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# Semiquantitative analysis of ECM molecules in the different cartilage layers in early and advanced osteoarthritis of the knee joint

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**Summary.** The study was conducted to examine the expression of collagen type I and II in the different cartilage layers in relation to other ECM molecules during the progression of early osteoarthritic degeneration in human articular cartilage (AC).

Quantitative real-time (RT)-PCR and colorimetrical techniques were used for calibration of Photoshop-based image analysis in detecting such lesions.

Immunohistochemistry and histology were performed with 40 cartilage tissue samples showing mild (ICRS grade 1b) respectively moderate/advanced (ICRS grade 3a or 3b) (20 each) osteoarthritis compared with 15 healthy biopsies. Furthermore, we quantified our results on the gene expression of collagen type I and II and aggrecan with the help of real-time (RT)-PCR. Proteoglycan content was measured colorimetrically. The digitized images of histology and immunohistochemistry stains were analyzed with Photoshop software. T-test and Spearman correlation analysis were used for statistical analysis.

In the earliest stages of AC deterioration the loss of collagen type II was associated with the appearance of collagen type I, shown by increasing amounts of collagen type I mRNA. During subsequent stages, a progressive loss of structural integrity was associated with increasing deposition of collagen type I as part of a natural healing response. A decrease of collagen type II is visible especially in the upper fibrillated area of the advanced osteoarthritic samples, which then leads to an overall decrease. Analysis of proteoglycan showed losses of the overall content and a loss of the classical zonal formation. Correlation analysis of the proteoglycan Photoshop measurements with the RT-PCR revealed strong correlation for Safranin O and collagen type I, medium for collagen type II, alcian blue and glycoprotein but weak correlation with PCR aggrecan results.

Photoshop based image analysis might become a valuable supplement for well known histopathological grading systems of lesioned articular cartilage.

The evidence of collagen type I production early in the OA disease process coupled with the ability of chondrocytes to up-regulate collagen type II production suggests that therapeutic agents that suppress collagen type I production and increase collagen type II production may enable chondrocytes to generate a more effective repair response.

**Key words:** Cartilage, Osteoarthritis, RT-PCR, Photoshop image analysis

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## Introduction

In bradytrophic tissues like hyaline cartilage, the homeostasis between production and breakdown of the matrix is exceptionally sensitive. Chondrocytes have microvilli-like cytoplasmic extensions that allow them to recognize changes in the mechanics and composition of the surrounding matrix, and up to an extent they can repair damage by synthesizing new matrix material (Musumeci et al., 2011). When the physicochemical

balance is disturbed, in the collagen-proteoglycan relationship for example, biomechanical properties quickly change and problems like cartilage stiffness develop. The mechanical strength of cartilage is primarily maintained by intracellular signaling affecting gene expression for ECM components. Proteoglycan content determines stiffness (Kempson et al., 1973) and collagen fibrils offer resistance against cartilage deformation. This requires the maintenance of a delicate balance. On the one hand there is the intrachondral swelling pressure that keeps the cartilage "stretched" and results from osmotic pressure and electrostatic repulsion between neighboring glycosaminoglycans and polysaccharides on proteoglycans, and on the other there is the collagen network to provide resistance against deformation. The balance itself determines the degree of pressure on the proteoglycans and thus the number of negative charges exposed, which in turn determines the degree of tissue hydration. Since the superficial tangential zone (the outermost cartilage layer) is impermeable to water, when compressed, water stored in interfibrillary spaces flows horizontally (Teshima et al., 2004). Since chondroitin sulfate (glycosaminoglycan) resists this flow, water bears the main portion of weight placed on the cartilage. Animal experiments have demonstrated that, even in early subclinical phases, once degeneration has begun it is not reversible. Various stimuli (mechanical, cytokines, etc.) and the uncoordinated imbalance of matrix components like aggrecan and collagen type II help arthritic processes to develop further (Young et al., 2005). Collagen type II is the main collagen component of healthy hyaline cartilage in many mammals and is distributed relatively uniformly throughout cartilage zones-from superficial to transition. It has been shown that collagen type II mRNA is produced in vivo and that in the early stages of degeneration there is a switch to collagen type I (mRNA). This can be observed in the superficial regions of a meniscectomy model (Young et al., 2005), for example, or in the interterritorial matrix of osteoarthritis sufferers (Miosge et al., 2004).

