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Title: Multimodal Approach to Assess Bone Regeneration and Scaffold Performance

Authors and Affiliations:

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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? **No**

- 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: 38

Introduction

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**.

INTRODUCTION:

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

- 1.1. **Camila Wendt:** Our research evaluates how different biomaterials influence bone architecture and structural organization during the regeneration process.
 - 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. **Camila Wendt:** Bone regeneration requires multiscale evaluation, as bone is hierarchically organized; this protocol enables integrated structural analysis across length scales.
 - 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., Marcos Farina:** This multimodal workflow provides complementary structural insights that cannot be achieved using a single imaging technique alone.
 - 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. **Marcos Farina:** This workflow requires significant time, technical expertise in equipment operation, and access to specialized instrumentation.

- 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. **Marcos Farina:** Future studies may investigate functional correlations, long-term bone remodeling and optimize biomaterial design.
 - 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.

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**.

NOTE: Authors have added the following answers after their shoot in English and Portuguese

What problem in your field of research does your JoVE article address?

- 1.6. **Camila Wendt, Post-Doctoral Researcher:** (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
 - 1.6.2. Our article presents a workflow for multimodal analysis of mineralized samples that can be adapted to biomaterials, bone, and other biominerals such as mollusk shells and sponge spicules.
 - 1.6.3. Nosso artigo apresenta um fluxo de trabalho para análise multimodal de amostras mineralizadas que pode ser adaptado para biomateriais, osso e outros biominerais, como conchas de moluscos e espículas de esponjas.

What value do you expect this video article to have for other researchers working in your field?

- 1.7. **Camila Wendt, Post-Doctoral Researcher:** (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
 - 1.7.2. This video presents a workflow that integrates the observation of the same sample using different imaging approaches, enabling visualization across multiple length scales.
 - 1.7.3. Este vídeo apresenta um fluxo de trabalho que integra a observação da mesma amostra por diferentes técnicas de imagem, permitindo sua visualização em múltiplas escalas.

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

Videographer: Please film the testimonials in both English and Portuguese

Protocol

2. Sectioning the Resin-Embedded Bone Containing the Implanted Biomaterial

Demonstrator: Camila Wendt

2.1. To begin, position the fixated and resin-embedded tibia containing the implanted biomaterial on the worksurface and orient it as required to select the sectioning direction [1-TXT].

2.1.1. WIDE: Talent placing the fixated-resin-embedded bone sample on the work surface and adjusting orientation. **TXT: Section either transversely or longitudinally as required** **Author's NOTE:** As the procedure following sample attachment and adjustment is linear and fast, the steps 2.1, 2.2, 2.3, 2.4 and 2.5 were recorded together. In the following sections, close-up shots of the same process were captured.

2.2. Obtain a low-speed diamond saw disk to section the bone into approximately 0.7-millimeter slices [1].

2.2.1. Talent positioning the sample on the diamond saw disk

2.3. Fill the liquid reservoir until the fluid level completely covers the bottom edge of the blade [1].

Added shot: Overview filming of the complete process regarding sample positioning
Video editor: I think this covers 2.1 to 2.4 steps, in close-ups. Use the footage as needed according to the shot descriptions

2.3.1. Talent pouring liquid into the reservoir and checking that the blade edge is submerged.

2.4. Now, secure the sample to the specimen holder and attach it to the instrument arm [1]. Ensure the sample is stable and horizontally aligned with the disk surface [2]. Adjust the specimen position using the positioning knobs until the target cut region is aligned with the blade [3].

