TECHNICAL NOTE

Novel Procedure for High-Fidelity Tendon Histology

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ABSTRACT: A novel method to obtain high-quality histologic sections of rat tendon for light microscopic study is presented. This approach utilizes non-deleterious dehydrating and clearing solvents and a hydrophobic acrylic (methyl methacrylate) resin. This methodology avoids processing and microtomy artifacts common with routine paraffin wax techniques and overcomes specimen size limitations associated with hydrophilic (e.g., glycol methacrylate) resin histologic methods. These novel histologic processing techniques facilitate the reliable assessment of tendons' cellular and matrix components and can be readily adapted to morphologic studies of damage, healing, and repair. © 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 25:390–395, 2007
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INTRODUCTION

Tendon is a dense, fibrous tissue connecting muscle to bone. It is characterized by a hierarchical arrangement of type I collagen fibers embedded within an extracellular matrix consisting principally of proteoglycans. Due to its dense architecture, tendons are susceptible to numerous histologic processing artifacts that compromise qualitative and quantitative morphologic assessment. Such artifacts may confound experimental results in histologic studies of matrix damage and repair. Rodent tendons, commonly used in animal models of healing and repair,[1] are especially challenging tissues to process well with standard histologic methods such as dehydration using graded alcohols, clearing with hydrocarbons, and embedding in paraffin. Ethyl alcohol (ethanol) dehydration causes extensive shrinkage and hardening of tissue, while displacement of ethanol with xylene or toluene can exacerbate this hardening effect.[2] Furthermore, paraffin embedding of these hardened, shrunken specimens does not provide adequate tissue stabilization for sectioning. Collectively, the aforementioned approaches markedly compromise morphological detail while making seamless microtomy difficult. Techniques used to soften tissue en bloc, meanwhile, can lead to swelling artifacts and shredding during sectioning. Processing samples to glycol methacrylate (GMA), a hydrophilic resin,[3] can improve morphology and reduces microtomy artifact, but processing versatility (e.g., ability to work with larger isolated tendons or tendon-bone insertions) is greatly compromised. This article describes novel tendon processing methods which provide improved morphologic fidelity through modification of dehydration and clearing techniques combined with acrylic resin embedding methods typically used for mineralized bone histology.

MATERIALS AND METHODS

Tissue Source and Primary Fixation

All tissue processing protocols were developed using freshly harvested, normal flexor digitorum longus (FDL) tendons from adult (5–8 months old) female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), and then adapted to tendons with bony insertions (rat Achilles, supraspinatus, and patellar tendons). Tendons were harvested immediately following animal euthanasia under approval of our institution's Animal Care and Use Committee.

During dissection and preparation for histologic processing, specimens were kept moist with phosphate-buffered saline (PBS). Following tissue harvesting,

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samples were laid flat and affixed to acrylic plates to maintain tendon tension (approximately 2 Newtons) during fixation. Tendons were fixed with 10% neutral buffered formalin or 4% paraformaldehyde. Fixation times were dependent on the tissue volume (range: 24 h for FDL tendons to 96 h for patellar tendons with entire tibiae intact). Samples were rinsed well with deionized water after primary fixation. Tendons were processed as described below for both paraffin and acrylic embedding (see Table 1 for summary).

Processing Technique

Decalcification

Tendons with bony attachments were decalcified in formic acid;[4] FDL tendons, which lack bony insertions, did not require decalcification. As with the fixation procedure, processing duration was dependent on sample size (typically 48 h with one change of solution per day for rat Achilles).

Dehydration I

To avoid the deleterious effect of ethyl alcohol on tissue, the tendon dehydration sequence was initiated with ethylene glycol monoethyl ether (EGME, Fisher Scientific, Pittsburgh, PA). This reagent does not require graded dilutions and does not cause distortion. Tissues may remain in this reagent for long periods (i.e., months) without injury.[4] Dehydration times using this reagent are determined by specimen size and the number of samples being processed, while the volume of reagent used should be 20 times that of the specimens. Tendons were placed in EGME for 24 h (with four changes of solution).

Dehydration II

EGME is too viscous to ensure adequate dehydration of rodent tendons. We used acetonitrile (Fisher Scientific), a gentle, low-viscosity solvent (0.38 cP at 5°C), to complete the dehydration process. This reagent, which is also used in electron microscopy processing, does not solubilize charged phospholipids[5] and is freely miscible with EGME. Because of its low viscosity, acetonitrile readily penetrates dense specimens and ensures thorough dehydration. Samples were placed in acetonitrile for 24 h with three changes.

