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EVALUATING THE COLLAGEN NETWORK OF ARTICULAR CARTILAGE USING CONTRAST-ENHANCED X-RAY MICROSCOPY

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INTRODUCTION

Affecting 1 in 8 Canadians in 2010 and expecting 1 in 4 Canadians in 2040 [1], osteoarthritis (OA) is a common chronic joint disease characterized by degenerative changes of joint cartilages. Articular cartilage is comprised of chondrocytes and extracellular matrix which is a composite of water and water-imbibing proteoglycans that resist compression when restrained by a network of tensile-resisting fibrillar collagen. Early diagnosis of OA requires the understanding of OA pathology and sensitive imaging, ideally at the cellular-molecular level. A key step is to evaluate the collagen network structure in both normal and injured articular cartilage, where the collagen network is disrupted typically as superficial fibrillation or clefts.

Traditional cartilage histopathology uses phosphotungstic acid (PTA) to stain connective tissue such as the collagen network, and was developed in 1900 [2]. Non-staining options for viewing cartilage collagen after histological sectioning include Fourier Transformed Infrared imaging (FTIR) [3] or polarized light microscopy [LM] [4]. These gold standard histological techniques produce excellent images, but the process is destructive, time-consuming and limited to two dimensional views. Contrast-enhanced Magnetic Resonance Imaging [5] can image collagen distribution in three dimensions (3D), but with limited image resolution compared to the structures.

Recently micro-computed tomography (micro-CT) technology [6] provides the possibility to image the cartilage collagen network non-destructively in high resolution and 3D using the same PTA contrast agent used in the traditional histological staining.

X-ray microscopy (XRM) is a novel imaging technique, which is distinct from the traditional micro-CT because it combines both geometric magnification and optical objectives of microscopy to achieve higher spatial resolution at a relatively longer working distance. By simply adjusting working distances, XRM can produce edge-enhancement by introducing a propagational phase contrast effect. The purpose of this study is to establish a correlation between the PTA-enhanced XRM and the gold-standard histology for the evaluation of the cartilage collagen network.

MATERIALS AND METHODS

Specimens

Two healthy bovine knee joints (1 left & 1 right) were collected from a local market in a vacuum sealed container. Three cartilage disks of 6 mm diameter were incised from the medial femoral condyle of the right knee (denoted as AC discs). A small articular cartilage cube of 1.5 x 1.2 mm^2 cross-sectional area and 1.2 mm thickness was incised from the medial femoral condyle of the right knee with subchondral bone (denoted as small ACB). A bigger articular cartilage cube of 5 x 3 mm^2 cross-sectional area and 1.6 mm thickness was incised from the middle of the medial femoral condyle of the left knee with subchondral bone (denoted as big ACB).

All these tissue samples were put into neutral buffered formalin (NBF) overnight for XRM imaging next day. The AC discs samples were put into 70% ETOH containing 1% PTA solution for the predefined staining time of 0, 18, 36, 54, 72, 91 and 1151 hours and taken out for XRM imaging. The first AC disc was imaged at 0, 18 and 91 hours; the second AC disc was imaged at 36, 54 and 72 hours; and the last AC disc was imaged at 1151 hours. The small ACB sample was only imaged after 91 hours of PTA staining time. The big ACB sample was imaged after 0, 18 and 36 hours of PTA staining time.

XRM Imaging

The AC disc sample was sandwiched between two pieces of foam and wrapped in Parafilm inside a sample tube (10 mm OD, Scanco Medical) with padding on both sides. The tube was filled with a 70% ETOH solution and sealed by Parafilm. X-ray imaging was performed on a pre-clinical XRM scanner (Versa 520, Carl Zeiss X-ray Microscopy, USA) using the settings of 80 kVp, 7 W with a custom filter at 6 micron isotropic pixel size. 3 second of exposure (2s for 91 hrs) with 2501 projections took 3 hours of scan time (0, 18, 36 hrs); 3 second of exposure with 1601 projections took 2 hours of scan time (54, 72, 1151 hrs).

The small ACB sample was sandwiched in a hard foam cylinder that tightly fitted in a Kapton straw (5 mm ID). The Kapton straw was secured by a metal stub (7 mm OD, Scanco Medical) from the bottom, filled with 70% ETOH, capped by a foam plug and sealed by Parafilm from the top. The imaging settings using the same XRM scanner were 80 kVp, 7 W with another custom filter at 1.3 micron isotropic pixel size. 1 second of exposure with 1601 projections took 1.7 hours of scan time.