2.4.1. Talent placing the sample into the holder and locking it in place.

2.4.2. Talent pointing to the alignment of the sample surface relative to the saw disk.

2.4.3. Talent adjusting the knobs to precisely align the target region with the blade.

- 2.5. Begin cutting the sample using a higher speed setting and apply low pressure until an indentation forms [1]. Then, reduce the speed to complete the sectioning smoothly and minimize vibration [2].
 - 2.5.1. Talent turning the dial to increase saw speed and gently pressing the blade into the sample.
 - 2.5.2. Talent adjusting the speed setting lower and cutting smoothly.
- 2.6. Stop the saw immediately after the cut is complete [1].
 - 2.6.1. Talent pressing the stop button as the blade finishes the cut. **Author's NOTE:** The video is long; it can be shortened by focusing on the final section, highlighting sample slice detachment from the bulk specimen.
- 2.7. Now, remove the sample from the diamond saw disk [1] and examine the sample using a stereomicroscope [2]. ~~Begin at a low magnification such as 5x to 10x to inspect the entire specimen [2]. Then, increase the magnification to between 20x and 40x to identify the region of interest [3]. Select regions with high interface density between new bone and biomaterial for further analysis [4].~~ **NOTE: VO is adjusted**
Added shot: Talent removing the sample from the diamond saw disk
 - 2.7.1. Talent placing the sectioned sample under a stereomicroscope. **NOTE: This might have been slated as 2.7.2. and 2.7.2-bis is the close up video of the sample**
 - 2.7.2. ~~Talent adjusting magnification of the scope~~
 - 2.7.3. ~~Talent viewing the sample through the scope.~~
 - 2.7.4. LAB MEDIA: Figure 5B

3. Mounting the Sectioned Bone Sample for Thinning

Demonstrator: Marcos Farina

- 3.1. For mounting, use a homemade assembly consisting of an inner solid stainless-steel cylinder and an outer hollow cylinder that provides mechanical support during polishing [1].
 - 3.1.1. Talent showing the inner solid and the outer hollow cylinder components side-by-side.
- 3.2. Next, place the inner cylinder on a hot plate and heat it to approximately 120 to 140

degrees Celsius [1].

3.2.1. Talent placing the inner aluminum cylinder on a hot plate and turning on the heat setting.

3.3. Choose a suitable wax [1] and apply a small amount of wax onto the surface of the heated cylinder and allow it to melt completely [2].

Added shot: Talent showing the wax box and picking up the wax

3.3.1. Talent adding wax to the cylinder surface.

3.4. Then, place the sample with the surface of interest facing down onto the wax-coated area [1] and gently press the sample to ensure full contact with the melted wax [2].

3.4.1. Talent positioning the sample face-down onto the melted wax.

3.4.2. Talent pressing gently to secure even contact with the wax surface.

Author's NOTE: The item 3.4.2 was filmed together with the 3.4.1

3.5. Now, remove the aluminum cylinder from the hot plate and allow the wax to solidify at room temperature [1].

3.5.1. Talent lifting the cylinder from the hot plate and setting it aside to cool.

4. Thinning and Polishing the Mounted Bone Sections

Demonstrator: Camila Wendt

4.1. For coarse and intermediate polishing, select sandpaper with grit sizes of 1200, 2500, and 4100 to reduce the sample thickness to approximately 0.15 millimeters [1].

4.1.1. Talent selecting sandpapers of increasing grit size and placing them on the polishing station.

4.1. Start with 1200 grit sandpaper, followed by 2500 grit while applying light pressure [1]. Periodically monitor the sample thickness during polishing using reflection microscopy [2].

4.1.1. Talent polishing the sample with 1200 grit sandpaper and switching to 2500 grit.

4.1.2. Talent viewing the sample through a reflection microscope.

4.2. Once the target thickness is reached, polish both sides of the sample using 4100 grit sandpaper [1]. Continuously clean the sample with distilled water during each polishing step [2].

4.2.1. Talent polishing the sample using 4100 grit sandpaper.

4.2.2. Talent rinsing the sample with distilled water during polishing. **NOTE: The item 4.3.2 was filmed together with 4.3.1**

4.3. For final polishing, choose a diamond paste with 6 or 3-micrometer particle size based on the surface roughness of the sample [1]. Apply a small amount of diamond paste onto a felt polishing cloth mounted on a polishing disc [2].

4.3.1. Talent looking at the sample and picking up an appropriate paste from the table.

4.3.2. Talent applying diamond paste to a felt cloth on the polishing disc. **NOTE: The item 4.4.2 was filmed together with 4.4.1**

4.4. To detach the sample from the cylinder, warm the wax on a hot plate [1]. After removing the unit from the hot plate [2], take the sample out from the cylinder [3].

4.4.1. Talent placing the mounted sample on a hot plate to soften the wax.

4.4.2. Talent removing the cylinder from the hot plate

Added shot: Talent removing the sample from the cylinder

4.5. Now, gently polish both sides of the sample using minimal pressure [1] and dry the polished surface with filter paper prior to microscopy analysis. [2].

4.5.1. Talent gently polishing both sides of the sample.

4.5.2. Talent using filter paper to dry the sample surface. **NOTE: The action was modified**

4.6. Mount the sample between a glass slide and coverslip using immersion oil as the mounting medium [1]. Examine collagen fiber orientation using polarized light microscopy, starting with low magnification between 5x and 10x [2].

