Propagation-Based Phase Contrast Computed Tomography as a Suitable Tool for the Characterization of Spatial 3D Cell Distribution in Biomaterials

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1. Introduction

Cell-based therapeutic approaches to treat articular cartilage defects can regenerate into fibrous rather than hyaline tissue, which has a lower capacity to withstand mechanical stress. Articular cartilage is a 3D structured tissue with a hierarchical ordering and limited self-regeneration capacity, rendering the treatment of complex lesions such as deep chondral or osteochondral defects a clinical challenge. In each zone, the chondrocytes exhibit a zone-specific different morphological shape, which must also be realized in matrix-supported therapeutic approaches to ensure the same mechanical properties of regenerated tissue compared with native articular cartilage. Several studies have shown the positive effects of electrical stimulation on bone regeneration and cartilage tissue. In this context, it is important to understand how the migration, shape, and also differentiation of the chondrocytes in combination with a scaffold material can be altered by the application of electrical fields.

However, the investigation of cartilage tissue and cell migration happens via different techniques, each having specific...
Advantages and shortcomings. In clinical diagnostics, magnetic resonance imaging (MRI) is used to visualize directly changes and defects in the cartilage tissue.\cite{8} It is a noninvasive technique based on the coupling of the hydrogen spin to strong magnetic fields and radio waves. Quantitative MRI can provide structural information in the submillimeter range to assess parameters such as the volume and thickness of the tissue.\cite{9,10} However, it is limited by the achievable spatial resolution; thus, cell distribution within 3D tissue volumes cannot be examined by MRI. Although histological examinations using light microscopy are considered the method of choice for cell distribution analysis, this approach does not provide a comprehensive overview of the total number and distribution of cartilage cells within a 3D biomaterial (matrix), as the provided information is only 2D.\cite{11}

Light-based techniques to visualize 3D cell distributions in tissues, such as multiphoton microscopy, require for either cartilage sectioning\cite{12} or additional clearance steps to achieve sufficient penetration depth to receive tissue image data in three dimensions.\cite{13}

An established technique for the generation of 3D information with high spatial resolution is X-ray-based micro computed tomography (μCT), as this technique allows the imaging of volumes in a nondestructive fashion down to the micrometer and even nanometer scale. The imaging of soft tissue is challenging, as such samples exhibit low attenuation contrast at high X-ray energies, as they are mainly made of carbon, oxygen, and hydrogen. Furthermore, imaging under aqueous conditions requires the X-ray energies of about 30 keV to have sufficient transmission and minimized radiation damage. The application of X-ray computed tomography for soft matter samples has become possible with the development of X-ray phase contrast tomography, which utilizes the phase shift of the X-ray wave.\cite{14} X-rays interact with matter not only by attenuation of their amplitude, but also by a shift of their phase. For coherent X-rays, this change in phase can be utilized as a contrast mechanism for weakly attenuating samples. Thus, phase contrast imaging at synchrotron radiation facilities with limited access opens the possibility to investigate cell distribution within an opaque soft matter matrix with micrometer resolution, which is hardly possible for other techniques such as optical or fluorescence microscopy.

Therefore, this technique is ideally suited to investigate the 3D distribution of chondrocytes in biomaterials without any embedding. The aim of this study was to assess the parameters and feasibility if the spatial distribution of chondrocytes within a collagen scaffold can be determined under aqueous condition with high resolution. Two scaffolds with chondrocytes seeded on top were investigated, where one was exposed to an electrical field during in vitro cell culture. These experiments aimed to test if phase contrast tomography can fill the gap between MRI and histology to reveal micrometer-sized information on the cell distribution within the biomaterial.

2. Results: 3D Assessment of Chondrocyte Distribution

The samples were imaged by propagation-based phase contrast tomography, and the 3D volume was reconstructed. As the samples were larger than the field of view (FOV), a region of interest scan was performed concentrating on the center of the samples. Figure 1a shows a 3D rendering of the reconstructed volume. In the next step, the cells were segmented, which is needed for the visualization, see Figure 1b, and the following spatial distribution analysis (Figure 2).

