protocol for 3D visualization of distinct types of cells and microstructures within a highly intact tissue architecture (Figures 11 and 12).

Preservation of fluorescent proteins (including GFP, YFP, CFP and RFP) facilitates the use of novel and versatile combinations and multiplexing of various dyes (Figures 12 and 13).

Fixed and pre-stained organs can be stored long-term at −80°C (at least up to five years) for subsequent imaging by iFAST3D (Figure 14).

LIMITATIONS

Samples should be imaged 1–3 days after the staining procedure, due to the low stability of some fluorescently-labeled antibodies. There is the possibility for fast photobleaching of some types of primary labeled antibodies (e.g., CD150-PE) by confocal laser exposure. This restricts for some samples the adjustment of scanning or repeated scanning. The protocol is not working well for immunohistochemistry staining protocols. The protocol only offers a static view of the tissue and does not allow for direct intravital imaging.
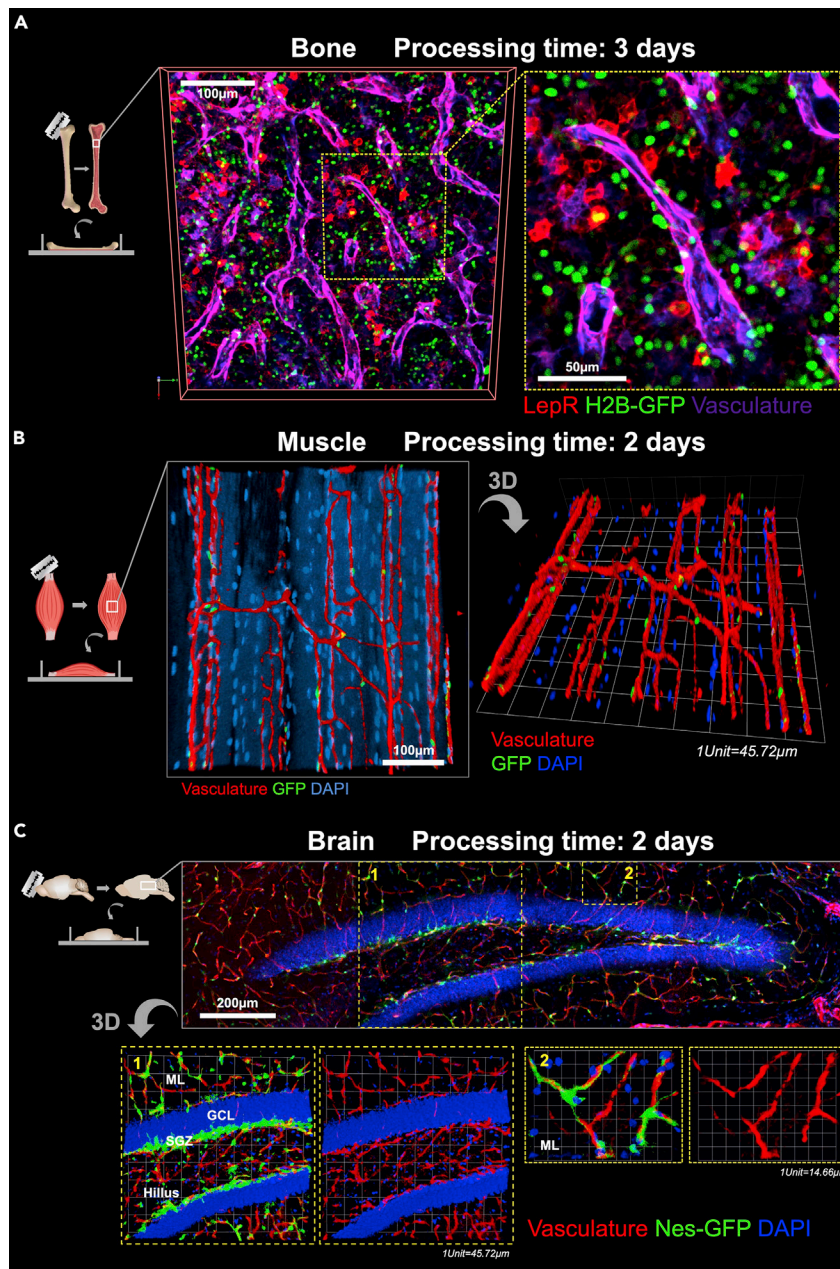
Figure 11. iFAST3D enables FAST and high-fidelity 3D confocal IF imaging

(A) Whole-mount confocal image of label retaining (LR) mouse BM vasculature (magenta) and Leptin Receptor[+] (LepR^{+}) cells (red) with LR-cells (GFP[+], green) after only 3 days of workflow. Vasculature was stained intravenously with anti-PECAM1 (CD31) and anti-VE-Cadherin (CD144) antibodies.