~~Increase the intermediate magnification between 20 and 40x to locate regions for potential electron microscopy analysis [3].~~ Finally, prepare the sample for scanning electron microscopy [3] to further analyse the bone-biomaterial interface [4].

4.6.1. Talent mounting the sample on a slide containing immersion oil and placing coverslip.

4.6.2. Talent placing the sample under a polarized light microscope.

NOTE: The item 4.7.2 was filmed together with 4.7.1

4.6.3. Talent ~~adjusting the magnification of the microscope~~ preparing the sample for scanning electron microscopy

4.6.4. Talent working at the electron microscopy unit (may be placing the sample or looking at the microscope collected system). **Author's NOTE:** 4.7.4 bis detailed filming of the sample positioning at the microscope chamber 4.7.4 bis bis close up filming of the microscope chamber.

Results

5. Results

5.1. After polishing and initial optical microscopy examination, intrinsic sample features allowed the region identified under light microscopy [1] to be matched to the corresponding location in the microcomputed tomography dataset [2].

5.1.1. LAB MEDIA: Figure 5B.

5.1.2. LAB MEDIA: Figure 5A.

5.2. Polarized light microscopy differentiated preexisting bone, shown in blue [1], from newly formed bone during healing, depicted in orange [2].

5.2.1. LAB MEDIA: Figure 5C. *Video editor: Highlight the blue-colored region representing the original bone.*

5.2.2. LAB MEDIA: Figure 5C. *Video editor: Highlight the orange region inside the yellow box.*

5.3. Higher magnification polarized light microscopy revealed an interface where bone grew in close apposition to preexisting cortical bone, with type 1 collagen fibers oriented perpendicular in a thin region [1].

5.3.1. LAB MEDIA: Figure 5D. *Video editor: Zoom in on the yellow dashed circle marking the bone interface area.*

5.4. Scanning electron microscopy imaging enabled precise localization of areas of interest [1].

5.4.1. LAB MEDIA: Figure 5E.

5.5. A volume of the selected region was further examined using slice-and-view tomography [1]. The face of the trench displayed bone morphology on a growing trabecula [2].

5.5.1. LAB MEDIA: Figure 5F.

5.5.2. LAB MEDIA: Figure 5G. *Video editor: Zoom in on the area inside the big grey rectangle*

5.6. Selected Area Electron Diffraction patterns indicated that both the biomaterial region [1] and the bone region were composed of hydroxyapatite crystals [2].

5.6.1. LAB MEDIA: Figure 8BC. *Video editor: Focus B*

5.6.2. LAB MEDIA: Figure 8BC. *Video editor: Focus C*

5.7. Energy-Dispersive X-ray Spectroscopy mapping of the imaged interface region [1] showed phosphorus [2] and calcium [3].

5.7.1. LAB MEDIA: Figure 8DEF. *Video editor: Focus D.*

5.7.2. LAB MEDIA: Figure 8DEF. *Video editor: Focus E.*

5.7.3. LAB MEDIA: Figure 8DEF. *Video editor: Focus F*

1. Sectioning
Pronunciation link: <https://www.merriam-webster.com/dictionary/sectioning>
IPA: /'sek.fə.nɪŋ/
Phonetic Spelling: sek·shuh·ning
2. Resin-embedded
Pronunciation link: <https://www.merriam-webster.com/dictionary/resin>
IPA: /'rez.ɪn/
Phonetic Spelling: rez·in
3. Tibia
Pronunciation link: <https://www.merriam-webster.com/dictionary/tibia>
IPA: /'tɪb.i.ə/
Phonetic Spelling: tib·ee·uh
4. Biomaterial
Pronunciation link: <https://www.merriam-webster.com/dictionary/biomaterial>
IPA: /ˌbaɪ.oʊ.mə'tɪr.i.əl/
Phonetic Spelling: bye·oh·muh·teer·ee·uhl
5. Transversely
Pronunciation link: <https://www.merriam-webster.com/dictionary/transversely>
IPA: /trænz'vɜ:s.li/
Phonetic Spelling: tranz·vers·lee
6. Longitudinally
Pronunciation link: <https://www.merriam-webster.com/dictionary/longitudinally>
IPA: /ˌlɑ:n.dʒə'tu:.dɒn.əl.i/
Phonetic Spelling: lon·juh·too·duh·nuh·lee

