Analysis of Bone Tissue using Xenium v1 In Situ Gene Expression Assay

Introduction

Research of healthy and diseased bone tissues often requires investigation of bone gene expression. However, the analysis of bone using spatial imaging techniques is challenging due to difficulty of obtaining high-quality RNA from samples, the ease of sectioning which typically necessitates decalcification, as well as high rates of tissue detachment from tissue slides. Data quality is dependent on many factors, including species (mouse vs. human), bone type, sample quality, post-mortem interval (PMI), and tissue processing. This Technical Note describes methods for decalcifying and preparing human and mouse bone tissue samples for use in 10x Genomics Xenium v1 In Situ Gene Expression assays.

For this study, healthy mouse femur, tibia, sternum, and neonatal mouse bone, as well as human tibia, femur, and bone marrow tissue samples were analyzed. The methods section outlines the sample preparation techniques for calcified bone and soft bone for formalin fixed & paraffin embedded (FFPE) mouse and human tissue sections. Decalcification methods were tested and are compatible with Xenium v1, but other assays, such as Xenium Prime, have not been tested.

This Technical Note is a proof-of-concept document and not intended for use as a Demonstrated Protocol. Methods described in this Technical Note may require further optimization for which 10x Genomics Support cannot provide additional guidance. Even when implementing the guidance listed in this Technical Note, 10x Genomics cannot guarantee assay performance.

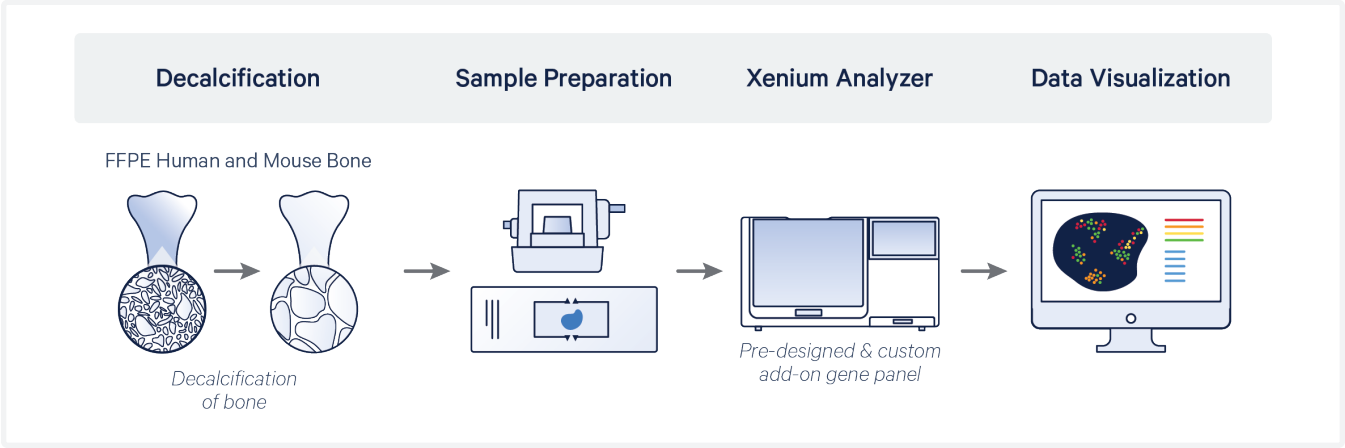

Figure 1. Decalcification overview for Xenium v1 In Situ Gene Expression assays.

Decalcification

Decalcification is essential for histological, immunological, and molecular studies of bone tissue. However, decalcifying agents can degrade RNA, therefore making it crucial to optimize conditions that soften the bone while minimally affecting RNA integrity.

Decalcification can be carried out on the surface of an FFPE block or during or after fixation, prior to embedding. Decalcification methods may vary depending on the species of interest.

In this Technical Note, decalcification was tested immediately after fixation, though other methods may require further optimization.

Both EDTA and acid-based decalcification methods (e.g., formic acid) were tested, while other acid-based methods, such as hydrochloric and nitric acid, were not evaluated.

Calcified Tissue

Multiple decalcification methods were tested in this Technical Note to remove calcified bone. For mouse tissue, EDTA, EDTA/Paraformaldehyde (PFA), and formic acid performed similarly. For human tissue, EDTA or EDTA/PFA was the most effective decalcification method that also preserved RNA quality, leading to optimal data.

Always decalcify the tissue prior to sectioning, as residual calcified bone can damage surrounding tissue and blades during sectioning, resulting in suboptimal data. While data can be obtained without decalcification by sectioning with a diamond blade, this technique presents numerous challenges and is outside the scope of this Technical Note.

