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PROTOCOL

Multiplexed tape-stabilized cryohistology of mineralized large animal specimens

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Abbreviations: AP, alkaline phosphatase; IACUC, institutional animal care and use committee; OCT, optimal cutting temperature; PBS, phosphate-buffered saline; PVP, polyvinylpyrrolidone; ROI, region of interest; TB, Toluidine blue O; TMJ, temporomandibular joint; TRAP, tartrate-resistant phosphatase; Tris-HCl, Trizma® hydrochloride; UV, ultraviolet

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ABSTRACT

Tape-stabilized cryohistology is a powerful histological method to reinforce tissue samples during and after sectioning, enhancing the overall image quality. This technique has widely been applied to section mineralized small animal (*i.e.*, mice, rat, rabbit) specimens, but has only been sparsely implemented for large animal samples that have a greater tendency to tear due to their increased surface area. Here, we present an optimized protocol for tape-stabilized cryohistology of undecalcified minipig vertebral body, femoral head, and temporomandibular joint samples. This protocol further develops a pipeline for sequential staining and imaging of the tape-stabilized cryosections. Images from multiple rounds of staining (endogenous bone mineral labels, aligned collagen (polarized light), tartrate resistant phosphatase (TRAP), alkaline phosphatase (AP), and toluidine blue) are overlaid to provide insight into dynamic bone remodeling. Overall, the established multiplexed tape-stabilized cryohistology protocol provides step-by-step instructions and guidance to cryosection large, mineralized tissues, and maximize data output from a single histological section.

Keywords: mineralization, bone, cryohistology, large animal, tissue interface

BACKGROUND

Historically, histological assessment of mineralized tissues required decalcification prior to traditional paraffin or frozen sectioning. Decalcification was required in order to maintain tissue morphology during sectioning on a paraffin microtome or cryostat. The main limitation of sectioning decalcified tissues is that by removing the mineral from the tissue, key features of the biology and structure are lost. Alternatively, undecalcified specimens could be embedded in resin for plastic sectioning. While plastic sectioning maintains the mineral content, it is more time consuming, utilizes harsh solvents, and is expensive compared to either paraffin or frozen histology. Plastic (and paraffin) sectioning can also disrupt certain biological features within the tissue, yielding reduced signal of fluorescent proteins, attenuated activity of certain enzymes, and often requiring harsher antigen retrieval steps for immunostaining. To address many of these limitations, researchers have developed tape-stabilized cryosectioning techniques to maintain section morphology of mineralized tissues.

Tape-stabilized cryosectioning was first introduced with the CryoJane system (originally from Instrumedics (Hackensack, NJ), but now Leica Biosystems (Deer Park, IL), which uses an adhesive tape that can be adhered to the frozen tissue block on the cryostat [1]. The tape serves to maintain tissue quality during the sectioning process. The section is then transferred from the tape to special adhesive-coated slides (*i.e.*, tape transfer), while the tissue is still frozen. This technique was transformational, in that it allowed researchers to maintain biological features within the tissue while not sacrificing section quality. However, the CryoJane system had a few challenges. First, to purchase the system there was a substantial upfront equipment cost to purchase the system. Additionally, there were some complications associated with the tape transfer process requiring fine temperature control to successfully transfer the complete tissue section from the cryotape to the slide. In the early 2000's, Kawamoto developed an

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alternative adherent tape (*i.e.*, cryofilm) to stabilize cryosections [2]. Since the Kawamoto cryofilm is transparent and minimally autofluorescent, unlike the tape in the CryoJane system, it can be glued to a glass slide (with the tissue facing upward), followed by staining and imaging. With the cryofilm-tissue units glued to the slides, the coverslips can be easily removed without dislodging the section. Hence, the Kawamoto cryofilm allows for several rounds of staining and imaging of the same section. Pairing this process with automated tiled slide scanning microscopes, we and others developed a high-throughput multiplexed cryohistological platform to generate multilayer, high-content fluorescent and chromogenic images to assess cell/matrix morphology and map expression of specific genes and proteins on a per cell basis – all within the same section [3–7].

Within the field of orthopaedic research, tape-stabilized cryohistology is particularly useful for reinforcing soft tissue-to-bone interfaces with incongruous material properties [6,8,9]. Tissue interfaces are commonly investigated in tissue injury and repair studies [10–12], but notoriously challenging to cryosection. These challenges are exacerbated as tissue size increases. Tissues harvested from larger species (*e.g.*, human, cow, pig, goat, sheep, dog, horse) have a greater surface area for tearing and folding to occur, in comparison to rodent and rabbit tissues. Despite the utility of tape-stabilized cryohistology for large animal samples, only a few studies have applied this method to section such tissues [13–15].

In addition to improving section quality, tape-stabilized cryohistology could greatly benefit the large animal research community because this method maximizes data output while minimizing sample utilization. Due to housing and budget constraints, sample number is often limited in large animal studies, and samples are carefully divided between outcome measures [16]. A single tape-stabilized cryosection can be repeatedly stained, imaged, and washed, permitting the overlay of images from multiple staining rounds, as previously established with rodent tissues [3]. The overlaid images provide insight into the co-localization (or lack thereof) of deposited minerals, aligned collagen, matrix elements, and specific cell populations [6], which can be quantified [3,4,14,15].

To enable the widespread adoption of multiplexed tape-stabilized cryohistology within the large animal research community, this work establishes a detailed pipeline for mineralized sample harvest, preparation, sectioning, staining, and imaging (**Fig. 1A, B, C**). The established workflow is validated using vertebral body, femoral head, and temporomandibular joint samples from adult Yucatan minipigs. Prior to euthanasia, these animals received intravenous injections of xylenol orange and calcein green bone fluorochrome labels, administered two weeks apart [14]. The developed protocol enables the overlay of the incorporated mineral labels, aligned collagen (polarized light), tartrate resistant phosphatase (TRAP), alkaline phosphatase (AP), and toluidine blue staining on a single histological section. Most importantly, this protocol provides step-by-step instructions to create tear-free histological sections from large, mineralized samples, and perform sequential staining to maximize data output from these cost and time intensive large animal studies.