This study was conducted to investigate the expression of collagen type I and II in relation to other ECR components in different cartilage layers during the progression of early osteoarthritic degeneration and to analyze the associated immunohistochemical changes in tissue metabolism. Quantitative real-time PCR and colorimetric techniques were used to calibrate our semiquantitative Photoshop-based image analysis (Lehr et al., 1997; Matkowsjyj et al., 2003; Lahm et al., 2007), used to document early osteoarthritic changes and aid in the development of simpler, more economical means of serial analysis for the future.

## Materials and methods

We took tissue samples from 40 patients undergoing implantation of total knee endoprosthesis. The samples were drawn at a right angle from as deep as the subchondral bone. In 20 cases lesions were mild (ICRS grade 1b with recognizable cracks and fissures), and the biopsy was taken from the trochlea region and adjacent areas (group A). In 20 cases of more severe lesions (macroscopic ICRS grade 3a or 3b), the biopsy was taken from the direct region of the lesion itself and adjacent areas (group B). Demographic differences within the group were minimal: group A: 56-72 years (average: 64 years; 14 women, 6 men) vs. group B: 58-71 years (average: 66 years; 13 women, 6 men). 15 control biopsies with macroscopically healthy cartilage (ICRS grade 0) were taken from the same region, 8 taken during an ACT, 6 during endoprosthesis implantation and 1 during major amputation (age: 28-68 years, average: 48 years; 7 women, 8 men). We excluded patients with rheumatoid arthritis, ankylosing spondylitis, psoriasis or similar arthritides, and malignancies or infections with inflammatory arthritides. The control group included subjects with a Collins and McElligott "histopathological grading" of 0 (Miosge et al., 2004). Grade I was used for group A and grade III for group B. Vertical zonal formation of hyaline cartilage was defined as suggested by Lahm et al. (2007) and Fukui et al. (2008). Approval was given by the institutional review board and informed consent was obtained from each patient.

Tissue prefixation, decalcification, dehydration, Epon (1:1) embedding (Epon 812, Fa. Carl Roth GmbH, Karlsruhe) formalin fixation, slicing into semithin sections (5 $\mu$m), and paraffin staining were performed according to standardized procedures (Lahm et al., 2004). All histological sections (x50) with safranin O (Chroma-Gesellschaft Schmid & Co), PAS (Chroma-Gesellschaft Schmidt & Co) Alcian blue (Merck), and immunohistochemical stains were examined with light and polarization microscopy, photographed (Axioskop, Zeiss, Oberkochen, Germany and Kodak Elite 400) and scanned.

Monoclonal mouse antibodies (anti-human Collagen I and Collagen II) were used for immunohistochemical analysis (Immunodiagnostika und Biotechnologie, Berlin, Germany). Hyaluronidase digestion was performed to identify collagen fibers (10000U Hyaluronidase/ml; 37°C for 2.5 hours; Sigma Chemicals, St. Louis, USA). Enzyme activity was stopped by rinsing three times with PBS buffer.

The cryo-sections were then pre-incubated at room temperature for an hour in a humid chamber with 1% BSA. After suctioning off the BSA, primary antibody was added to each object (ca. 50 $\mu$l/object). The antibody added (fluorescein-conjugated secondary antibody) was diluted 1:100 in BSA.

### *Image analysis*

After embedding and staining the digitized images of histology and immunohistochemistry stains were imported and analyzed with Photoshop software as described in detail by Matkowsjyj et al. (2003) and

Lahm et al. (2007).

*PCR*

For the actual measurements 15 samples of healthy cartilage and 20 samples showing various stages of degeneration were used.

*RNA extraction*

RNA isolation from human cartilage tissue was performed according to Oncoscreen instructions, Baelde et al. (2001) and Ruettger et al. (2010). For each sample, 0.03 $cm^3$ of frozen material was ground and mixed with 5 ml of Trizol (Invitrogen) and 5 $\mu$l of glycogen (Invitrogen). Using the syringe and various disposable cannulas (sizes 21 and 14, Fisher Scientific) each sample was then homogenized. 1 ml chloroform (Roth) was added, and the suspension was mixed thoroughly and then centrifuged for 10 minutes at 13200xg. RNA was precipitated with isopropanol (Roth). The pellet was washed twice with 70% ethanol (Roth), dried and then placed in 45 $\mu$l of highly purified water (Invitrogen).

