2-Photon Laser Scanning Microscopy on native Cartilage and Collagen-Membranes for Tissue-Engineering

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ABSTRACT

In our experiments 2-Photon laser scanning microscopy (2PLSM) has been used to acquire 3-dimensional structural information on native unstained biological samples for tissue engineering purposes. Using near infrared (NIR) femtosecond laser pulses for 2-photon excitation and second harmonic generation (SHG) it was possible to achieve microscopic images at great depths in strongly (light) scattering collagen membranes (depth up to 300 µm) and cartilage samples (depth up to 460 µm). With the objective of optimizing the process of chondrocyte growth on collagen scaffolding materials for implantation into human knee joints, two types of samples have been investigated. (1) Both arthritic and non-arthritic bovine and human cartilage samples were examined in order to differentiate between these states and to estimate the density of chondrocytes. In particular, imaging depth, fluorescence intensity and surface topology appear promising as key information for discriminating between the non-arthritic and arthritic states. Human chondrocyte densities between $2 \cdot 10^6/cm^3$ and $20 \cdot 10^6/cm^3$, depending on the relative position of the sample under investigation within the cartilage, were measured using an automated procedure. (2) Chondrocytes which had been sown out on different types of I/III-collagen membranes, were discriminated from the scaffolding membranes on the basis of their native fluorescence emission spectra. With respect to the different membranes, either SHG signals from the collagen fibers of the membranes or differences in the emission spectra of the chondrocytes and the scaffolding collagenes were used to identify chondrocytes and membranes.

Keywords: 2-photon laser scanning microscopy, 2PLSM, second harmonic generation, SHG, tissue engineering, chondrocyte, collagen, native fluorescence, cartilage

1. MOTIVATION & INTRODUCTION

Hyaline cartilage is formed and maintained by chondrocytic cells (chondrocytes) which are embedded in their extracellular matrix (ECM), i.e. chondrocytes are populate cavities (lacunae) in the ECM with reduced ECM density. The ECM mainly consists of collagen II, proteoglycans like chondroitin sulfate and keratan sulfate, glucosamine and water[9; 13]. Due to the task as a shock absorber and joint material, cartilage has unique properties[11] such as high flexibility and a low dynamic friction coefficient. These characteristics are due its dense structure of the ECM and its capability to retain water. This dense structure, however, makes optical examinations of the cartilage on the microscopic level difficult, as it strongly scatters but does not absorb much light. At the same time it also prevents chondrocytes from rebuilding the ECM following a cartilage injury. This is due to the fact that only chondrocytes directly located in the lesion can contribute to the process of ECM regeneration. This limitation in the healing capability of articular cartilage therefore often results in its arthritic degeneration following an injury. To enable successful cartilage healing, different techniques[7; 16] e.g. microfractures, autologous chondrocyte implantation (ACI) and matrix-induced autologous chondrocyte implantation (MACI) have been developed. We are particularly interested in membrane associated treatments like MACI, where chondrocytes are extracted from the patient by an (articular) endoscopical cartilage biopsy. Afterwards, they are isolated from their ECM, proliferated and finally sown on a I/III-collagen membrane scaffold structure, where they can adhere and start rebuilding new cartilage(-like) tissue. This membrane is then implanted to the patients lesion where the chondrocytes regenerate new ECM while the scaffolding I/III-collagen

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membrane is catabolized within approximately one year. To achieve optimal growth conditions for chondrocytes on the collagen membrane, it is necessary to vary important growth factors such as temperature, cell culture medium composition, temperature etc. Furthermore, a promising strategy[12] is to apply mechanical stress (similar to stress in cartilage) to the chondrocytes that are adhered to the collagen fibers in order to promote redifferentiation. The evaluation of these biomechanical techniques requires long-term, labeling-free examinations. We are investigating both the original cartilage and different types of cell-populated collagen scaffold materials, not only to provide this feedback mechanism, but also to estimate the number of viable chondrocytes from the primal biopsy.
Because strongly light scattering and (optically) dense materials have to be examined with respect to their 3-dimensional structure, we have chosen near infrared (NIR) induced 2-photon excitation of native fluorescence as an examination tool. 2PLSM[1; 4; 5; 18; 19] offers the advantage of intrinsic 3-dimensional sectioning properties combined with great penetration depths into biological samples when using NIR (780 nm-920 nm) excitation wavelengths[3]. Furthermore, native fluorescence of e.g. NAD(P)H, flavine, elastin and collagen[10; 18] as well as SHG, especially of collagenes[2; 6; 17], can be induced by 2PLSM. This allows for an examination procedure which does not require mechanical slicing or the use of fluorescent probes. Entirely sterile long term studies on the growth process of chondrocytes on scaffolding collagen membranes under various conditions are currently being started.