MATERIALS AND METHODS

Reagents

- ✓ 10% neutral buffered formalin (Sigma, Cat #HT501320)
- ✓ Sucrose (Sigma, Cat #S7903)
- ✓ Polyvinylpyrrolidone (PVP) (Sigma, Cat #P5288)
- ✓ Phosphate-buffered saline (PBS) (Fisher Scientific, Cat #14190136)
- ✓ Chitosan, high molecular weight (Sigma, Cat #419419)
- ✓ Glycerol (Fisher Scientific, Cat #15514011)
- ✓ TO-PRO™-3 Iodide (642/661) (Fisher Scientific, Cat #T3605)
- ✓ ELF 97 Phosphatase Substrate (Fisher Scientific, Cat #E6588)
- ✓ Sodium acetate anhydrous (Sigma, Cat #W302406)
- ✓ Sodium tartrate dibasic dihydrate (Sigma, Cat #71995)
- ✓ Glacial acetic acid (Sigma, Cat #695092)
- ✓ Vector® Blue Substrate Kit, Alkaline Phosphatase (AP) (Vector Labs, Cat #SK5300)
- ✓ Trizma® hydrochloride (Tris-HCl) (Sigma, Cat #T3253)
- ✓ Hoechst 33342 (ThermoFisher Scientific, Cat #H3570)
- ✓ Toluidine blue O (TB) (Electron Microscopy Sciences, Cat #22050)
- ✓ D-Fructose (Fisher Scientific, Cat #S25332A)

Supplies

- ✓ Tissue-Plus™ O.C.T. Compound (Fisher Scientific, Cat #23730571)
- ✓ Disposable base molds for macrocassettes (Fisher Scientific, Cat #5030334)
- ✓ Cryofilm (Section-Lab, Cat Cryofilm type 2C (10))
- ✓ Disposable microscope slides, plastic (Fisher Scientific, Cat #S67112A)
- ✓ Platinum line adhesive slides, glass (Electron Microscopy Sciences, Cat #71887-100)
- ✓ Platinum line coverglass, 24 × 50 mm (Electron Microscopy Sciences, Cat #71887-21)

Recipes

- ✓ Sucrose/PVP: 20% sucrose, 2% PVP dissolved in 1x PBS
- ✓ Chitosan adhesive: Dissolve 0.25 mL concentrated acetic acid in 100 mL deionized (DI) water (0.25% v/v). Dissolve 0.75 g of chitosan powder in 100 mL acetic acid solution. Stir solution overnight to dissolve. **NOTE:** Store solution at room temperature.
- ✓ TRAP buffer: 9.2 g sodium acetate anhydrous and 11.4 g of sodium tartrate dibasic dihydrate in 1000 mL DI

water. Adjust the pH to 4.2 with concentrated glacial acetic acid (1.5 – 2 mL). **NOTE:** Store solution at 4°C for up to 12 months.

- ✓ TRAP staining solution: Dilute the yellow fluorescent substrate 1:50 in the TRAP buffer.
- ✓ AP buffer: 100 mM Tris-HCl (pH 8 – 8.5).
- ✓ AP staining solution: 5 mL AP buffer, 80 µL Reagent 1, 80 µL Reagent 2, 45 µL Reagent 3 (Reagents denoted in Vector® Blue Substrate Kit).
- ✓ Toluidine blue stain: 0.025% (w/v) TB solution in DI water.
- ✓ Glycerol-based mounting medium: 50% glycerol, 50% 1x PBS.
- ✓ Fructose-based mounting medium: 30% fructose dissolved in DI water. **NOTE:** Heat at 60°C to dissolve.