*Determining RNA concentration*

RNA concentration was measured with a nanophotometer (Implen).

*Characteristics of analytical primer*

The sequence for the reference gene HPRT1 corresponded to that used by Vandesompele et al. (2002). COL1A1 and COL2A1 sequences corresponded to those used by Miosge et al. (2004). The aggrecan primer was a new design that could be used for human cartilage tissue (see Table 1). For all primers searches for similar sequences were performed using BLAST. It was also confirmed that there were exon overlapping primers for all amplicons.

*Primer Testing and Gel electrophoresis*

The amplicons HPRT, COl1A1, COl2A1 and Agg for PCR were tested in classical PCR with the test samples c60095/1 and c60095/2. Therefore RNA was extracted, reverse transcription was performed and cDNA was produced, 2 $\mu$l of which were used for the classical PCR. PCR products were placed on 1.2% agarose gel. All amplicon bands lay in the expected range with no sign of double bands.

Extracted RNA samples were tested for quality by applying 350 ng of RNA per track to 1.2% agarose gel and separating at 130V for 60 min.

*Reverse Transcription*

RNA samples were converted into cDNA (10 ng/$\mu$l).

*Quantitative PCR*

Beginning with a final sample of 25$\mu$l we added the following: 12.5 $\mu$l 2x QuantiFast SYBR Green PCR Master Mix (Qiagen), 6.5 $\mu$l RNAse-free water (Qiagen), 1 $\mu$l primer mix (6 mM) and 5 ng cDNA. For each gene being measured, 4 dilution series were made.

ABI 7500 (Applied Biosystems) was used for quantification.

After initial denaturation at 95°C, cDNA products underwent PCR 40 cycles of amplification (5 minutes). This included denaturation (95°C for 15 seconds) and then extension/annealing (60°C for 60 seconds). Validity of the PCR results was verified by sequencing and melting curves.

*Proteoglycan /PG content*

The cartilage digests were measured colorometrically in classical technique (Squires et al., 2003) using a dimethylene blue assay. α-chymotrypsin and proteinase K digests were diluted in distilled water prior to analysis 1:20 and 1:5. Shark chondroitin sulphate was used as a standard, Cumulative release was expressed as the total amount at that specific time point.

## Results

*Results of delta Ct method*

In the first analysis we calculated the difference

**Table 1.** Characteristics of analytical PCR Primers.

| Gene/Amplicon Identific. | Identification | Accession Number | Amplicon Length | Sequence Forward/Reverse | Annealing Temperature |
|---|---|---|---|---|---|
| HPRT I | HPRT496F | NM_000194 | 94bp | TGA CAC TGG CAA AAC AAT GCA | TM = 50.5°C |
| HPRT496A | HPRT589R | | | GGT CCT TTT CAC CAG CAA GCT | TM = 54.4°C |
| Collagen type I | Col1A1_81F | NM_000088 | 256bp | TCC CCA GCC ACA AAG AGT C | TM = 53.2°C |
| Col1A1A | Col1A1_336R | | | CGT CAT CGC ACA ACA CCT | TM = 50.3°C |
| Collagen type II | Col2A1_2976F | NM_033150 | 149bp | CTC CTG GAG CAT CTG GAG AC | TM = 55.9°C |
| Col2A1A | Col2A1_3124R | | | ACC ACG ATC ACC CTT GAC TC | TM = 53.8°C |
| Aggrecan | Agg32512F | NC_000015 | 170bp | ACT TCA GAC CAT GAC AAC TCG | TM = 52.4°C |
| Agg32512A | Agg35067R | | | ACA CGG CTC CAC TTG ATT CTT | TM = 52.4°C |

between target and reference genes (ΔCt). COL1A1, COL2A1 and Agg were the target genes. From the 4 resulting ΔCt values, the mean and standard deviation were calculated.

### *Level of expression compared to reference gene*

To determine the level of expression the ΔCt values were converted into linear values in the equation $2^{-\Delta Ct}$ and presented in table 2. The table also shows the relationship between the values of the control group and group A respectively those of the control group and group B as quotients.

Aggrecan turnover in damaged areas showed significant variation but tended to be lower than in healthy cartilage and correlated weakly with proteoglycan content.