The big ACB sample was wrapped with Parafilm and glued (by Loctite instant adhesive) to the top surface of a metal stub (7 mm OD, Scanco Medical). The imaging settings using the same XRM scanner were 80 kVp, 7 W with no filter at 5 micron isotropic pixel resolution. The x-ray source and the detector were pulled 1.5 times the absorption distances for a phase contrast edge-enhancement effect. 2 seconds of exposure with 1601 projections took 1.7 hours of scan time.

Histology

After XRM imaging, the small ACB sample went through the standard histology procedures including de-calcification, processing, embedding and sectioning into thin slices (15 micron for polarized LM and 8 micron for histology). Slides of the small ACB section were imaged under the polarized LM (Xiopaln2, Carl Zeiss Microscopy, USA). After hematoxylin and eosin staining for histomorphology, the slides were imaged using the same microscope.

Data Analysis

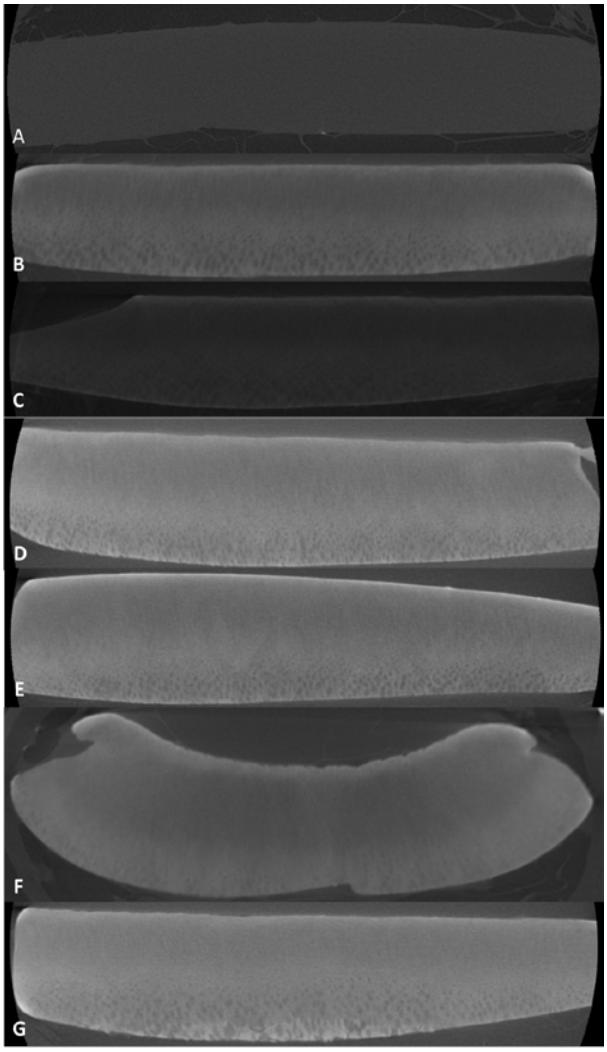

Figure 1: Images of AC discs with A-G as PTA-stained for 0, 18, 36, 54, 72, 91 and 1151 hrs.

Custom developed Matlab (v.R2014a) code was programed to analyse each of the AC disc's reconstructed image data. 200 image slices from the same AC disc region were visually selected and the volume of interest was selected to include as much cartilage as possible. For each image slice, using threshold-based edge detection, the center line of the cartilage was found and was rotated to the horizontal direction (Fig 1). After edge detection and refinement, the cartilage region between the articular surface and subchondral bone surface was resized to 300 pixels deep and 1000 pixels wide as the region of interest (ROI) (Fig 2). Averaging along the 1000 pixel width and 200 slices produced the averaged image intensity profile along the cartilage depth at each PTA staining time (Fig 3).