Clearing

For clearing, we use methyl salicylate (Sigma-Aldrich, St. Louis, MO), i.e., oil of wintergreen, which is an exceptionally gentle clearing solvent that does not overharden tissue[6] and works well for tendon-bone insertions. Tendon samples were placed in methyl salicylate overnight, or up to 24 h, until they were totally transparent in appearance.

Embedding

Tendons processed using the aforementioned steps were then embedded in either normal paraffin, or in methyl methacrylate (MMA) prior to sectioning. MMA is widely used for undecalcified bone histology. Standard MMA kits provide detailed instructions on infiltration and embedding. The infiltration and polymerization times for MMA will vary depending on the kit manufacturer. In our laboratory, we typically infiltrate for 4 days (3 changes) and the polymerization process requires 3–4 days.

Chemical Safety

All of the aforementioned compounds should be handled in the same manner as other volatile solvent chemicals such as xylene. Material Safety Data Sheets (MSDS) should be reviewed before working with these reagents and institutional chemical safety guidelines should be consulted. Methyl salicylate is less hazardous than xylene. Waste methacrylate solutions with catalyst are polymerized under a fume hood, and, once solidified completely, the solid plastic can be placed in the conventional trash.

Sectioning

Once embedded, FDL tendons were sectioned on a heavy duty, motorized rotary microtome (Leica RM2165, Nussloch, Germany). A sledge microtome (e.g., Leica

Table 1. Summary of Processing Steps in Rat Tendon Histology Protocol[a]

Procedure	Reagent/Media	Processing Time for Tendon Only (e.g., Rat FDL)	Processing Time for Tendon with Bone Insertion (e.g., Rat Achilles)
Fixation	Formalin	24 h	72 h
Decalcification[b]	Formic acid	n/a	48 h
Dehydration I	EGME	24 h (3 changes)	24 h (4 changes)
Dehydration II	Acetonitrile	24 h (2 changes)	24 h (3 changes)
Clearing	Methyl salicylate	Overnight	24 h
Embedding	MMA	6 days	7 days

[a]Note that longer processing times are generally required when working with larger-sized tissues.
[b]For tendons with bone attachments.
EGME, ethylene glycol monoethyl ether; FDL, flexor digitorum longus; MMA, methyl methacrylate.

SM2500) normally used to cut mineralized bone samples embedded in MMA must be used to section larger MMA blocks (i.e., tendon-bone insertions). Thin (4–6 μm) sections cut longitudinally on a microtome are sectioned using a D-profile tungsten carbide knife, and sections should be mounted on charged or silane-coated slides. Sections were covered with a thin, nonstick plastic, and section slides were then clamped together using a slide press or C-clamp and dried in an incubator at 37°C overnight. Tissue sections were deplasticized in toluene for 4 h (two changes) before staining.

RESULTS

Photomicrographs of Toluidine blue-stained rat tendons are provided in Figures 1–3. In comparison to paraffin sections, MMA sections of FDL tendons show markedly improved cellular preservation, matrix fidelity, and maintenance of the synovial tissue (Figs. 1 and 2). The MMA sections of rat patellar tendon shown in Figure 3 further demonstrate the morphologic detail afforded by plastic embedding of tendon insertions. Lower magnification MMA images demonstrate high-fidelity morphologic detail of the synovium and collagen fibers, as well as the tendon midsubtance and insertion sites, while the higher magnification MMA images reveal improved cellular details. Unlike the case for paraffin embedding, no artifactual splitting or fraying was observed when using MMA embedding.

DISCUSSION

Tendons are dense collagenous tissues that are difficult to dehydrate, clear, embed, and then section without distortion or fraying. These issues

