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TITLE:

Optical Sectioning and Visualization of the Intervertebral Disc from Embryonic Development to Degeneration

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KEYWORDS:

intervertebral disc, spatial chondrocyte organization, embryonic development, disc degeneration, chondrocyte, cell cluster formation

SUMMARY:

We present a method to investigate spatial chondrocyte organization in the anulus fibrosus of the intervertebral disc using an optical sectioning method.

ABSTRACT:

Intervertebral disc (IVD) degeneration is a leading cause of low back pain and it entails a high degree of impairment for the affected individuals. To decode disc degeneration and to be able to develop regenerative approaches a thorough understanding of the cellular biology of the IVD is essential. One aspect of this biology that still remains unanswered is the question of how cells are spatially arranged in a physiological state and during degeneration. The biological properties of the IVD and its availability make this tissue difficult to analyze. The present study investigates spatial chondrocyte organization in the anulus fibrosus from early embryonic

development to end-stage degeneration. An optical sectioning method (Apotome) is applied to perform high resolution staining analyses using bovine embryonic tissue as an animal model and human disc tissue obtained from patients undergoing spine surgery. From a very high chondrocyte density in the early embryonic bovine disc, the number of cells decreases during gestation, growth, and maturation. In human discs, an increase in cellular density accompanied the progression of tissue degeneration. As had already been demonstrated in articular cartilage, cluster formation represents a characteristic feature of advanced disc degeneration.

INTRODUCTION:

The intervertebral disc (IVD) is a cartilage-based structure that biochemically and with respect to cellular architecture, at first sight, resembles in many ways the articular cartilage[1]. Indeed, both IVD degeneration and osteoarthritis (OA) of articular cartilage are characterized by joint space narrowing due to cartilage wear, subchondral cyst and osteophyte formation, and subchondral sclerosis[2,3]. Despite these seeming similarities architecture and functional role of both tissues differ. While the matrix of articular cartilage is mainly formed of an arcade-forming collagen type II network, the IVD consists of three different types of tissue: the collagen type II-rich nucleus pulposus in the center takes up axial loads and transmits them to an encompassing ring of densely packed circular collagen type I fibers which is called anulus fibrosus. Their function is to absorb the translated axial pressures received by the proteoglycan - and water-rich nucleus with their tensile longitudinal fiber strength. At the top and bottom of each nucleus and anulus a hyaline cartilaginous endplate forms the junction to the adjacent vertebrae[4] (**Figure 1**).

In articular cartilage, four distinct spatial chondrocyte patterns can be found: pairs, strings, double strings, small respectively big clusters[5–7] (**Figure 2**). Changes in this pattern are associated with OA onset and progression[8,9]. Spatial chondrocyte organization is also indicative for a direct functional property of cartilage, namely its stiffness, underlining the functional relevance of this image-based grading approach[10,11]. These patterns can additionally be identified with already existing clinically available technology[12]. Due to the similarities between the IVD and articular cartilage, it can be hypothesized that characteristic chondrocyte patterns are also present in the IVD. Cluster formation is a phenomenon also observed in the degenerate IVD[13,14].

When trying to analyze spatial cellular organization in the IVD, it is necessary to overcome several technical difficulties that are not present when investigating articular cartilage:

First, processing of the tissue itself is much more challenging than with the homogeneous hyaline cartilage which is largely composed of collagen type II. The IVD's main fiber component is collagen type I, which makes it much more difficult to generate thin histologic sections. While in the hyaline articular cartilage even thick sections can easily be analyzed due to the "glass-like" nature of the tissue, the collagen type I network of the IVD is optically highly impenetrable. For this reason, a strong background noise is a common problem in the histology of the IVD. A fast and cheap way to penetrate this optically dense tissue is the use of an optical sectioning device e.g., by means of an Apotome. In such an Apotome, a grid is inserted in the illumination pathway of a conventional fluorescence microscope. In front of the grid a plane-

parallel glass plate is placed. This tilts back and forth thus projecting the grid in the image in three different positions. For each z-position, three raw images with the projected grid are created and superimposed. By means of special software, the out of focus light can be calculated out. The underlying principle is that, if the grid is visible, that information is in focus, if not it is considered to be out of focus. With this technique, well focused and high-resolution images can be acquired in a reasonable amount of time.

Secondly, the tissue is hard to come by from human donors. When doing total knee replacement, the entire surface of the joint can be obtained for further analysis during surgery. Although osteoarthritis of a diarthrodial joint is also a disease of the whole joint, there are nevertheless strong focal differences in the quality of the cartilage with usually some areas of the joint still being intact, for example due to reduced loading in that area. This situation is different in the IVD, where surgery is usually only performed when the disc is globally destroyed. When obtaining tissue from human donors from the operation room, the tissue is also highly fragmented and it is necessary to correctly allocate the tissue to one of the three cartilage types of the IVD before doing further analyses. To allow more detailed analyses of also larger tissue sections and to look into the embryonic development of the IVD the choice of an animal model organism is, therefore, necessary.

When choosing such a model organism it is important to have a system which is comparable with the human disc with respect to its anatomy and dimensions, its mechanical loading, the present cell population as well as its tissue composition. For the purpose of the presented technique here we suggest the use of bovine lumbar disc tissue: A critical property of the human disc resulting in its low regenerative potential is the loss of notochordal cells during maturation in the nucleus. However, in numerous model organisms notochordal cells can be detected their entire life long. Most of the few animals which lose their notochordal cells such as sheep, goats or chondrodystrophig dogs have an IVD that is much smaller than human discs. Only lumbar bovine discs present with a comparable sagittal disc diameter to those of human IVDs[15].

A key factor leading to early disc degeneration is excessive mechanical loading. The intradiscal pressures of a standing cow in the lumbar spine are around 0.8 MPa with the spine aligned horizontally. Surprisingly these pressures are comparable to the lumbar intradiscal pressures reported for the erect human spine (0.5 MPa)[15,16]. Also the amount of water and proteoglycans in bovine discs is comparable to that of the IVD from young humans[17]. Therefore, although the actual movement pattern of the motion segments might differ in quadrupedal animals from the bipedal human, with respect to total loading and disc characteristics, the cow is much closer to human biology than other established animal models for the IVD such as sheep and dogs.

In this protocol we present a technique how to analyze changes in the IVD from the point of view of spatial chondrocyte organization from early embryonic development to end stage degeneration.

PROTOCOL:

For the analysis of embryonic development and maturation, bovine discs were used. To evaluate degeneration of the IVD, human samples were analyzed.

Human IVD tissue was obtained from patients undergoing surgery for lumbar disc degeneration, disc prolapse, or spinal trauma in the Department of Orthopaedic Surgery, University Hospital of Tübingen and the BG Trauma Centre Tübingen. Full ethical committee approval was obtained before the commencement of the study (project number 244/2013BO2). Written informed consent was received from all patients before participation. The methods were carried out in accordance with the approved guidelines.
Bovine tissue was obtained from the Bavarian State Office for Health and Food Safety/Oberschleißheim and from a rendering plant in Warthausen (Germany). Local and veterinary authorities' approval was received for tissue from dead animals.

1. Sample harvest

1.1. Human IVD tissue: Place intraoperatively-obtained IVD samples immediately in Dulbecco's Modified Eagle Medium (DMEM) with 2% (v/v) of penicillin-streptomycin and 1.2% of (v/v) amphotericin B. Store at 4 °C until further processing. Process the tissue within 48 h. Alternatively, store the tissues at -20 °C for several weeks.

