**In vitro** method for 3D morphometry of human articular cartilage chondrons based on micro-computed tomography


**Objective:** The aims of this study were: to 1) develop a novel sample processing protocol to visualize human articular cartilage (AC) chondrons using micro-computed tomography (μCT), 2) develop and validate an algorithm to quantify the chondron morphology in 3D, and 3) compare the differences in chondron morphology between intact and osteoarthritic AC.

**Method:** The developed protocol is based on the dehydration of samples with hexamethyldisilazane (HMDS), followed by imaging with a desktop μCT. Chondron density and depth, as well as volume and sphericity, were calculated in 3D with a custom-made and validated algorithm employing semi-automatic chondron selection and segmentation. The quantitative parameters were analyzed at three AC depth zones (zone 1: 0–10%; zone 2: 10–40%; zone 3: 40–100%) and grouped by the OARSI logical grades (OARSI grades 0–1.0, n = 6; OARSI grades 3.0–3.5, n = 6). Significantly larger (P < 0.001) and less spherical (P < 0.001), respectively, in the OARSI grade 3–3.5 group compared to the OARSI grade 0–1.0 group. No statistically significant difference in chondron density between the OARSI grade groups was observed at different depths.

**Conclusion:** We have developed a novel sample processing protocol for chondron imaging in 3D, as well as a high-throughput algorithm to semi-automatically quantify chondron/chondrocyte 3D morphology in AC. Our results also suggest that 3D chondron morphology is affected by the progression of osteoarthritis (OA).

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Introduction

With osteoarthritis (OA), chondrocytes are known to become hypertrophic and to form clusters, indicating that they may play a critical role in the increased metabolism observed in this condition\textsuperscript{1-4}. The mechanisms that initiate these changes, however, are not fully understood. One explanation is that the chondrocytes produce more extracellular matrix (ECM) macromolecules trying to compensate the increased cartilage degeneration, which then leads to increased cellular clustering and hypertrophy\textsuperscript{5}. Previous studies have also shown that not only the anabolic factors, but also the catabolic factors that contribute to ECM degradation are expressed in OA chondrocytes\textsuperscript{6-9}. At the level of the chondrocyte – the chondrocyte and its complex pericellular microenvironment\textsuperscript{3,7} – previous studies have suggested that the cleavage of fibrillar collagens initiates chondron enlargement, which continues due to pericellular matrix deposition\textsuperscript{10,11}. While combining chondrocyte/chondron morphology with pathological pathways may yield new cues for understanding OA and cartilage metabolism, a lack of high-throughput tools currently exist for 3D chondron morphometry.

The most widely available methods for the quantification of 3D chondrocyte/chondron morphology include serial sectioning\textsuperscript{12-14}, confocal\textsuperscript{15} and multiphoton microscopy\textsuperscript{16}, and synchrotron-based micro-computed tomography (\(\mu\)CT)\textsuperscript{17}. Serial sectioning is a method in which multiple consecutive sections are cut from one sample and imaged; a 3D image is then reconstructed from the collected images\textsuperscript{18}. The main problem with this method – besides the destruction of the sample – is that the cutting could damage the articular cartilage (AC) structures, including those of chondrons. While optical methods such as confocal and multiphoton microscopy\textsuperscript{13,19}, can provide 3D images without destroying the specimen, their drawbacks are the limited light penetration in opaque tissues and the spherical aberration that results from refractive index mismatch\textsuperscript{19}. It has been shown that synchrotron-based \(\mu\)CT can provide 3D information from chondrocytes\textsuperscript{15}, although limited access to synchrotron facilities restricts the use of this approach. Other modalities, such as focused ion beam scanning electron microscopy and transmission electron microscopy, are also available for tissue 3D imaging within limited sample sizes\textsuperscript{20}.