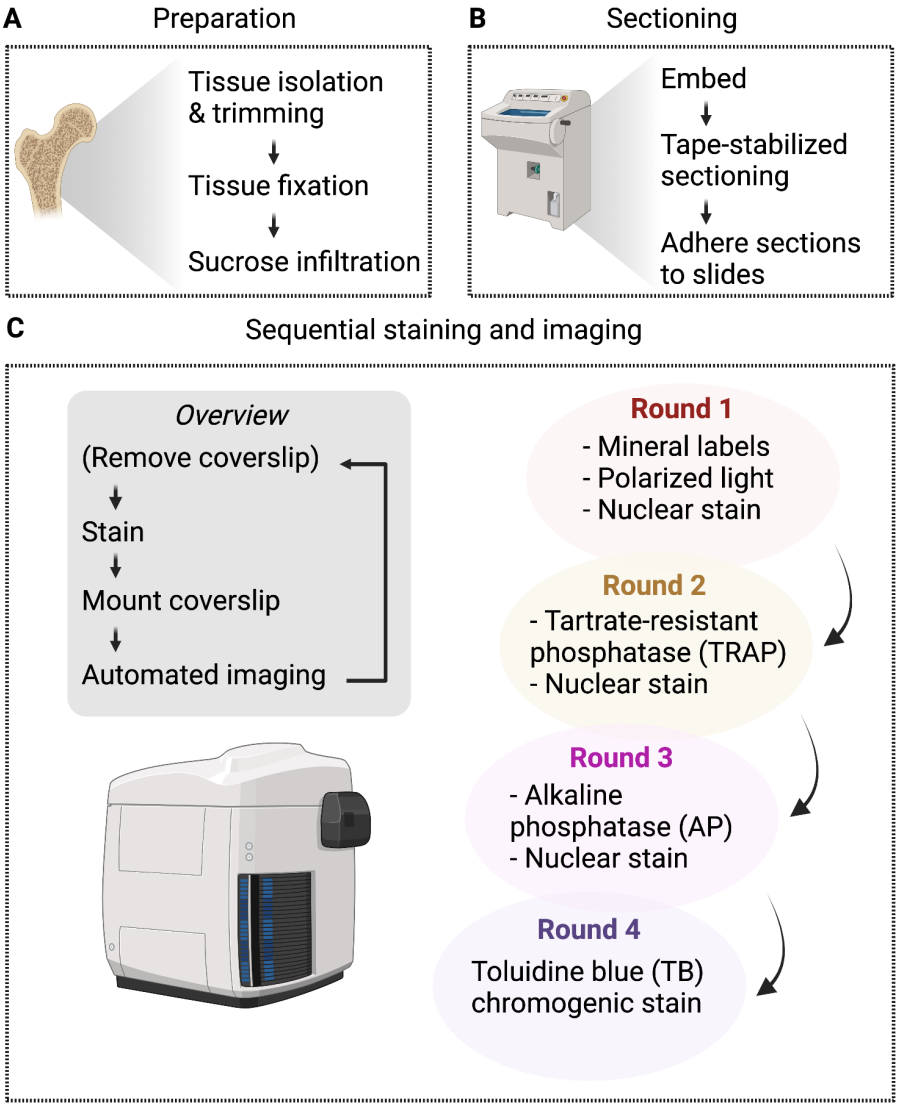

Figure 1 Overview of multiplexed tape-stabilized cryohistology. (A) Tissues are prepared for embedding and sectioning. **(B)** Trimmed tissues are embedded in cryo-embedding medium and sectioned using cryofilm. The following sections are glued to glass slides. **(C)** Sequential staining and imaging are performed using a slide scanner.

Equipment

- ✓ Electric saw
- ✓ Cryostat
- ✓ Handheld ultraviolet (UV) lamp
- ✓ Zeiss Axio Scan.Z1 digital slide scanner with Plan-Apochromat 10X/0.45 M27 objective
  - ○ Cameras:
    - ▪ Fluorescent camera (Zeiss Axiocam 506 mono)
    - ▪ Color camera (Hitachi Kokusai Electric America, Cat #HV-F202SCL)
  - ○ Filters:
    - ▪ DAPI/Hoechst 33342 filter (Chroma, Cat #49000 - ET - DAPI)

- Calcein filter (Chroma, Cat #49020 - ET - Narrow Band EGFP to Minimize Autofluorescence)
- Xylenol orange filter (Chroma, Cat #49004 - ET - CY3/TRITC)
- TO-PRO™-3 Iodide filter (Chroma, Cat #49009 - ET - Cy5 Narrow Excitation)
- TRAP filter (Chroma, ELF97 Custom Set_ AT350/50x T400lp AT550/40m)
- AP filter (Chroma, Chroma, Cat #49009 - ET - Cy5 Narrow Excitation)

Animals

✓ Yucatan minipig bones were collected from a prior IACUC-approved study at the University of Pennsylvania [14]. The animals (12-month-old castrated male minipigs) received intravenous injections of the mineral labels xylenol orange (4 weeks before euthanasia) and calcein green (2 weeks before euthanasia).

PROCEDURE

Sample fixation and embedding

1. Euthanize animals according to IACUC protocol.
2. Harvest the mineralized tissues of interest, remove unwanted soft tissues, and trim samples into manageable units using a handheld or electric saw (**Fig. 2**).

   NOTE: The maximum sample size is set by the cryomolds (37 × 24 × 10 mm). Smaller samples will be easier to section and handle.

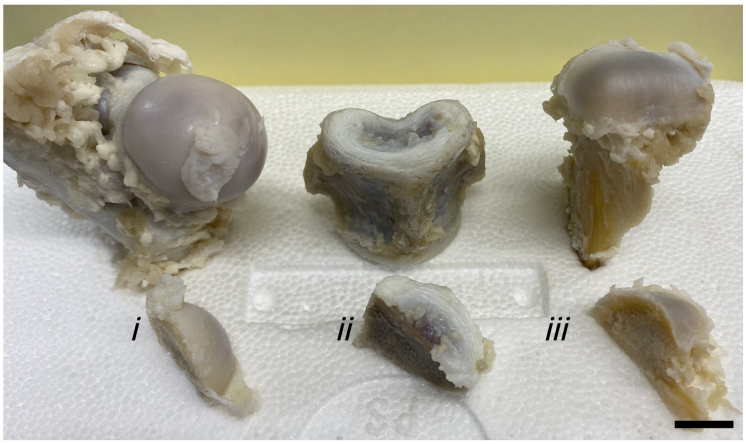

Figure 2 Representative harvested bones, and smaller trimmed bony units from one year old Yucatan minipigs. The smaller bony units were embedded for sectioning. i – Femoral head, ii – vertebral body, iii – temporomandibular joint (TMJ). Scale bar = 1 cm.

3. Place samples in 10% neutral buffered formalin at 4°C until properly fixed. Fix samples for 3–5 days.
4. Transfer specimens from formalin to sucrose/PVP solution for 7 days at 4°C.

   NOTE: The sucrose/PVP infiltration functions as a cryoprotectant and will make it easier to section the mineralized specimens, enhancing section quality.

5. Embed samples in Tissue-Plus™ O.C.T. Compound (cryo embedding medium), or other comparable polyvinyl alcohol/polyethylene glycol-based embedding mediums. Partially fill the cryomold with cryo embedding medium, and carefully place the intended cut plane parallel with bottom of mold.
6. Place the cryo mold on a base of dry ice to freeze the bottom of the sample, add more cryo embedding medium to fully cover the sample, and continue freezing on dry ice. Store samples at −20°C until use.

   NOTE: For long-term storage (greater than 1–2 months), store samples at −80°C and cover in plastic wrap to prevent them from drying out within the cryo embedding medium [3].

Tape-stabilized cryosectioning

7. Remove the samples from the freezer, and place in the cryostat with a temperature between −20°C and −25°C.
8. Remove the sample from the cryomold, and adhere to the specimen disc with cryo embedding medium. Wait at least 5 minutes for the embedding medium to freeze, 'gluing' the sample to the disc.
9. Load the specimen disc into the specimen head of the cryostat (**Fig. 3A**). Insert a disposable ceramic-coated blade into the cryostat. Adjust the specimen head so that the blade evenly cuts the sample block.
10. Trim the block using the manual cutting wheel until you reach the region of interest. Brush off the initial sections to create a smooth, clean block face.

    NOTE: Reduce the trim thickness and sectioning speed if the sample is difficult to cut.