1.2. Bovine IVD tissue: Ensure to harvest the tissue from the animals within 24 h after death. Resect the bovine discs with the surrounding vertebrae from the dead animals en-bloc. Transport the frozen tissue on dry ice and store at -20 °C until further processing.

NOTE: If only fluorescence analyses are intended and no further biochemical quantification methods such as ELISA or PCR are planned, perform the tissue fixation as explained below. This allows to keep the tissue longer in storage before it needs to be processed. To prevent deterioration of the tissue matrix, perform fixation within 48 h after harvest unless the tissue is frozen directly.

2. Sample preparation

2.1. Thaw the frozen tissue at room-temperature. Process the tissue as soon as no ice-crystals can be felt any more upon digital gentle compression of the tissue.

NOTE: Perform preparation of the tissue in DMEM in a Petri dish.

2.2. Identify the origin of the human IVD tissue (anulus fibrosus, intermediate zone, nucleus pulposus, or cartilaginous endplate) based on macroscopic properties such as collagen density and orientation.

2.3. Take the motion segment consisting of the bovine IVD disc with its two adjacent vertebrae and dissect the disc as a whole from the subchondral bone using a surgical blade (blade number 15).

2.3.1. Use two anatomic forceps to flip the disc to reach areas more centered. Perform the dissection. Ensure to resect the nucleus pulposus last as it is much thinner than the anulus, prone to tear, and it does not come off in a defined fashion easily.

2.3.2. Identify the different areas of the cartilage.

2.3.3. Cut out the area of interest from the whole disc using a surgical blade (blade number 20 or 22). Alternatively, use a cryotome blade.

NOTE: As bovine discs come en-bloc as a part of the spine, the discs can be prepared in-toto. This makes correct identification of the different types of cartilage much easier. When dissecting the disc in the fashion described above, the cartilaginous endplate remains on the vertebrae. If this area shall be investigated, it is best taken off the underlying bone with a chisel working in a slightly bent tangential direction.

2.4. Ensure to process the IVDs from the bovine embryos with a crown-rump length of smaller than 20 cm in toto to preserve the tissue architecture. Do not perform any dissection of the vertebrae in these cases.

2.5. Once the disc has been resected in-toto, identify the different areas of the cartilage.

2.5.1. Perform the decalcification in ethylenediaminetetraacetic acid (EDTA) (20% (w/v); pH = 7.4) at room temperature. Choose the volume depending on the sample size - the entire tissue must be well covered with EDTA.

2.5.2. Change the decalcification solution daily which can go for up to 5 days depending on the size of the tissue.

2.5.3. Verify that the decalcification is successful with a 20 Gauge needle that penetrates the vertebrae without notable resistance.

NOTE: The daily change of decalcification solution is important to prevent the chelate binder EDTA from saturation to maintain reaction effectivity. It also prevents bacterial colonization.

3. Grading of sample age, integrity, and degeneration

3.1. Classify the human disc tissue into one of the five following categories with the help of clinical information as well as X-ray and magnetic resonance imaging[18] (**Figure 3)**.

NOTE:
Category I: To serve as near-healthy sample use anulus without any radiological sign of IVD degeneration derived from acute spinal trauma.
Category II: To illustrate a situation of acute inflammation with beginning degeneration use

tissue from the intermediate zone from patients with clinical symptoms with a duration of less than 4 weeks.
Category III: To describe a situation where the inflammatory reaction had already had time to affect the tissue and cells take tissue from patients who were operated for a nuclear prolapse but with a symptom duration exceeding 4 weeks.
Category IV: For moderate disc degeneration select anulus obtained from surgery with interbody fusion for degenerative disc disease with a Pfirrmann score of 3 or 4 in magnetic resonance imaging[19].
Category V: For end stage degeneration process anulus obtained from surgery with interbody fusion for degenerative disc disease with a Pfirrmann score of 5.

3.2. Classify the bovine tissue based on the developmental stage/age of the animal into one of the eight categories, as displayed in **Table 1**.

3.2.1. Calculate the gestation age on crown-rump length of the embryos based on the formula suggested by Keller:

$$\text{Gestation age in months} = \sqrt{Crown - rumplength(cm) + 1} - 1$$

NOTE: Animals in the first 4 weeks of gestation present with a crown-rump length of 0.8–2.2 cm[20].

4. Tissue fixation

4.1. Fixate the samples in 10x the volume of the sample of 4% (w/v) formaldehyde solution in phosphate buffered saline (PBS) over night at 4 °C.

NOTE: The formaldehyde solution penetrates tissue at a rate of about 1 mm/hour from each direction. For very small or very big samples an adjustment of the exposure time could be necessary.

4.2. Store the tissue in PBS at 4 °C until further processing.

5. Histologic sectioning

5.1. Embed the samples in water-soluble embedding medium on the cryotome knob.

5.1.1. Place the tissue onto the knob such that either an axial plane is generated or a plane that cuts the collagen type I lamellae perpendicular (e.g., a median sagittal sectioning plane).

NOTE: The tissue has to be fully covered by the embedding medium.

5.2. Section the embedded tissue at a thickness of 70 µm in human samples and 40 µm in bovine samples using a standard cryotome.

NOTE: The difference in section thickness is due to the difference in tissue integrity between the intact bovine disc and the highly degenerate human tissue.

5.3. Collect the sections on a glass slide.

5.3.1. Encircle the tissue sections with a hydrophobic pen.

5.3.2. Rinse the sections 3 times with phosphate-buffered saline (PBS) to remove the water-soluble embedding medium.

6. Fluorescence staining

6.1. Add 60 µL of 1% (v/v) of DAPI (Exmax 358 nm, Emmax 461 nm) as well as 1% (v/v) of Actin Tracking staining (Exmax 540 nm, Emmax 565 nm) in PBS and incubate for 5 min at room temperature.

NOTE: The staining protocol described here is to visualize the nucleus using DAPI nuclear staining (blue) and the cytoplasm using Actin Tracking Stain (red). The IVD has a strong autofluorescence due to the collagen fibers in the green channel. The amount of fluid added to the sections in this protocol is intended for sections of a size of about 5 mm x 5 mm. For larger sections, this amount needs to be increased accordingly. Perform all the works in rooms without direct sunlight exposure and with dimmed lights to prevent bleaching the dye.

6.2. Remove the staining fluid with a pipette and wash three times with 60 µL of PBS each time.

6.3. Add a suitable mounting medium and cover the sections with a cover slip.

NOTE: Ensure that there are no air bubbles entrapped when adding the cover slip. This is best done by starting contact of the slip with the slide on one rim and then let the slip come down slowly.

7. Microscopic imaging and processing

7.1. Place a slide with a stained section on the sample holder of the microscope.

NOTE: Due to the dense collagen type I network of the IVD, the scattered light makes the tissue difficult to visualize using conventional fluorescence microscopy. One way to address this problem is to perform optical sectioning using structured illumination. This also allows to render a three-dimensional projection of the entire specimen in both channels (blue and red). This is best done using the structured illumination setting and the Mosaic-mode with a 10x

magnification objective lens to obtain an overview of the sample as well as 3D reconstructions of individual patterns.

7.2. Start the structured illumination device.

7.2.1. Perform single field-of-view imaging with a suitable fluorescence microscope, fluorescence filters and adequate illumination.

NOTE: Adjust the exposure time for all the filters used in order to standardize the imaging acquisition. To get an accurate representation of the specimen at a higher resolution image the sections with a higher magnification (e.g., 20x objective).

7.2.2. Postprocess the pictures by optimizing the intensity and brightness using an image optimization software compatible with the fluorescence microscope.

