Use of commercially available adhesive tapes to preserve cartilage and bone tissue integrity during cryosectioning

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The use of fluorescent tags to monitor protein expression and to lineage-trace cells has become a standard complement to standard histological techniques in the fields of embryology, pathology and regenerative medicine. Unfortunately, traditional paraffin embedding protocols can substantially diminish or abolish the native emission signal of the fluorophore of interest. To preserve the fluorescent signal, an alternative is to use cryosectioning; however, this can often result in undesirable artefacts such as tearing or shattering – particularly for mineralized tissues such as bone and cartilage. Here we present a method of using a commercially available tape to stabilize murine femur tissue, thus allowing for cryosectioning of cartilage and bone tissues carrying fluorescent tags without the need for demineralization.

The availability of genetic tools to identify and track cells within the skeletal system as well as to manipulate gene expression has increased in the past 10 years [1,2]. The ability to rapidly and cost-effectively analyze tissues is essential for the *in situ* analysis of musculoskeletal tissue histology and gene expression, particularly when carrying out medium- to high-throughput phenotyping analysis [3]. Generating high-quality histological sections of animal tissues that preserve tissue integrity and morphology can be technically challenging. These challenges are especially appreciated when sectioning mineralized tissues, such as cartilage and bone. Standardized cryosectioning protocols typically provide an effective and rapid system for visualizing tissue histology; however, when mineralized tissues are sectioned in this manner, researchers frequently struggle with tissue distortion, tearing, wrinkling, and shattering of bone and cartilage. To alleviate these issues, protocols have been developed that include demineralization prior to sectioning. Indeed, tissue decalcification improves the ease of

sectioning mineralized tissues; however, decalcification protocols can distort tissue morphology (i.e., demineralization by acidic solutions) or require long-term decalcification by chelating agents (i.e., EDTA) for up to several weeks [4,5]. These caveats may be unacceptable for some experimental analyses and disrupt fast-paced workflows in certain laboratory environments. In addition, many protocols include paraffin embedding prior to sectioning. Paraffin embedding can be problematic because the fixation and/or embedding process can diminish or abolish the native emission signal of fluorophores of interest [6,7]. Consequently, transgenic fluorescent proteins within the tissue must be visualized by subsequent immunohistochemistry protocols; however, due to the high cost of antibodies, the additional time requirement, and potential diminished signal-to-noise, this approach may not be optimal.

With these technical limitations in mind, we set out to develop a protocol for inexpensive and effective cryosectioning of mineralized tissues. Our approach

was to evaluate the feasibility of using widely available commercial adhesive tapes to stabilize skeletal tissues during cryosectioning. A product carried by Section-lab currently exists on the market (Section-lab [8], Hiroshima, Japan). Section-lab tape works by attaching an adhesive tape directly to the front of the tissue block so that sectioned tissue adheres to the tape and remains in its natural conformation and orientation during cryosectioning. Section-lab tape is excellent and has been used with great success [9–11]; however, the cost of materials is considerable when sectioning a large number of samples. Therefore, the focus of this project was to discover a more cost-effective alternative for routine cryosectioning use. An optimal tape for our cryosectioning purpose must be inexpensive, easy to work with, nonautofluorescent and adhere strongly to tissue samples. Additionally, an ideal tape will retain these qualities during post-sectioning processing such as H&E staining protocols where solvents that could disrupt the integrity of the adhesive are used.

METHOD SUMMARY

This study presents a method of using commercially available tape to stabilize murine skeletal tissues during cryosectioning without the need for demineralization.

From our analysis of 12 commercially available tapes, we discovered that several tapes can indeed be used to stabilize skeletal tissues during cryosectioning, enabling rapid and cost-effective generation of histological sections without the need for demineralization. Here, we show that this method can reliably be used to generate high-quality sections of the murine femur and preserves anatomical structures. Additionally, we show that these sections can be used for the evaluation of native fluorescence and histochemical staining.