Figure 2 shows the number of chondrocytes as a function of the distance from the surface of collagen scaffolds with and without electrical stimulation. Both depth profiles show the same behavior, i.e., a high number of chondrocytes within the first 60 μm followed by a decrease in their number with increasing distance from the scaffold surface to a maximum depth of 580 μm. To understand the depth profile, we have plotted vertical cuts from the tomographic reconstruction in Figure 3. These images reveal a thick layer of chondrocytes, which corresponds to the observed high accumulation at the near surface. For the unstimulated sample, no chondrocytes were detected after 300 μm sample depth, whereas chondrocytes can be visualized down to a migration depth of 580 μm for samples stimulated with electric fields.

To show this more closely, the percentage increase of the chondrocytes as a function of the distance from the interface is plotted in Figure 2. We note that the data were cut at the x-axis, as counts for the chondrocytes with electrical stimulation were dropped to zero. This representation shows that the number of cells for the interface region is increased by 20% and constantly increases down to the bulk of the sample where it reaches 100%.

Figure 4 shows the volume distribution of the chondrocytes for samples without and with electrical stimulation. For the samples, no direct correlation with respect to the chondrocyte size can be drawn. We argue that the oscillations in the size

![Figure 1](image-url)
**Figure 2.** Depth profile of the chondrocytes evaluated from the tomographic reconstruction for collagen scaffold with and without electric stimulation. a) Total number of human chondrocytes as a function of the distance from the interface. b) Percentage increase as a function of the distance from the interface.

**Figure 3.** Slices through the 3D reconstruction of tomographic measurement of a cell-seeded collagen-based matrix a) without and b) with electrical stimulation. The bottom row shows the segmentation of the chondrocytes in the respective slice grown on the collagen-based scaffold c) without electrical stimulation and d) with electrical stimulation.

**Figure 4.** 2D maps correlating the size distribution of chondrocytes with the distances from the interface. The color code represents the number of chondrocytes. For samples a) without electric stimulation and b) with electric stimulation. c) Size distribution averaged along the distance from the interface.
distribution occur due to the behavior of the chondrocytes forming columns composed of single cells. It seems that the overall number of cells is increased, whereas no preferential volume size can be deducted from the data. This is also shown in Figure 4c. Here, the volume size distribution is averaged along the depth.

3. Discussion

Due to the complexity of cartilage tissue and the fact that injured hyaline cartilage cannot regenerate itself, the treatment of cartilage lesions remains a major therapeutic challenge. Therefore, there is still a great need for research in this field.[2,4,15,16]

In addition to the approach of using endogenous chondrocytes or stem cells, methods have also been developed to anchor these cells in the lesion.[2,15] For this purpose, a variety of different biomaterials have been developed in recent years for potential application in the treatment of cartilage injuries.[17] The aim is that the used biomaterials are transformed into physiological structures through the migration of cells into the materials and subsequent cellular processes. Biophysical stimuli such as electrical stimulation appear to be suitable for regulating the processes that lead to the formation of hyaline articular cartilage.[6,7,18]

In clinical routine, cartilage lesions are diagnosed by arthroscopy or MRI, as MRI is a noninvasive method.[8] MRI could be used to characterize the conversion of biomaterials with regard to changes in collagen and proteoglycan content in, e.g., ex vivo or in vivo experiments.[10,19] The advantage of MRI is that the biomaterials could remain in position, thus enabling continuous investigations. The disadvantage of MRI is that no statement can be made about the distribution of the cells with respect to their zonal position within the matrix. However, this information is important to assess whether the utilized biomaterial can resemble the cellular distribution as found in healthy hyaline cartilage in vivo.