7.3. To visualize the section as a whole use the mosaic imaging technique

7.3.1. Open the acquisition settings (press on **6D- Acquisition**) from the toolbar panel.

7.3.2. Adjust the mosaic settings (in **MosaiX Register**) and define the number of columns and rows of field-of-view images that shall later be merged to one overview image.

7.3.3. Press **Setup** and adjust the focus correction of individual tiles.

NOTE: It is almost impossible for a large tissue section to have the whole tissue surface in a single focus plane. Image tiles at different focal levels can be taken by 'MosaiX Acquisition'.

7.3.4. To start the acquisition of the image tiles, press **Start**.

7.3.5. Stitch the imaged tiles by using the stitching function (**Stitching** button) with 20% overlap incorporated in the software.

7.3.6. Postprocess the pictures by optimizing the intensity and brightness using an image optimization software compatible with the fluorescence microscope.

7.4. To analyze the spatial chondrocyte organization, use the 3D function incorporated in the software.

7.4.1. Adjust the z-stack settings. Define the scanning parameters: define the start and stop positions in the z-axis and the slice distance by activating **Start/Stop** button.

NOTE: The software automatically calculates the number of slices.

7.4.2. To start acquisition of the image z-stacks, press **Start**.

7.4.3. Postprocess the pictures by optimizing the intensity and brightness using an image optimization software compatible with the fluorescence microscope.

7.5. Export the pictures with a file format compatible with the image processing software.

NOTE: Export the 3D reconstructions as individual images or/and as an interactive 3D model or in a video format.

8. Cellular pattern identification and density assessment

8.1. Open the exported mosaic pictures of the entire tissue section in an appropriate image processing program.

8.2. Define the areas subjected to cell density assessment by defining regions of interest of 500 µm x 500 µm in the pictures.

NOTE: All tiles that do not have an adequate image quality are excluded from analysis.

8.3. Identify individual cellular patterns.

NOTE: Single cells are defined as individual cells - fully encapsulated within the adjacent matrix. Pairs are defined as two adjacent cells in close proximity (<25 µm) whereby the cells are interconnected through their matrixes (see **Figure 2**.) String-formations are at least three chondrocytes aligned in line (from the middle of the nuclei <25 µm). These cells are encompassed by an intact matrix and matrix interconnections can be seen between each cell. Clusters represent multiple cells that are located in direct proximity to each other (<25µm) and are encapsulated in a large lacuna devoid of matrix.

8.4. Use a cell count plug-in for the quantitative analysis of the cellular patterns.

NOTE: The cytoplasm staining represents a verification method to identify the different spatial patterns.

8.5. Calculate the cell density by dividing the counted cells by the size of the chosen region of interest.

REPRESENTATIVE RESULTS:

Using mosaic images, the architecture of the IVD with its dense collagen fiber network in the anulus and the softer nucleus can clearly be recognized (**Figure 4**). A continuous decrease in cellular density can be observed during embryonic development (**Figure 5**). While in the early stages of IVD development a cell density of 11,435 cells/mm² in the bovine anulus fibrosus and 17,426 cells/mm² in the bovine nucleus pulposus can be found, these numbers decrease rapidly to 1,011 cells/mm² (bovine anulus fibrosus) and 488 cells/mm² (bovine nucleus pulposus) until

birth. In the adult cattle 71 cells/mm² (anulus fibrosus) resp. 106 cells/mm² (nucleus pulposus) are seen (**Figure 6 A–B**). Using bi-channel imaging with the Apotome allows to visualize the 3D architecture of the spatial patterns (**Figure 7**).

FIGURE AND TABLE LEGENDS:

Figure 1. Macroscopic anatomy of the intervertebral disc. Schematic drawing of the intervertebral disc showing the nucleus pulposus (red), directly around it the intermediate zone (pink), and then in circular layers around it the anulus fibrosus. Note the ply-angle direction of the collagen type I fibers in the anulus fibrosus. The axial load to the nucleus can thus be translated to axial tensile forces of the collagen fibers.

Figure 2. Schematic illustration of different spatial organizational patterns of chondrocytes. Depending on the tissue chondrocytes are found as single cells, pairs or strings in healthy cartilage. With beginning degeneration these patterns change to form double strings, small clusters and then big clusters in end-stage degeneration. This figure has been modified from Danalache, M. et al.[21].

Figure 3. Different conditions of the intervertebral disc (IVD). (**A–D'**) Sagittal T2-weighted magnetic resonance imaging of the human lumbar spine (**A–D**), with the magnified motion segment L4/L5 (**A'–D'**). The ventral side of the patient faces left, the dorsal side faces right with the spinal canal with its white signal for the cerebrospinal fluid. (**A,A'**) Intact IVD with the anulus displayed with a hypointense (black) signal due to the high collagen type I content and the nucleus displayed much brighter (hyperintense) due to the high content of water-binding proteoglycans (Pfirrmann grade [19]). (**B,B'**) Beginning IVD degeneration with a loss of the water signal from the nucleus pulposus and where the distinction between the anulus and nucleus is lost (Pfirrmann grade [19] IV). (**C,C'**) Acute nuclear prolapse with still a prominent water signal in the area of the nucleus indicative for an otherwise intact IVD and the disc tissue protruding dorsally into the spinal canal. (**D,D'**) Advanced disc degeneration with a largely destroyed IVD with a complete loss of the water signal within the disc, ventral and dorsal spondylophyte formation and subchondral sclerosis of the vertebrae corresponding to a Pfirrmann score [19] of 5.

Figure 4. Mosaic fluorescence imaging of the intervertebral disc (IVD). The architecture of the IVD with its dense collagen fiber network in the anulus fibrosus and the softer nucleus can clearly be recognized. DAPI-nuclear staining (white) in the axial (**A1**) and sagittal (**A2**) plane shows the cell distribution and arrangement within the IVD. Magnified representative areas from these mosaic images are displayed in **B1–B4** and **C1–C4** illustrating the spatial chondrocyte organization - in this case single cells (green box), pairs (blue box) and strings (yellow box). **A**: scale bar 1,000 µm, **B1–B4**: scale bar 200 µm, **C1–C4**: scale bar: 50 µm. This figure has been modified from Bonnaire, F. C. et al.[22].

Figure 5. Different developmental and maturation stages of bovine anulus fibrosus and human degenerating anulus fibrosus. DAPI nuclear staining. The mosaic images show a whole early embryonic disc in an axial section (**A1**) and representative images of the bovine anulus

during embryonic development, maturation, and beginning degeneration (**A2–A8**). (**B1–B3**) Anulus from human IVDs was obtained intraoperatively. A continuous decrease in cellular density can be observed during embryonic disc development. A higher spatial organization cellular pattern seems to be present especially around birth. In the adult human disc during degeneration, the cellular density increases again and increasing cluster formation can be observed. Scale bar 100 µm. Wog: weeks of gestation. This figure has been modified from Bonnaire, F. C. et al.[22].

Figure 6. Reduction in cellular density throughout development and maturation of the bovine intervertebral disc. The mean (standard deviation) cell count per mm^2 is illustrated by bar diagrams for the bovine anulus fibrosus (**A**) and the bovine nucleus pulposus (**B**). A clear reduction of cellular density can be observed especially during the embryonic period which continues to a lesser extent at least until full maturation of the disc (n=72). Wog: weeks of gestation. This figure has been modified from Bonnaire, F. C. et al.[22].