7. Reservoir
Pronunciation link: <https://www.merriam-webster.com/dictionary/reservoir>
IPA: /'rɛz.ə.vwɑ:r/
Phonetic Spelling: rez·er·vwahr
8. Specimen
Pronunciation link: <https://www.merriam-webster.com/dictionary/specimen>
IPA: /'spɛs.ə.mən/
Phonetic Spelling: spes·uh·muhn
9. Indentation
Pronunciation link: <https://www.merriam-webster.com/dictionary/indentation>
IPA: /,ɪn.dən'teɪ.fən/
Phonetic Spelling: in·den·tay·shuhn
10. Stereomicroscope
Pronunciation link: <https://www.merriam-webster.com/dictionary/stereomicroscope>
IPA: /,stɛr.i.oo'maɪ.krə'skoʊp/
Phonetic Spelling: ster·ee·oh·my·kruh·skohp
11. Magnification
Pronunciation link: <https://www.merriam-webster.com/dictionary/magnification>
IPA: /,mæg.nə.fɪ'keɪ.fən/
Phonetic Spelling: mag·nuh·fih·kay·shuhn
12. Assembly
Pronunciation link: <https://www.merriam-webster.com/dictionary/assembly>
IPA: /ə'sɛm.bli/
Phonetic Spelling: uh·sem·blee
13. Stainless-steel
Pronunciation link: <https://www.merriam-webster.com/dictionary/stainless>
IPA: /'stɛn.ləs/
Phonetic Spelling: stayn·luhs
14. Cylinder
Pronunciation link: <https://www.merriam-webster.com/dictionary/cylinder>
IPA: /'sɪl.ɪn.də/
Phonetic Spelling: सिल·in·der
15. Polishing
Pronunciation link: <https://www.merriam-webster.com/dictionary/polish>
IPA: /'pɑː.lɪʃ/
Phonetic Spelling: pah·lish
16. Reflection
Pronunciation link: <https://www.merriam-webster.com/dictionary/reflection>
IPA: /rɪ'flɛk.fən/
Phonetic Spelling: rih·flek·shuhn
17. Distilled
Pronunciation link: <https://www.merriam-webster.com/dictionary/distilled>
IPA: /dɪ'stɪld/
Phonetic Spelling: dih·stild

18. Immersion
Pronunciation link: <https://www.merriam-webster.com/dictionary/immersion>
IPA: /ɪˈmɜː.ʒən/
Phonetic Spelling: ih·mer·zhuhn
19. Coverslip
Pronunciation link: <https://www.merriam-webster.com/dictionary/coverslip>
IPA: /ˈkʌv.ə.slɪp/
Phonetic Spelling: kuv·er·slip
20. Polarized
Pronunciation link: <https://www.merriam-webster.com/dictionary/polarized>
IPA: /ˈpoʊ.lə.aɪzd/
Phonetic Spelling: poh·luh·ryzd
21. Microcomputed
Pronunciation link: No confirmed link found
IPA: /ˌmaɪ.kroʊ.kəmˈpjuː.tɪd/
Phonetic Spelling: my·kroh·kuhm·pyoo·tid
22. Tomography
Pronunciation link: <https://www.merriam-webster.com/dictionary/tomography>
IPA: /təˈmɑː.grə.fi/
Phonetic Spelling: tuh·maa·gruh·fee
23. Apposition
Pronunciation link: <https://www.merriam-webster.com/dictionary/apposition>
IPA: /ˌæp.əˈzɪʃ.ən/
Phonetic Spelling: ap·uh·zish·uhn
24. Trabecula
Pronunciation link: <https://www.merriam-webster.com/dictionary/trabecula>
IPA: /trəˈbɛk.jə.lə/
Phonetic Spelling: truh·bek·yuh·luh
25. Diffraction
Pronunciation link: <https://www.merriam-webster.com/dictionary/diffraction>
IPA: /dɪˈfræk.ʃən/
Phonetic Spelling: dih·frak·shuhn
26. Hydroxyapatite
Pronunciation link: <https://www.merriam-webster.com/dictionary/hydroxyapatite>
IPA: /haɪˌdrɑːk.siˈæp.ə.taɪt/
Phonetic Spelling: hy·drok·see·ap·uh·tyte
27. Spectroscopy
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectroscopy>
IPA: /spekˈtrɑː.skə.pi/
Phonetic Spelling: spek·traas·kuh·pee
28. Phosphorus
Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphorus>
IPA: /ˈfɑːs.fə.əs/
Phonetic Spelling: faahs·fer·uhs