**Table 2.** Calculation of the ΔCt values (means) and quotients between expression values for the genes Col1A1, Col2A1 and Agg and the reference gene.

| | Col1A1 $2^{\Delta Ct}$ | Col2A1 $2^{\Delta Ct}$ | Agg $2^{\Delta Ct}$ |
|---|---|---|---|
| Control (Mean) | 0.015 | 0.0025 | 0.0091 |
| Degenerative Group A (Mean) | 0.108 | 0.0009 | 0.0053 |
| Quotient of ΔCt values Group A ·/· Control | 7.2 | 0.36 | 0.58 |
| Degenerative Group B (Mean) | 0.3006 | 0.0004 | 0.0049 |
| Quotient of ΔCt values Group B ·/· Control | 20.04 | 0.16 | 0.54 |

### *Results of photoshop analysis*

Table 3 shows the results of the Photoshop analysis for safranin O for proteoglycans, Alcian blue for polysaccharide components, PAS for glycoproteins, and immunohistochemical staining for collagen type I and II. Fig. 1 shows an example of Photoshop based analyses of healthy and degenerative cartilage. With PAS staining reduction in standard deviation was smaller than with safranin O or Alcian blue (mean and median in Table 3). This means that loss of zonal differentiation (with differences in ECM distribution) is especially evident with safranin O staining. Here the standard deviation of color uptake capability is especially low as the damaged cartilage areas are more homogenous overall. Smaller vertical cracks were frequent in samples of severely damaged tissue.

Here in contrast to the other staining characteristics, we observed an increase in safranin O uptake into the subchondral bone, suggestive of remodeling activity. In healthy cartilage the Photoshop based analysis showed uniform distribution of collagen type II in the different cartilage layers. The more advanced the degenerative process, the greater the decrease in collagen type II in the upper fibrillary areas. In samples with especially advanced degeneration this reduction was so pronounced that the overall collagen type II content was also

**Table 3.** Results (mean ± standard deviation) of Photoshop based image analysis for Safranin O, PAS, Alcian blue and immunostainings for collagen I and II.

| | Group A ICRS Grade 1b | Group B ICRS Grade 3a and b | Control ICRS Grade 0 |
|---|---|---|---|
| Safranin-O (proteoglycans) | 146,77±21,77 | 167,38±15,30 | 111,52±25,90 |
| PAS (glycoproteins) | 224,80±17,34 | 263,22±12,12 | 212,19±19,46 |
| Alcian blue (polysaccarides) | 163,29±5,33 | 198,08±10,45 | 156,88±18,93 |
| Collagen I (immunostaining) | 152,69±20,05 | 133,96±27,69 | 162,20±17,62 |
| Collagen II (immunostaining) | 131,29±21,23 | 170,91±26,06 | 122,74±18,33 |

NB: Higher absolute values (mean) correspond to reduction in colour uptake.

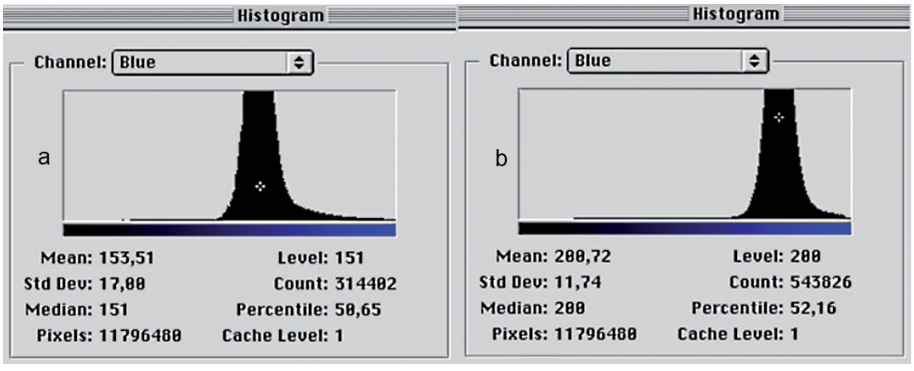


**Fig. 1.** Examples of "Photoshop based image analysis" of Alcian blue stained specimens from healthy and grade III damaged cartilage. Diagrams in "Image menu" with "Mean", "Median" and "Standard deviation" of individual image. A low mean value with relatively high standard deviation indicates a high dye uptake capability and intactness of zonal differentiation (healthy cartilage). The grade III example shows a decreased dye uptake capability (higher mean) and reduction in zonal differentiation (lower SD).