Soft Tissue

Soft tissue that is derived from human or mouse is compatible with Xenium v1 In Situ Gene Expression assays with no protocol modifications. Soft tissue includes tissue dissected completely away from any surrounding bone or biopsies where no residual bone is remaining.

Soft tissue containing any amount of calcified bone may require decalcification. Calcified bone within soft tissue can cause damage during sectioning when the blade encounters bone shards, dulling the blade and resulting in tissue tears or inconsistent tissue thickness. Additionally, the bone shards can shear surrounding tissue during sectioning.

Process samples immediately following tissue harvesting, as waiting longer can compromise soft tissue. For FFPE bone marrow without calcified bone, refer to the Xenium In Situ for FFPE - Tissue Preparation Guide (CG000578) for more details. For FF bone marrow without calcified bone, refer to Xenium In Situ for Fresh Frozen Tissues – Tissue Preparation Guide for more details (CG000579).

Methods

Overview

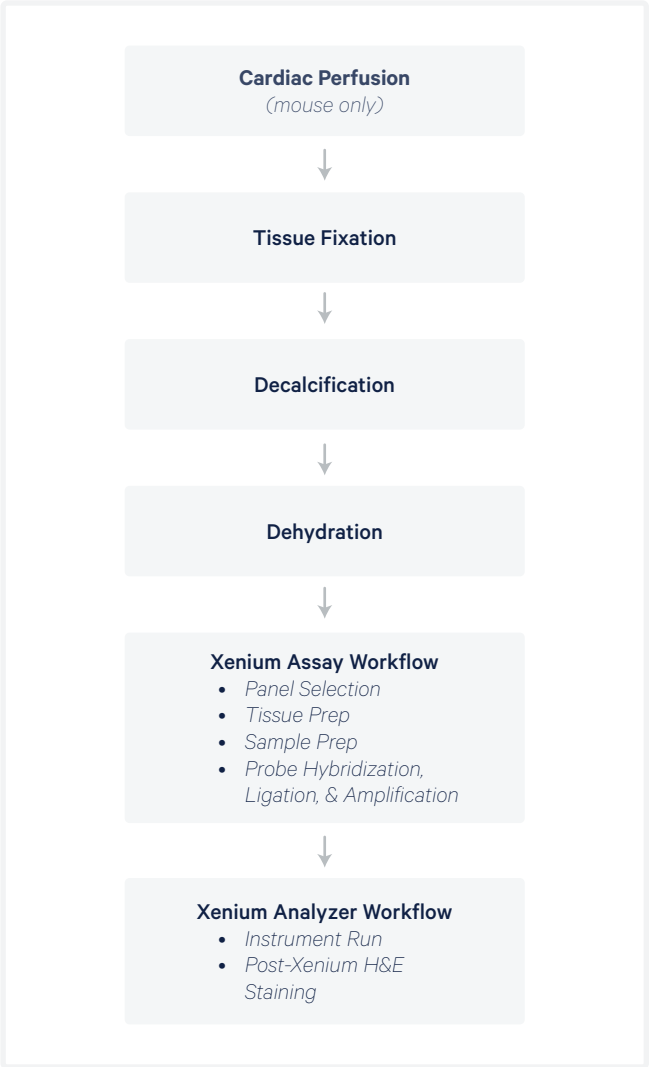

Figure 2. Methods overview for processing of mouse and human bone.

Materials

Item	Supplier	Part Number (US)
Paraformaldehyde, 16% w/v aq. soln., methanol free	Thermo Scientific	AA433689L (requires dilution to 4%)
Paraformaldehyde Solution, 4% in PBS	Thermo Scientific	AAJ19943K2
Formalin solution, neutral buffered, 10%	Millipore Sigma	HT501128
Epredia 10% Neutral Buffered Formalin	Epredia	5701
UltraPure 0.5M EDTA, pH 8.0	Thermo Fisher Scientific	15575020
Formic Acid	Millipore Sigma	PHR3559-5X1ML
Phosphate buffered saline (10X), pH 7.4	Invitrogen	AM9625
Nuclease-free Water (not DEPC-treated)	Thermo Fisher Scientific	AM9932

Not all materials listed are required. Choose one fixative and one decalcification method based on the Methods outlined below. One of the two PFAs listed can be used for cardiac perfusion (mouse only). 10% neutral buffered formalin (NBF) is used for tissue fixation. For decalcification, either EDTA or formic acid can be used.