Figure 7. Apotome imaging of the intervertebral disc (IVD). Bi-channel image showing the cytoplasm (red, Actin staining) and the nucleus (blue, DAPI nuclear staining). (**A**) In the intact IVD, in addition to the predominant spatial pattern of single chondrocytes, pairs are also found. (**B**) In degenerated anulus cell clusters can be found. Scale bar 20 µm.

Video 1. Apotome imaging of a pair in the intervertebral disc (IVD) as a 3D Model. Bi-channel images showing the cytoplasm (red, Actin staining) and the nucleus (blue, DAPI nuclear staining) of a pair.

Video 2. Apotome imaging of a cluster in the intervertebral disc (IVD) as a 3D Model. Bi-channel images showing the cytoplasm (red, Actin staining) and the nucleus (blue, DAPI nuclear staining) of a cluster.

Table 1: Bovine embryonic development, maturation, and growth after birth with its different milestones. Wog - weeks of gestation. Gestation period ca. 283 days, natural life-expectancy 20–25 years[20,23,24].

DISCUSSION:

Using fluorescence microscopy augmented by mosaic imaging and optical sectioning, we evaluated the spatial arrangement of chondrocytes in the anulus of the lumbar IVD throughout development, maturation, and degeneration. While degenerative tissue could be harvested from patients receiving spine surgery for disc degeneration, analysis of the embryonic period and maturation phase required the use of a model organism (bovine). High cellular densities were noted in the anulus during early embryonic development. In the further course of development, postnatal growth and maturation a pronounced decrease in cellular density could be observed. In human tissue with advanced disc degeneration, we could then quantify an increase in cellular density in the anulus fibrosus.

The rapid increase in tissue volume combined with actively dividing and biosynthetically active

notochordal cells are probable reasons for the changes in cellular density observed in the embryo[25]. The mechanisms by which cellular density again increases with degeneration remain, however, still unclear. Degenerative processes of the anulus lead to a series of pathological changes including increased tissue innervation and inflammation, upregulation of matrix-degrading enzymes and growth factor production, and also changes in cellularity[26–28].

When considering cell presence as a function of cellular spatial organization based on patterns known from articular cartilage[7,9] we found no recognizable spatial organization in early embryonic discs where the cells seem to be densely packed and which we considered to be present as single cells[22]. This finding is consistent with the results from embryonic articular cartilage[7]. In the healthy mature anulus, single cells represent the predominant spatial pattern[22]. Pairs and string-formations can, however, also be observed[22]. The more degenerate the tissue in human discs, the higher the proportion of cells that can be found in clusters[22]. The physiopathological model suggested by Rolauffs in articular cartilage[7,9,29] shows an intriguing similarity to our findings. Previous studies on IVD degeneration had also already outlined cluster formation as a hallmark of disc degeneration[14,30–33]. Since these clusters can mostly be found in highly degenerated tissues, cluster formation might indicate a failed attempt of the tissue to repair the degenerative damage[34]. Establishing a strong correlation between the locally predominant chondrocyte pattern and tissue elasticity, a high functional relevance of these patterns could be demonstrated in articular cartilage[10,11]. It can be speculated that such a functional relevance also applies to spatial chondrocyte organization in the IVD.

Fluorescence histologic analyses are an easy to come by and attractive means to analyze morphologic changes to tissues. When trying to histologically analyze the IVD, there are distinct technical difficulties that need to be overcome: First, the limited availability of human tissue makes it important to choose an adequate animal model organism with which those aspects of the disease can be studied where human samples cannot be obtained. For the research question addressed in this study, we chose a bovine animal model.

Secondly, processing of the collagen type I-rich IVD is much more challenging than for most other human tissues. The dense collagen type I-fiber network strongly scatters the fluorescing light creating a high background noise signal. This problem is best addressed by using a technique that allows the subtraction or elimination of such background signal. A well-known method to do so is confocal microscopy. While the image quality of confocal acquired LASER-images is usually excellent, the disadvantages of this technique are that it is relatively time-consuming and as such it does not allow the analysis of larger tissue areas by means of mosaic imaging. Secondly confocal microscopes are relatively expensive and not available everywhere. A much faster and cheaper way to filter out background noise is to perform optical sectioning e.g., by means of an Apotome.

To be able to correctly interpret the findings, it is essential to also correctly allocate the tissue that is to be analyzed to its origin in the IVD. While this task is relatively simple when having an entire bovine disc to select from, it can be very challenging when receiving human tissue from the operation theatre. The characteristic feature of the anulus fibrosus is its very dense

collagen type I network in an angle-ply fiber orientation. The nucleus, in contrast, is an amorphous gelatinous structure where no higher collagen architecture is visible to the naked eye. The intermediate zone lies between those two extrema and also possesses a clear collagen fiber architecture, but it is much softer and less dense than the anulus fibrosus. The cartilaginous endplate consists of hyaline cartilage, does not present with a collagen architecture recognizable by the naked eye but it is rather "glass-like" as the term "hyaline" suggests. Unlike the nucleus pulposus it is, however, also very stiff, cannot be deformed, and is situated on the subchondral bone which can often still be recognized by inspection of the tissue.

Once the tissue origin has been correctly identified, the tissue still needs to be correctly oriented for sectioning to obtain standardized images which also allow a proper qualitative and quantitative read-out. In orientation to other imaging techniques such as magnetic-resonance imaging we suggest two standard analysis-planes: one median-sagittal section and one in the axial plane. These two planes will also provide a good impression of the architecture of the collagen type I-fiber network of the anulus. When interpreting the findings obtained from the F-actin staining it needs to be kept in mind that freezing of the tissue samples may cause cytoskeletal structure alterations[35] which should, however, not affect the spatial chondrocyte organization.

To fully comprehend the cellular biology of the IVD is one of the still open challenges in our understanding of disc degeneration and regeneration[36]. Questions that sometimes seem trivial, such as the physiological arrangement of cells in healthy tissue or the question of cellular reorganization during degeneration, remain, to date, unanswered. The obtained knowledge may allow us to use this image-based degeneration marker to evaluate tissue quality, investigate the reversibility of cluster formation and thus possibly define a therapeutic window in which the degenerating processes can still be successfully targeted.

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DISCLOSURES:

The authors have nothing to disclose.