Materials & methods

We procured 12 different tapes from standard stationery suppliers and from

a tape warehouse (FindTape.com) that supplies tape for a wide range of applications including book repair, greenhouse repair, packing, sealing and construction (Figure 1A). These tapes were tested with mouse muscle and femur tissue obtained from C57Bl/6 and double transgenic *Col2.3:GFP;ColIX:mCherry* mice [12,13]. Prior to sectioning, individual tape strips were prepared on a cutting mat that had been cleaned of dust with 70% EtOH (Figure 1B). Tape strips of approximately 30 cm were loaded onto the cutting mat, cut into ~3 cm segments with a razor blade and tape ends were folded onto themselves with forceps to create a non-sticky tab for subsequent tape handling (Figure 1B).

Our protocol involved these steps:

1. Tissue fixation

- Fix tissues in 4% paraformaldehyde overnight at room temperature.
- Cryo-protect samples in 30% sucrose overnight at 4°C.

2. Embedding

- Embed sample within OCT (Optimal Cutting Temperature) compound (Tissue-Tek®, 4583, Sakura Finetek, CA, USA), within a cryomold (255608–925, VWR, PA, USA), ensuring that the entire sample (properly oriented) is covered by OCT.

- Create a cold bath for sample freezing by adding dry ice to a bath of 95% EtOH.
- Gently place the cryomold into the EtOH bath so that the cryomold floats on top of the EtOH.

- Leave sample floating in EtOH bath until the entire sample freezes (the OCT will change from clear to white). Note: at this point the sample can be stored at –80°C for 2–3 months.

3. Cryo-sectioning

- Mount the sample onto the cryostat chuck using OCT.
- Begin sectioning according to the instructions for the device used. We use a Leica Cryostat (CM 3050S).
- We set the cryostat to section at 12 µm thickness; however, we have had success at all thicknesses between 8 µm and 16 µm.
- Peel tape off the mounting board (9952 6"×8" OLFA self-healing rotary mat) kept at room temperature and press the adhesive side of the tape onto the sample block using a wide and flat tool such as the end of a wooden paint stir-stick. Ensuring that the sample is entirely covered by the tape.
- Section the sample in one slow, but continuous movement. If the sectioning motion is uneven the sample can fail to section cleanly and is unlikely to adhere to the tape.
- Use forceps to lift the freshly-sectioned tissue by the tab on the tape and place the section onto a glass slide (any brand; we used 48311-703 Superfrost Plus, VWR), with the tissue adhered to the tape and the tape placed adhesive side up on the slide (see Figure 1C).
- Using laboratory tape, secure the sections onto the slide by taping the tab of the sectioning tape to the glass slide. Alternatively, the tape can be affixed to the glass with a chitosan or UV-curing liquid adhesive [10]. Note: at this point, slides can be stored at –80°C for up to 6 months.

Figure 1. Tapes evaluated and the method of storage before use. (A) The tapes evaluated are listed and were assigned a unique identifier during testing. They ranged in thickness from 10–140 microns (manufacturer dimensions often in 1/1000 of an inch). The thickness, adhesives and other characteristics for some tapes were not available from the manufacturer. (B) Tape strips are shown loaded onto a cutting mat in preparation for sectioning. A mat with texture is desirable as highly adherent surfaces cause the tape to curl upon removal. Dust is removed with a 70% EtOH wash and tape strips are loaded in a dust-free environment (left panel). A tab can be created by folding over one side for easy pick-up (right panel). (C) Schematic diagram to illustrate the physical orientation of the tape system components. Note that the tissue and tape is placed adhesive-side up and imaging should be carried out through the coverslip.

4. Preparing slides for imaging

- If the tissue sample harbors native fluorescent markers, the following steps should be conducted in a light-reduced environment such as an aluminum foil-covered chamber.
- Remove residual OCT from tissue sample by submerging the slide in 1x PBS for 20 min at room temperature.
- Remove slide from 1x PBS bath and tip slide at an angle for 5 min to pour off residual liquid.
- For immediate imaging, mount tissue with mounting media (Vectashield with DAPI, H1200, Vector Laboratories, CA, USA) and apply the coverslip (16004–330, VWR) so that it rests on the tissue sample with the adhesive side of the tape face-up and the slide underneath (see Figure 1C).
- For Hematoxylin and Eosin staining, the typical initial steps (de-paraffin and rehydration) are skipped and the slide can be immersed in the Hematoxylin stain step after removing the OCT with 1x PBS (skip step 1 and 2 and proceed to step 3 in ref [14]). Note: at this point the sample can be stored at 4°C, protected from light.