Key Considerations

Tissue Fixation

- Optimal fixation will vary depending on the composition and size of the sample.
- Tissue should be fixed with fresh formaldehyde.
- Fixative penetration is key; generate a coronal or axial section as segmenting the bone into smaller pieces achieves more consistent fixation. Coronal sections of bone ensure the marrow becomes exposed to fixative.

Decalcification

- Decalcification times may vary depending on species, bone type, and amount of calcified bone present. Assess bone for adequate decalcification before processing and embedding.

Processing and Embedding

- To minimize RNA fragmentation, use low-melting paraffin wax for embedding.

Block Selection

- During sectioning, if there is evidence of insufficient decalcification (e.g. sectioning difficulty), selecting a different block is suggested.

Sectioning

- Clean workstation, microtome, tools, and blades with RNase Decontamination Solution before sectioning to minimize exogenous RNases.
- Utilize a clean, new blade for sectioning.
- 5 µm section thickness is recommended and has been validated for FFPE samples using the Xenium In Situ Gene Expression assay workflow.
- If the block chatters during sectioning or the block is too hard to generate a good quality section, the block may not be sufficiently decalcified.
  - Warm the block using lukewarm water (37°C) to soften and hydrate it prior to sectioning.
  - After tissue sections have been placed onto Xenium slides, tissues should be dried according to the Xenium In Situ for FFPE - Tissue Preparation Guide (CG000578) and then stored in a room temperature desiccator until ready to proceed to the staining protocol.

Sample Preparation

- Follow recommended drying steps outlined in the Xenium In Situ for FFPE - Tissue Preparation Guide (CG000578).
- Follow the protocol according to the Xenium In Situ for FFPE - Deparaffinization & Decrosslinking Demonstrated Protocol (CG000580). As bone tissue is more prone to detachment, follow best practices.
  - During deparaffinization and decrosslinking, exercise caution during immersion steps so as to not disrupt the tissue.
  - When pipetting into Xenium Slide Cassettes, dispense and remove reagents along the side of the well without touching or pipetting on top of the tissue.
  - If tissue detachment occurs, tissue drying time post-sectioning may be extended further than standard recommendations.
  - Different slide coatings may also minimize tissue detachment. While not tested for bone samples, Poly-L-Lysine increased adherent cell adhesion to Xenium slides.

Sample Storage

- For optimal assay performance, proceed quickly from sample collection to fixation to dehydration/clearing to embedding.
- Samples should not be stored long-term in PBS at any stage in the workflow.
- If it is not possible to proceed immediately to dehydration and clearing, a sample can be transferred from fixative to 70% ethanol for short-term storage (potentially up to 3 months as long as ethanol is refreshed weekly to ensure concentration remains at 70% throughout storage time).

Panel Selection

- Pre-designed panels for mouse and human can be used, and add-on panels are also recommended to maximize bone-specific and bone marrow-specific cell types and genes. Refer to the Panel Design page on the 10x Genomics Support Website for a complete list of available panels and information on how to start the panel design process for custom panels.
- Refer to the 10x Genomics Datasets page for a preview of human and mouse bone data as well as the add-on gene list for the datasets (available as supplemental files that also list the panel gene name, transcript ID, and cell type annotation in CSV format).
- Consider targeting more highly expressed genes when designing a custom panel as RNA with low expression may be degraded during decalcification.
- Panel to tissue match may impact high level metrics such as transcripts per cell, so careful consideration of custom add-on panels is key.

Mouse Bone Workflow

Cardiac perfusion with 4% PFA was performed on healthy adult *C57bl/6* mice. Mouse femur bones were collected and fixed in 10% NBF at 4°C overnight (18-24 h).

Samples were prepared using two decalcification methods:

- 0.5M EDTA (~3 days)
- 10% formic acid (24 h)

Representative data for mouse bone presented in the Results section is from the 10% formic acid method. Both methods showed comparable assay performance.

EDTA-based decalcification:

0.5M EDTA (pH 8.0) was prepared. Muscle and soft tissue were removed from bone samples before starting the decalcification process. Tissue samples were submerged in a sufficient volume of EDTA solution and a stir bar was utilized to agitate the solution during decalcification.

Decalcification was performed with 0.5M EDTA (pH 8.0) for ~3 days at 4°C. The time for decalcification may vary depending on bone thickness. A time course may be required for optimization. Probe the tissue to determine whether the bone is still rigid. Decalcification is complete once the bone is pliable as determined by gently probing the tissue with sharp metal forceps.