(B) Representative stacked whole-mount and 3D reconstructed confocal images of myofibrils from Gluteus Maximus showing the muscle microvasculature and the localization of GFP^{+} satellite cells (Nestin-GFP, Day et al., 2007).

(C) Dentate Gyrus in Hippocampus, brain microvasculature, neuronal stem cells in the SGZ (1) and perivascular cells (2) in the ML (Nestin-GFP, Mignone et al., 2004) (SGZ = Subgranular zone; ML = Molecular layer, GCL = Granule cell layer).

TROUBLESHOOTING

Problem 1

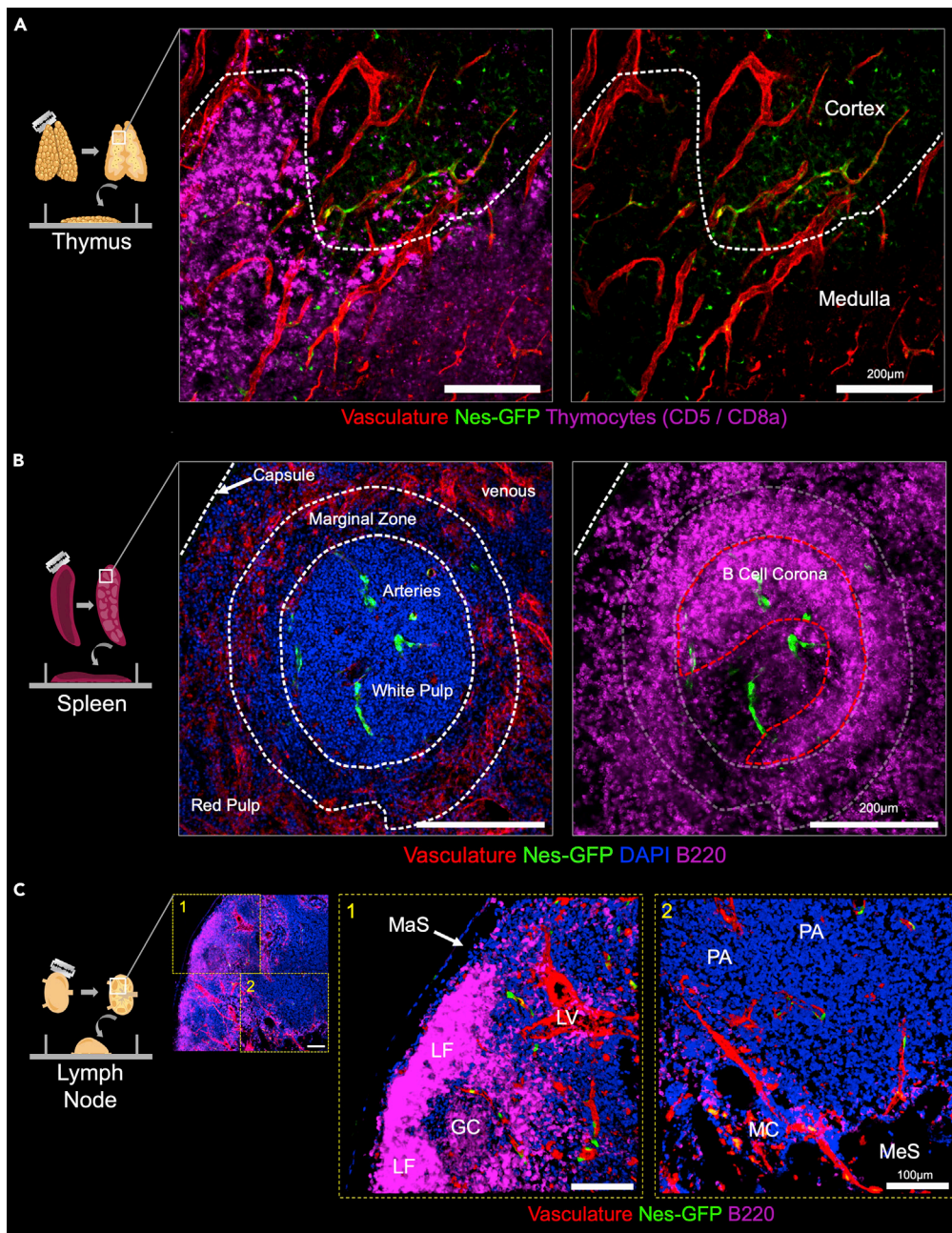Vasculature (intravital) staining issues. Confocal microscopy.