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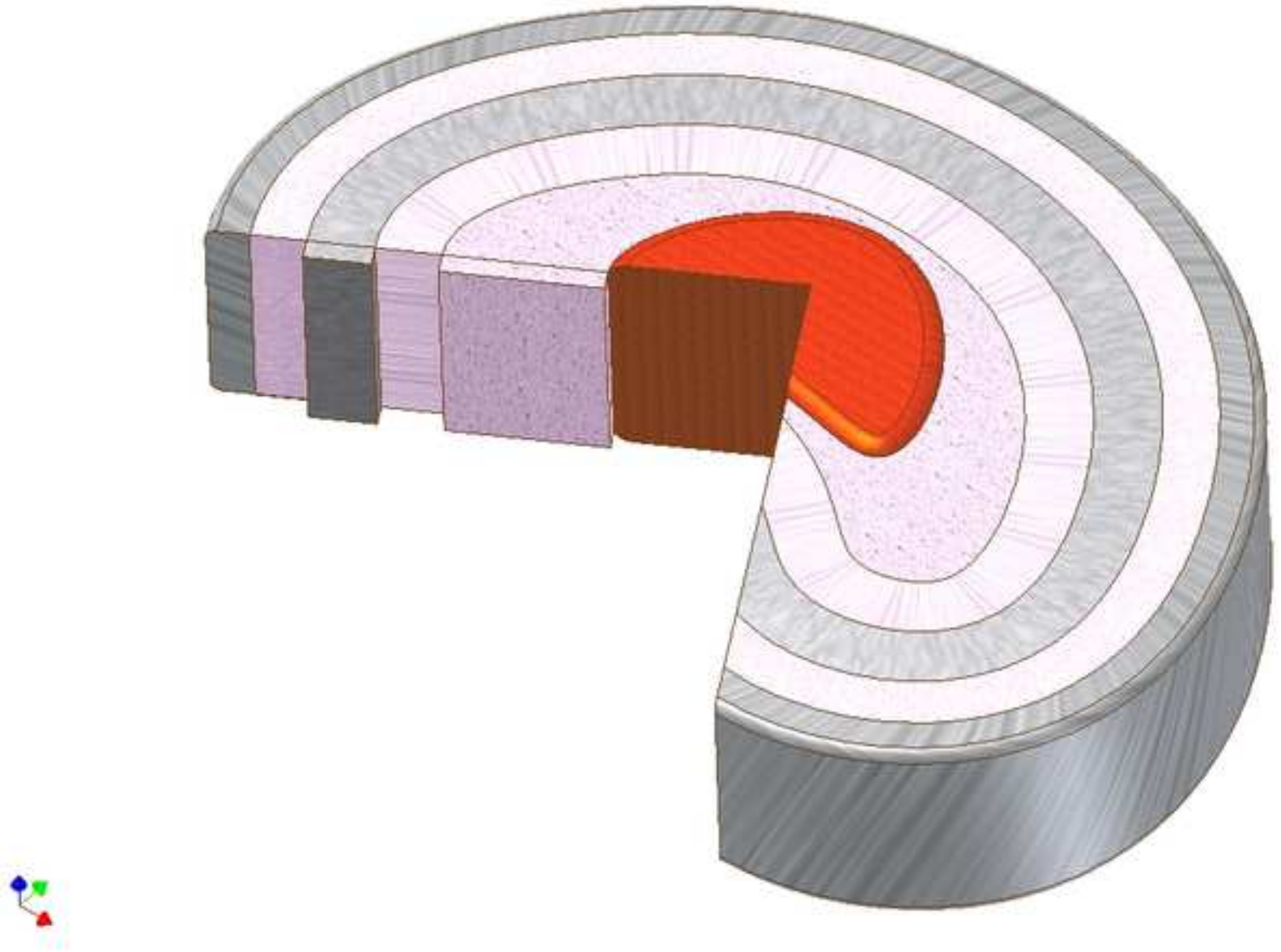

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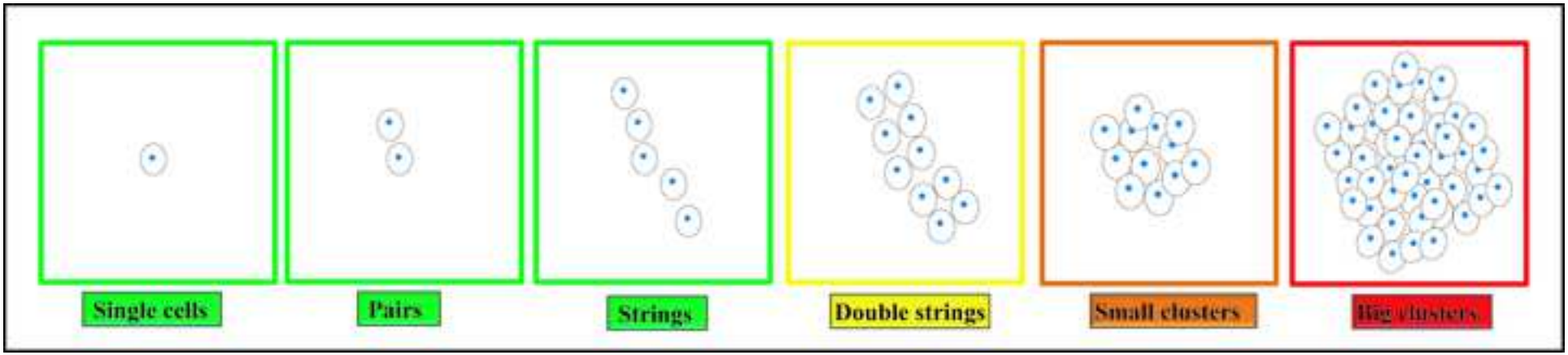

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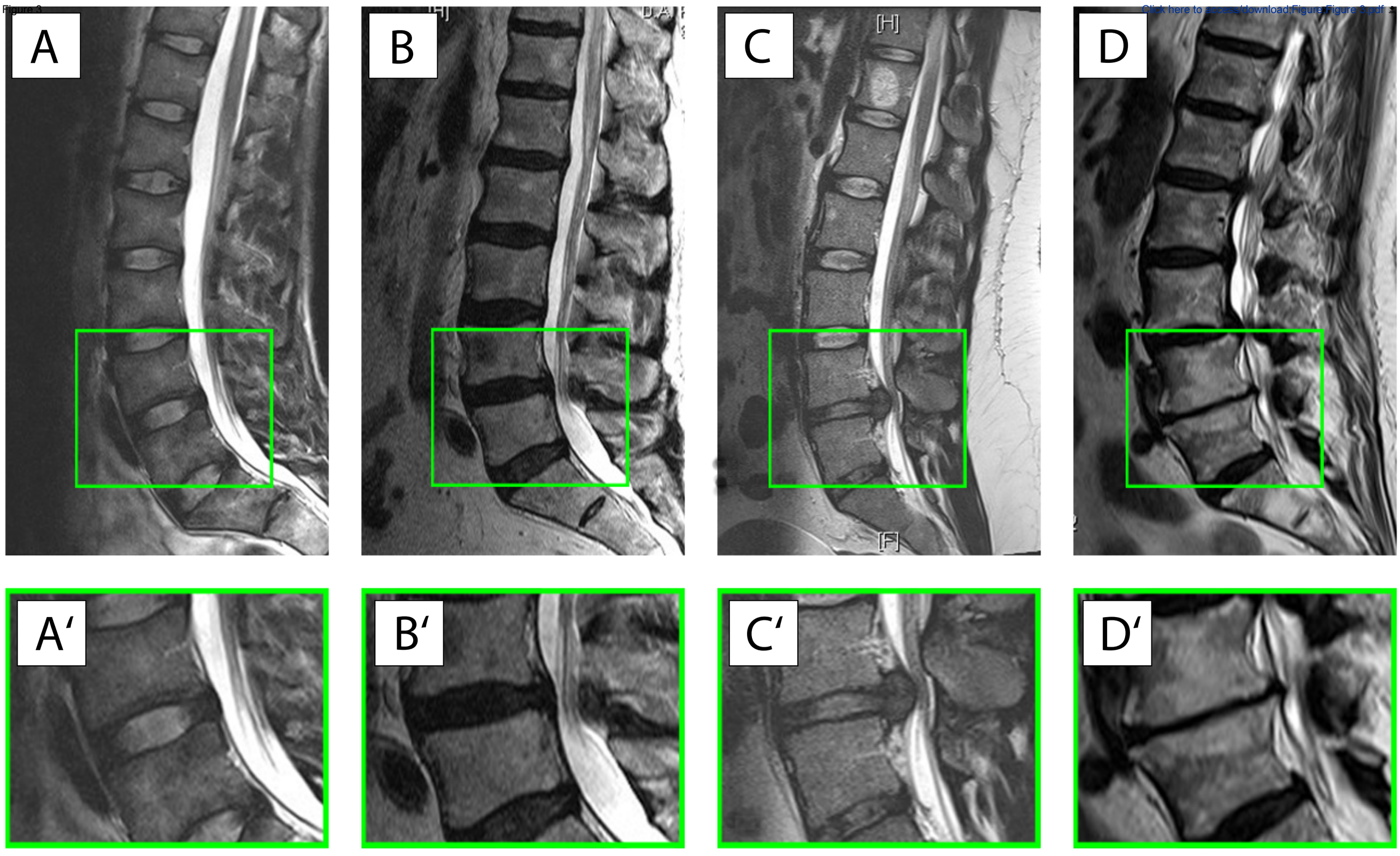