Images were collected using a Nikon AZ100 Macroscope and a Nikon DSFi1 camera with brightfield illumination (see Figure 1A for the set-up). For fluorescence imaging, illumination was provided by a Nikon Hg Intensilight (C-HGF), while filter cubes were used to filter the emission and excitation wavelengths (B-2E/C FITC, G-2E/C TRITC and UV-2E/C DAPI, Nikon). To obtain better depth of field, higher magnification images were collected and processed with the Nikon Extended Depth of Focus (EDF) module.

Results & discussion

We procured 12 types of widely available commercial tape to evaluate for use during cryosectioning. These 12 tapes were chosen to represent a range of thicknesses, widths and adhesive materials (Figure 1A). Each tape was assigned a unique identifier during testing. Tape thickness ranged from 10 to 140 microns and width ranged from 6.3 to 38.1 mm. All 12 tapes were evaluated in comparison to the Cryofilm 3C tape produced by Section-lab, our 'gold standard' for stabilizing tissues during cryosectioning due to its optical clarity, lack of autofluorescence, and workability. Importantly, all new tapes tested cost less than \$0.01 per cm².

Figure 2. Assessment of optical clarity, autofluorescence and workability. (A) Evaluated tapes are shown in brightfield to show transmitted light optical clarity and under three different common epifluorescence imaging settings to demonstrate the qualitative level of autofluorescence (tape thickness is indicated in microns, scale bar = 100 microns). **(B)** Based on the images in A, five tapes were chosen to test workability in comparison with the SL tape. A 12-micron section through DAPI-stained mouse muscle is shown, mounted with Vectashield and a coverslip. Tapes 1 and 9 are the easiest to use and mount and were comparable to the Section-lab (SL) tape (scale bar = 50 microns).

Prior to sectioning, individual tape strips were prepared on a cutting mat (Figure 1B) and tape ends were folded onto themselves with forceps to create a non-sticky tab for subsequent tape handling (Figure 1B). After testing several cutting surfaces, we determined a cutting mat with a rugged texture is desirable, as smooth highly adherent surfaces cause the tapes to curl upon removal.

We suspect that the most important characteristics of a successful tape are 1) low auto-fluorescence of the tape (both the base material and adhesive) and 2) the ability to adhere to frozen tissues. Therefore, each

tape was first assessed for optical clarity, autofluorescence and workability. To evaluate optical clarity, tapes were imaged by brightfield microscopy (see Figure 1C for slide arrangement). Tapes #1–11 were observed to be relatively optically clear; however, tape 12 showed significant optical distortion (Figure 2A). Tapes were then tested for autofluorescence in three commonly used fluorescent channels (DAPI, FITC, and TRITC). Tapes 1, 5, 7, 8 and 9 showed low autofluorescence in all three channels. Tapes 2, 3, 4, 6, 10, 11 and 12 all showed significant autofluorescence in at least one channel and were therefore not

Figure 3. Use of commercially available tape for histology. We chose to test the tape on sections of the proximal adult mouse femur, which has a challenging ultrastructure including cortical and trabecular bone, and bone marrow voids. While the SL tape produces a superior tissue preparation, tapes 1 and 9 are acceptable with little shattering or loss of bone marrow tissue. Dense tissue (growth plate, enlarged to the right) was well preserved. Alizarin-red stained sections are shown on the far right to demonstrate tissue mineralization. For comparison, a section obtained without the use of tape for tissue stabilization during cryosectioning is shown in the bottom panel. Left panels, scale bar = 500 microns; Right panels, scale bar = 50 microns. SL: Section-lab.