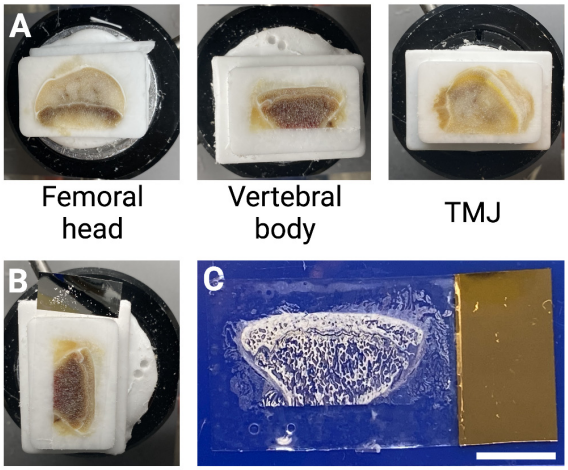

Figure 3 Tape-stabilized cryosectioning of mineralized large animal specimens. (A) The femoral head, vertebral body, and temporomandibular joint (TMJ) fit within the 37 × 24 × 10 mm cryo molds. **(B)** Cryofilm applied to the block face. **(C)** Tape-stabilized section of vertebral body collected on plastic slide (prior to gluing on glass slide). Scale bar = 12 mm.

11. Set the section thickness to 18 µm.

12. Cut a piece of cryofilm slightly bigger than the sample size. Keep cryofilm chilled in the cryostat.

13. Using chilled forceps, grab the silver/gold tab on the cryofilm and peel off the non-adherent backing. Place the sticky side of the cryofilm over the sample (**Fig. 3B**). Use a roller or paintbrush to apply pressure to the block face, further adhering the tape to the sample.

    NOTE: To prevent bubbles or folds in the tape, first place two corners of the cryofilm on the sample, and slowly lower the tape until the entire piece is stuck. The block face must be smooth for the tape to properly adhere. Avoid cutting the cryofilm larger than the block face.

14. Use forceps to grip the non-adherent gold tab of the cryofilm in one hand and use the manual cutting wheel to create an 18 µm section.

    NOTE: If you do not hold the cryofilm while sectioning, the sample may fall off the stage and curl up. Alternatively, you can use the plastic anti-roll plate to create flat sections.

15. Place the collected section on a plastic slide with the gold tab facing up (tissue side up) (**Fig. 3C**).

16. Repeat to acquire as many sections as desired.

17. Store sections at 4°C, −20°C, or −80°C depending on length of storage required. At 4°C, store for no longer than a month.

Adhesion of sections to glass slides

18. Remove a section from a plastic slide with forceps, and using scissors, carefully trim off the excess tape around the tissue. At minimum, trim off the gold/silver tab.

19. Pipette a drop of chitosan adhesive for each section onto a glass slide. Drop size varies (50–100 µL/section) based on section size.

20. Gradually lay the trimmed section TAPE SIDE DOWN onto the chitosan adhesive. The adhesive should cover the entire bottom of the sample to avoid bubbles between the slide and section.

21. Tilt the slide up on one of its long edges to allow excess chitosan adhesive to drain downward.

22. Place slides in this orientation on top of a paper towel in a slide box.

23. Place slide box with the lid propped open in the refrigerator for 2 days to allow the chitosan adhesive to fully dry.

NOTE: Drying at room temperature for ~24 hours can be used to expedite the drying process.

Staining and imaging Round 1: Mineral labels

NOTE: There are various fluorochrome labels (i.e. xylenol orange, calcein green, etc.) that can be intravenously injected into large animals [14,17]. These labels are incorporated into newly deposited mineral within 48 hours post-injection to track mineral deposition and apposition in bone and (fibro)cartilages. The mineral labels should be imaged first because the tartrate resistant phosphatase (TRAP) buffer used for TRAP staining will demineralize the sections, removing the fluorescent mineral labels. If your samples do not have mineral labels, skip this step, and proceed to the next stage.

24. Prepare glycerol-based mounting medium with TO-PRO™-3 Iodide nuclear counterstain (1:1000 dilution).

CAUTION: Hoechst 33342 or DAPI nuclear counterstain will interfere with subsequent TRAP staining using the ELF97 fluorescent substrate, so do not use that here.

25. Remove sections from the refrigerator and lay the slides flat so that the tissues face upward.

26. Hydrate the sections with PBS for 10 minutes.

NOTE: This step is important to reduce bubble formation in the subsequent coverslipping steps.

27. Remove PBS and cover the sections in the glycerol-based mounting medium (~500 µL/section).

28. Use the side of the pipette tip to evenly spread the mounting medium across the surface of the tissue.

29. Mount the coverslip. Tilt slides to remove excess glycerol and dry off the slides.

30. Load the slides into the microscope trays (**Fig. 4A**).

31. Insert the trays into the slide scanner (**Fig. 4B**), and close the outer door (**Fig. 4C**).

32. Select the appropriate scanning profile and exposure times for the fluorophores. You may also perform dark field imaging in this round to accentuate the mineralized tissue, and polarized light imaging to visualize aligned collagen.

33. Click preview scan to preview each slide.

34. Manually enter the slide names for each slide.

35. Use the tissue detection wizard to select a region of interest (ROI) on each slide, corresponding with the tissue. Save each ROI with the sample name. These ROI's will be reused for subsequent imaging rounds.