Formic Acid-based decalcification:

Formic acid was prepared by diluting formic acid to 10% in Nuclease-free water. Decalcification was performed with 10% formic acid for ~24 h at 4°C. Similar to EDTA-based decalcification methods, bone rigidity was tested with a probe to avoid under- or over-processing.

Following decalcification, the mouse bone samples were washed in 3x with 1X PBS (pH 7.4), dehydrated with several ethanol wash steps (store in 70% ethanol at -20°C if needed), and proceeded with processing and embedding. For more guidance on sample storage, see Key Considerations.

The following documents were followed for processing and embedding of the mouse bone samples:

- **Tissue Preparation:** Xenium In Situ for FFPE - Tissue Preparation Guide (CG000578)
- **Sample Preparation:** Xenium In Situ for FFPE Tissues - Deparaffinization & Decrosslinking Demonstrated Protocol (CG000580)
- **Probe Hybridization, Ligation, and Amplification:** Xenium In Site Gene Expression - Probe Hybridization, Ligation & Amplification User Guide (CG000582)
- **Instrument Run:** Xenium Analyzer User Guide (CG000584). The on-instrument analysis was run with Xenium Onboard Analysis version 1.9.0
- **H&E Staining:** Xenium In Situ Gene Expression - Post-Xenium Analyzer H&E Staining Demonstrated Protocol (CG000613)

The Xenium Mouse Tissue Atlassing Panel, a pre-designed panel targeting 379 genes of interest, was used. The panel was designed using reference data from CZ Biohub San Francisco and the Tabula Muris Consortium[1], and has been validated in the whole mouse pup and in a variety of adult tissues (liver, lung, heart, skin, kidney, spleen, colon, brain, and thymus).

To enhance detection of bone cell types, a 100-gene custom add-on panel was added to supplement the identification of osteoblasts, osteocytes, osteoclasts, and chondrocytes.

Human Bone Workflow

FFPE tissues were purchased from Discovery Life Sciences (Acute Lymphoid Leukemia bone marrow), Precision Biospecimens (non-diseased bone marrow), and Avaden Biosciences (non-diseased femur bone). The femur sample was delivered in fixative and the decalcification, processing, and embedding were performed in-house by 10x Genomics.

Human bones were fixed in 10% NBF at 4°C overnight (18-24 h). The thickness of the tissue significantly affects fixative penetration. Tissue sections should be no larger than a standard tissue cassette and no thicker than 5-10 mm.

The human bones were washed 3x in PBS and decalcified in 15% EDTA (pH 8.0)/0.4% PFA in a 50 ml conical vial for ~3-4 weeks at 4°C. A probe was used to determine if the bone was less rigid (or more gelatinous). EDTA was changed to fresh solution every 2-3 days; samples were gently agitated during the decalcification process.

Following decalcification, the human bone samples were washed 3x with 1X PBS (pH 7.4), dehydrated with several ethanol wash steps (store in 70% ethanol at -20°C if needed), and proceeded with processing and embedding. For more guidance on sample storage, see Key Considerations.

Human bone may take months for optimal decalcification depending on the size and region of bone selected. No decalcification was necessary for the human bone marrow. This sample was processed and embedded as an aspirate, with only minimal calcified bone regions present at the time of collection.

The following documents were followed for processing and embedding of the human bone samples:

- **Tissue Preparation:** Xenium In Situ for FFPE - Tissue Preparation Guide (CG000578)
- **Sample Preparation:** Xenium In Situ for FFPE Tissues - Deparaffinization & Decrosslinking Demonstrated Protocol (CG000580)
- **Probe Hybridization, Ligation, and Amplification:** Xenium In Site Gene Expression - Probe Hybridization, Ligation & Amplification User Guide (CG000582)
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- **H&E Staining:** Xenium In Situ Gene Expression - Post-Xenium Analyzer H&E Staining Demonstrated Protocol (CG000613)

The Xenium Human Multi-Tissue and Cancer Panel, a pre-designed gene panel targeting 377 genes of interest, was used. The panel was designed using single cell RNA sequencing data curated and reprocessed for standardization by the Human Protein Atlas[2]. Genes were chosen to accurately type cells, and identify select immune, proliferation, and tumor markers in human breast, lung, skin, liver, colon, kidney, lung cancer, and heart.

To enhance detection of bone cell types, a 100-gene custom add-on panel was added to supplement the identification of blood progenitors, endothelial cells, macrophages, stromal cells (smooth muscle and fibroblasts), T cells, and B cells detected in bone marrow.