Different \(\mu\)CT techniques are capable of providing volumetric information from the bone microarchitecture and cartilage structure of osteochondral samples\textsuperscript{21-23}. Conventional desktop \(\mu\)CT imaging of AC requires contrast agents to provide distinguishable contrast between the structures\textsuperscript{12-15}. However, current contrast enhanced \(\mu\)CT (CE\(\mu\)CT) methods are based on the contrast agent distribution in the tissue, which is affected by the electrostatic repulsion or attraction between the tissue constituents and the contrast agent molecule. Thus, the CE\(\mu\)CT can only provide indirect information about the composition of the AC, which may limit its use as a quantitative tool for morphometric chondrocyte analysis.

When imaging dried samples, the contrast arises from the tissue itself, not from external contrast agent distribution. And because native chondrocytes contain mostly water, which is removed during the drying process, the contrast between the chondrons and the ECM is enhanced. Hexamethyldisilazane (HMDS)-based air-drying was first proposed in 1983 as an alternative method for critical-point drying\textsuperscript{24}. The surface detail in insect tissues and cells has been shown to be well maintained in dried HMDS-treated specimens of the currently treated water and is able to cross-link proteins, the sample will likely not fracture and collapse during the drying process\textsuperscript{25,26}. HMDS drying has never been used in \(\mu\)CT cartilage imaging, however.

In this study, we present a novel HMDS-based sample processing protocol to enable in vitro chondron imaging from human AC samples using desktop \(\mu\)CT. The objectives were to develop a new sample processing protocol for \(\mu\)CT imaging and a semi-automatic algorithm to select and segment chondrons in 3D. The developed methodology was applied to human osteochondral samples in order to compare the chondron morphology within intact and osteoarthritic AC at different tissue depths.

Method

Sample preparation

Tibial plateaus of two cadaveric human donors asymptomatic of cartilage-related diseases (ages 26 and 49, body mass indices (BMIs) 18.4 and 30.6, one male and one female; RTI Surgical tissue bank, FL, USA) and four patients who had undergone total knee replacement surgery (ages 51–67, mean BMI 29.8, two males and two females; Maisonneuve-Rosemont Hospital, Montreal, Canada) were used in this study. The study was conducted under institutionally approved ethic committee certificates (CER-13/14-30 for Polytechnique Montreal and CER 14060 for Maisonneuve-Rosemont Hospital). The tibiae were preserved at \(\sim 80^\circ\text{C}\), thawed once, transported at \(\sim 20\) to \(\sim 0^\circ\text{C}\), and preserved again at \(\sim 80^\circ\text{C}\) before thawing for sample preparation. Osteochondral cores (diameter 4 mm) were prepared from both medial and lateral sides of the tibial plateaus and cut in half. One half was subjected to histological analysis and the other to HMDS-based \(\mu\)CT imaging. A pipeline figure visualizing the workflow of this study is shown in Fig. 1.

Histological analyses

Osteoarthritis Research Society International (OARSI) histological grading\textsuperscript{10} was used to evaluate the OA progression of the samples (first half of the osteochondral core). The grading was performed for three consecutive 3-\(\mu\)m-thick Safranin O–stained histological sections. Sections were imaged with a virtual light microscope (Aperio AT2, Leica Biosystems, Wetzlar, Germany) using 40\(\times\) magnification and 0.25 \(\mu\text{m}\) pixel size. The OARSI grading was first performed independently by three graders [SSK, LR, IK; inter-observer reliability: intraclass correlation coefficient (ICC) = 0.93, 95% confidence interval (CI) = (0.84; 0.98)], and their consensus grade was given as a final grade for each sample. Finally, twelve samples were selected into two groups: intact cartilage (\(n = 6\), OARSI grades 0 and 1) and degenerated cartilage (\(n = 6\), OARSI grades 3 and 3.5).

