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Title: Preparation of Formalin-Fixed, Paraffin-Embedded Bone Marrow Samples for Spatial Transcriptomics Using EDTA-Based Decalcification

Authors and Affiliations:

Lorea Campos-Dopazo¹, Miguel Cócera^{1,2}, Paula Aguirre-Ruiz^{1,2}, Purificación Ripalda-Cemborain¹, Sarai Sarvide¹, Amaia Vilas-Zornoza^{1,2}, Ana Rosa López-Pérez³, Emma Muiños-López¹, David Gómez-Cabrero⁴, Felipe Prosper^{1,2,3}, Isabel A. Calvo^{1,2}

¹ Hematology and Oncology Program, Center for Applied Medical Research, Instituto de Investigaciones Sanitarias de Navarra, Cancer Center Clínica Universidad de Navarra

² Centro de Investigación Biomédica en Red de Cáncer

³ Hematology and Cell Therapy Department, Instituto de Investigaciones Sanitarias de Navarra, Cancer Center Clínica Universidad de Navarra

⁴ Bioscience Program, Biological and Environmental Sciences and Engineering Division, King Abdullah University of Science and Technology

Corresponding Authors:

Isabel A. Calvo icalvoa@unav.es

Email Addresses for All Authors:

Lorea Campos-Dopazo lcamposdopa@unav.es

Miguel Cócera mcocerafern@unav.es

Paula Aguirre-Ruiz paguirreruiz@unav.es

Purificación Ripalda-Cemborain pripalda@unav.es

Sarai Sarvide ssarvide@unav.es

Amaia Vilas-Zornoza avilaszo@unav.es

Ana Rosa López-Pérez alopez.1@unav.es

Emma Muiños-López emuiños@unav.es

David Gómez-Cabrero david.gomezcabrero@kaust.edu.sa

Felipe Prosper fprosper@unav.es

Isabel A. Calvo icalvoa@unav.es

Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. Filming location: Will the filming need to take place in multiple locations? **It will take place in different labs in the same building, on the same floor.**

If **Yes**, how far apart are the locations? Less than 100m

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

Current Protocol Length

Number of Steps: 20

Number of Shots: 46

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

The abbreviations are expanded in the answers, please check if they are all correct

INTRODUCTION:

REQUIRED: Summarize your research focus and the main questions it aims to answer.

- 1.1. **Lorea Campos-Dopazo:** Our bone marrow preparation method enables the spatial characterization of transcriptionally heterogeneous malignant plasma cells and their surrounding microenvironment *in situ*.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Lorea Campos-Dopazo:** Overcoming the challenges of working with mineralized bone tissue, our EDTA-based workflow preserves morphology and mRNA integrity for spatial transcriptomics.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Lorea Campos-Dopazo:** This protocol enables effective analysis of FFPE Bone marrow samples, providing spatial insights into multiple myeloma pathogenesis by mapping PCs and niche organization.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Lorea Campos-Dopazo:** The main challenge is achieving a proper decalcification, because under- or over-decalcification compromises morphology and RNA integrity, hindering spatial transcriptomic analysis.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Lorea Campos-Dopazo:** Future studies can use this protocol to investigate bone marrow cellular composition, spatial organization, and malignant niche interactions during disease progression and aging.
- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

NOTE: Testimonial questions were not recorded

Testimonial Questions (OPTIONAL):-

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

What is the key takeaway you hope viewers remember after watching your video article?

~~1.6. Lorea Campos Dopazo, PhD Candidate at CIMA Universidad de Navarra: (authors will present their testimonial statements live)~~

~~1.6.1. INTERVIEW: Named talent says the statement above in an interview style shot, looking slightly off camera~~

How does the video format of your article help you to share your work with the broader scientific community?

~~1.7. Lorea Campos Dopazo, PhD Candidate at CIMA Universidad de Navarra: (authors will present their testimonial statements live)~~

~~1.7.1. INTERVIEW: Named talent says the statement above in an interview style shot, looking slightly off camera~~

Authors: Could you please also deliver the above statements in Spanish?

Videographer: Please film the testimonials in both English and Spanish

Ethics Title Card

All the animal experiments have been approved by the Animal Ethics Committee at the University of Navarra

The human study has been approved by the Research Ethics Committees of the University of Navarra School of Medicine and the Navarra Health Service

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

Authors: Please let us know if you would use the human or the mouse bone on the shoot day?

Author's Response: If it is possible, we would like to use both, human and mouse bones, working in parallel with both samples.