2. METHODS

2.1. 2-Photon laser scanning microscope

The 2PLSM consists of a mode locked Tsunami Ti:Sa laser (Fig. 1; 1) that generates 100 fs laser pulses between 760 nm and 960 nm pumped by a Millenia X solid state laser (both Spectra-Physics), a TriM Scope (Fig. 1; 2-4) multi focal scanning unit (LaVision BioTec) and an inverted microscope (IX 71, Olympus: Fig. 1; 5). Detection of the fluorescence signal in the non-descanned optical pathway is realized by a back illuminated EMCCD camera (IXON BV887ECS-UVB, Andor Technology: Fig. 1; 8) or a photomultiplier (H7422-40, Hamamatsu) for a single exciting laser focus (Fig. 1; c). The exciting NIR laserbeams are directed via a dichroic mirror (2P-Beamsplitter 680 DCSPXR, Chroma: Fig. 1; 9) onto the back aperture of the objective lens (Fig. 1; 6) while stray light from the NIR in the detection path is blocked by a short pass filter (2P-Emitter E 700 SP, Chroma). In order to perform spectrally resolved measurements, a filter wheel (LaVision BioTec), a spectrograph (SpectraPro 2300i, Acton Research Corporation) or a home built prism based spectrometer (Fig. 1; 7) can be introduced into the non-descanned detection pathway. The scanning unit consists of an integrated pre-chirp section (Fig. 1; 2) to compensate for laser pulse dispersion and two galvanometric mirror scanners (Fig. 1; 4) to scan the laser foci in one optical x-y-plane (i.e. one depth) of the sample. The focus multiplexing section of

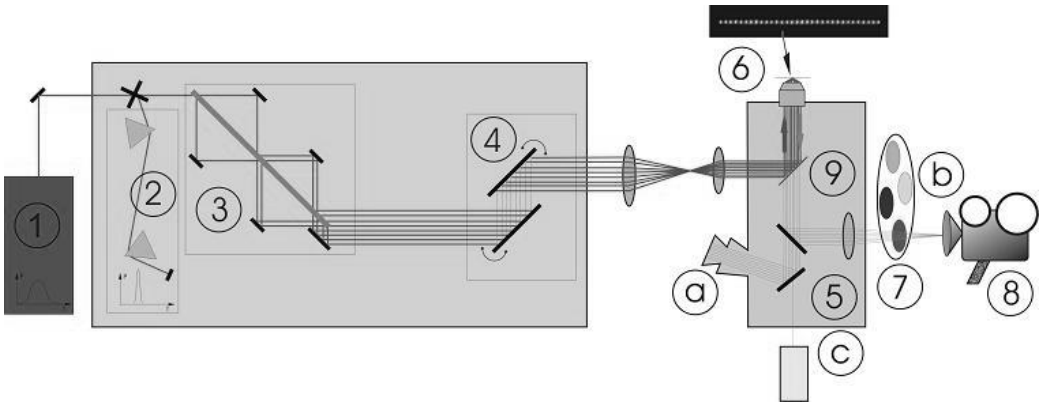

Figure 1: Schematic setup of 2PLSM, for details see text

the TriM Scope (Fig. 1; 3) splits the incidenting laser beam into a variable number of beams of the same average power. This section consists of a set of ten 100% reflective mirrors and one (adjustable) 50% mirror. By introducing the 50% mirror between the set of 100% mirrors, the laser beam can be split into 1,2,4,..,64 beams resulting in an adjustable number of excitation foci (Fig. 1; 6) in the sample. In addition, laser power can be adjusted in order to achieve short acquisition times with sufficient fluorescence while keeping the photodamage minimal. A mechanical focus and sample drive (MFD, Märzhäuser) in combination with a NIR coated objective lens (XLUMPLFL20XW, Olympus) with a large working distance (WD = 2 mm) allows for the acquisition of depth resolved fluorescence x-y-scans inside the sample.