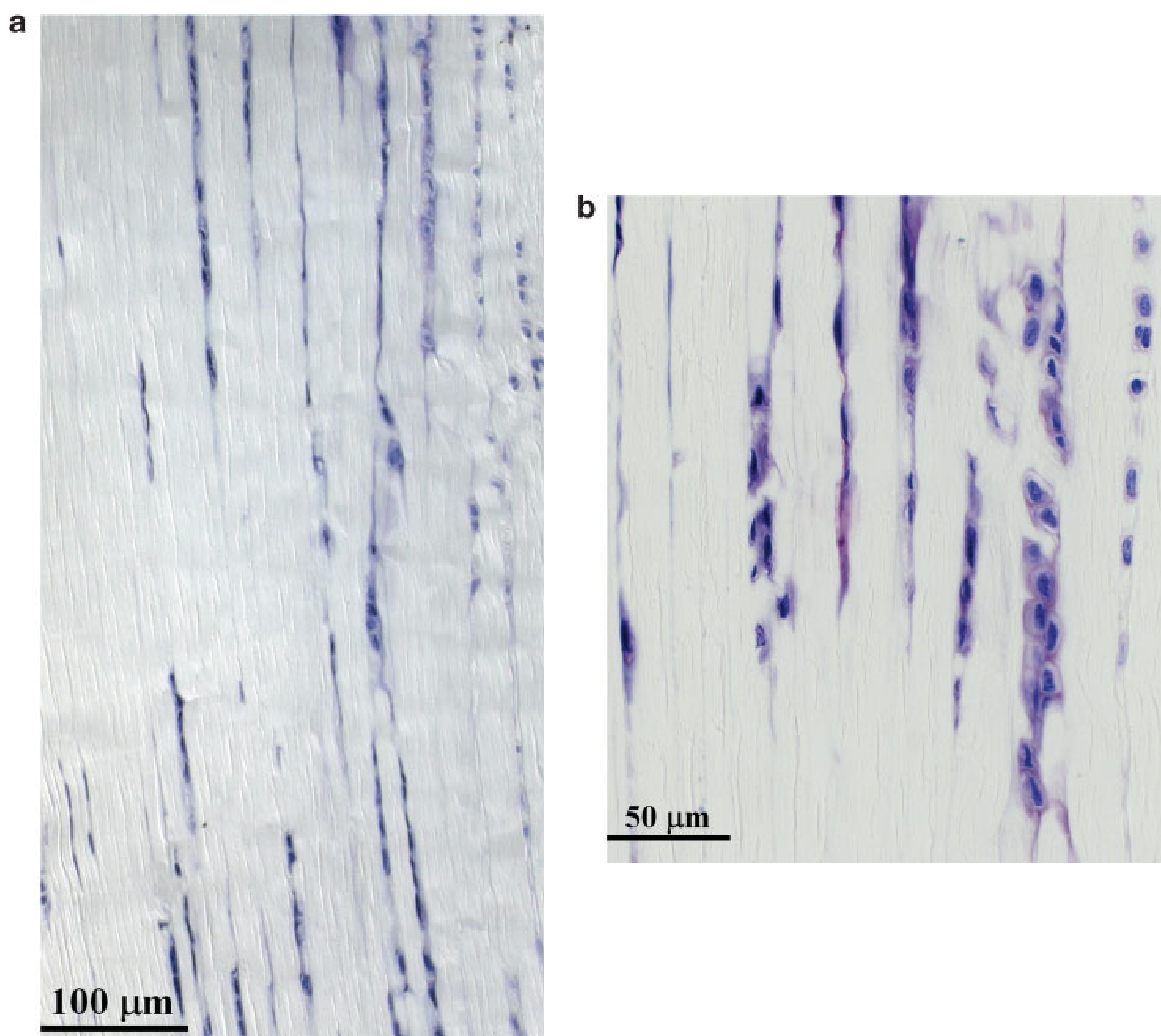

Figure 1. Paraffin sections (5 μm thickness) of normal rat flexor digitorum longus (FDL) tendon processed using modified dehydration and clearing reagents and stained with Toluidine blue. (a) low magnification; (b) high magnification.