Figure 12. iFAST3D enables multicolor IF for mouse organs and tissues

(A) Extended focus projection images revealing the microvasculature (red) of the thymus in the cortex and medulla with different locally concentrated thymocytes (CD5/CD8a magenta) in longitudinal whole-mounts from Nes-GFP mouse thymus, which imply a special role for Nes-GFP$^+$ perivascular cell ensheathed microvessels in the cortex of the thymus.

(B) Longitudinal spleen whole-mounts from Nes-GFP mice. Extended focus projections depicting the splenic capsule, the marginal zone, the B cell corona with frequent B cells (B220, magenta), the microvasculature (red) with arterioles (indicated by periarteriolar Nes-GFP^{high} cells) located frequently in the white pulp region and venous vasculature (red) concentrated in the red pulp zone.

(C) Z-stacked whole-mount image (overview) and 3D reconstructions (image 1 and 2) of a mesenteric lymph node from Nes-GFP mouse, showing the marginal and medullary sinus (MaS and MeS), the follicles (LF), the germinal center (GC), the paracortical area (PA) the medullary cords (MC) and the lymphatic vessels (LV). To note the distribution of B cells (B220, magenta) and periarteriolar Nes-GFP^{high} cells (green).
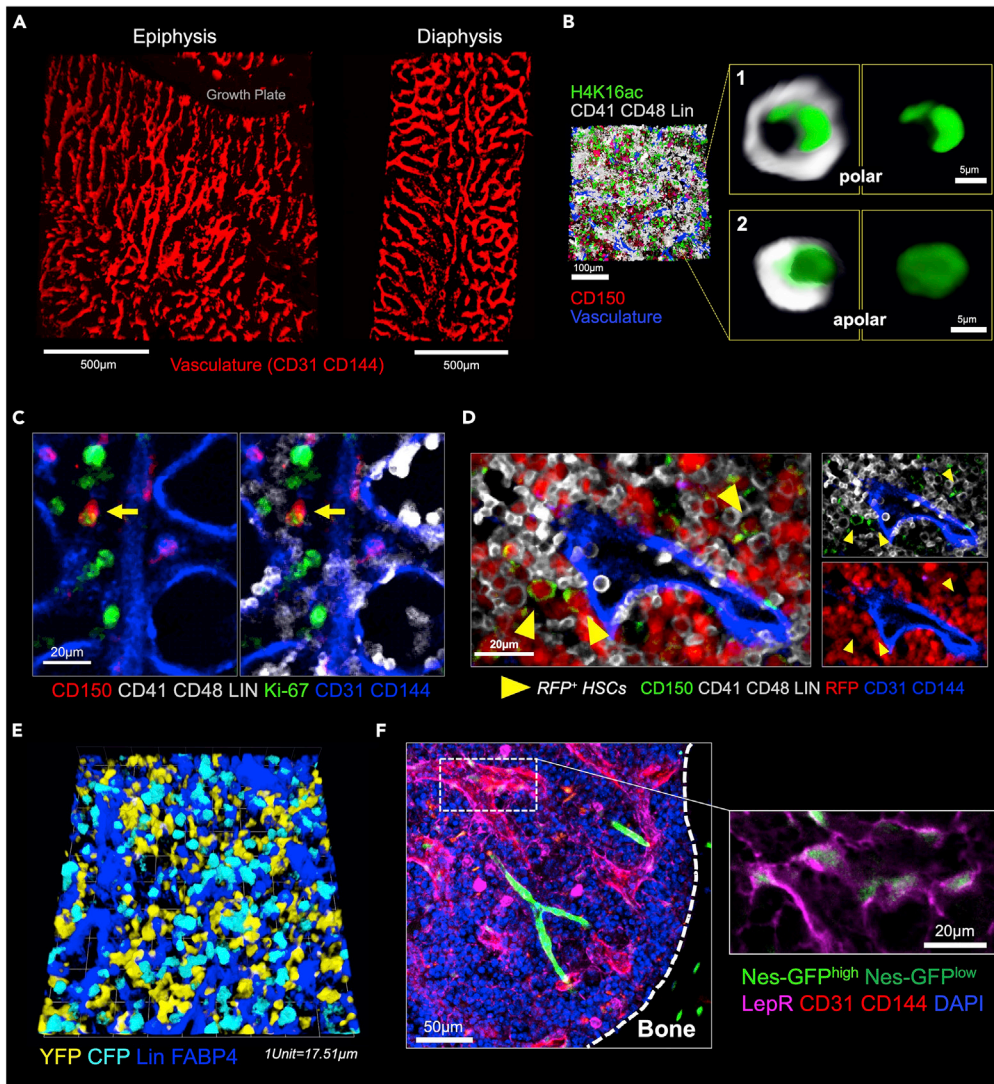
Figure 13. Highly intact tissue architecture and preservation of molecular integrity