Data acquisition and experiment control is performed by the TriM scope's software package (Imspector, LaVision BioTec). 5-dimensional fluorescent data sets (including spectral and temporal data axis) are handled and processed with Imspector, ImageJ[15] or Imaris 4.0 (Bitplane AG) software packages.

2.2. Cartilage

Human articular cartilage in both healthy and arthritic form was investigated directly (within six hours) after orthopedic surgery. The samples were kept in Ringer solution at room temperature during transport and examination. No additional labeling was applied to the samples. After removal from the transport container, the cartilage samples were cut with a scalpel, when necessary, placed in glass bottom petri dishes and immediately examined with 2PLSM.

2.3 Chondrocytes on collagen membranes

Healthy human and bovine chondrocytes were sown out on different types of I/III-collagen membranes at a density of approx. 280000 per cm². The cell-populated membranes were kept under cell culture conditions for 4 to 6 days before examination. The membranes then were transported in culture medium for 2 to 14 hours under ambient conditions, comparable to real implantation situations such as those for MACI membranes.

3. RESULTS

3.1. Cartilage

Native fluorescence of human and bovine cartilage was examined with 2PLSM within six hours after extraction. Both types of samples exhibited autofluorescence after 2-photon excitation with 800 nm 100 fs laser pulses, making a 3-dimensional reconstruction of the ECM and chondrocyte distribution possible. Imaging depths of up to 460 µm for arthritic bovine cartilage were achieved. Our experimental findings of great optical penetration depth (exceeding approx. 200 µm independent of laser power, as scattering of the fluorescence is the limiting factor) and a reduced fluorescence intensity are consistent with macroscopical findings of a loss of ECM in arthritic cartilage. Furthermore, the surface structure of arthritic cartilage is more fibrous and less smooth than that of non-arthritic cartilage, which again correlates with macroscopical results of increased friction between two joint surfaces[14].

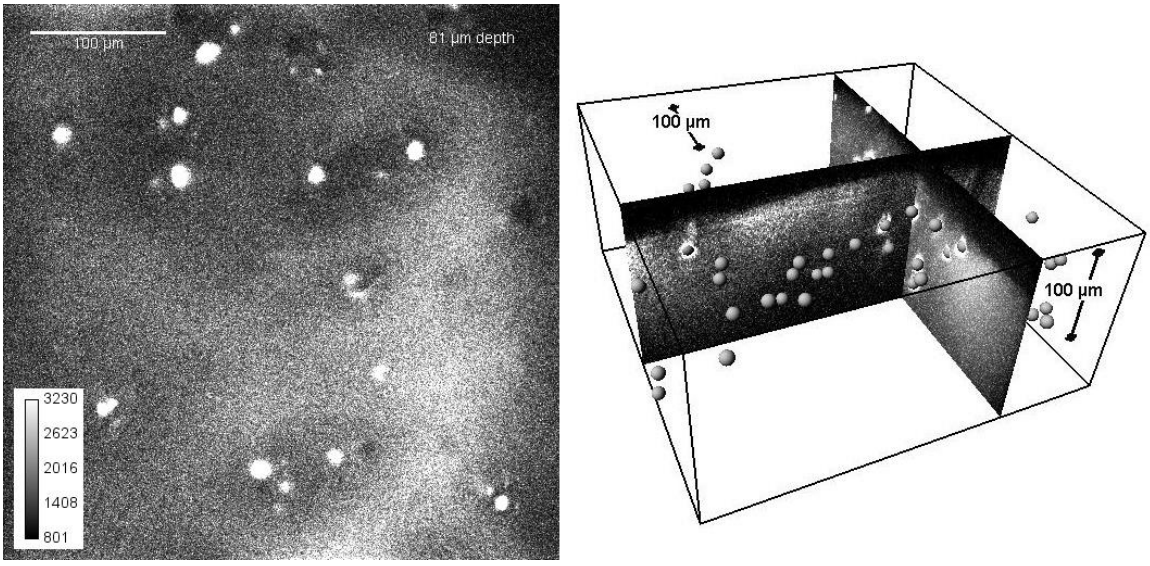

Figure 2: Native fluorescence of human cartilage after 2-photon excitation with 800 nm 100 fs laser pulses, 64 foci à 4 mW, 560 ms exposure time. Left: Optical plane 81 µm inside the sample with HQ575/50 detection filter, chondrocytes are visible as bright spots surrounded by areas of low fluorescence intensity (lacunae). Right: Same dataset with two sectioning planes, colocalized fluorescence intensity of HQ525/50 and HQ575/50 detection filters allowed for automated detection of chondrocytes (represented by balls)