Results

Mouse Bone - Data Highlights

High quality data was sucessfully generated from mouse bone samples with ~24x10^6 decoded trascripts across ~253,560 cells (Table 1). Post-Xenium H&E staining of mouse human femur passed histological assessments for sample quality. Add-on gene panel assisted in cell type resolution of the mouse bone.

Key Metrics	Value
Number of Cells Detected	253,560
Median Transcripts / Cell	70
Median Pre-designed Transcripts / Cell	53
Median Custom Transcripts / Cell	15
Nuclear Transcript Density / 100 µm²	115.7 µm²
Total High Quality Decoded Transcripts	~24 x 10^6

Table 1. Key metrics data generated for mouse femur bone.

Post-Xenium H&E staining aligned to transcript data showed tissue architecture of distinct cell types in trabecular bone (Figure 3A) and cortical bone (Figure 3B). Cell types were identified by a range of markers (Table 2).

Cell Type	Marker	
	Trabecular Bone	**Cortical Bone**
Endothelial Cells	Cdh5, Mcam	Cdh5, Mcam
Hematopoietic stem cells (HSCs)	Cxcr4, Flt3, and Runx1	Cxcr4, Flt3, and Runx1
Immune Cells	Fcgr1, Rnase4, and Slamf1	-
Chondrocytes	Col10a1m, Fgfr3, and Mmp13	Col10a1m, Fgfr3, and Mmp13
Erythroid cells	Alas2, Gata1, and Car1	Alas2, Gata1, and Car1
Monocytes	Ccr2	-
Osteocytes	-	Dmp1, Fgf23, Mepe
Osteoblasts	Col1a1, Phex, and Pon3	Col1a1, Phex, and Pon3
Platelets	Itga2b	Itga2b
Osteoclasts	-	Ctsk, Tmem178

Table 2. Cells types identified by associated markers for trabecular and cortical bone.

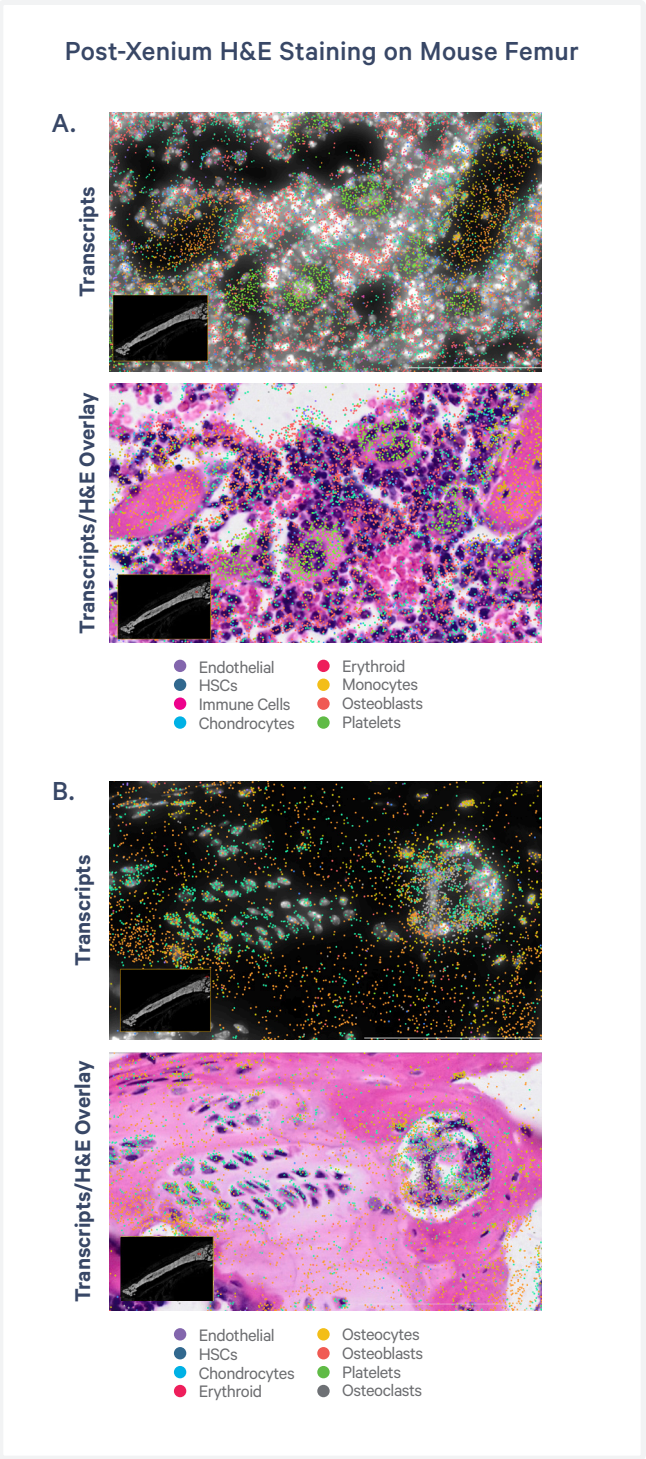

Figure 3. Erythroblastic niche in mouse femur bone marrow shows high proximity to platelets and chondrocytes **(A)**. Proximal femur shows distinct organization of bone niche cell types **(B)**.