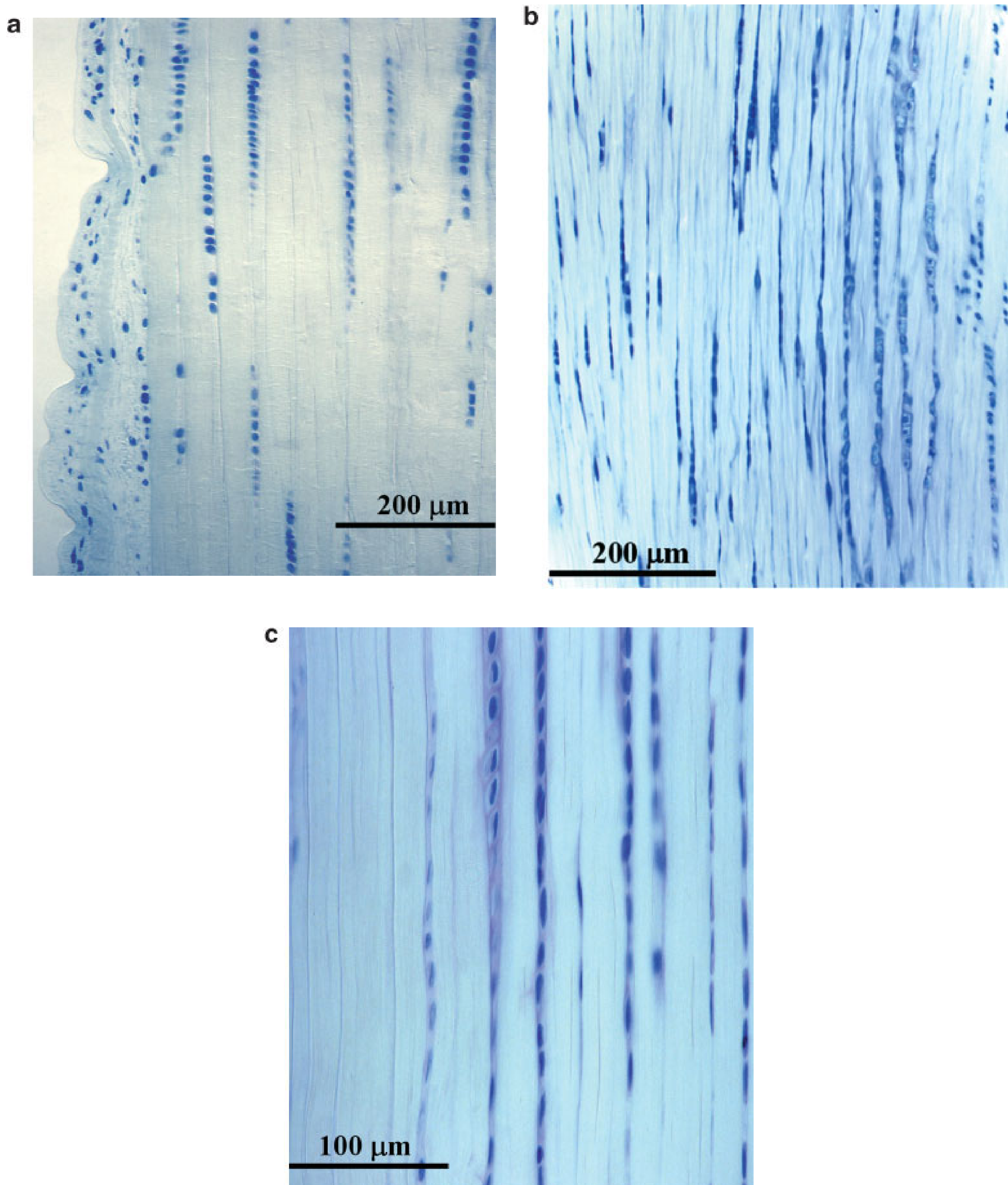

Figure 2. Low (a, b) and high (c) magnification views of methyl methacrylate (MMA) sections of normal rat FDL tendon stained with Toluidine blue. In comparison to paraffin sections (Fig. 1), MMA sections demonstrate improved morphological and cellular detail including preservation of synovial tissue.

are particularly problematic for small rodent tendons. We have developed novel approaches for dehydration (using EGME and acetonitrile) and clearing (with methyl salicylate) that alleviate many of the artifact problems inherent to traditional histological methods for tendon. Relative to paraffin processing, morphological fidelity was considerably improved when using MMA embedding