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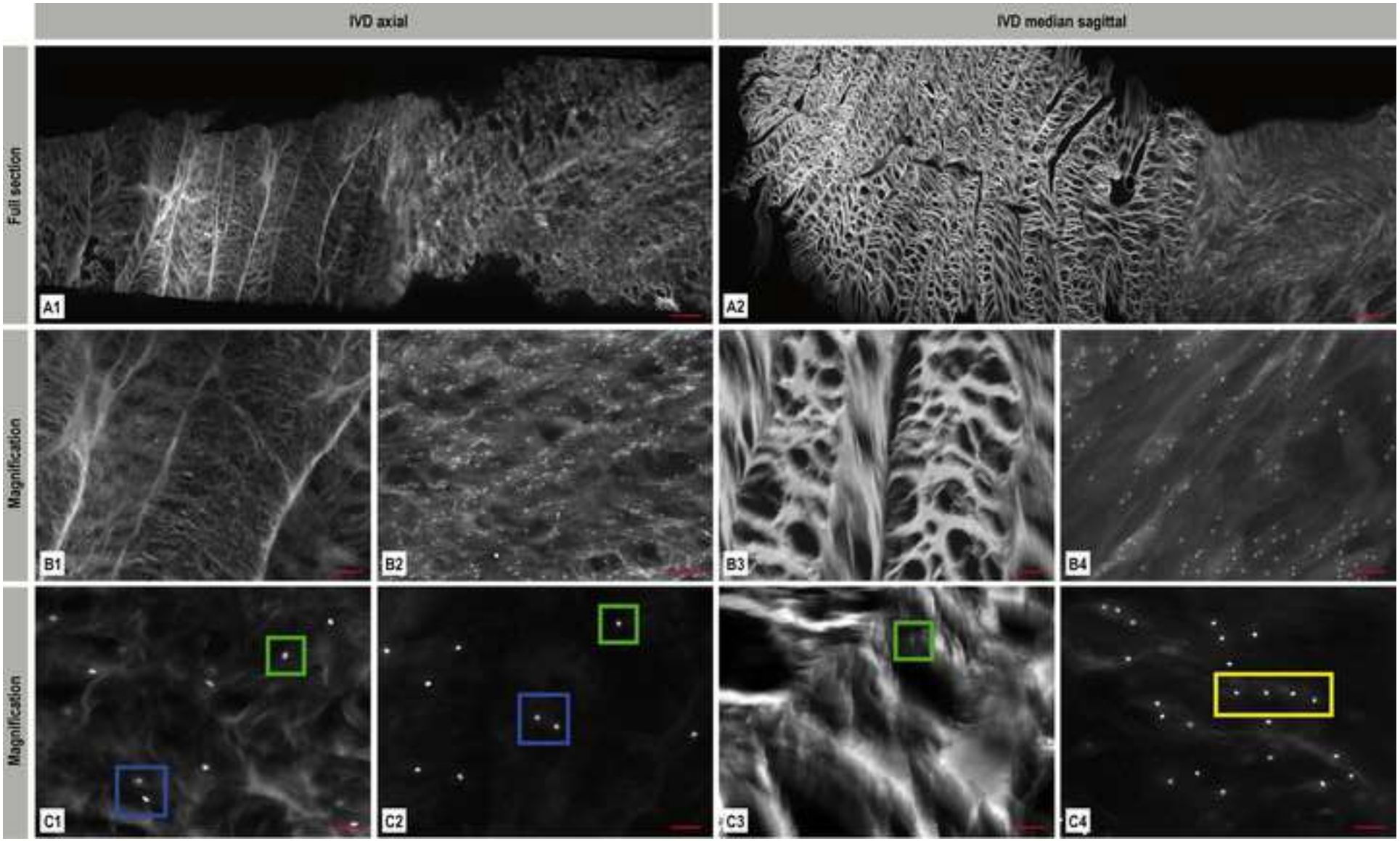

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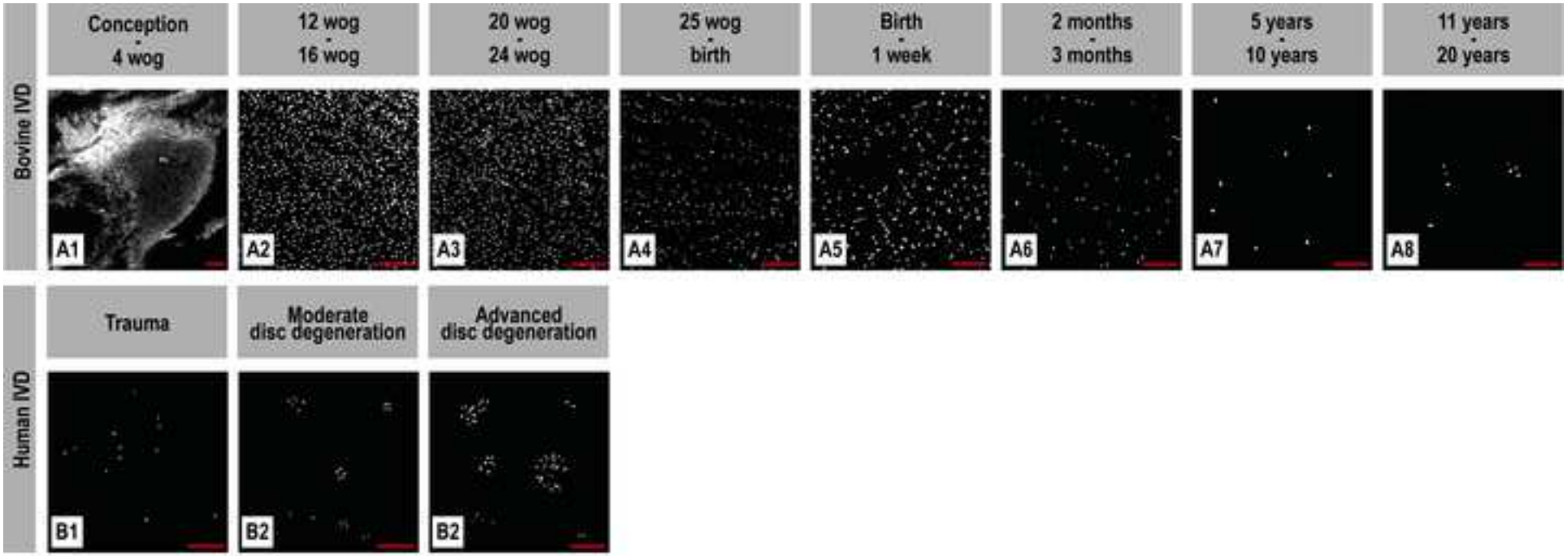