Using 2PLSM fluorescence datasets, it was possible to automatically (spot detection option of Imaris) detect and count chondrocytes inside human cartilage samples as can be seen in Fig. 2. This required a colocalization of HQ525/50 and HQ575/50 emission filter datasets, that can be seen as "bright coronas" around the balls (representation of detected chondrocytes) emerging from the two sectioning planes in Fig. 2. With this method, it was possible to quantify chondrocyte densities in the sample under investigation, which was approx. $2 \cdot 10^6/cm^3$ for the dataset presented in Figure 2. Depending on relative distance to the bone within identical samples, densities of different cartilage samples varied between $2 \cdot 10^6/cm^3$ and $20 \cdot 10^6/cm^3$. These findings are well in keeping with results of classical "stain and cut" methods[8].

3.2 Chondrocytes on fleece collagen membranes

Extracted and proliferated chondrocytes are being sown out on fleece I/III-collagen membranes (Fig. 3) in MACI procedures. Figure 3 clearly shows the rough fibrous structure of the fleece collagen material. Thick (up to 80 µm)loosely assembled collagen fibers constitute the top layer (up to 200 µm) of the collagen membrane. The chondrocytes adhere to these fibers and are blocked from migrating deeper into the membrane by a tight network of thin regularly assembled fibers beneath the top layer. This second layer is approx. 1.5 mm thick and provides not only a barrier for the chondrocytes but also protects them from destructive mechanical stress after implantation and ensures that the scaffolding fibers are not catabolized before sufficient ECM material has been built up by the chondrocytes. In order to perform long term studies on chondrocytes, it is necessary to detect them on the scaffolding collagen fibers without staining protocols. We found that SHG provides a good contrast mechanism as it occurs on the collagen fibers but not on the chondrocytes. Using colocalization of the collagen fibers SHG and fluorescence emission, it was possible to spectrally separate them from the chondrocytes by means of emission filters (Fig. 4). Using complete emission spectra it was also possible to separate chondrocytes and collagen fibers, but the significantly higher data volume and extended acquisition times for these measurements make a filter based spectral separation more practical.