Authors: We **CANNOT** film both bones in parallel for all the steps, as it will confuse the viewers. **Please use 1 bone for all the common steps.**

Use both bones for the following shots that differ according to the bone type:

Shot 2.1.1 (use both), 2.2.2 (use both), 3.6.1 (mouse femur only), 5.4.2 (human bone), 5.4.3 + 5.4.4 (mouse bone). For all the other steps, please demonstrate using 1 bone sample only

Author's NOTE: The protocol was performed using mouse sample only, except in the shots that differ according to the bone type.

2. Bone Sample Collection and Fixation

Demonstrators: Lorea Campos-Dopazo and Miguel Cócera

2.1. To begin, obtain mouse hind-limb bones and human bone samples in cold DPBS on ice [1-TXT].

2.1.1. WIDE: Talent placing the container with cleaned bone samples in DPBS on ice.
TXT: See the text protocol for bone isolation

2.2. Discard the DPBS [1] and place the bones into labeled individual plastic cassettes [2]. Close the lids securely to prevent tissue loss during processing [3].

2.2.1. Talent discarding DPBS from the container.

2.2.2. Talent placing a bone sample into a labeled plastic cassette.

2.2.3. Talent closing the cassette lid securely.

2.3. Under a chemical fume hood, add 4 percent buffered formaldehyde solution at a 1 to 20 sample-to-fixative ratio [1] and incubate for 24 hours at 4 degrees Celsius [2].

- 2.3.1. Talent adding 4 percent buffered formaldehyde solution in the chemical fume hood.
- 2.3.2. Talent placing the container at 4 degrees Celsius.

3. Bone Decalcification Using EDTA

- 3.1. Discard the formalin and add ultrapure water to the bone [1]. Briefly agitate the container, then remove the water to eliminate residual formalin [2].
 - 3.1.1. Talent discarding the formalin and adding ultrapure water to the container holding the bone sample.
 - 3.1.2. Talent agitating the container and discarding the water.
- 3.2. Prepare a 0.25 molar EDTA solution in ultrapure water, and adjust the pH to 6.95 [1].
 - 3.2.1. Talent adding EDTA to water and mixing.
- 3.3. Add enough EDTA solution to completely immerse the bone [1]. Incubate the sample for 5 days at 4 degrees Celsius with gentle agitation [2]. **Authors:** I am saying 5 days here to maintain a flow. So, we say incubate for 5 days, change EDTA, and then incubate again for 5 more days (instead of saying incubate 10 days at the beginning, which can be a bit confusing).
 - 3.3.1. Talent adding EDTA to the bone sample to completely immerse it.
 - 3.3.2. WIDE: Talent placing the container on the shaker at 4 degrees Celsius.
- 3.4. After 5 days, replace the EDTA solution with the same volume of fresh EDTA to maintain chelating capacity [1]. Continue the incubation under the same conditions for 5 more days [2].
 - 3.4.1. Talent discarding the used EDTA and adding fresh EDTA solution to the container.
 - 3.4.2. WIDE: Talent returning the container to the shaker at 4 degrees Celsius.
Author's NOTE: We placed it on a shaker that is in a room that is at 4°C. This involves all the step that require agitation at 4°C.
- 3.5. After complete decalcification, discard the EDTA solution [1] and place the bone on a sterile Petri dish [2]. Insert a fine sterile needle into a peripheral area away from the region of interest. If the needle passes through with minimal resistance, the sample is adequately decalcified [3].

- 3.5.1. Talent discarding the EDTA solution from the container.
- 3.5.2. Talent placing the sample in a sterile petri dish.
- 3.5.3. Close-up of Talent inserting a fine sterile needle into a peripheral region of the bone on the Petri dish.

Authors: Please specify which of the 2 checks described here you would prefer, the scalpel cut or the needle insertion, so that we can film the correct one.

Author's Response: We will perform the needle insertion method.

- 3.6. For mouse femurs, gently bend the bone with forceps. Increased flexibility indicates adequate mineral removal [1].

- 3.6.1. Close-up of Talent bending the decalcified mouse femur with forceps.

Authors: If the mouse femur is not used on the shoot day, we can delete this step.

Author's Response: We will also use mouse bone samples.

