Novel Method for Preservation of Poly-Caprolactone Scaffolds in FFPE Histology

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INTRODUCTION: Osteoarthritis (OA) is an orthopedic condition that induces chronic degeneration in tissues of the joint in more than 600 million people worldwide. Despite its high prevalence, treatment options for this condition are not yet ideal for patients. To overcome the challenges that current treatment with total joint replacements or early surgical intervention provides, the field has turned towards novel biomaterials as a solution. The use of biodegradable scaffolds composed of substances such as polycaprolactone (PCL) to support osteochondral repair has been widely investigated in recent years. However, evaluation of these implants by traditional histological methods after *in-vivo* studies is impacted by the implant's low melting point of 60°C and ready degradation in Xylenes. As a result, histology examining PCL-implanted tissue constructs often is only able to show small fragments of PCL remaining within the tissue. This inability to visualize PCL on traditional Formalin-Fixed Paraffin Embedded (FFPE) histology sections limits the ability of researchers to examine the interactions that PCL and newly formed tissues may have on the cellular level. To address this, we developed a novel tissue processing protocol that sought to both preserve the integrity of PCL implants in high-quality FFPE sections while also reducing processing time. We hypothesized that combining the use of low-melting-temperature paraffin wax, *d*-limonene clearing agents, and warm tissue infiltration would enable visualization of PCL on FFPE histology sections with minimal impact to tissue quality, as seen in previous methodologies [1,2]. We evaluated representative sections of PCL implants within cartilage lesions following processing in our protocol for preservation of PCL filaments.

METHODS: *Scaffold fabrication and placement:* PCL implants were synthesized using methods previously described.[3,4] Inks were printed using a customized solvent-cast printer into scaffolds (3mm depth, 4mm thickness). Under IACUC approval, full-thickness defects of identical size in 4 rabbits (female only, due to animal availability) were prepared in the trochlear groove of both hind limbs of each animal. Prior to implantation, scaffolds were incubated in bone marrow aspirate concentrate obtained from the same animal during defect creation. Samples were harvested at 3 months after surgery.
Tissue Processing and Microtomy: Tissues were fixed immediately after harvest in 10% Neutral Buffered Formalin for a minimum of 2 weeks before being decalcified in Immunocal Decalcifier (StatLab) until easily cut with a microtome blade. Tissues were trimmed to ~3mm thickness and dehydrated in a progressive gradient using times, temperatures, and reagents described in Table 1. After embedding in Paraffin 46-48 (Millipore Sigma), tissues were sectioned at 5µm on a rotary microtome (Leica Biosystems) and allowed to dry overnight. Sections were rehydrated in a progressive gradient without Xylene and stained with Hematoxylin and Eosin (H&E), and Safranin-O counterstained with Fast Green (SafO). Slide mounting was performed with Cover-Safe (StatLab), a Toluene-free mounting medium, and analyzed via bright field light microscopy.

RESULTS SECTION: Representative histology (Fig. 1) of different treatment groups (left, mid, and right image panels, respectively) demonstrate broad regeneration of osteochondral tissues within the implanted defect (1A, rows 1&2: scale bars = 500µm). High-magnification images show preserved PCL fibers in both superficial and deep regenerated tissue segments without damage or distention of regenerated tissues (Fig. 1B, scale bars = 100µm).

DISCUSSION: Histological results indicate that this novel processing protocol preserved PCL implant material while reducing manual processing times compared to previously reported protocols. In addition, results show protocol provides simultaneous evaluation of both implant and regenerated tissue within the same slides. These findings underscore the efficacy of our protocol in the evaluation of osteochondral tissue implant efficacy and provide a strong foundation for further study in the field of biomaterial research.

SIGNIFICANCE/CLINICAL RELEVANCE: This work advances technical capability in the evaluation of biomaterials in osteochondral tissue regeneration strategies, driving potential clinical relevance as treatment options in early-stage osteoarthritis.

REFERENCES: [1] Dębski+, *Materials* 15(5):1732, 2022. [2] Hegazy+, *Ann Int Med Dent Res* 1:57-61, 2015. [3] Camacho+, *Biomater Sci* 7:4237-4247, 2019. [4] Camacho+ *Biomater Sci* 9:6813-6829, 2021

Processing Step	Immersion Solution	Item of Technique	PCL-Preserving FFPE Method
Dehydration	Alcohol 70%	Duration Temperature Stirring	45 Minutes 30°C-35°C 100rpm
	Alcohol 80%	Duration Temperature Stirring	45 Minutes 30°C-35°C 100rpm
	Alcohol 90%	Duration Temperature Stirring	45 Minutes 30°C-35°C 100rpm
	Alcohol 100%	Duration Temperature Stirring	45 Minutes 30°C-35°C 100rpm
Clearing	Histoclear I	Duration Temperature Stirring	45 Minutes 30°C-35°C 100rpm
	Histoclear II	Duration Temperature Stirring	45 Minutes 30°C-35°C 100rpm
Infiltration	Paraffin	Duration Temperature Stirring	2 Hours to O/N 48°C-50°C 100rpm
Total Time in Protocol			***6.5 Hours Minimum***

Table 1: Sequence of tissue processing in novel PCL-preserving FFPE method. Total processing times are listed for this method.

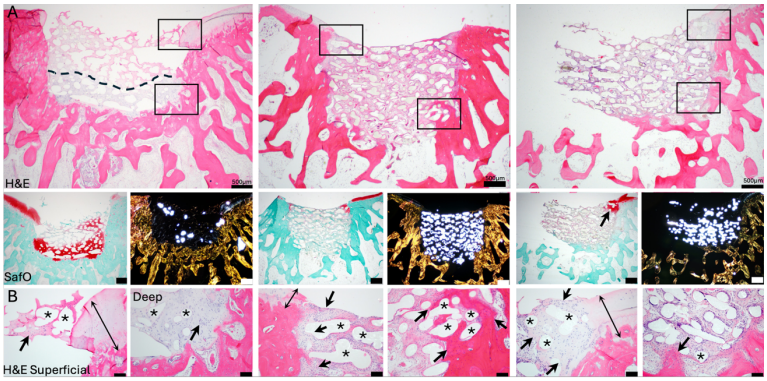

Fig. 1: Low magnification imaging (Rows 1 & 2, scale = 500µm) shows concentrations of proteoglycan-rich matrix within superficial regions of the defect (upper frame) and osteogenesis concentrated at the base of the defect (lower frames). High magnification imaging (Row 3, scale = 100µm) of superficial and deep peripheral margins of the defect corresponding to above frames show preserved PCL implant (asterisks) surrounded by primitive mesenchyme (single-headed arrow) at interface of the flanking articular cartilage (double-headed arrows), while others demonstrate areas of chondrogenesis and osteogenesis, respectively. Central areas of all images show preserved PCL material surrounded by primitive mesenchymal cells mixed with adipose islands, multinucleate giant cells and lymphoplasmacytic infiltrates.