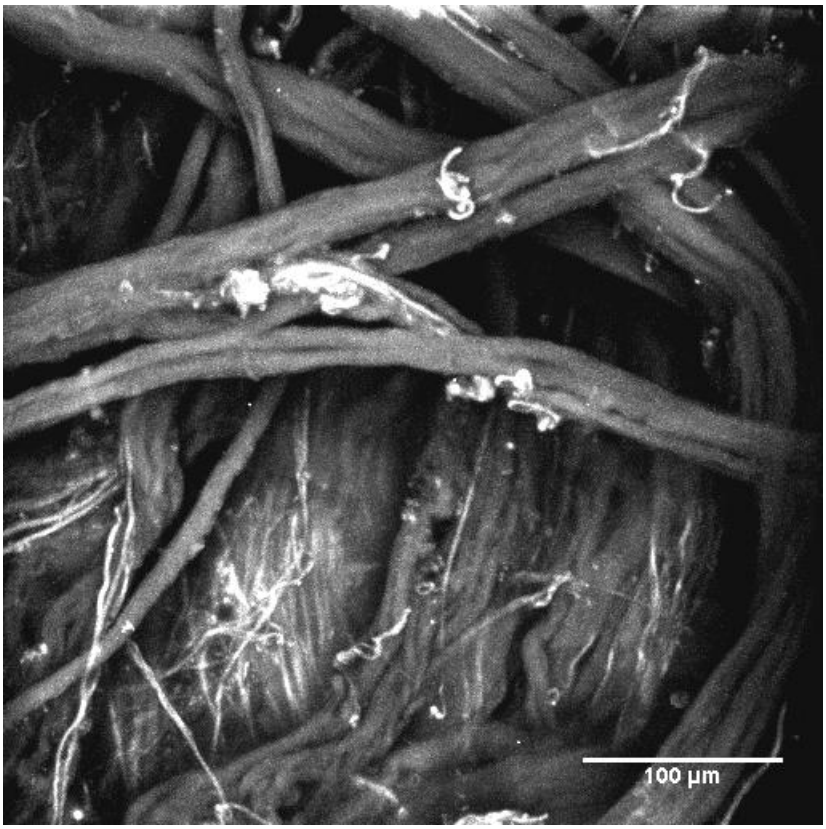

Figure 3: I/III-collagen fleece membrane, 2-photon (810 nm, 64 foci à 4.4 mW, 1 s exposure time) excited native fluorescence, Filter: HQ575/50, maximum intensity projection of 240 optical sections à 1 µm.

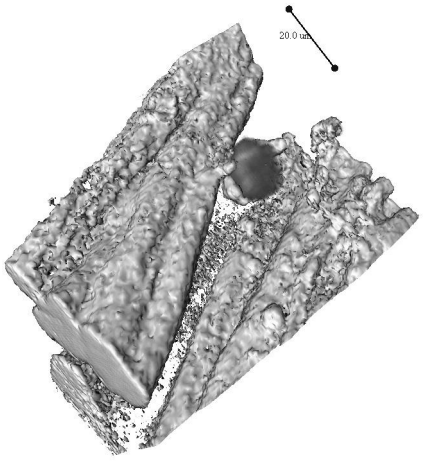

Figure 4: Digital reconstruction based on 2-photon induced (820 nm, single focus < 15 mW, PMT detection) native fluorescence (HQ 525/50 and HQ 575/50 emission filters) and SHG (HQ 410/20 emission filter), scale bar represents 20 µm.

3.3 Chondrocytes on sponge-like collagen membranes

As the improvement of growing conditions for chondrocytes on collagen membranes is an important goal, different types of membranes have been tested. Sponge-like collagen membranes (s. fig. 5) showed beneficial effects on the

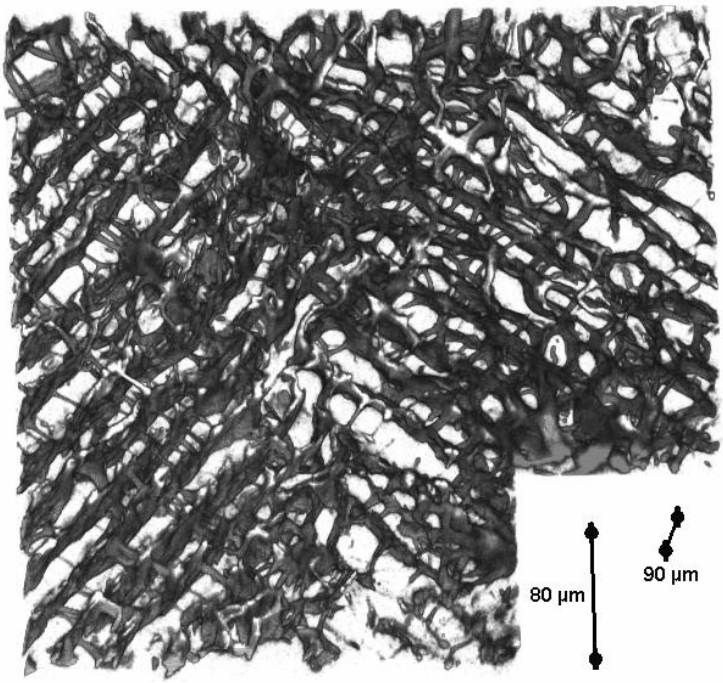

Figure 5: Digital reconstruction based on native 2-photon induced (820 nm, PMT detection) fluorescence, no emission filter, dataset contains images of up to 90 µm inside the sample

viability of chondrocytes on these membranes in dead/alive staining procedures. Their basic structure is comparable to honey combs into which the cells were expected to migrate while being supplied with cell culture medium through the