4. Dehydration of the Decalcified Bone

- 4.1. Wash the decalcified bone by adding ultrapure water to the container [1], then incubate for 5 minutes at 4 degrees Celsius with agitation [2].
 - 4.1.1. Talent adding water to the sample container.
 - 4.1.2. Talent placing the container on the shaker at 4 degrees Celsius.
- 4.2. Discard the water and replace it with 70 percent ethanol [1]. Incubate for 1 hour at 4 degrees Celsius [2]. Continue dehydration in 80% and 96% ethanol for 1 hour each at 4 degrees Celsius. Then, incubate the sample in 100% ethanol overnight [3].
 - 4.2.1. Talent discarding water and adding 70 percent ethanol to the container.
 - 4.2.2. Talent placing the sample at 4 degrees Celsius.
 - 4.2.3. Talent discarding old ethanol and adding fresh ethanol from a labeled container.

Authors: The sample is first incubated with 70% ethanol for 1 hour in step 1.5.2 of the manuscript, then again it is incubated with 70, 80 and 90% ethanol as per step 1.5.3 of the manuscript. Is the incubation in 70% ethanol done twice? Although the Figure 1 schematic shows only 1-time incubation in 70% ethanol. Please clarify.

Author's Response: The 70% ethanol incubation is performed only once.

- 4.3. Then, discard the 100 percent ethanol and replace it with xylene. Incubate for 4 hours at room temperature [1]. After incubation, remove the xylene [2].
 - 4.3.1. Talent discarding ethanol and adding xylene to the container inside the chemical fume hood and placing the sample aside.
 - 4.3.2. Talent discarding the xylene.

Authors: All the ethanol and xylene steps must be performed inside a chemical fume hood.

Videographer: Please ensure the fume hood is visible in at least some of the frames

5. Paraffin Infiltration and Embedding

Demonstrator: Lorea Campos-Dopazo

- 5.1. Place the sample cassette in a glass containing melted paraffin [1] and incubate overnight at 60 degrees Celsius for thorough infiltration [2].
 - 5.1.1. Talent placing the sample cassette in a glass containing melted paraffin.
Author's NOTE: 5.1.1 and 5.1.2 were filmed together.
 - 5.1.2. Talent placing the sample in the 60 degrees Celsius oven for paraffin infiltration.
- 5.2. Clean the surfaces of the embedding station with an RNase decontamination solution [1]. Preheat the paraffin reservoir, cassette, mold paraffin baths, and mold warming plate to 60 degrees Celsius [2]. Then, activate the cold plate and allow it to cool [3].
 - 5.2.1. Talent wiping the surfaces of the embedding station with RNase decontamination solution.
 - 5.2.2. Talent setting the embedding station temperatures to 60 degrees Celsius.
 - 5.2.3. Talent activating the cold plate.
- 5.3. Next, place the cassette containing the infiltrated sample in the paraffin bath, and incubate for 2 hours to remove residual xylene [1]. Then, place all the required molds in the designated paraffin bath [2].
 - 5.3.1. Talent picking up the sample cassette and placing it in the cassette paraffin bath.
 - 5.3.2. Talent placing the metal and the plastic molds in the mold paraffin bath.

Authors: The note specifies standard metal molds for human samples and Visium-compatible plastic molds for mouse samples, and states that whole mouse bones must be

cut immediately before embedding. Please confirm which you would show on the shoot day, and whether the cutting step should be added.

Author's Response: We will perform it using plastic molds for mouse samples and metal molds for human samples. The sectioning of the mouse bones will be required at this step.

- 5.4. Dispense melted paraffin from the embedding station to form a thin base layer in the mold [1]. Open the cassette and gently transfer the human bone sample into the metal mold using warm forceps [2]. Using a sterile scalpel, cut the whole mouse bone into appropriately sized fragments [3] and place them in a square plastic mold compatible with spatial transcriptomics slides [4].
 - 5.4.1. Close-up of Talent dispensing a thin base layer of paraffin into the mold.
 - 5.4.2. Close-up of Talent opening the cassette and transferring the human bone fragment into the metal mold with warm forceps.
 - 5.4.3. Talent cutting the mouse bone into fragments with a scalpel.
 - 5.4.4. Talent placing the mouse bone fragments into plastic mold.
- 5.5. Place the paraffin-filled mold on the warming plate [1]. While the paraffin remains liquid, use warm forceps to orient the sample in the desired cutting plane [2]. Transfer the mold to the cold surface of the embedding station to hold the bone fragment in position [2].
 - 5.5.1. Talent placing the paraffin-filled mold on the mold-warming plate.
 - 5.5.2. Close-up of Talent orienting the bone fragment in the mold with warm forceps.
 - 5.5.3. Talent placing the mold on the cold surface of the embedding station.
- 5.6. Fill the mold with melted paraffin until the tissue is completely covered. Ensure that no large bubbles remain [1]. Position the labeled cassette on top of the mold so that it forms the base of the paraffin block [2].
 - 5.6.1. Close-up of Talent filling the mold with melted paraffin, avoiding bubbles.
 - 5.6.2. Talent positioning the labeled cassette on top of the mold.
- 5.7. Transfer the mold and cassette to the cold plate for 10 minutes, until the paraffin block is opaque and firm [1]. Then, remove the paraffin block from the mold [2], and store it at 4 degrees Celsius until sectioning [3].
 - 5.7.1. Talent transferring the mold to the cold plate of the embedding station.