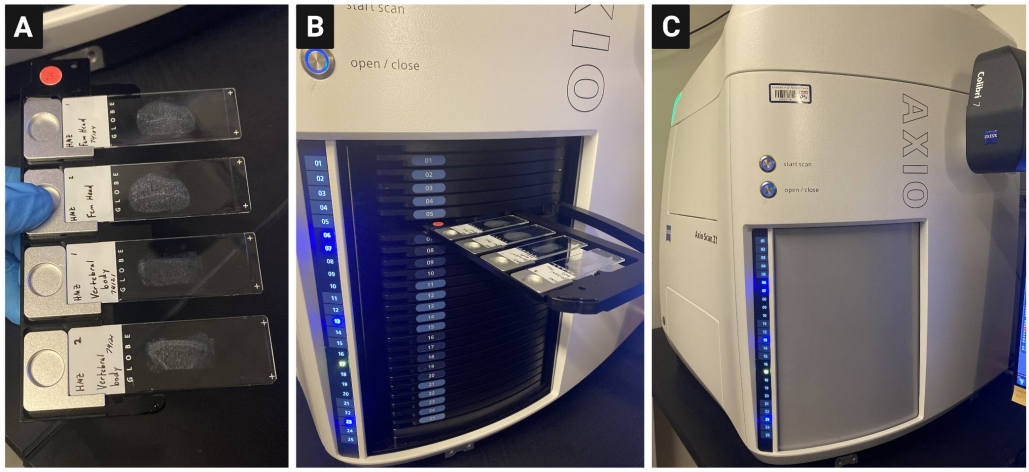

Figure 4 High-throughput imaging of mineralized cryosections. (A) Load slides into microscope trays. Each tray holds four slides. **(B)** Load trays into the machine. **(C)** Close the outer door and proceed to setup the scan in the Zeiss software.

36. Press the start scan button on the slide scanner to initiate scan.

Staining and imaging Round 2: Tartrate resistant phosphatase (TRAP) enzymatic activity

37. Submerge slides in a coplin jar filled with 1x PBS until the coverslips fall off.

38. Apply the TRAP buffer to each of the slides.

NOTE: The typical volume is ~500 µL to cover each section.

39. Check the slides under a fluorescent microscope to determine with the sections are sufficiently decalcified (*i.e.*, the mineral labels disappear). This usually takes 2 h 30 min at room temperature.

40. Prepare the TRAP staining solution by diluting the yellow fluorescent substrate in the TRAP buffer 1:50.

41. Apply the TRAP staining solution to the slides. Again, make sure to cover the entire tissue with the solution.

42. Place the slides under a UV light for 50 minutes.

43. Rinse slides with 1x PBS for 5 minutes 3 times.

44. Prepare glycerol-based mounting medium with TO-PRO™-3 Iodide nuclear counterstain (1:1000 dilution). Use this solution to coverslip the slides.

CAUTION: Do not use Hoechst 33342 as the nuclear counterstain here. The fluorescent signal will overlap with the custom yellow filter set.

45. Repeat Steps 30 – 36 to image the slides in the slide scanner. Load the saved region of interest for each respective slide from the previous imaging round. Image slides with the ELF97 filter (TRAP stain) and the Cy5 (TO-PRO™-3 Iodide nuclear counterstain) channel.

Staining and imaging Round 3: Alkaline phosphatase (AP) enzymatic activity

46. Submerge slides in a coplin jar filled with 1x PBS until the coverslips fall off.

NOTE: If you stained the slides with TO-PRO™-3 Iodide nuclear counterstain in Round 2, incubate the slides in the acidic TRAP buffer to remove this nuclear stain before proceeding. This will open the Cy5 channel for AP staining. Visually check the slides under the microscope to confirm that the nuclear stain has been washed away.

47. Prepare the AP buffer and the AP staining solution.

48. Incubate the slides for 20 min in the AP staining solution at room temperature.

49. Rinse the slides in 1x PBS for 5 min 3 times.

50. Prepare glycerol-based mounting medium with Hoechst 33342 nuclear counterstain (1:1000 dilution). Use this solution to coverslip the slides.

51. Repeat steps 30 – 36 to image the slides in the slide scanner. Load the saved region of interest for each respective slide from the previous imaging round. Image the DAPI (Hoechst stain) and Cy5 (AP stain) channels.

Staining and imaging Round 4: Toluidine blue (TB)

52. Submerge slides in a coplin jar filled with DI water until the coverslips fall off.

53. Rinse the slides DI water for 5 min 3 times to sufficiently remove the PBS.

NOTE: Salts will cause the following TB stain to leach out, so it is important to rinse with DI water before staining.

54. Prepare the TB stain in DI water.

55. Incubate slides for 2 min in the TB solution.

56. Remove TB and rinse the slides DI water for 5 min 3 times.

57. Prepare fructose-based mounting medium. Use this solution to coverslip the slides.

58. Repeat steps 30 – 36 to image the slides in the slide scanner using a brightfield profile. Load the saved region of interest for each respective slide from the previous imaging round.

Image export

59. Open the .czi files for a given sample from each round of imaging in the Zen software.

60. For the 'Round 1' image, set a region of interest that you are interested in exporting. Save this ROI in the Zen software.

61. Load the saved ROI onto the images from Rounds 2 – 4.

NOTE: Depending on your camera setup, you will likely have to shift the ROI on the 'Round 4' (chromogenic) image to roughly match the fluorescent images. Fine corrections will be made later during image overlay.

62. Export the desired ROI's as .jpg files. Create single channel files for each image (not composite images). Rename each image file with an appropriate description, *i.e.*, Animal1_VertebralBody_DarkField.

Image overlay

63. Using Affinity Photo, create a 'New Stack', and open the single channel exported ROI .jpg files from all rounds of imaging.

NOTE: Other imaging software programs that can handle multiple layers (e.g., Photoshop, GIMP, Pixelmator, etc.) can also be used.

64. Align images: Check 'Automatically Align Images' which will reposition the images to align them.

65. Create layers: In the right-hand menu under 'Layers' there will be a 'Live Stack Group'. Right-click on the 'Live Stack Group' and click 'Ungroup' to split each image file into a layer.

66. Select all the layers and click on the blend mode dropdown to then click 'Screen'. This feature will let you see through each layer.

67. If you would like to overlay the fluorescent layers onto the TB layer, change the opacity of the TB layer to 25% – 40%.

If your color and fluorescent cameras have different resolutions, you will need to transform the color camera image. The scaling per pixel information can be found by opening the image in the Zen software and clicking 'Info'. To determine the scale factor, divide the color camera resolution by the fluorescent camera resolution. Multiply by 100 to get a %.

68. Transform TB layer: In Affinity Pro, select the TB layer, and click the lock icon to lock the aspect ratio, and type *=percentage% in W: text box, and press 'Enter'.