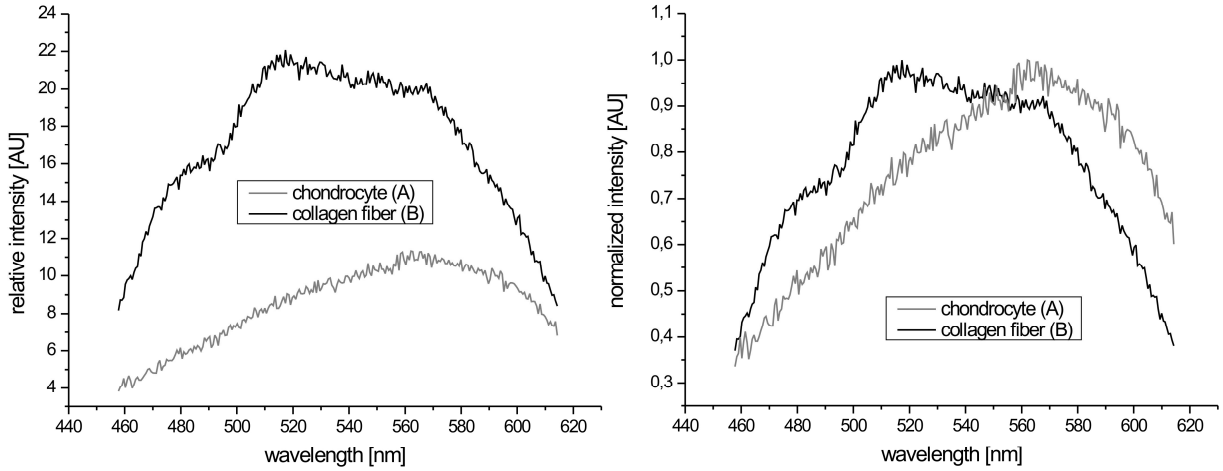

Figure 6: Emission spectra of I/III collagen fibers and chondrocytes on sponge-like membranes after 2-photon excitation (32 foci à 5.5 mW, 785 nm, 650 ms exposure time), prism based home build spectrometer with ±7 nm wavelength accuracy, spectra taken from regions in Fig.7 which clearly could be identified as collagen fiber and as chondrocyte, left: relative intensity, right: normalized intensity.