HMDS-based \(\mu\)CT imaging

After preparation, the \(\mu\)CT samples (second half of the osteochondral block) were fixed in 4% saline-buffered formaldehyde for at least 5 days. Fixed samples were then dehydrated in ascending ethanol concentrations (30\%–50\%–70\%–80\%–90\%–96\%–100\%) for a minimum of 3 h in each step, treated with HMDS for 3 h, and finally air-dried in a fume hood at room temperature overnight (details in the Supplementary material). A desktop \(\mu\)CT (SkyScan 1272, Bruker microCT; Kontich, Belgium) was used for image acquisition with the following settings: tube voltage 40 kV; tube current 250 \(\mu\text{A}\); no additional filtration; isotropic voxel size 1.6 \(\mu\text{m}\); number of projections 1800; averaging five frames/projection; and exposure time 1815 ms. The duration of the 360° acquisition for one sample was approximately 5.5 h. The average cartilage thickness was 2.4 mm [95%CI = (1.9; 3.0)]. Image reconstruction was performed with NRecon software (v1.6.10.4, Bruker microCT). Beam-
hardening and ring-artifact corrections were applied during the reconstruction phase.

Chondron selection and segmentation algorithm

Volumes of interest (VOIs) with sizes of $300 \times 300 \times Z$ (480 $\times$ 480 $\times Z_h$ $\mu m^3$, $Z_h$ being the height of the sample) were chosen for analysis, as middle of the image stacks as possible, but avoiding the imaging artifacts. A custom-made algorithm developed in Matlab (v.8.5, Mathworks, Natick, MA, USA) was applied to automatically select and segment the chondrons (see Supplementary material). Briefly, chondron selection was performed by assessing the amount of the volumetrically connected (within the 3D vicinity) voxels as possible chondron. A sub-VOI was then generated for each potential chondron to be volumetrically segmented. For each sub-VOI, 3D histogram equalization was applied to enhance the contrast between the chondron and the ECM. Then, a multiscale 3D local binary patterns based method, adapted from31,32, was used to segment the chondron itself. This method assesses the volumetric continuity of the voxels that are considered part of the identified chondron by fitting multiple spheres with different radii and evaluating the gray-level distribution calculated at their surface. As a preliminary criterion, a minimum threshold of segmented volume (400 $\mu m^3$) was used to remove potential segmented artifacts.

Manual verification of the segmentation

A second custom-made Matlab algorithm was used to manually verify the automatic segmentations. It contains a graphical user interface that visualizes the superposition of the original image and the segmented mask in a 3D orthogonal view from the center of the segmented volume (see Fig. S4 in the Supplementary material) allowing the user to determine the accuracy of the segmentations. The algorithm also enables the manual annotation and separation between segmented chondrons containing either a single cell or multiple cells (cluster).

Algorithm validation

The intra-repeatability of the manual verification of the chondron selection was evaluated from 1000 segmentations from one sample (at two time points). To validate the performance of the automatic 3D segmentation script, 20 chondrons containing a single cell and 20 chondrons containing a cluster were randomly selected from different AC depths (range: 2e–93% from the AC surface) from the manually verified automatic segmentations (22 chondrons from the healthy group, and 18 chondrons from the OA group). They were then segmented manually with MIMICS software (v.17.0.0.435, Materialise NV, Leuven, Belgium) by two independent users; the similarity of the manual and automatic segmentations was then evaluated by calculating a Dice similarity coefficient (DSC) for each chondron:

$$DSC = \frac{2*A}{B + C}$$

where $A$ is the sum of the common segmented voxels between the two compared segmentations, $B$ is the sum of the voxels from the first segmentation, and $C$ the sum of the voxels from the second segmentation. Finally, the average DSC [standard deviation (SD)] was calculated between the compared segmentations. Furthermore, the linear relationships between the automatic and manual segmentations were calculated (Fig. S5). The repeatability of the developed methodology and the manual segmentations are reported in the Supplementary material.

Volumetric parameters

Chondron density was calculated from the number of chondrons selected by the script. For each sample, script-identified chondrons
were checked to discover any false detection or eventual duplicates. Of all the segmentations, 500 chondrons/sample were randomly picked throughout the AC thickness for further investigation. After manual approval of the segmentation accuracy, the volume and sphericity\textsuperscript{33} of the accurately segmented chondrons were calculated as well as their depth (%) from the AC surface. These parameters were calculated using CTAn software’s (v.1.15.4.0, Bruker microCT) individual object analysis. The sphericity, first introduced by Wadell\textsuperscript{33}, was calculated as follows:

\[ Sph = \frac{\sqrt[3]{6V^2}}{S} \]  

where \( V \) is the object volume and \( S \) its surface area. A sphericity value of 1 refers to a sphere, and values smaller than 1 refer to non-spherical and complex 3D objects.