5.7.2. Close-up of Talent removing the solidified paraffin block from the mold.

5.7.3. Talent placing the sample in a refrigerator.

Authors: I added the following step with representative shots to provide full context for the results, per our journal guidelines. Please check if the narration is correct.

5.8. Following paraffin embedding, section the tissues to obtain 5- and 20-micrometer-thick samples [1]. Assess tissue morphology and integrity by hematoxylin and eosin staining [2], and optionally perform immunofluorescence imaging to verify specific cell types [3]. Finally, process suitable sections for spatial transcriptomics and subsequent data analysis [4-TXT].

5.8.1. Shot of sectioning in tissue.

5.8.2. Talent adding the stain on the sample slide.

5.8.3. Talent placing the sample under a fluorescence microscope.

5.8.4. Talent placing the sample on CytAssist for spatial transcriptomics. **TXT: See text protocol for spatial transcriptomics**

Results

6. Results

- 6.1. Successful EDTA-based decalcification was indicated by increased flexibility of the mouse femur [1] and effortless needle penetration into the human bone sample [2]. Both samples were then embedded successfully in paraffin [3].
 - 6.1.1. LAB MEDIA: Figure 2ABDE *Video editor: Please emphasize B*
 - 6.1.2. LAB MEDIA: Figure 2ABDE. *Video editor: Please emphasize E*
 - 6.1.3. LAB MEDIA: Figure 2C and Figure 2F. *Video editor: Please emphasize the embedded bone fragment in each panel*
- 6.2. Hematoxylin and eosin staining showed better-preserved marrow architecture in the mouse sections [1], whereas both human samples showed structural limitations that may reflect differences in pre-analytical handling and processing [2].
 - 6.2.1. LAB MEDIA: Figure 3A and Figure 3B.
 - 6.2.2. LAB MEDIA: Figure 3C and Figure 3D.
- 6.3. In the EDTA-processed mouse multiple myeloma tissue, Endomucin staining identified well-defined vascular structures [1], while CD271 staining demonstrated preserved mesenchymal stromal networks [2].
 - 6.3.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the images showing red fluorescence.*
 - 6.3.2. LAB MEDIA: Figure 4A. *Video editor: Highlight the image showing green signals.*
- 6.4. In healthy elderly human tissue, the endothelial cells were detected by CD31 [1] and the mesenchymal cells by PRRX1 [2], suggesting that the tested niche markers remained identifiable after EDTA decalcification [3].
 - 6.4.1. LAB MEDIA: Figure 4B. *Video editor: Highlight the image showing red signals.*
 - 6.4.2. LAB MEDIA: Figure 4B. *Video editor: Highlight the image showing green signals.*
 - 6.4.3. LAB MEDIA: Figure 4B
- 6.5. The degraded, acid-decalcified human multiple myeloma specimens had limited downstream utility and were used for baseline transcriptomic profiling and

immunohistochemical detection of CD138-positive malignant plasma cells [1].