Figure 4. Use for fluorescence imaging. Femur tissue from mice carrying transgenic reporters was used for native fluorescence imaging. Expression of the *Col2.3:GFP* reporter (green) can be seen in the developing trabeculae, while the *ColX:mCherry* reporter (red) can be found in hypertrophic chondrocytes within the growth plate. DAPI allows for visualization of the nuclei (blue). In this region of the mouse, SL tape, tape 1 and tape 9 all performed very well with respect to resolving the signal-to-noise for each fluorophore. Left panels, scale bar = 500 microns; Middle panels, scale bar = 200 microns; Right panels, scale bar = 100 microns. SL: Section-lab.

evaluated further (Figure 2A). We next tested the workability of tapes 1, 5, 7, 8 and 9 during cryosectioning of adult murine muscle tissue. Testing revealed that the physical properties of tapes 5, 7 and 8 were suboptimal for use during cryosectioning due to tape curling or excessive tape thickness, which made it difficult to mount tissue with a coverslip. We therefore selected tapes 1 and 9 for further testing (Figure 2B).

Next, we directly compared tapes 1 and 9 to the Section-lab tape during cryosectioning of a mineralized adult murine femur. We chose the murine femur because it is a large, mineralized bone with a detailed ultrastructure that can reveal subtle differences in the ability of a tape to produce high-quality histological sections [15]. We first compared H&E-stained sections at several magnifications and found that the SL tape produced the highest-quality images, followed by tape 1, and then tape 9 (Figure 3). The difference in section quality is best observed in the epiphyseal trabecular bone (black arrows). These delicate structures are mostly preserved by SL tape and tape 1; however, some fragmentation is visible when using tape 9. At high magnification, we observed that all three tapes produce high-quality images of the cartilaginous growth plate. The quality of section through bone marrow in tape 1 was comparable to the SL tape. Additionally, we show excellent preservation of mineralized tissue as indicated by alizarin red staining. Thus, these results demonstrate that the adhesives of tapes 1 and 9 tolerate a variety of histological reagents, including: EtOH, Eosin Y stain, Hematoxylin stain, alizarin red stain, Citrisolv™ and Cytoaseal™. These results suggest that tapes SL and 1 are best-suited for preserving the integrity of delicate structures; however, all three tapes are acceptable for preserving more robust skeletal structures such as cortical bone and growth-plate cartilage. To preserve bone marrow integrity, some users may wish to try the alternative CryoJane tape transfer system, which requires a specialized instrument to be installed inside the Cryostat.

We additionally evaluated tapes during fluorescence imaging. We procured femora from mice that express two transgenes: *Col2.3:GFP* [11] (GFP driven by osteoblast-specific Collagen I promoter) and *ColX:mCherry* [12] (RFP driven by Collagen X promoter, specific to hypertrophic chondrocytes). We found that

tape SL and tape 1 are indistinguishable from each other at all magnifications, while tape 9 produces an image plagued by air bubbles at low magnification (white arrows, Figure 4). This is due to issues during coverslip mounting, where we observed that the adhesive used in tape 9 prevents the mounting media from spreading evenly across the section, resulting in the observed bubbles. These bubbles are not problematic if nonaffected regions of tissue are imaged at high-magnification (Figure 4, middle and right columns). All three tapes produced high-quality fluorescent images with no excessive autofluorescence in all three imaged channels. Together, these results suggest that tapes SL and 1 are best suited for low-magnification fluorescence imaging; however, all three tapes are suitable for high-magnification fluorescence imaging. After fluorescence imaging, we found that the same sections can be subsequently processed for other protocols including H&E or Alizarin Red staining. Indeed, the sections shown in Figure 4 were processed to generate Figure 3 after imaging.

Our work has demonstrated that at least two widely available and economical tapes can be used to preserve murine femur tissue integrity during cryosectioning. Although the gold standard cryotape developed by Kawamoto [8] (SL) was the easiest to work with and produced the highest quality images of the tapes we tested, we show that a Circuit Plating/Splicing tape produced by JVCC (tape 1) can produce high-quality histological sections for H&E, Alizarin Red, and fluorescent imaging at a reduced cost. Ultimately, this work has inspired our laboratory to adopt a system where we use a combination of different tapes during sectioning. The SL tape is reserved for every third to fifth section to ensure the generation of publication-quality images, while the Circuit Plating/Splicing tape is used for intervening sections or for preliminary and pilot experiments.

Author contributions

FVM and MAS conceived of the project. MAS and DDP carried out the experiments with help from JWH. FVM, MAS and DDP wrote the manuscript.

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Ethical conduct of research

The use of animals in this research was approved by the University of Southern California's Animal Use Committee.

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