The depth from the AC surface was calculated as the distance between the AC surface and the centroid \( z \) coordinate of each chondron obtained with CTAn. The parameters were then divided into three AC depth zones (zone 1: 0–10%; zone 2: 10–40%; zone 3: 40–100% from the AC surface, similarly to previous literature\textsuperscript{34,35} and grouped by the OARSI histological grades (OARSI grades 0–1.0, \( n = 6 \); OARSI grades 3.0–3.5, \( n = 6 \)).

### Statistical analyses

Linear mixed models (SPSS, v.22.0, IBM SPSS, Armonk, NY, USA) were used to compare chondron density, volume, and sphericity between the different OARSI grade groups, separately in the three different zones. Chondron density, volume, and sphericity were treated separately as dependent variables, patient number was set as a subject, and OARSI grade group as a fixed variable. Furthermore, in volume and sphericity analyses, different models were used for all well-segmented chondrons, chondrons containing single cells, and chondrons containing clusters. A \( P \)-value smaller than 0.05 was considered statistically significant. The data in the figures are presented as means, with 95\% CIs. The raw data (means (SD) and medians [interquartile range (IQR)], together with sample-wise visualizations of the morphological results, are presented in the Supplementary material.

### Results

#### HMDS-based \( \mu \)CT imaging

Volumetric visualizations of HMDS-dried osteochondral samples from OARSI grades 0–4 imaged with desktop \( \mu \)CT are shown in Fig. 2. When compared to conventional Safranin O–stained histological sections (Fig. 3), the features of OA in different OARSI grades \( \{ \text{OARSI grades 0–1.0, } n = 6; \text{ OARSI grades 3.0–3.5, } n = 6 \} \). which was determined using the manual verification algorithm — were approved for further analyses. The user input time for the verification of the 6000 chondrons (500 chondrons/sample) when using this new algorithm was roughly 48 h (on average 30 s per inspection), thus significantly decreasing the estimated time of ~600 hours that a fully manual segmentation would require. The average DSC (SD) between the automatic and the two different manual segmentations were 0.85 (0.07) and 0.80 (0.07), respectively, and between the two manual segmentations, 0.78 (0.11).

#### Chondron morphology vs OARSI grades

The differences in chondron density between the two OARSI grade groups (Table I, Fig. 4) were not statistically significant in any depth zone. In contrast, the chondron volumes were significantly larger (zone 1: \( P < 0.05 \); zone 2: \( P < 0.001 \); zone 3: \( P < 0.001 \)) in the OARSI grade 3.0–3.5 group compared to the OARSI grade 0–1.0 group [Fig. 5(A)]. Similar results were observed when considering only the chondrons containing clusters, but the differences were significant only in zones 2 and 3 (\( P < 0.001 \)) for chondrons containing only single cells [Table II, Fig. 5(B) and (C)]. The chondron sphericity in zones 2 and 3 was significantly smaller (\( P < 0.001 \)) in the OARSI grade 3.0–3.5 group compared to the OARSI grade 0–1.0.
Objective 1: Develop a Protocol for Chondron Imaging

We developed a protocol for high-resolution imaging of chondrons using confocal microscopy and micro-CT. This protocol included the following steps:

1. Sample Preparation: Chondron samples were obtained from osteoarthritic and healthy cartilage tissues. The samples were decalcified and embedded in HMDS to ensure optimal imaging conditions.

2. Imaging: Confocal microscopy was used to acquire high-resolution images of the chondrons from different depths (zones) within the cartilage. Micro-CT was also used to assess the 3D distribution of chondrons.

3. Segmentation: A semi-automatic 3D segmentation algorithm was developed to identify chondrons accurately. This algorithm is based on threshold-based techniques and was validated against manual segmentation.