Cells were annotated manually with assistance from Annotation of Cell Types (ACT)[3] and Tabula Muris (Figure 4A). A range of immune cells in various states of differentiation was observed throughout the bone marrow tissue. The erythroblastic niche in the mouse femur bone marrow showed proximity of platelets to erythroid cells as expected, as well as chondrocytes. Cell annotation on the bone/joint interface revealed specific connective tissue cell types (Figure 4B). Chondrocytes, fibroblasts, and smooth muscle cells (SMCs) were observed in stratified regions. The identified cell types (using ACT and manual annotation) included chondrocytes, fibroblasts, skeletal muscle cells, endothelial cells, and SMCs. Cell types were depicted by UMAP in Figure 4C. These results demonstrate the successful identification and localization of cell types within the mouse femur tissue, providing valuable insights into the cellular composition and organization of the bone marrow microenvironment.

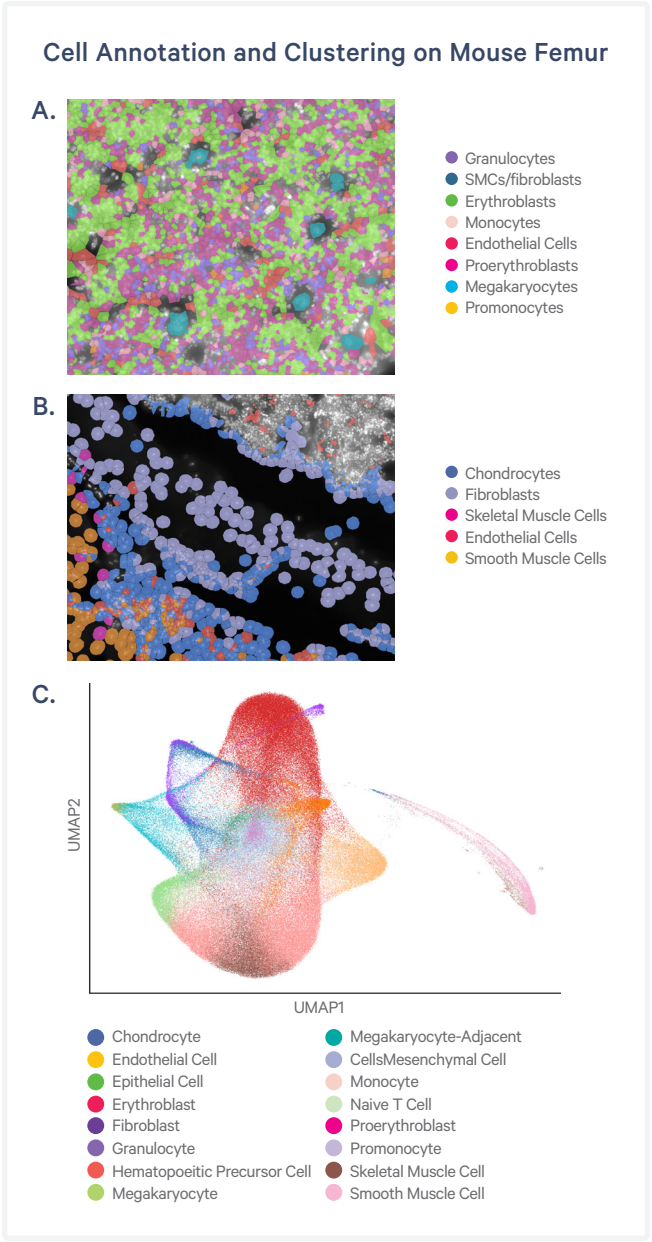

Figure 4. Cells were annotated manually with assistance from ACT and Tabula Muris softwares. A range of immune cells in various states of differentiation was observed throughout the bone marrow tissue. Custom add-on gene panel to the Mouse Atlas Panel generated enough specificity to annotate cells in complex environments **(A)**. Cell annotation on bone/joint interface revealed specific connective tissue cell types. Chondrocytes, fibroblasts, and SMCs are seen in stratified regions **(B)**. UMAP of cell types present in mouse femur bone sample. Lineage and expression similarities are reflected in the UMAP region containing marrow cells (left) and differentiation from bone cells clustering as a discrete population with no expression similarity with marrow cells (right) **(C)**.