(A) Representative 3D reconstructed images showing vasculature (red) in tibial epiphysis/metaphysis and diaphysis.

(B) Representative confocal BM images showing *in situ* single polar (1) and apolar cell (2) for the epigenetic marker H4K16ac (green).

(C) *In situ* subcellular Ki-67 BM cell staining with endogenous HSC 4 days after 5-FU treatment. Arrows indicate Ki-67^{+} HSC. To note the dilated sinusoidal vessels after the chemotherapy with 5-FU.

(D) Representative extended focus projection of femoral BM from recipient mouse transplanted with RFP[+] HSCs. Donor-derived RFP[+] HSCs are marked by yellow arrowheads. Lineage markers, CD41 and CD48, are shown in white, CD150 in green and endothelial cells are in blue. All donor-derived cells are RFP[+] (red).

(E) 3D reconstructed confocal images of a whole-mounted femur from recipient mouse transplanted with CFP[+] young and YFP[+] aged HSCs showing donor-derived CFP[+] and YFP^{+} BM cells 6 weeks after transplantation. Hematopoietic markers (CD41, CD48 and lineage) and FABP4^{+} in blue.

(F) Whole-mount confocal images revealing periarteriolar Nes-GFP^{high} cells (bright green) and overlap of perisinu-soidal LepR^{+} cells (magenta) and Nes-GFP^{low} cells (dim green) in BM of Nes-GFP mice. Dashed lines denote the endosteum.

Potential solution

Ensure to inject the antibody solution into the lateral tail vein (before you begin step 1c).

Ensure proper perfusion (before you begin step 2b) and fixation (before you begin step 4).
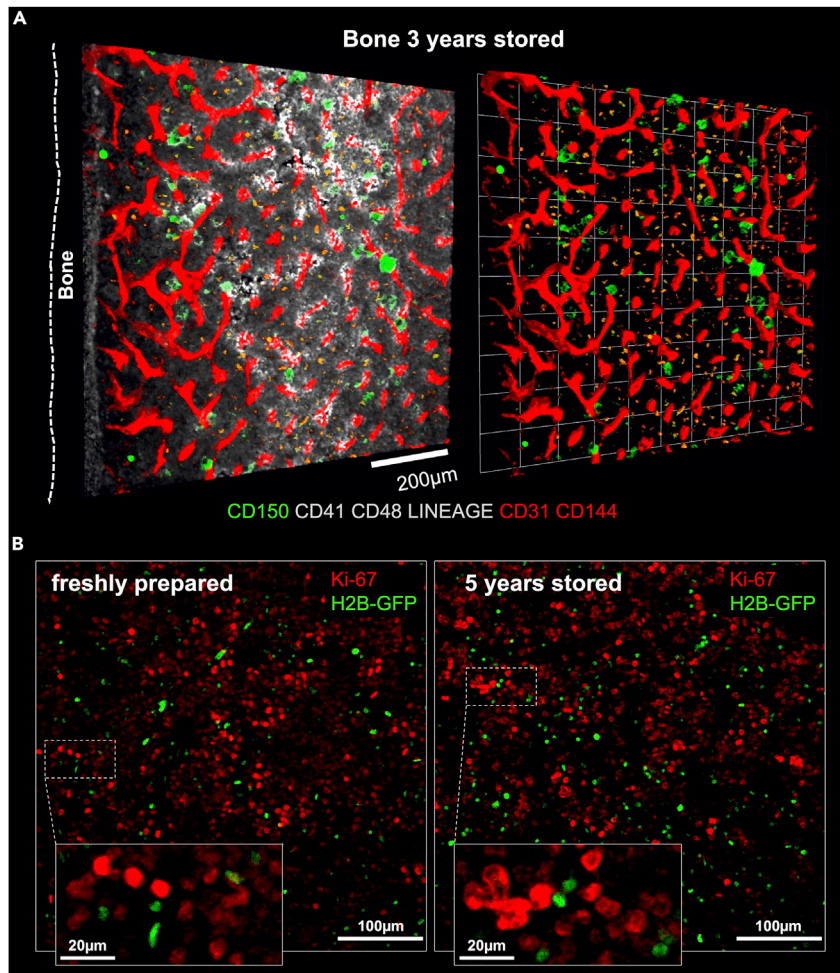
Figure 14. Preservation of structure and antigenicity even after long-term storage