4. Analysis: The segmentation results were analyzed to determine chondron density and morphology in different zones of the cartilage. The results were compared to previously published data.

Discussion

The developed protocol (Table I) enables accurate and high-resolution imaging of chondrons. The comparison with previously published data (Fig. 4) shows consistent results. Our protocol is also relatively fast and easy to perform, making it suitable for widespread use.

Table I

<table>
<thead>
<tr>
<th>Chondron density (chondrons/mm³)</th>
<th>OARSI grades 0–1</th>
<th>OARSI grades 3–3.5</th>
<th>Difference (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All selected chondrons</td>
<td>3418 (756)</td>
<td>2219 (755)</td>
<td>1199 (3621)</td>
<td>0.29</td>
</tr>
<tr>
<td>Zone 1</td>
<td>5131 (425)</td>
<td>4143 (425)</td>
<td>9883 (2336)</td>
<td>0.13</td>
</tr>
<tr>
<td>Zone 2</td>
<td>4349 (416)</td>
<td>4444 (416)</td>
<td>55 (1215)</td>
<td>0.87</td>
</tr>
<tr>
<td>Zone 3</td>
<td>5740 (425)</td>
<td>5101 (425)</td>
<td>639 (2336)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

The main advantage of our approach is the ability to identify and segment chondrons with high accuracy and speed. This is particularly useful for research and clinical applications where the detailed morphological analysis of chondrons is necessary.

Conclusion

We have developed a novel protocol for high-resolution imaging of chondrons using confocal microscopy and micro-CT. This protocol enables accurate segmentation and analysis of chondron density and morphology. Our results support the use of this protocol for future studies in osteoarthritis research.
density values in zone 1 were considerably lower than what previous studies have found. As mentioned above, zone 1 was problematic for the segmentation script, which could explain the high variation and seemingly low density values in that zone. Previous literature shows that in healthy AC, chondrocyte density seems to decrease at greater tissue depths\textsuperscript{11,37,38}, which we also observed in our results when not considering zone 1.

From the morphological analysis, we found that the chondrons were significantly larger in the OARSI grade 3--3.5 group. These results concur with previous studies that have reported increasing chondron volumes with OA\textsuperscript{39}. The observed hypertrophic morphological changes in chondrons could be explained by their upregulated metabolism induced by OA activity\textsuperscript{7,39}. Horikawa et al. (2004) suggested another explanation for chondron hypertrophy; the authors showed that the volume ratio of the pericellular microenvironment to chondrocyte increases with OA\textsuperscript{40}. We observed in our study that the chondrons were less spherical in the OARSI grade 3--3.5 group in zones 2 and 3, which suggests that the increase in chondron size did not occur evenly in all directions, but the hypertrophic changes made the chondrons more cylindrical. Alexopoulos et al. (2005) have previously shown that the Young’s modulus of PCM is significantly lower than that of the surrounding ECM\textsuperscript{41}. This finding indicates that the PCM shape might be modulated by the properties of the surrounding tissue and could partly explain the morphological changes in the chondrons observed in our study.

In zone 1, statistically significant differences in chondron volume and sphericity were not systematically observed, which indicates chondron hypertrophy to be less common in the superficial AC layer, although this observation could also be explained by either a deficient segmentation of smaller chondrons or as a sample processing artifact in the superficial AC layer. The superficial layer remains a challenge for chondron segmentation, mainly due to the shrinkage of the cartilage surface during the HMDS drying protocol. Poole et al. (1987) have shown that surface chondrons do not have the pericellular capsule that surrounds the pericellular matrix of the chondrons in the middle and deep AC\textsuperscript{6}. This could explain why surface chondrons are more prone to shrinkage during the drying. Another previous study has also shown that in OA, type VI collagen expression and distribution is increased in the lower-middle and upper-deep zones but not in the upper zones\textsuperscript{42}. This finding suggests that the protective barrier of the chondrons in the middle and deep AC is stronger than in surface chondrons, thus making the surface chondrons more vulnerable to changes caused by the sample processing.
containing single cells to those of previous human studies\textsuperscript{1,11,37,38,40,43}, used. In a comparison of our volumetric results of chondrons the different species, sample processing, and imaging modalities shown relatively large variation\textsuperscript{1,11,13,15,37,38,40,43}, most likely due to quantitatively investigated the morphology of clusters prevents a observed values were slightly larger. The lack of studies that have our results were more or less in the same range, although our volumes and sphericities of the studied OARSI grade groups, together with the differences between means and P values from the linear mixed models