6.5.1. LAB MEDIA: Figure 4C.

1. Formaldehyde
Pronunciation link: <https://www.merriam-webster.com/dictionary/formaldehyde>
IPA: /fɔr'mældəˌhɑd/
Phonetic Spelling: for·mal·duh·hyde
2. Decalcification
Pronunciation link: <https://www.merriam-webster.com/dictionary/decalcification>
IPA: /diˌkælsəfəˈkeɪʃən/
Phonetic Spelling: dee·kal·suh·fuh·kay·shuhn
3. EDTA
Pronunciation link: <https://www.merriam-webster.com/dictionary/EDTA>
IPA: /ˌiːˌdiːˌtiːˈeɪ/
Phonetic Spelling: ee·dee·tee·ay
4. Molar
Pronunciation link: <https://www.merriam-webster.com/dictionary/molar>
IPA: /ˈmoʊlər/
Phonetic Spelling: moh·ler
5. Chelating
Pronunciation link: <https://www.merriam-webster.com/dictionary/chelate>
IPA: /ˈkiːleɪtɪŋ/
Phonetic Spelling: kee·lay·ting
6. Femur
Pronunciation link: <https://www.merriam-webster.com/dictionary/femur>
IPA: /ˈfiːməɹ/
Phonetic Spelling: fee·mer
7. Ethanol
Pronunciation link: <https://www.merriam-webster.com/dictionary/ethanol>
IPA: /ˈɛθəˌnɔl/
Phonetic Spelling: eth·uh·nawl
8. Xylene
Pronunciation link: <https://www.merriam-webster.com/dictionary/xylene>
IPA: /ˈzaɪˌliːn/
Phonetic Spelling: zy·leen
9. Paraffin
Pronunciation link: <https://www.merriam-webster.com/dictionary/paraffin>
IPA: /ˈpærəfɪn/
Phonetic Spelling: pair·uh·fin
10. RNase
Pronunciation link: No confirmed link found

- IPA: /'ɑr, ɛn, eɪs/
Phonetic Spelling: ar·en·ays
11. Visium
Pronunciation link: No confirmed link found
IPA: /'vɪziəm/
Phonetic Spelling: viz·ee·uhm
12. Transcriptomics
Pronunciation link: No confirmed link found
IPA: /,træn,skrɪp'tamɪks/
Phonetic Spelling: tran·skrip·tah·miks
13. Micrometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
IPA: /maɪ'kræmətər/
Phonetic Spelling: my·krah·muh·ter
14. Hematoxylin
Pronunciation link: <https://www.merriam-webster.com/dictionary/hematoxylin>
IPA: /,hi:mə'tæksəlɪn/
Phonetic Spelling: hee·muh·tok·suh·lin
15. Eosin
Pronunciation link: <https://www.merriam-webster.com/dictionary/eosin>
IPA: /'i:əsɪn/
Phonetic Spelling: ee·uh·sin
16. Immunofluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/immunofluorescence>
IPA: /,ɪmjənoʊ'flʊə'reɪsəns/
Phonetic Spelling: im·yuh·noh·floor·ess·uhns
17. Myeloma
Pronunciation link: <https://www.merriam-webster.com/dictionary/myeloma>
IPA: /,maɪə'loʊmə/
Phonetic Spelling: my·uh·loh·muh
18. Endomucin
Pronunciation link: No confirmed link found
IPA: /,ɛndoʊ'mju:sɪn/
Phonetic Spelling: en·doh·myoo·sin
19. Vascular
Pronunciation link: <https://www.merriam-webster.com/dictionary/vascular>
IPA: /'væskjələr/
Phonetic Spelling: vas·kyuh·ler
20. Mesenchymal
Pronunciation link: <https://www.merriam-webster.com/dictionary/mesenchymal>
IPA: /mɛ'zɛŋkəməl/
Phonetic Spelling: meh·zeng·kuh·muhl
21. Stromal
Pronunciation link: <https://www.merriam-webster.com/dictionary/stromal>

- IPA: /'stroʊmə/
- Phonetic Spelling: stroh·muhl
22. Endothelial
- Pronunciation link: <https://www.merriam-webster.com/dictionary/endothelial>
- IPA: /,ɛndəʊ'θi:liəl/
- Phonetic Spelling: en·doh·thee·lee·uhl
23. PRRX1
- Pronunciation link: No confirmed link found
- IPA: /,pi:,ɑr,ɑr'ɛks wʌn/
- Phonetic Spelling: pee·ar·ar·eks·wun
24. Transcriptomic
- Pronunciation link: No confirmed link found
- IPA: /,træn,skrip'təmɪk/
- Phonetic Spelling: tran·skrip·tah·mik
25. Immunohistochemical
- Pronunciation link: <https://www.merriam-webster.com/dictionary/immunohistochemistry>
- IPA: /,ɪmjənoʊ,hɪstoʊ'kɛmɪkəl/
- Phonetic Spelling: im·yuh·noh·his·toh·kem·ih·kuhl
26. Malignant
- Pronunciation link: <https://www.merriam-webster.com/dictionary/malignant>
- IPA: /mə'liɡnənt/
- Phonetic Spelling: muh·lig·nuhnt
27. Plasma
- Pronunciation link: <https://www.merriam-webster.com/dictionary/plasma>
- IPA: /'plæzmə/
- Phonetic Spelling: plaz·muh