Phase contrast tomography offers the possibility to investigate soft tissue samples under near physiological conditions with high spatial resolution allowing visualizing the cell distribution or even single cells with high accuracy.[20] Size, position, and shape of cells can be identified at differentiation stages.[21] Furthermore, in more advanced approaches, specific immunostaining could additionally allow the detection of important structural markers, such as collagen II, as already shown in the μCT study of Metscher and Müller.[22] In addition, the high achievable resolution is only possible for samples having a size of a few millimeters or even smaller. Furthermore, due to the coherent X-ray beam needed, such investigations are restricted to advanced X-ray sources such as synchrotrons, and a high radiation dose is applied to the samples during measurements. However, a perspective to yield similar information at laboratory-based sources is using grating-based phase contrast tomography.[23] Different studies show the applicability and versatility, but further developments have to be done to reach a similar quality as with synchrotron measurements.

Histological examinations are the method of choice when it is necessary to characterize cells within different tissues or biomaterials. The main advantage of histological examination is the combination of staining cells specifically and either marking special cell components such as collagen II for cartilage tissue[24] or in parallel use of different staining. Although histology provides valuable insight into the morphology and cell migration, there is a lack in 3D information.[11] Figure 5 shows a histological image of a cell-seeded collagen scaffold with some typical artefacts caused by sample preparation, such as fold or cutting artefacts. Furthermore, a shrinking of the sample may happen due to the embedding procedure during histological preparations. For
histological investigations, the samples have to be sectioned before staining, and image acquisition has to be done by optical microscopy, thus yielding only 2D information. Volumetric information of samples can only be obtained using serial slices, imaging them and aligning these multiple sequential sections, thus generating a major effort of sample preparation and time.[11,25]

To be able to make an all-encompassing statement on the suitability of used biomaterials and stimuli, a multimodal approach combining the techniques mentioned earlier is recommended.

4. Conclusion and Outlook

In the present work, we could show the feasibility to investigate chondrocyte distribution by means of propagation-based phase contrast tomography, which allows assessing the cell distribution in 3D volumes with a resolution of 3.65 μm. The shown measurements allow observing an increased cell migration in the presence of an applied external electric field at 1 V m⁻¹ compared with unstimulated controls. The data indicate that an alternating electric field is fostering cell migration. These experiments allow for the assessment of cell size and the number of cells as a function of the distance to the interface in the full 3D volume. Based on this experience in terms of measurement properties and data evaluation approach, we are able to carry out a comprehensive study on the influence of electrical fields on cell migration.

In contrast to histology, which is the “gold standard” for the analysis of soft tissue samples, the collagen matrix can be investigated under aqueous conditions without any embedding or slicing, reducing preparation artefacts. However, only spatial information can be deduced from phase contrast tomography, requiring additional techniques to assess extracellular matrix composition, quality of collagen matrix, or hyaline cartilage component deposition, which is possible using histology or MRI. Thus, a comprehensive analysis of the cartilage microstructure that requires a multimodal investigation is needed, as each of the mentioned techniques has its advantages allowing to address specific questions. None of these methods alone can provide comprehensive characterization. Such combination of techniques enables to understand fully the connection of cartilage quality, as determined by MRI or histology, to the chondrocyte distribution, size and shape, as determined by inline phase contrast tomography.

5. Experimental Section

Cell Culture on 3D Scaffolds and Capacitively Coupled Electrical Stimulation: Human chondrocytes of a 30 year old man (NHAC-kn 31343, CC-2550; LONZA Walkersville Inc., Walkersville, MD, USA) were expanded at 37°C, 5% CO₂, and 5% O₂ and cryopreserved at passage 3. After thawing, the chondrocytes were cultivated as described previously.[7]