69. Manually adjust layers from the different round if they are not properly aligned.

70. Click 'File' > 'Export' to export the image as a Photoshop file (.psd), .tif, .jpg, or .png.

Anticipated results

Multiplexed tape-stabilized cryohistology creates tear-free sections from large, mineralized specimens, and provides insights into mineral dynamics of bone, fibrocartilage, and cartilage. Toluidine blue staining (**Fig. 5A, 6A, 7A**) highlights both the overall cartilaginous and bony structure of the samples. This chromogenic stain also serves as a background image for later image overlay, if so desired.

For the first round of fluorescent imaging, aligned collagen can be visualized using polarized light (**Fig. 5B, 6B, 7B**, *cyan*). There is the greatest polarized light signal in dense fibrous tissues. For example, at the head of the femur, the ligamentum teres insertion site into bone shows highly aligned collagen, as seen in the first image inlay (**Fig. 5B**).

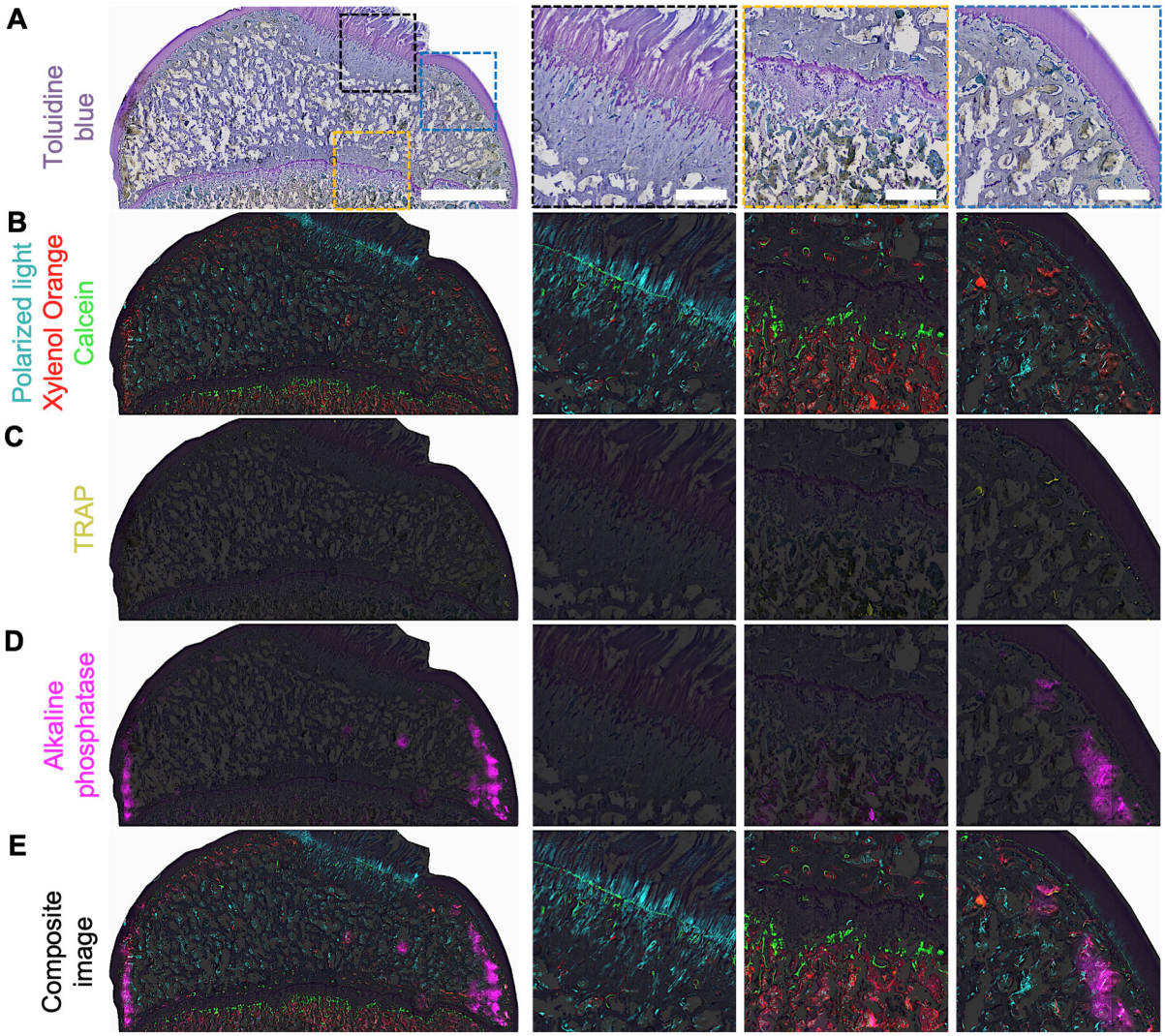

Figure 5 Multiplexed cryohistology of minipig femoral head. (A) Toluidine blue chromogenic staining. Full image scale bar = 5 mm. Zoom-in images scale bars = 1 mm. **(B)** Polarized light (cyan), xylenol orange (red), calcein (green) overlaid onto toluidine blue image. **(C)** Tartrate-resistant phosphatase (TRAP) (yellow) enzymatic staining overlaid onto toluidine blue image. **(D)** Alkaline phosphatase (AP) (magenta) enzymatic staining overlaid onto toluidine blue image. **(E)** Composite image.

Also, within the first round of fluorescent imaging, any endogenous bone mineral labels

are captured (**Fig. 5B, 6B, 7B**, *red & green*), before section decalcification in the subsequent staining steps. Bone mineral labels, or fluorochrome labels, commonly include xylenol orange, calcein green, alizarin red, tetracycline, and demeclocycline [17]. These fluorescent labels are incorporated into newly formed bone within 48 hours post-injection. Therefore, label uptake (or label signal) is dependent on animal age (growth rate), animal health (*i.e.*, injury, bisphosphonate usage, etc.), and the skeletal location (*i.e.*, mechanical and metabolic demands). Skeletally mature animals without any bony injury may have minimal label uptake. The animals used in this study were approaching skeletal maturity, as reflected by the reduced mineral uptake in the femoral head and vertebral body samples (**Fig. 5B, 6B**). However, in the temporomandibular joint sample, there was considerable label incorporation (**Fig. 7B**). Interestingly, a previous study in 1-year-old dogs showed significantly higher bone turnover in the jaw in comparison to the shaft of the femur [18].