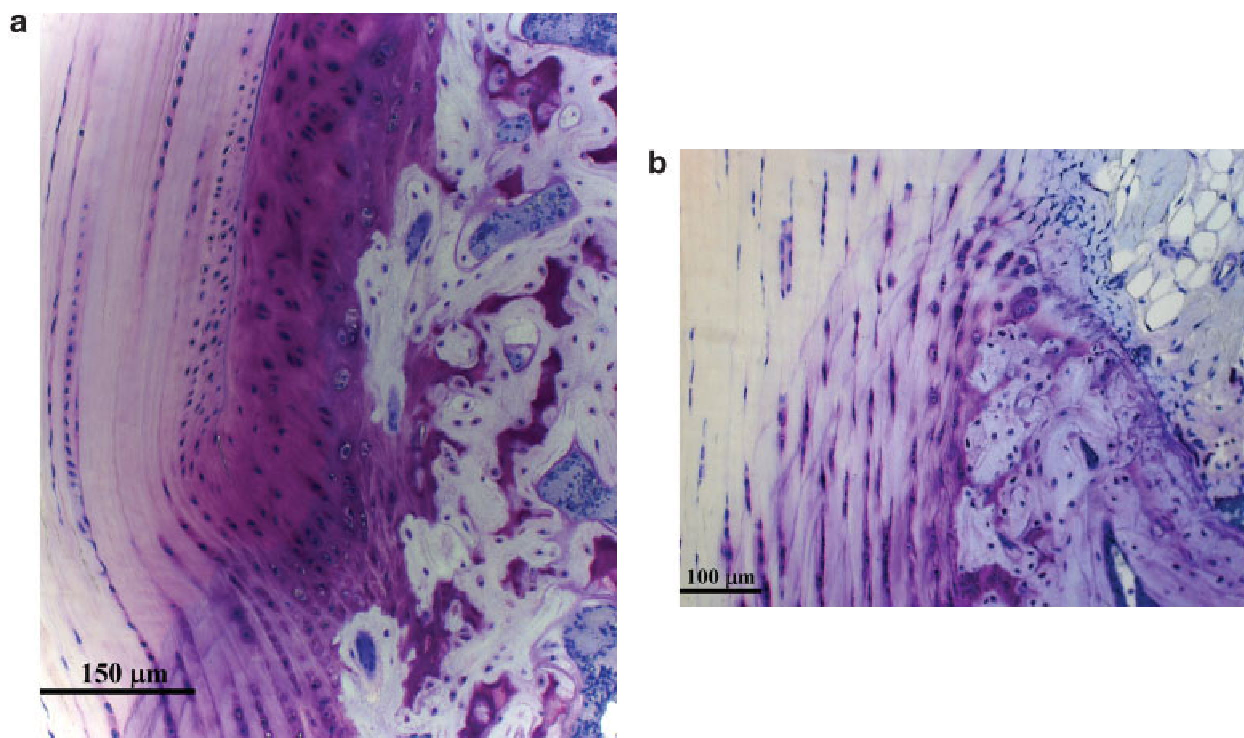

Figure 3. Toluidine blue-stained MMA sections of normal rat patellar tendon highlighting morphologic detail of collagen matrix, fibrocartilage, and calcified components of insertion sites at low (a) and high (b) magnification.

(Figs. 1 and 2). Our protocols for methacrylate (i.e., plastic) embedding of tendon tissues are quite effective in delineating tissue constituents within the tendon matrix and bone insertion sites. Specifically, we have found that adapting MMA embedding for use in tendon presents significant advantages over paraffin embedding in terms of 1) preservation of cells, synovium, and their relationship to the adjoining collagenous matrix, and 2) the dimensional stability of MMA which allows better assessment of normal collagenous architecture.

As stated above, paraffin is not well suited for high-fidelity rat tendon histology. Glycol methacrylate (GMA) offers improved morphologic detail for small, isolated tendon samples, but this media is very viscous and has proved inconsistent for embedding larger tendon samples. Methyl methacrylate has the rigidity and versatility to support a variety of isolated and tendon insertion samples. This method constitutes an improvement over paraffin techniques and presents an alternative to glycol methacrylate processing. The use of gentle dehydrating and clearing reagents, combined with the improved cellular detail offered by methyl methacrylate embedding, yields a more refined picture of tendon microstructure. However, two primary considerations for using this new protocol are the processing time requirements and cost. Because this procedure requires manual processing, time requirements are considerably increased (Table 1) relative to those of conventional paraffin techniques. Furthermore, the processing cost for the entire procedure is approximately twice that of the standard paraffin technique, in part due to the fact that MMA should not be reused. In addition, microtome sectioning of MMA-embedded tendon is technically difficult, requiring the skill of an experienced histotechnologist.

Our newly established protocols for tendon histology considerably improve section quality relative to standard paraffin approaches and offer versatility for processing both the tendon proper (e.g., flexor tendons) and bony insertion sites. We have begun to apply the methods described above in assessing low-level matrix damage in fatigue-loaded rat tendons[7]; in these studies, it is essential that histological approaches minimize tissue artifacts so that mechanisms of tissue micro-failure can be understood. Other potential applications requiring high-fidelity tissue morphologic study include investigations of tendon healing, repair, and regeneration following suture repair and/or cell-based therapy (e.g., growth factor administration).

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