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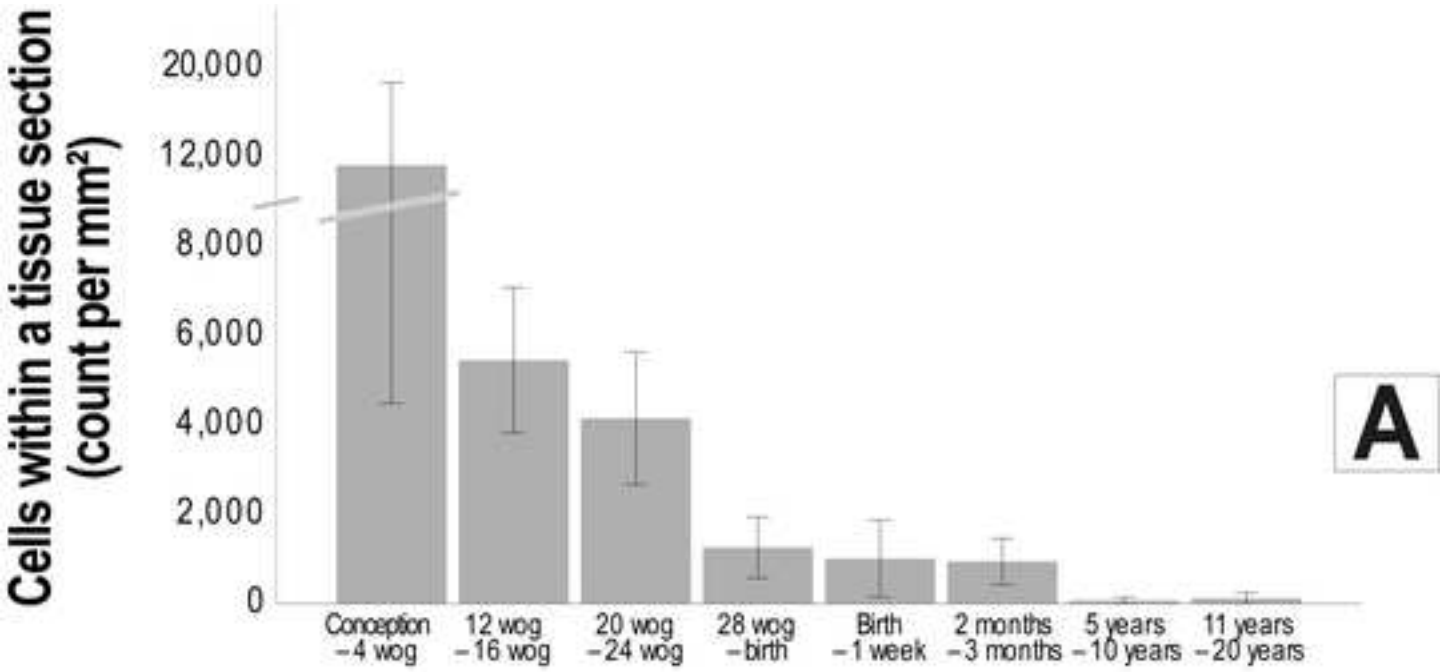