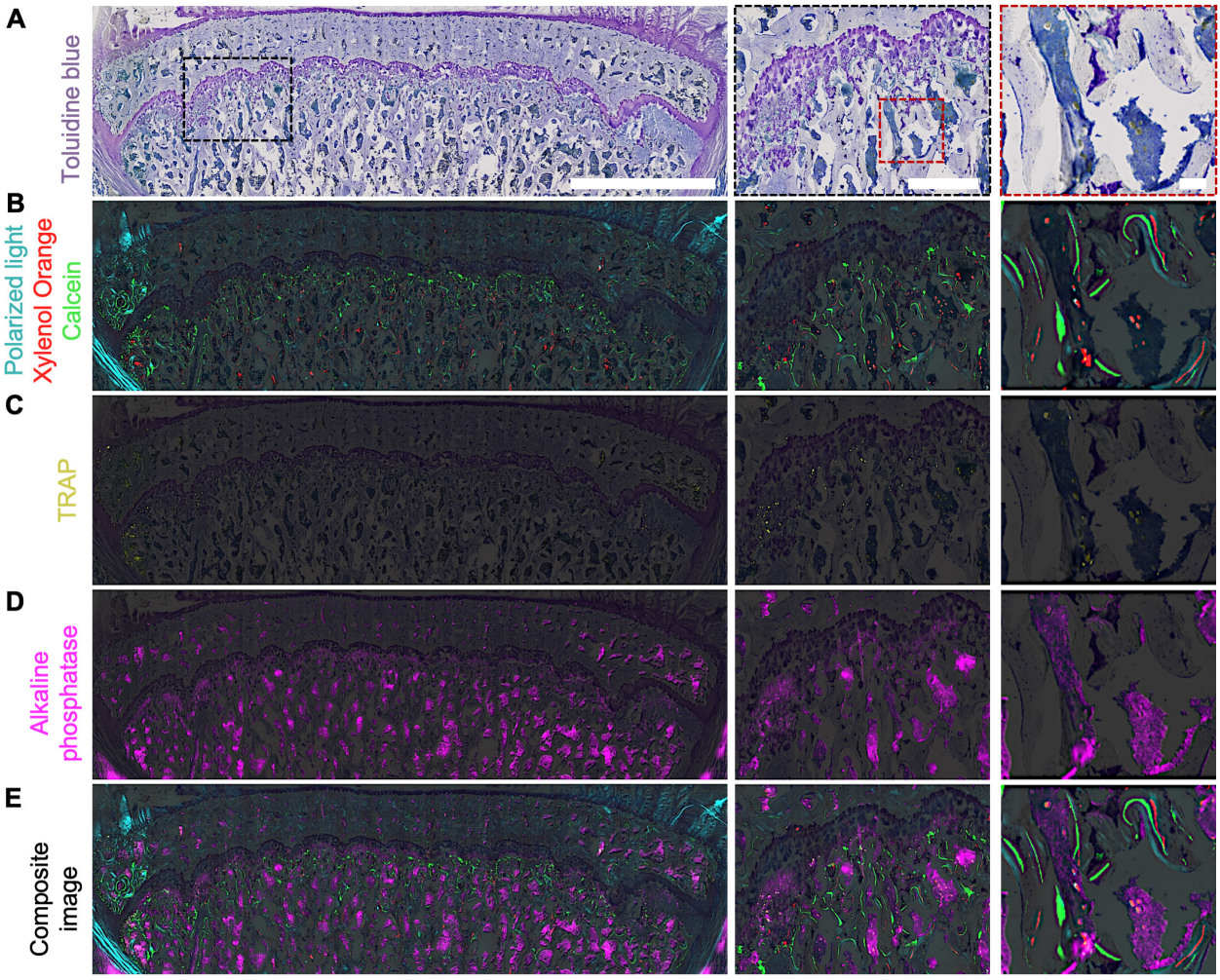

Figure 6 Multiplexed cryohistology of minipig vertebral body. (A) Toluidine blue chromogenic staining. Full image scale bar = 5 mm. Middle column scale bar = 1 mm. Right column scale bar = 100 µm. **(B)** Polarized light (cyan), xylenol orange (red), calcein (green) overlaid onto toluidine blue image. **(C)** Tartrate-resistant phosphatase (TRAP) (yellow) enzymatic staining overlaid onto toluidine blue image. **(D)** Alkaline phosphatase (AP) (magenta) enzymatic staining overlaid onto toluidine blue image. **(E)** Composite image.