**Table 4.** Correlation of Real Time PCR resp. colorimetrical measurement (proteoglycan GAG) with Photoshop image analysis.

| | Correlation Coefficient (Spearman) | p-Value |
|---|---|---|
| Safranin-O | 0.815 | 0.001 |
| Glycoprotein | 0.622 | 0.001 |
| Alcian blue | 0.646 | 0.001 |
| Collagen I | 0.730 | 0.001 |
| Collagen II | 0.617 | 0.018 |
| Aggrecan | 0.220 | 0.095 |

reduced. In 6 cases this was found even when deeper areas showed a slight increase and in all remaining layers and samples no significant changes were present. The superficial layers instead showed greater color uptake for collagen I, which increased significantly with more severe degeneration. In some biopsies that already demonstrated significant fiber remodeling there were still parts with clear signals for collagen type II. In 2 specimens however, almost no collagen type II signal was present, correlating with a nearly complete fibrocartilaginous transformation.

Correlation analysis for real-time PCR with dimethylene blue assays or Photoshop analysis, using Spearman correlation analysis, showed strong correlation for safranin O and collagen type I, medium correlation for collagen type II, Alcian blue and PAS (glycoprotein), and a weak correlation for aggrecan (Table 4). It appears that when degenerative changes are primarily located in the pericellular matrix, the correlation between methods is weaker.

## Discussion

The current literature still contains many different observations and interpretations of distribution, number, and modes of change of collagens and extracellular matrix molecules (ECM) in hyaline joint cartilage during degenerative processes. Differences depend largely on the study method employed (immunohistochemistry, biochemical analysis, gene expression analysis etc.). Chondrocytes in the different cartilage zones also demonstrate significant variation in characteristics, such as matrix synthesis activity (Martin et al., 2001; Lorenz et al., 2005; Lorenz and Richter, 2006).

Progressive functional and anatomical cartilage deterioration during arthritic processes can no longer be considered a measure of excessive cartilage degradation alone. It is also a sign of uncoordinated and insufficient synthesis response, meaning that the type, number, and organization of extracellular molecules produced are no longer favorable (Martin et al., 2001). Neuropathological and oncological studies of hormone receptor quantification have already demonstrated a good correlation between Adobe Photoshop based analysis and biochemical techniques such as enzyme immunoassays (Matkowsjyj et al., 2003). More recent studies of cartilage layers and their extracellular matrix molecules have applied Photoshop analysis as well (Lehr et al., 1997; Matkowsjyj et al., 2003; Lahm et al., 2004).

In general, studies like this one, that are performed exclusively with reliable semiquantitative comparisons of Photoshop analyses produce very good information on biomechanically and biologically relevant content of collagens, proteoglycans, etc as among other factors the latter coincide with tensile strength, failure strain and other biomechanical factors of biomechanical integrity (Temple-Wong et al., 2009).

In an experimental Photoshop analysis of hyaline joint cartilage, the medium-term influence of subchondral damage on the cartilage was investigated. Even in the experimental set-up, safranin O analysis not only showed the progressive loss of proteoglycan in the course of degeneration; it also showed the loss of zonal differentiation as well. PAS analysis had a medium significance for reflecting glycoprotein content, but information on zonal differentiation was somewhat less reliable (Lahm et al., 2007). Alcian blue also showed only a medium grade correlation with polysaccharide component content (using quantitative real-time PCR as the gold standard). Proteoglycan loss is represented in various Photoshop analyses but best of all by decreased uptake of safranin O. With progressive degeneration, this change reaches into the deep zones of the cartilage, producing a disintegration of the "tidemark" between calcified and uncalcified cartilage, and also to a lesser degree the mineralization zone between calcified cartilage and subchondral bone (Lahm et al., 2007).

According to our results, the content and regulation of aggrecan in RT-PCR cannot serve as a standard for proteoglycan content. This is despite the fact that these large proteoglycans, produced by covalent bonds between "protein skeletons" and glycosaminoglycans dominate and with condroitin sulfate compose a significant portion of cartilage mass, so that a catabolic situation would thus lead to a loss of the glycosaminoglycan bearing fragments, which in turn would compromise cartilage function (Bluteau et al., 2001).