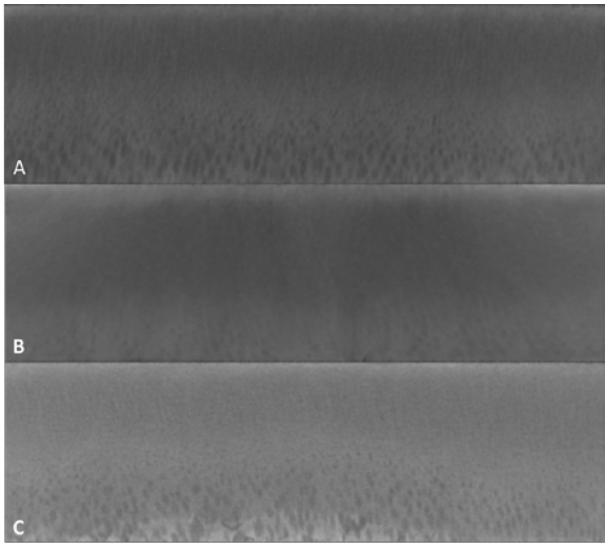

Figure 2: The first ROI slices of the AC discs with A-C as PTA-stained for 18, 91 and 1151 hours, respectively.

RESULTS

Figures 1 and 2 clearly showed that the PTA stained cartilage nicely highlighted the internal structure as compared with the even intensity distribution in the non-stained cartilage (Fig 1A). Figure 3 indicated that more PTA was absorbed throughout the depths in longer staining time, with the exception that at 0-7% cartilage depth 36 hrs had higher image intensity than 54 hrs. This may indicate that PTA accumulated more at the first contacting surface at the early stage of its penetration

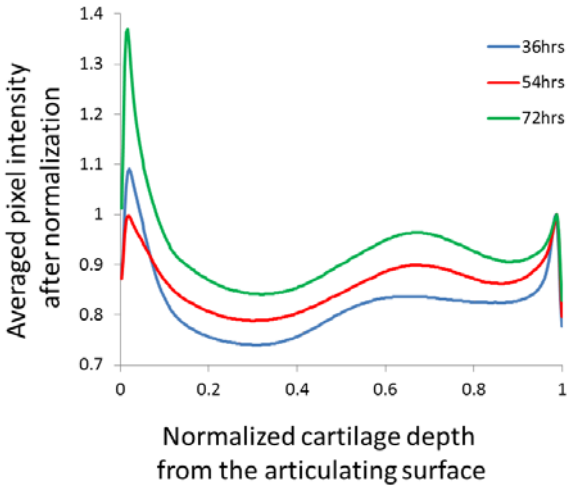

Figure 3: Averaged image intensity variations along cartilage depth, normalized by maximal value on the subchondral bone side.

process. The overall distribution of the intensity variation matched well with the collagen density expectation along the depth, with most dense packed collagen in the surface zone (0 – 20%), most loose packed collagen in the transitional zone (20%-50%), denser packed in the deep zone (50%-95%) before the tide-mark (close to 100%). The XRM image (Fig 4B) of the small ACB sample visually matched well with its histology slice (Fig 4E) on both the distribution and size of the chondrocyte clusters and the tide-marks. The XRM images (Fig 4B) visually matched well with the collagen fiber

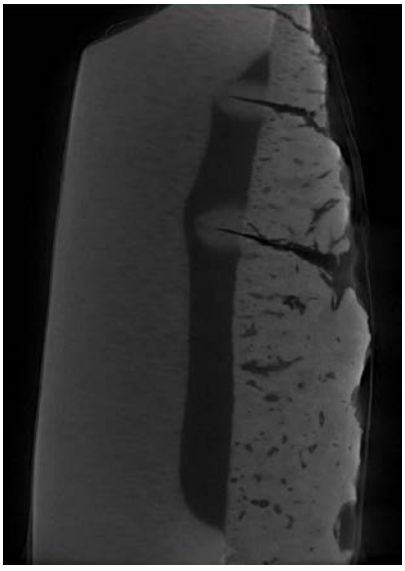

Figure 5: A XRM image of the 18 hrs PTA-stained big ACB sample.