At passage 4, cells were seeded on a 3D collagen-based scaffold (Chondrofile, Medix GmbH, Esslingen, Germany), mainly consisting (90%) of rat-tail tendons collagen type I. Chondrofile is in the article referred to as collagen scaffold. Scaffolds with plane-parallel surfaces were 10 mm in diameter and 5 mm in height. After washing, scaffolds were placed in 24-well plate (Corning, Glendale, AZ, USA), and cells were seeded with a density of 1.05 × 10⁵ cells mm⁻². Cell-seeded scaffolds were cultivated with the chondrogenic growth factors insulin-like growth factor (IGF)-1 (R&D Systems, Minneapolis, MN, USA) and transforming growth factor (TGF)-β1 (Peprotec, Hamburg, Germany) each 50 ng mL⁻¹ under hypoxic conditions as described in the previous study.[7] After 3 days, the medium was replaced by medium without added growth factors. Subsequently, cell-seeded scaffolds were placed in an electrical stimulation device (Patent DE 10 2018 114 019 A1). The cell-seeded scaffolds were stimulated with an alternating capacitively coupled electric field (≈1 V m⁻¹ at 60 kHz) three times each day for 45 min within 8 h over a period of 7 days with media changing every 2 days. Unstimulated cell-seeded scaffolds served as controls. After stimulation, the scaffolds were washed with (0.1 mol L⁻¹) phosphate buffer and fixed with a mix of glutaraldehyde (2%) and paraformaldehyde (4%) in phosphate buffer and cut to a cylindrical sample of ≈5 mm in diameter using a razor blade (Wilkinson Sword GmbH, Solingen, Germany) to achieve a suitable sample size for tomography measurements. However, the preparation of smaller sample size was not possible without inducing artifacts. To increase the attenuation contrast of the cells in the surrounding collagen matrix, the cells were stained with 1% osmium tetroxide solution (Science Service GmbH, Munich, Germany). The attenuation and phase contrast are both influenced by the staining, as it affects both, the real part decrement, α, and the imaginary part, β, of the refractive index. As the α to β ratio is smaller than on the attenuation contrast. By comparing the data from an unstained to a stained sample, an overall increase of the image contrast (electron density with respect to unstained areas) by 54% can be deduced. Due to the experimental setting, i.e., finite propagation distance, no estimation on the pure attenuation contrast can be made.

Tomography Measurements: The tomography measurements using propagation-based phase contrast were performed at microtomography end station of the imaging beamline P05 at PETRA III at DESY (Hamburg, Germany) operated by the Helmholtz-Zentrum Geesthacht.[26]

Figure 6 shows an image of the experimental setup along with a sample holder with a collagen-based scaffold sample. A camera system based on a complementary metal-oxide semiconductor (CMOS) with a pixel size of 6.5 μm and an array size of 5120 × 3840 pixel was used. The magnification was ≈9.954 with an effective pixel size of 0.642 μm. The resolution was determined to be 3.56 μm by doing a knife edge scan and calculating the mutual transfer function (MTF). The energy of the incoming, monochromatic beam was 30 keV. The propagation distance was chosen to be 234 mm. For each sample, 2400 projects were taken in the angular range from 0° to 180°. As the samples were larger than the FOV, a region of interest scanning was performed. Along with the radiographs of the
sample, reference images of the X-ray beam were taken to be able to perform a flat field correction.

The reconstruction was performed by a Matlab (MathWorks, Natick, USA) code using the ASRTA toolbox. In the first step, radiographs of the sample and the images of the beam profiles were correlated to find the respective flat field for each image. Afterward, the flat field correction and the reconstruction were performed. In contrast to attenuation contrast tomography, phase retrieval had to be performed prior to tomographic reconstruction to yield (relative) phase shifts from the acquired intensity images. For this, the Paganin algorithm was used. During the reconstruction, a binning was performed, resulting in images with a size of $2560 \times 1920$ with a pixel size of $1.29 \mu m$. The reconstructed volumes were segmented, making use of the software package Ilastik. For the segmentation, the 3D option of Ilastik was used. Thus, the cells were marked in all 3D directions, and Ilastik was trained on the dataset. As feature, the intensity, the detected edge (Laplacian, gradient, difference), and the texture (tensor eigenvalues, hessian eigenvalues) were used. In the following, the segmented label sets were analyzed with the 3D particles analyzer of ImageJ to extract the distribution, number, and size of the human chondrocytes. As the sample surface was bend, it was necessary to determine the distance of the chondrocytes from the interface to allow a quantification of the migration distance into the scaffold. This procedure was done in MATLAB yielding the following parameters: chondrocyte density and size distribution as the function of migration distance.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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