(A) 3D confocal whole-mount images of a 3-year-stored (−80°C) long bone processed by iFAST3D showing the preservation of the antigenicity for the highly sensitive CD150 protein and CD41/CD48/LIN (gray), and intravital stained vasculature (red).

(B) Representative extended focus projection of the BM of freshly prepared and 5 years stored (−80°C) H2B-GFP mouse bones processed by iFAST3D showing the proliferation marker Ki-67 (red). H2B-GFP (green).

Problem 2

No fluorescence signal detection in big BM areas or holes in BM. Confocal microscopy.

Potential solution

This problem could be due to loss of BM parts and due to uneven positioning of the sample. Also, nonspecific tissue between the shaved tissue surface and the microscope glass or non-planar cryostat shaving of the sample can cause these problems.

Ensure proper perfusion (before you begin step 2b) and fixation (before you begin step 4).

Remove non-specific tissue between the shaved tissue surface and the microscope glass (before you begin step 3b).

Shift the blade to an undamaged part or use a new one during cryostat shaving (steps 7 and 8).

Avoid touching the exposed BM (step 11).

Ensure that the sample is placed evenly on the microscope glass (step 24).

Problem 3
Low fluorescence signal. Confocal microscopy.

Potential solution
This issue could have several causes: low concentration of primary or secondary antibodies and insufficient antibody incubation times.

Optimize the antibody concentrations and incubation times, especially for novel antibodies, or use fresh ones (steps 16, 18, and 20).

Increase the setting of the laser or/and the detector (laser power, gain) (step 31).

Problem 4
High fluorescence signal or high background staining. Confocal microscopy.

Potential solution
High concentrations of primary or secondary antibodies and too long antibody incubation times could cause these problems.

Optimize the antibody concentrations and incubation times especially for novel antibodies (steps 16, 18, and 20).

Use a negative control with only the secondary antibody (without the primary antibody) to detect unspecific binding and optimize the concentration of the secondary antibody (steps 16 and 18).

Decrease the setting of the laser or/and the detector (laser power, gain) (step 31).

Extend the antibody washing times or add some surfactant (e.g., Triton X-100, Tween 20) to the washing buffer (steps 17 and 19).

Problem 5
Formation of air bubbles between the sample and the bottom glass. Confocal microscopy.

Potential solution
Carefully lift the sample and replace it cut-face down onto the drop in the 4-well-µ-slide (Figures 7B and 7C) and check again from the bottom side that the exposed surface is completely touching the glass planar (Figure 7D) (step 24).

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mehmet Saçma, mehmet.sacma@uni-ulm.de.

Materials availability
This study did not generate any new reagents.

Data and code availability
This study did not generate any dataset or code.

AUTHOR CONTRIBUTIONS

M.S. performed and analyzed laboratory and histological experiments and microscopy imaging and analysis. F.M. performed polar/apolar histological experiments. A.H. performed functional endosteal/central HSC transplantation experiments. K.S., G.M., and A.V. assisted in transplantation procedures and supported in cell sorting and flow analysis procedures. V.S. supervised the mouse work and took care of breeding and preparation of the mice used for experiments. M.D.M. and R.B. supported the experiments involving SCL-tTAxH2B-GFP double heterozygous mice. M.S., M.C.F., and H.G. designed and interpreted the experiments. M.A.M. supported 4D analysis and performed computational biology approaches. M.C.F. and F.M. supported in writing the manuscript. M.S. and H.G. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Saçma, M., Pospiech, J., Bogeska, R., de Back, W., Mallm, J.-P., Sakk, V., Soller, K., Marka, G., Vollmer, A., Karns, R., et al. (2019). Haematopoietic stem cells in perisinusoidal niches are protected from ageing. Nat. Cell Biol. *21*, 1309–1320. https://doi.org/10.1038/s41556-019-0418-y.