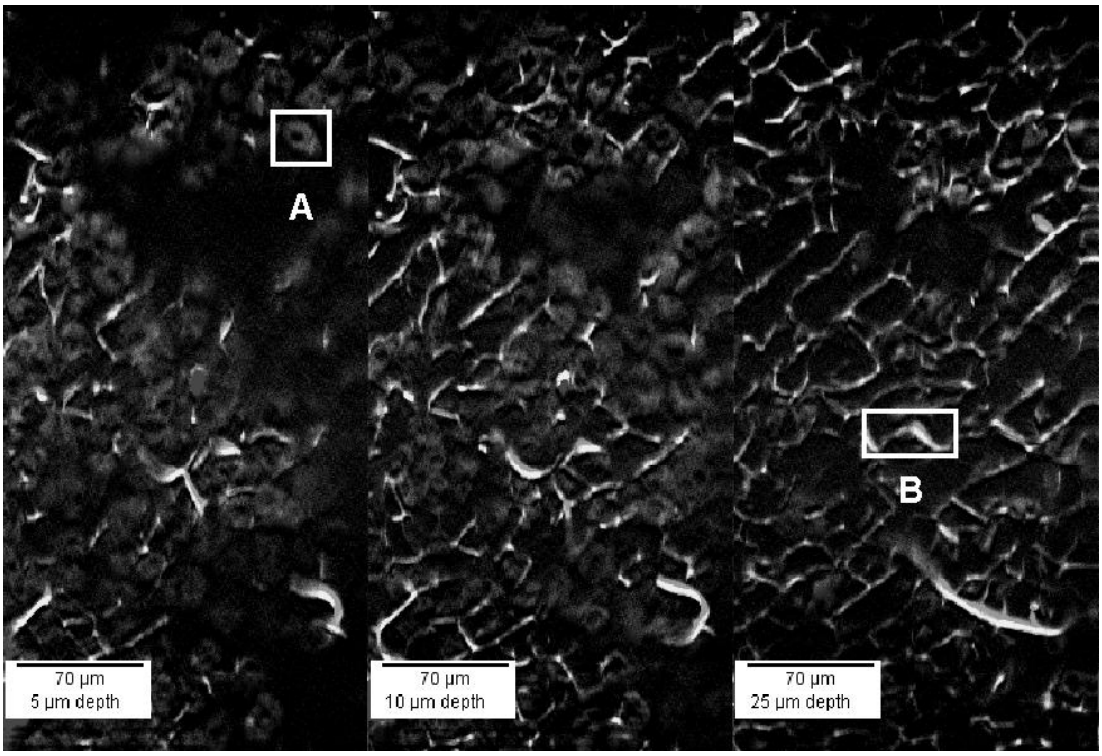

Figure 7: Spectrally unmixed (linear dependency of spectra in Fig. 6) dataset of 3 dimensionally resolved native fluorescence spectra, 2-photon excitation with 32 foci à 5.5 mW, 785 nm, 650 ms exposure time, region A shows a chondrocyte, region B shows a collagen fiber, original representation in red (fit to spectrum A in Fig. 6) and green (fit to spectrum B in Fig. 6)

membrane's tubes. We found that SHG on the sponge-like collagen fibers could not be detected and therefore was not applicable as a contrast mechanism to separate chondrocytes from the scaffolding membrane. Complete 3-dimensionally resolved emission spectra (s. Fig. 6) were acquired and a spectral unmixing fit (test on linear dependency for each 3-dimensinal voxel with respect to the 4^{th} spectral dimension) was applied to the complete dataset. As a result, chondrocytes could be discriminated from collagen fibers (s. Fig.7) throughout the complete dataset. Furthermore the validity of the spectral unmixing algorithm and the differences in the emission spectra was successfully tested with a real time staining procedure (propidium iodid and syto 83) only labeling cells. As a control experiment the same unstained and stained region in the sample were compared it could be verified that cells were correctly detected with a spectrally resolved and unmixed native fluorescence dataset. As it becomes apparent from Fig. 7, chondrocytes can only be found on the surface of sponge-like collagens, whereas migration deeper than approx. 30 µm into the membrane could not be detected. This is probably due to limited tube diameters of the membrane. Therefore alteration of the average tube diameter to approx. 100 µm is necessary to ensure a functional 3-dimensional chondrocyte distribution for cartilage repair procedures.

4. CONCLUSION AND OUTLOOK

Native and unlabeled human and bovine cartilage as well as cell populated collagen scaffold membranes for tissue engineering have been successfully examined with 2PLSM and SHG imaging microscopy. Structural information about the 3-dimensional distribution of chondrocytes in/on these strongly scattering biological materials has been quantified by different spectroscopic contrast mechanisms. No additional fluorescent labeling or sample fixation was required, as all samples have be kept in cell culture medium or physiological buffers during examination. Even completely sterile imaging procedures are possible. Photodamage of the samples has been kept to a minimum[10] by using a multi focal 2-photon laser scanning microscope which at the same time ensured sufficient fluorescent yield while keeping acquisition times acceptable (500 ms to 5 s per optical plane, 650 ms to 1 s per spectrally resolve line scans). Chondrocytes in human and bovine cartilage have been detected and it was possible to spectrally separate them from their surrounding ECM via adequate fluorescence emission filters. An automated spot detection then allowed for a calculation of the chondrocyte densities, between $2 \cdot 10^6/cm^3$ and $20 \cdot 10^6/cm^3$, with respect to the different positions in the cartilage sample. Furthermore, characteristics of the ECM (e.g. lacunae) and distinctive features of arthritic and non-arthritic cartilage have been identified. For arthritic cartilage significantly higher 2PLSM imaging depths and lower fluorescent yield are consistent with macroscopical findings of a loss in ECM mass and density. The surface of arthritic cartilage additionally showed a much rougher and more fibrous structure in 2PLSM than the surface of non-arthritic cartilage. These features are again in keeping with macroscopical diagnosis of an increased friction between cartilage surfaces for arthritic patients. In summary, 2PLSM is a valuable tool for a non-invasive, staining free and potentially sterile (therefore suitable for clinical use) 3-dimensional examination of cartilage, that provides information on its arthritic state.
For tissue engineering the same advantages make 2PLSM an attractive method of controlling cell growth on scaffolding collagen membranes. Various spectrally resolved measurements of cell and collagen autofluorescence / SHG allowed for their discrimination using either fluorescence emission filter or complete 3-dimensionally resolved emission spectra. Spectral unmixing of fluorescence emission spectra enabled the classification of fluorescent voxel with different emission characteristics and therefore a separation mechanism for collagens and chondrocytes. The intrinsic 3D sectioning capability of 2PLSM provided valuable information on the design of the pore size of sponge like collagen membranes, which is important for the optimization of the growing conditions for chondrocytes on these membranes and their potential use as implants.