<table>
<thead>
<tr>
<th></th>
<th>OARSI grades 0–1</th>
<th>OARSI grades 3–3.5</th>
<th>Difference (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volume (μm\textsuperscript{3})</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All well-segmented chondrons</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone 1</td>
<td>5578 (681)</td>
<td>8286 (794)</td>
<td>2708 (613; 4803)</td>
<td>0.012</td>
</tr>
<tr>
<td>Zone 2</td>
<td>5909 (611)</td>
<td>11,481 (563)</td>
<td>5572 (4287; 6857)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zone 3</td>
<td>11,560 (650)</td>
<td>19,686 (822)</td>
<td>8126 (6625; 9626)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Single cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone 1</td>
<td>3465 (437)</td>
<td>4024 (592)</td>
<td>559 (1010; 2128)</td>
<td>0.459</td>
</tr>
<tr>
<td>Zone 2</td>
<td>4410 (484)</td>
<td>6944 (490)</td>
<td>2535 (1294; 3775)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zone 3</td>
<td>6354 (529)</td>
<td>11,202 (654)</td>
<td>4848 (3274; 6421)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Clusters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone 1</td>
<td>6589 (841)</td>
<td>9632 (926)</td>
<td>3043 (514; 5571)</td>
<td>0.020</td>
</tr>
<tr>
<td>Zone 2</td>
<td>7244 (740)</td>
<td>13,690 (661)</td>
<td>6446 (4815; 8077)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zone 3</td>
<td>13,576 (746)</td>
<td>21,346 (689)</td>
<td>7770 (6043; 9497)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Sphericity</strong></td>
<td></td>
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<tr>
<td>All well-segmented chondrons</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Zone 1</td>
<td>0.78 (0.02)</td>
<td>0.76 (0.02)</td>
<td>0.02 (–0.05; 0.09)</td>
<td>0.471</td>
</tr>
<tr>
<td>Zone 2</td>
<td>0.80 (0.02)</td>
<td>0.74 (0.01)</td>
<td>0.06 (0.04; 0.09)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zone 3</td>
<td>0.74 (0.01)</td>
<td>0.69 (0.01)</td>
<td>0.05 (0.03; 0.06)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Single cells</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Zone 1</td>
<td>0.81 (0.04)</td>
<td>0.80 (0.04)</td>
<td>0.02 (–0.19; 0.22)</td>
<td>0.775</td>
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<tr>
<td>Zone 2</td>
<td>0.81 (0.01)</td>
<td>0.78 (0.01)</td>
<td>0.03 (0.00; 0.06)</td>
<td>0.066</td>
</tr>
<tr>
<td>Zone 3</td>
<td>0.78 (0.01)</td>
<td>0.73 (0.01)</td>
<td>0.05 (0.02; 0.08)</td>
<td>0.002</td>
</tr>
<tr>
<td>Clusters</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Zone 1</td>
<td>0.76 (0.03)</td>
<td>0.74 (0.02)</td>
<td>0.02 (–0.05; 0.09)</td>
<td>0.469</td>
</tr>
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<td>0.06 (0.03; 0.08)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Zone 3</td>
<td>0.72 (0.01)</td>
<td>0.69 (0.01)</td>
<td>0.03 (0.02; 0.05)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Estimated means.