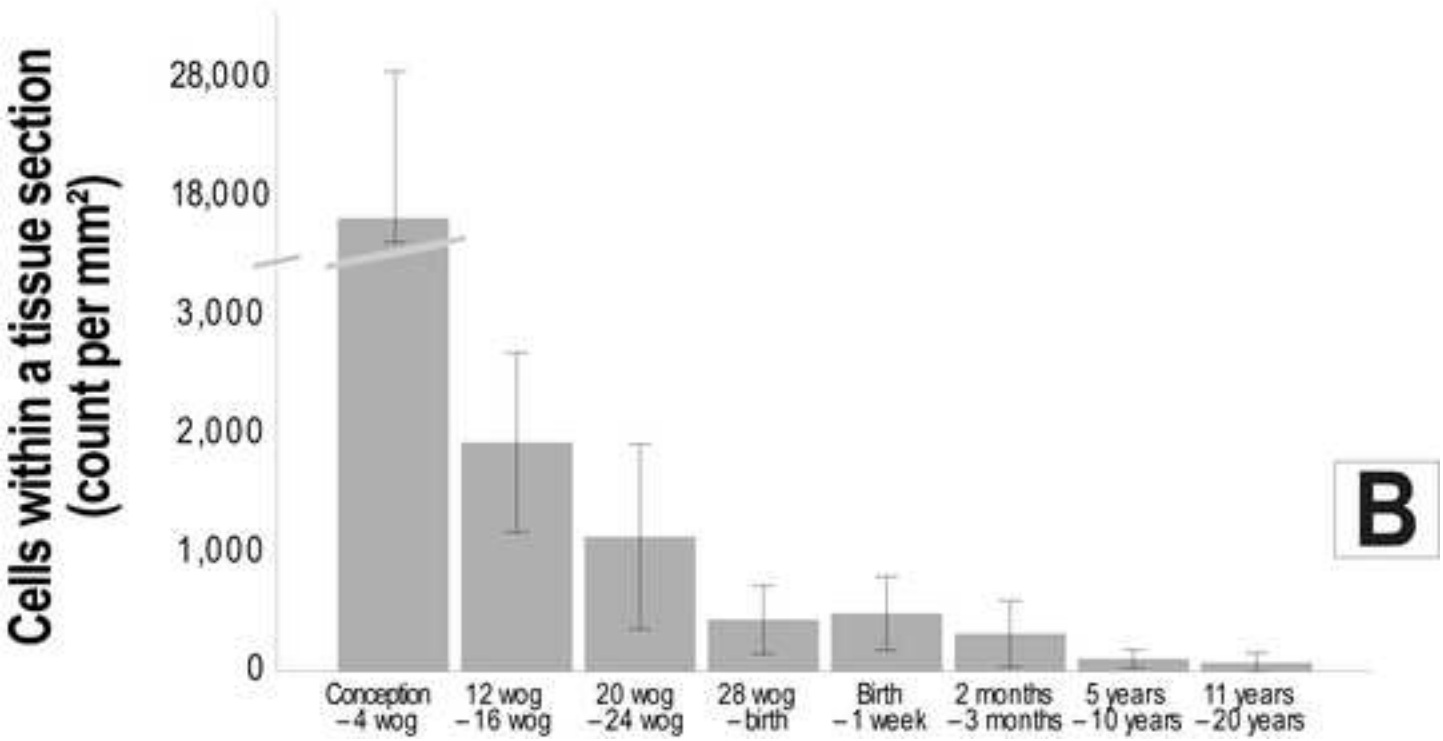

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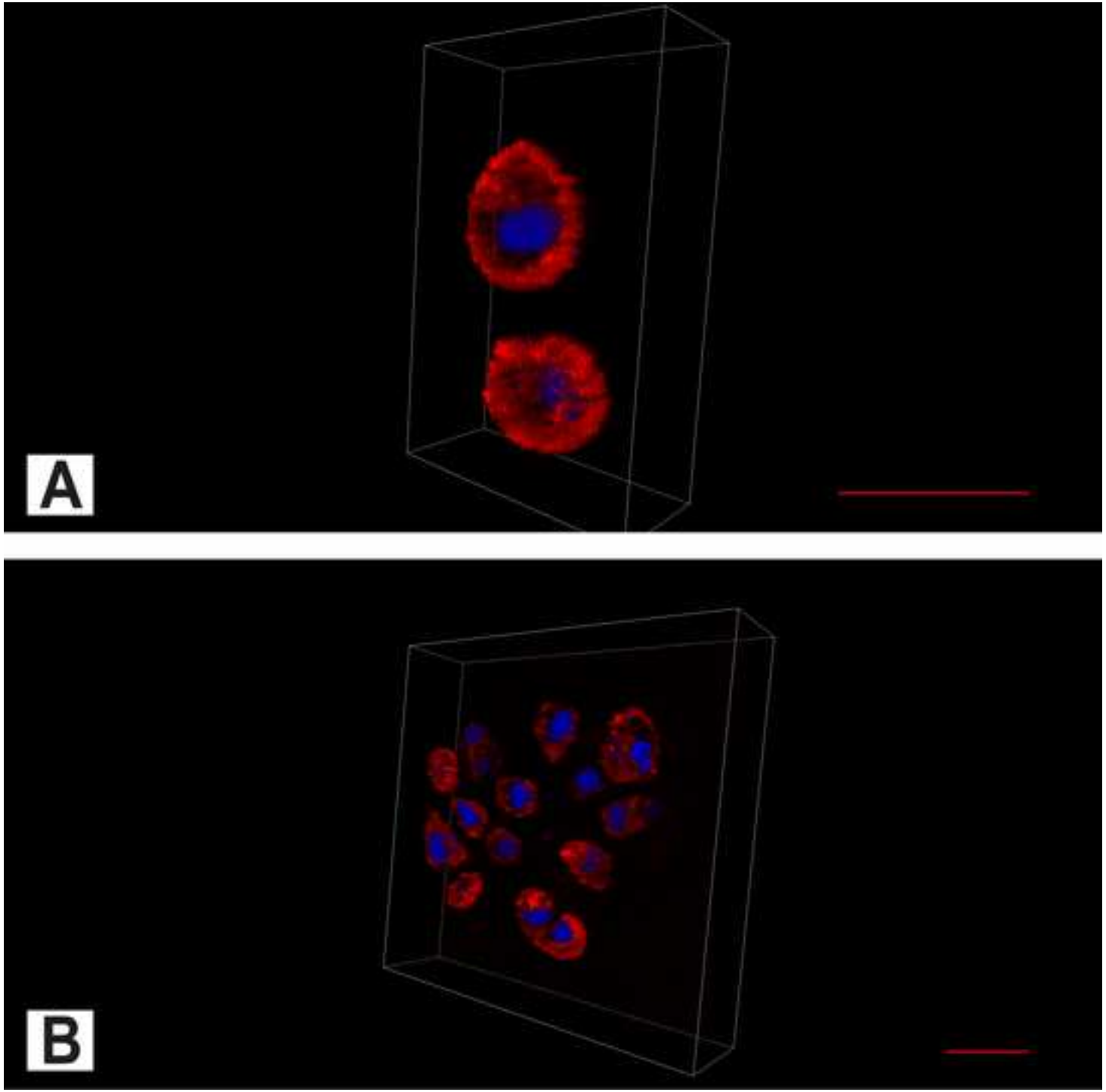

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Table 1:

Age group	Age	Landmarks of development and maturation
I	Conception – 4 wog	Germ layer formation and begin of organogenesis
II	12 wog – 16 wog	Organogenesis
III	20 wog – 24 wog	End of organogenesis and begin of fetogenesis
IV	28 wog – birth	Growth phase
V	Birth – 1 week	Final disc development and maturation without the influence of gravity
VI	2 months – 3 months	Early juvenile disc during growth and after adaptation to gravitational and gait loads
VII	5 years – 10 years	Fully mature disc
VIII	11 years – 20 years	Ageing disc and beginning degeneration

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Rebuttal Letter

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Point to point response file manuscript number: JoVE62594

Title: Optical sectioning and visualization of the intervertebral disc from embryonic development to degeneration

Florian Christof Bonnaire[1,2], Martina Feierabend[3], Julius Michael Wolfgart[1,4], Wolfram Breuer[5], Christian Walter[2], Ulf Krister Hofmann[1,2], Marina Danalache[1]

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Date: 03.06.2021

Author's response to reviews: printed in blue

Editorial comments:

Title: Optical sectioning and visualization of the intervertebral disc from embryonic development to degeneration

Version: 2

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please use American English throughout.

As suggested, we have made the required language adjustments and corrections and we now use American English.

2. Please revise the following lines to avoid previously published work: 39-42, 48-49, 58-63, 69-71, 111-120, 125-127, 211-223, 325-326, 459-460, 464-465, 468-471, 473-475,480-481, 483-487,492-495, 536-539.

As requested, we have modified the lines in question.

3. Line: 196-200: Please specify the volume of the EDTA used.

We thank the editor for this remark. We added the following sentence: “Choose the volume depending on the sample size - the entire tissue must be well covered with EDTA.”

4. Line 215-229: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

As suggested by the editor, we have thoroughly worked on this section again. In our opinion the description of the different categories should be written in der protocol section, as the correct classification of the tissue is an elementary component of the protocol and as such also relevant for the quality of the obtained results. We have rephrased the categorization to make it an active process. Please also see our comments in the letter to the editor.

5. Line 237: Please ensure that the cited references are appropriate.

We apologize for the inconvenience. The formula shown here was suggested by Keller in 1928 and is still valid and applied today. Unfortunately there are no English language references. For this reason we have to resort to the work of German veterinarians. We now not mention the year any more since it is of no relevance for the protocol.

6. Line 244-245: Please mention the volume of formaldehyde used. Are there any specific requirements in consideration of the size of the tissue?

We thank the editor for this remark. We added an additional NOTE for clarification: “The formaldehyde solution penetrates tissue at a rate of about 1 mm/hour from each direction. For very small or very big samples an adjustment of the exposure time could be necessary.”

We additionally suggest to use formaldehyde of a volume of 10x the volume of the sample.

7. Please ensure that the references are not duplicated. (7 and 31, 11 and 38, 23 and 30). Please complete reference number 35.

We apologize for the inconvenience. We double-checked the references and adjusted the erroneous parts.

Report of reviewer #1:

Title: Optical sectioning and visualization of intervertebral disc from embryonic development to degeneration

Version: 2

Reviewers' comments:

Manuscript Summary:

The authors use double fluorescent staining of both bovine and human intravertebral discs and visualise cell clusters using fluorescent microscopy. The authors addressed my suggestions and I think that the manuscript is of sufficient quality for publication.

Major Concerns:

No major concerns

Minor Concerns:

None

Report of reviewer #2:

Title: Optical sectioning and visualization of the intervertebral disc from embryonic development to degeneration

Version: 2

Reviewers' comments:

Manuscript Summary:

The manuscript by Bonnaire et al. is significantly improved. All my concerns have been addressed. I do now support publication of this protocol in JoVE.

Bonnaire et al. describe a method to investigate the degeneration of intervertebral discs. This is a very important topic. The authors use optical sectioning (immuno)fluorescence microscopy with an Apotome to determine the spatial organization of chondrocytes. The authors describe how to correctly prepare and allocate the tissue.

Most interestingly, the authors find first a decrease in cellular density during gestation, growth and maturation and then an increase in cellular density upon tissue degeneration, which they discuss in view of earlier findings on articular cartilage.

Major Concerns:

none

Minor Concerns:

With respect to query 21, I still believe that this information (number of images from animals and humans) should be given.

We thank the reviewer for pointing this out again. Our present manuscript aims at describing a technique. It is not primarily about our findings. We have, of course, referenced the original article so that the strategic experimental and statistical approach can be studied in detail. We believe that providing such information here would only mislead the reader and after thorough discussion we decided to not include these numbers in the protocol.

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Author: Marina Danalache, Anna-Lisa Erler, Julius M. Wolfgart, et al

Publication: Journal of Orthopaedic Research

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