There are contradictory results on regulation of aggrecan content in the catabolic situation. Matyas et al. (1999, 2002) and Young et al. (2005) found an up-regulation during the progression of osteoarthritic process, Lorenz et al. (2005) and Aigner et al. (1993) found no significant changes. Yagi et al. (2005) found a down-regulation. Squires et al. (2003), similar to this research team, found a variational relationship between proteoglycan content and aggrecan regulation.

In the early stages of osteorthritic processes slightly increased color intensity for collagen II in deeper layers is suggestive of a persisting but initially still intact repair process. When this repair mechanism is lost on the surface this is usually reflected in a significant decrease in collagen II content, leading (in this study) to an early reduction in total collagen II (Lorenz et al., 2005, 2006). Matyas et al. (1999, 2002) and Aigner et al. (1993) found an up-regulation of collagen II mRNA in early

degenerative stages. With further degeneration, however, chondrocytes seem to exhaust their capability to accumulate collagen II, and it can often only be found in clusters, leading to a decrease in total collagen II content in these studies.

Photoshop analysis showed almost uniform collagen II distribution in the zones of healthy cartilage. With advanced arthritis it found the same changes described previously, with a particularly sharp decrease in surface fibrillations. In the majority of cartilage samples, deeper layers showed clear signs of collagen II. This collagen however, is apparently no longer able to fulfill the biomechanical requirements of the matrix-besides a loss of the ability of anisotropic fibril reinforcement probably due to the changes in proteoglycan content observed (Gu and Li, 2011). The side chains of this molecule bind large quantities of water, producing swelling pressure and tension within the matrix and lending the cartilage its biomechanical properties. Proteoglycan loss is reversible, as long as its causes do not persist (Yagi et al., 2005). As with PAS staining, it is likely that when changes in color uptake are pronounced but spatially very circumscribed, the correlation between RT-PCR results and Photoshop analyses will also be weaker. This is the case with significant changes in the direct pericellular matrix.

Collagen type I is mainly found in advanced stages of arthritis, but is generally expressed in earlier stages with a pronounced expression of collagen type I mRNA. Even studies that applied other methods demonstrated largely similar relations. Miosge et al. (2004) found a factor 20 expression relation, which agrees strongly with our results of a COL I/HPRT1 ratio of 0.015 in healthy cartilage and a COL I/HPRT1 ratio of 0.29 in degeneratively modified cartilage. Our results differed in that, for each comparable stage, we found a 5 fold down-regulation of collagen II. In light of the early presence of collagen I as well as the basic ability of chondrocytes to up-regulate collagen II production, a therapy for stopping degenerative processes and strengthening chondrocyte response might involve a suppression of collagen I production, coupled with the promotion of collagen II production.

Absolute quantification methods, which offer information such as a definite number of Collagen type I mRNA molecules per cell or milligram of cartilage, need to be viewed critically: they present several problems, such as the need for a calibrated curve with a plasmid standard for every gene, a considerable number of steps, and a difficulty in quantifying losses (in grinding, dissolving in Trizol, loss of the watery phase, etc.). In addition, significant sequence dependant differences exist that prevent the conversion of mRNA to cDNA from being comparably efficient for each gene.

Finally, mRNA changes represent changes in collagen biosyntheses extremely well, so that the relative changes in aggrecan and collagen mRNA content described in this study can provide a valid index for chondrocyte response to joint damage. The potential of quantitative RT-PCR to replicate and quantify gene specific products makes it particularly useful for investigations of focal or regional cartilage lesions. Relative quantification with real-time PCR is a very effective method of quantifying mRNA and cDNA, requires no calibrated curve, and has a high degree of reproducibility and sensitivity and a low degree of variance. It is thus particularly suited to confirming the results of semiquantitative methods such DNA microarray, classical PCR, or Northern blot experiments.

Photoshop-based image analysis is an easily usable, commercially available instrument for a quick, serial semiquantitative analysis of cartilage samples. The sometimes extensive gene expressions in the various layers of cartilage (Fukui et al., 2008) can be elucidated with a few simple steps. However, the production of study specimens can be demanding. Subjective and partially insufficient (Barley et al., 2010) estimations of safranin O or Alcian blue color uptake in scores like HHGS (Mankin), Collins and McElligott or O'Driscoll can thus be fully replaced.

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