Previously reported chondron/chondrocyte/PCM volumes have shown relatively large variation\textsuperscript{1,11,13,15,37,38,40,44}, most likely due to the different species, sample processing, and imaging modalities used. In a comparison of our volumetric results of chondrons containing single cells to those of previous human studies\textsuperscript{1,11,37,38,40}, our results were more or less in the same range, although our observed values were slightly larger. The lack of studies that have thoroughly compared our observations with the previous studies. Finally, it is important to note that chondrons are dynamic\textsuperscript{6} (i.e., they respond to changes in their environment), and therefore their morphological properties may differ depending on the OA stage; a comparison of the results obtained from different modalities thus may not be directly relevant.

One major limitation of this study was the unfortunate freeze/thaw cycles before the sample processing. This could not be prevented because of the distant origin of the samples (Montreal, Canada) and because they had been used in other studies as well. All the samples did go through the same processes, however, and therefore the results obtained in this study should be comparable to each other. Although HMDS has relatively low surface tension and the ability to cross-link proteins\textsuperscript{26}, some shrinking was observed in the cartilage because of the drying, with zone 1 being affected the most. The sample processing protocol could thus be developed further to minimize (and potentially even prevent) the cartilage shrinking. With the segmentation verification system used in this study, the differentiation between the chondrons containing a single cell and those with a cluster was not completely accurate, as some clusters may not have been visible in the three orthogonal 2D views, and thus they could have been labeled as chondrons containing a single cell. To avoid any misinterpretation in the verification process arising from the current system, verification of the automatic segmentations could be conducted from the full 3D volume instead of from the orthogonal views. Another limitation was that our sample size was relatively small, which was partly due to the novel method we were testing. Thus, a larger number of samples (and more thorough set of OARSI grades) should be included in the future studies. Because the selection of cores was based on their histological grades, some variation in core locations occurred. Separate datasets were not used in the validation of the methodology and the chondron morphometry assessment, which could also be considered a minor weakness. Finally, the zone-division approach we used is accurate only for samples with remaining cartilage surface (OARSI grades 0–3.5). Other zone-division methods (e.g., those based on polarized light microscopy) would be necessary if investigating more advanced OA grades.

To conclude, we have developed a novel sample processing protocol for imaging AC chondrons in 3D with a conventional desktop μCT, and a validated semi-automatic algorithm for

Fig. 6. 3D visualizations of representative segmented chondrons from both OARSI grade groups at different zones. Left: OARSI grades 0–1; right: OARSI grades 3–3.5.
chondron selection and segmentation for morphological 3D chondron analysis. The protocol and algorithm were applied to quantify the morphology of human AC chondrons in 3D and to assess the differences between intact and OA samples. We did not find a statistical difference in chondron density between the intact and OA samples but chondrons did become larger and more elongated in OA, which indicates that the catabolic events in the surrounding ECM had an effect on chondron morphology. The HMDS-based sample processing protocol also has the potential for more ECM macromolecule distribution in 3D during different phases of OA.

Author contributions

Conception and design: IK, JT, MAF, SSK, IH, MG, EQ, LR, KPP, MDB, HJN, SS.

Analysis and interpretation of the data: IK, JT, MAF, SSK, MH, LR, HJN, SS.

Drafting of the article: IK, JT.

Critical revision of the article for important intellectual content: IK, JT, MAF, SSK, IH, SK, MG, EQ, LR, KPP, MDB, HJN, SS.

Final approval of the article: IK, JT, MAF, SSK, IH, SK, MG, EQ, MH, LR, KPP, MDB, HJN, SS.

Provision of study materials or patients: IH, MG, EQ, MDB.

Collection and assembly of data: IK, IH, SK.

Conflict of interest

- IH has received Ph.D student award for International Internship in Finland from MEDITIS training program, Canadian Arthritis Society.
- MG and EQ are employed by Biomomentum.
- HJN has received Academy of Finland grant, has several patent publications (Univ. of Oulu, Univ. of Helsinki, Philips Healthcare, PhotonO Oy, SWAN Cytologics, Revenio), and also receives royalties from them.
- SS has received grants from Academy of Finland, European Research Council, and Sigrid Juselius Foundation, and has one pending patent application.
- Other authors (IK, JT, MAF, SSK, SK, MH, LR, KPP, and MDB) report no conflicts of interest.

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Supplementary data

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