Human Bone - Data Highlights

Human bone samples generated high quality data with ~7x10^6 decoded trascripts across 225,906 cells in both calcified bone (RNA-poor) and marrow (RNA-rich) (Table 3). Despite decalcification, nuclear transcript density was high at 52.5 nuclear transcripts per 100 µm^2. Transcript density varied by morphology with high and low levels in marrow-rich and calcified regions, respectively. These results demonstrate that the current decalcification protocol preserves tissue integrity and RNA in both calcified and soft tissue regions.

Key Metrics	Value
Number of Cells Detected	225,906
Median Transcripts / Cell	27
Median Pre-designed Transcripts / Cell	19
Median Custom Transcripts / Cell	7
Nuclear Transcript Density / 100 µm^2	52.5 µm^2
Total High Quality Decoded Transcripts	~7 x10^6

Table 3. Key metrics data generated for human Acute Lymphoid Leukemia (ALL) bone marrow.

Post-Xenium H&E staining of human bone confirmed intact tissue morphology with visible and trabecular bone and marrow structures. Bone marrow H&E staining highlight hypercellularity, trabeculae, and common marrow cells. Add-on gene panel assisted in cell type resolution of the human bone.

H&E staining revealed tissue architecture and various cell types in human ALL bone marrow, as shown in Figures 5A (bone adjacent marrow) and 5B.

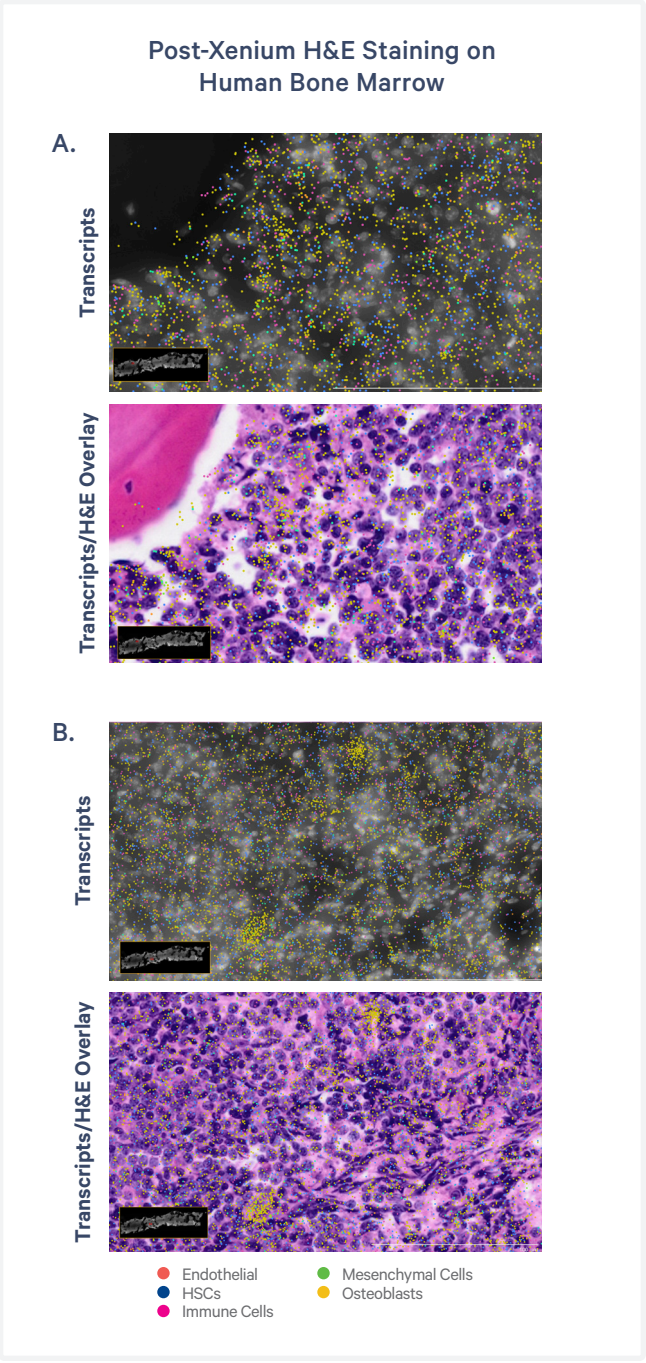

Figure 5. Human ALL sample shows even distribution of blood progenitors throughout the bone marrow **(A)**. Human ALL sample area with high enrichment of osteoblasts **(B)**.