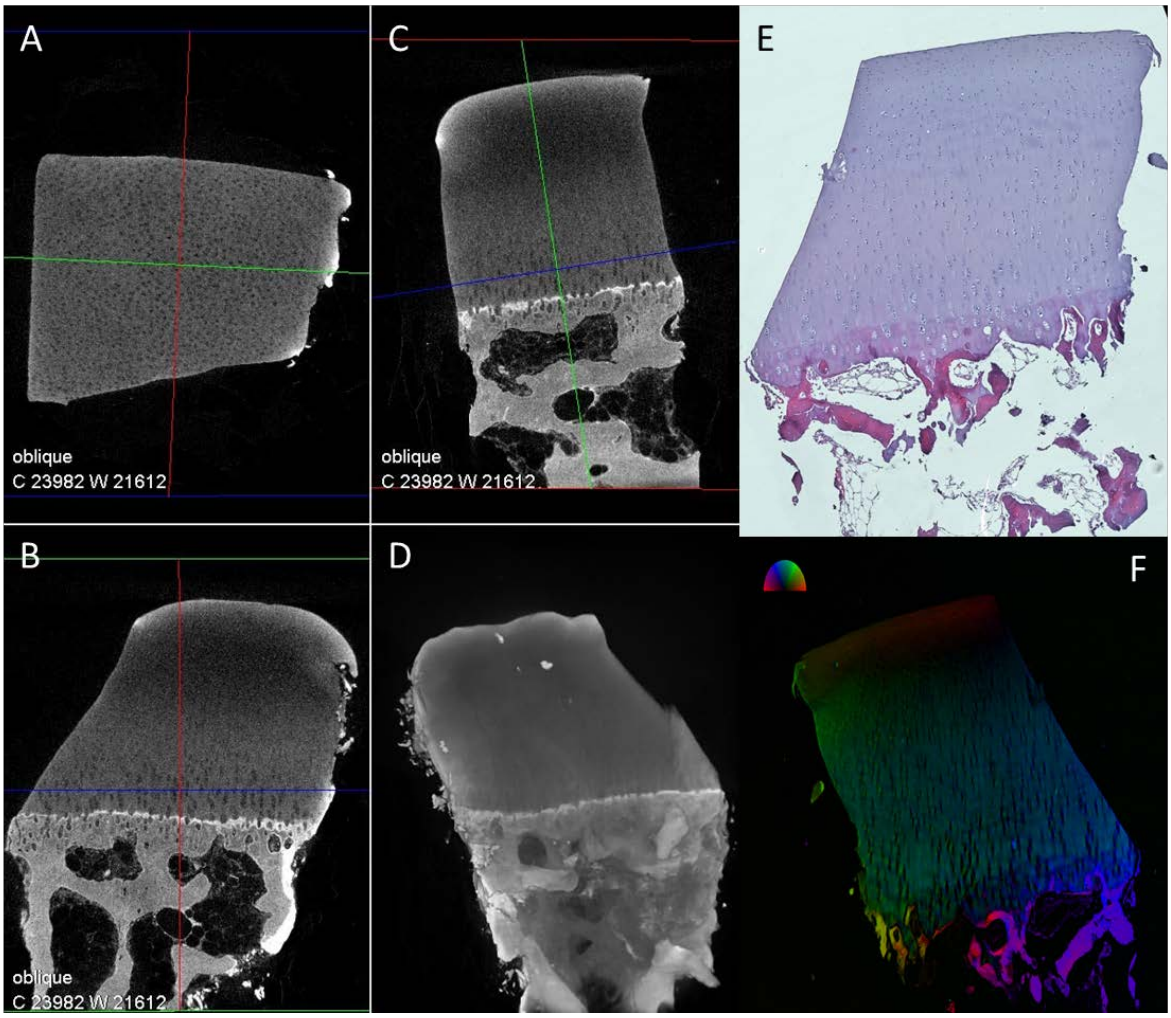

Figure 4: XRM images (3 orthogonal planes A-C and 3D view D) of the 91 hrs PTA-stained small ACB vs histology (E) & polarized LM (F).

directions as shown in the polarized image (Fig 4F). An interesting timestamp of the PTA penetration was captured in Figure 5 where the invading wave fronts from both sides meet.

DISCUSSION

The first AC disc was folded in 91 hrs scan (Fig 1F) after 0 hr and 18 hrs scanning, due to sample wrapping or shrinking or dehydrating during the process. This would lead to inaccurate presentation of the cartilage intensity variation along the depth (Fig 2B), because the present algorithm assumed the cartilage disc is flat and did not take into account the curvatures. Thus, the first AC disc was not used in the later scans and Figure 3 was only plotted based on the 36, 54 and 72 hrs staining performed on the second AC disc.

This preliminary study visually demonstrated that PTA-enhanced XRM is a novel approach for evaluating the collagen network in articular cartilage.

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