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REFERENCES

1. Cahalan,M.D., Parker,I., Wei,S.H., and Miller,M.J.: "TWO-PHOTON TISSUE IMAGING: SEEING THE IMMUNE SYSTEM IN A FRESH LIGHT", Nature, **2**, 872-880, 2002.

2. Campagnola,P.J. and Loew,L.M.: "Second-harmonic generation imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms", Nature Biotech., **21**, 1356-1360, 2003.

3. Cheong,W.F., Prahl,S.A., and Welch,A.J.: "A review of the optical properties of biological tissues", IEEE J.Quantum Electron., **26**, 2166-2185, 1990.

4. Denk,W. and Svoboda,K.: "Photon Upmanship: Why Multiphoton Imaging Is More than a Gimmick", Neuron, **18**, 351-357, 1997.

5. Denk,W., Strickler ,J.H., and Webb,W.W.: "Two-photon laser scanning fluorescence microscopy", Science, **248**, 73-76, 1990.

6. Fine,S. and Hansen,W.P.: "Optical Second Harmonic Generation in Biological Systems", APPLIED OPTICS, **10**, 2350-2353, 1971.

7. Hunziker,E.B.: "Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects", Osteoarthritis and Cartilage, **10**, 432-463, 2001.

8. Hunziker,E.B., Quinn,T.M., and Häuselmann,H.-J.: "Quantitative structural organization of normal adult human articular cartilage", Osteoarthritis and Cartilage, **10**, 564-572, 2002.

9. James,C.-B. and Uhl,T.L.: "A Review of Articular Cartilage Pathology and the Use of Glucosamine Sulfate", Journal of Athletic Training, **36**, 413-419, 2005.

10. König,K.: "Multiphoton microscopy in life sciences", Journal of Microscopy, **200**, 83-104, 2000.

11. Laasanen,M.S., Töyräs,J., Korhonen,R.K., Rieppo,J., Saarakkala,S., Nieminen,M.T., Hirvonen,J., and Jurvelin,J.S.: "Biomecanical properties of knee articular cartilage", Biorheology, **40**, 133-140, 2003.

12. Martin,I., Wendt,D., and Heberer,M.: "The role of bioreactors in tissue engineering", TRENDS in Biotechnology, **22**, 80-86, 2004.

13. Martinek,V.: "Anatomy and pathophysiology of articular cartilage", Deutsche Zeitschrift für Sportmedizin, **54**, 166-170, 2003.

14. Martini, J., Tönsing, K., Dickob, M., and Anselmetti, D. 2-Photon Laser Scanning Microscopy on Native Human Cartilage. Wilson, T. Proceedings of SPIE 5860(Confocal, Multiphoton, and Nonlinear Microscopic Imaging II), 16-21. 2005.

15. Rasband, W. S. ImageJ. (1997-2005). http://rsb.info.nih.gov/ij/, U. S. National Institutes of Health, Bethesda, Maryland, USA

16. Redman,S.N., Oldfield S.F., and Archer,C.W.: "CURRENT STRATEGIES FOR ARTICULAR CARTILAGE REPAIR", European Cells and Materials, **9**, 23-32, 2005.

17. Williams,R.M., Zipfel,W.R., and Webb,W.W.: "Interpreting Second-Harmonic Generation Images of Collagen I Fibrils", Biophys.J., **88**, 1377-1386, 2005.

18. Zipfel,W.R., Williams,R.M., Christie,R., Nikitin,A.Y., Hyman,B.T., and Webb,W.W.: "Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation", Proc Natl Acad Sci U S A, **100**, 7075-7080, 2003.

19. Zipfel,W.R., Williams,R.M., and Webb,W.W.: "Nonlinear magic: multiphoton microscopy in the biosciences", Nature Biotech., **21**, 1369-1377, 2003.