Cell types in ALL bone marrow were mainly composed of Neutrophils, B cells, and T cells (Table 4). Indicators of cell proliferation (MYC and MKI67) and apoptosis regulation (BCL2L11) were also detected, consistent with leukemic state (Figure 6). These results demonstrate the successful identification and localization of cell types within the human ALL bone marrow, providing valuable insights into the cellular composition, organization, and disease state in malignant bone marrow.

Cell Type	Marker
	Human ALL Bone Marrow
Endothelial Cells	ENG
Hematopoietic stem cells (HSCs)	CXCL12, CD24, and DYRK3
Immune Cells	CD40, FOXP4, and GAD1
Mesenchymal Cells	COL19A1, CYP1B1
Osteoblasts	ALPL, AXIN2, and BMP2

Table 4. Cells types identified by associated markers for ALL Human Bone Marrow.

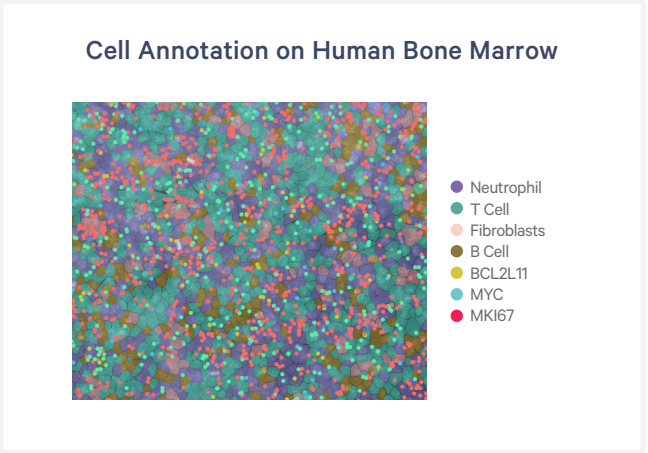

Figure 6. Cell types in human ALL bone marrow are highly constrained. Neutrophils, as well as highly variable B cells and T cells, compose most of the marrow population. MYC and MKI67 upregulation are hallmarks of unconstrained replication and are seen throughout the sample tissue. BCL2L11 and its polymorphs are associated with ALL and CLL.

Conclusion

Bone is a structurally complex and cellularly diverse tissue. High quality gene expression data is dependent upon effective preservation and decalcification of the bone to enable sectioning.

In this Technical Note, Xenium v1 In Situ Gene Expression assays are shown to be compatible with human and mouse bone samples using sample-dependent tissue preservation and decalcification methods, although human bone was more challenging due to increased calcification.

The following samples were tested in proof-of-concept experiments:

- Adult human femur, tibia bone, and bone marrow
- Adult mouse femur, tibia, and sternum
- Embryonic/neonatal mouse bone

These samples were tested using the Human Multi-Tissue Panel or the Mouse Tissue Atlassing Panel. A custom add-on panel is recommended to maximize bone and bone marrow-specific cell types.

Appropriate custom add-on panels enabled identification of specific cell types in bone, including: osteoblasts, osteoclasts, and osteocytes.

Additionally, specific cell types in bone marrow were also identified, including: SMCs and the expected hematopoietic cells.

In conclusion, decalcification enables successful spatial analysis of bone samples and can provide insights into the spatial organization of cells involved in bone formation, remodeling, and disease progression.

References

1. https://tabula-muris.sf.czbiohub.org/
2. https://www.proteinatlas.org/
3. http://xteam.xbio.top/ACT/
4. Xenium In Situ for FFPE – Tissue Preparation Guide (CG000578).
5. Xenium In Situ for Fresh Frozen Tissues – Tissue Preparation Guide for more details (CG000579).
6. Xenium In Situ for FFPE Tissues – Deparaffinization & Decrosslinking (CG000580).
7. Xenium In Situ Gene Expression – Probe Hybridization, Ligation & Amplification User Guide (CG000582).
8. Xenium In Situ Gene Expression – Post-Xenium Analyzer H&E Staining (CG000613).
9. Xenium Analyzer User Guide (CG000584).

Datasets

https://www.10xgenomics.com/datasets

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Contact:
support@10xgenomics.com
10x Genomics
6230 Stoneridge Mall Road
Pleasanton, CA 94588 USA