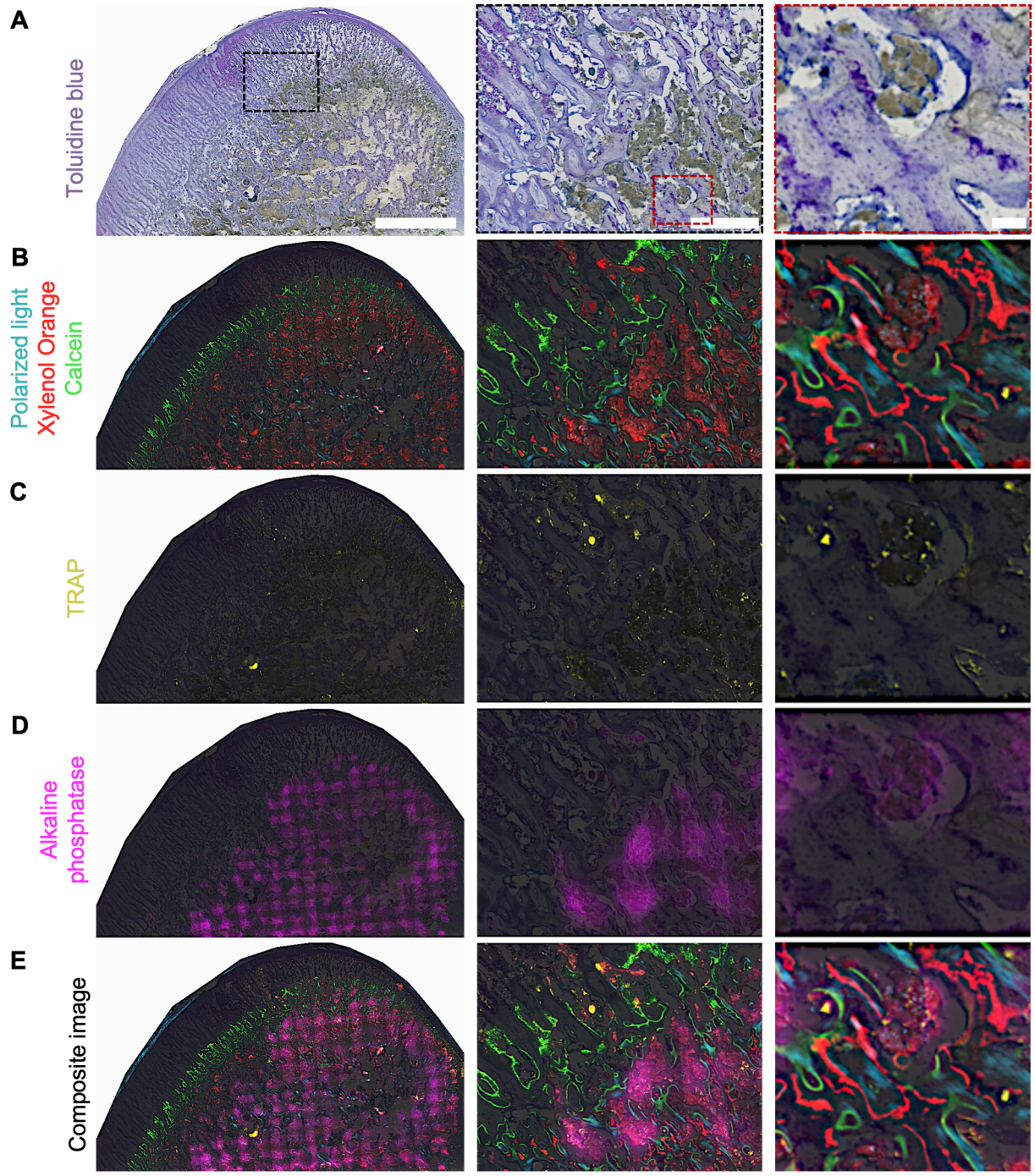

Figure 7 Multiplexed cryohistology of minipig temporomandibular joint (TMJ). (A) Toluidine blue chromogenic staining. Full image scale bar = 5 mm. Middle column scale bar = 1 mm. Right column scale bar = 100 μm. **(B)** Polarized light (cyan), xylenol orange (red), calcein (green) overlaid onto toluidine blue image. **(C)** Tartrate-resistant phosphatase (TRAP) (yellow) enzymatic staining overlaid onto toluidine blue image. **(D)** Alkaline phosphatase (AP) (magenta) enzymatic staining overlaid onto toluidine blue image. **(E)** Composite image.

For the second round of fluorescent imaging, tartrate-resistant phosphatase staining highlights areas of increased bone resorption (**Fig. 5C, 6C, 7C**, *yellow*). TRAP staining should appear as small puncta. In the case of bone injury, there should be increased TRAP, as seen in a prior large animal study investigating the impact of subchondral drilling and microfracture on the subchondral bone [14]. The specimens used in this protocol were not injured,

and so, TRAP staining is localized to the primary spongiosa of the growth plates and other osteochondral interfaces.

For the last round of fluorescent imaging, alkaline phosphatase staining indicates areas where mineralization is about to begin (**Fig. 5D, 6D, 7D**, *magenta*). Alkaline phosphatase is a major product of osteoblasts. While alkaline phosphatase staining correlates with fluorochrome label incorporation [19], it often overestimates the extent of the mineralizing bone surface [20]. Therefore, alkaline phosphatase staining is more diffusely observed in the minipig samples.

By using the same section for each round of staining, the images taken from the multiple staining rounds can be merged (**Fig. 5E, 6E, 7E**). Merged images allow for quantitative analyses of signal co-localization [6].

TROUBLESHOOTING

This protocol has been optimized for tissues up to 20 mm in the largest dimension, and a sectioning thickness of 18 µm. Tissues 20 mm in length allow for 2 mm of clearance on each side of the tissue within the cryomold. This border is needed to create even sections and prevent tissue shredding. It is best to trim the tissues as much as possible before embedding. Only embed the tissue volume that you are interested in imaging. Smaller tissues with less mineralization will be easier to section and clearly image. For rodent tissues (5–8 µm section thickness), it is best to follow the protocol previously developed by Dyment *et al*. [3]. If the large animal samples were significantly trimmed down, a thinner section thickness could be achieved.

While gluing the sections to microscope slides, you may find that the viscous chitosan adhesive can cause the tape-tissue sections to lie unevenly. To troubleshoot section waviness due to gluing, evenly spread the adhesive using the side of a pipette tip and re-position the cryofilm sections by pulling and not pushing the sections. If the sections do not dry in the appropriate orientation or if you observe waviness, you may remove the cryofilm and then re-glue the sections. Remember to let the slides dry for two days at 4°C after gluing.

To reduce bubble formation under the coverslips, hydrate the tissues before coverslipping. This is especially important after gluing the sections and imaging for the first time. You may increase the time of hydration, and/or use a higher volume of mounting medium to limit bubble formation.

For the best image quality, it is advised to complete the sequential imaging protocol as quickly as possible over the course of 1 week. The aqueous mounting medium will slowly evaporate over time, complicating coverslip removal. Therefore, it is advised to only glue down the tape-tissue sections when you are ready to complete the staining protocol. Otherwise, it is best to leave the sections in the refrigerator or freezer until further use.

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