Quantification of the Temporal Evolution of Collagen Orientation in Mechanically Conditioned Engineered Cardiovascular Tissues

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Abstract—Load-bearing soft tissues predominantly consist of collagen and exhibit anisotropic, non-linear visco-elastic behavior, coupled to the organization of the collagen fibers. Mimicking native mechanical behavior forms a major goal in cardiovascular tissue engineering. Engineered tissues often lack properly organized collagen and consequently do not meet *in vivo* mechanical demands. To improve collagen architecture and mechanical properties, mechanical stimulation of the tissue during *in vitro* tissue growth is crucial. This study describes the evolution of collagen fiber orientation with culture time in engineered tissue constructs in response to mechanical loading. To achieve this, a novel technique for the quantification of collagen fiber orientation is used, based on 3D vital imaging using multiphoton microscopy combined with image analysis. The engineered tissue constructs consisted of cell-seeded biodegradable rectangular scaffolds, which were either constrained or intermittently strained in longitudinal direction. Collagen fiber orientation analyses revealed that mechanical loading induced collagen alignment. The alignment shifted from oblique at the surface of the construct towards parallel to the straining direction in deeper tissue layers. Most importantly, intermittent straining improved and accelerated the alignment of the collagen fibers, as compared to constraining the constructs. Both the method and the results are relevant to create and monitor load-bearing tissues with an organized anisotropic collagen network.

Keywords—Mechanical straining, Collagen orientation, Tissue engineering, Multiphoton microscopy.

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INTRODUCTION

Load-bearing soft tissues have a well structured extracellular matrix, organized to perform the tissues' specialized functions. This extracellular matrix (ECM) is synthesized and organized by the cells under the (guiding) influence of external stimuli, such as tissue loading directions. It is composed of a network of fibrous proteins, predominantly collagen and elastin, embedded in a gel of proteoglycans, glycoproteins, and water. Collagen serves as the main load-bearing component of the matrix, whereas the elastin fibers give the tissue its resilience. Load-bearing tissues in general exhibit anisotropic, non-linear visco-elastic behavior, which is strongly dictated by the extracellular matrix organization, in particular the collagen fiber organization. In native heart valve leaflets, for example, collagen fibers are oriented primarily in the circumferential direction, resulting in stiffer and less extensible tissue in circumferential than in radial direction. Likewise, other cardiovascular tissues, such as blood vessels and pericardium, display similar complex (biaxial) mechanical properties, related to the particular collagen organization within these tissues.

Mimicking the native structural organization and hence biomechanical tissue behavior, forms a major goal in cardiovascular tissue engineering, in particular when creating tissues with high biomechanical demands, such as blood vessels or heart valves for systemic, high pressure applications. In its most successful paradigm, cardiovascular substitutes are engineered *in vitro* by using fast-degrading scaffolds, seeded with autologous myofibroblasts.[20,32] The so-obtained constructs were cultured in bioreactors under conditions that favor the production of *de novo* extracellular matrix components by the cells, including collagen fibers. However, these engineered tissues often lack sufficient amounts of properly organized matrix components and

consequently do not meet *in vivo* mechanical demands. To optimize the collagen architecture and hence improve the mechanical properties of engineered tissues, mechanical conditioning strategies are crucial.[22,27,39] Mol *et al.*, for instance, demonstrated that enhanced (anisotropic) mechanical properties can be obtained in tissue-engineered heart valves by conditioning with dynamic straining.[28] These results may be attributed to an upregulation of remodeling enzymes,[1,38] growth factors,[6] and matrix production,[3,16,33] but also to an improved collagen organization due to mechanical loading. Nevertheless, quantitative relationships between mechanical conditioning and resulting tissue structure and mechanical properties have not been fully established yet, mainly because of a lack of adequate tissue model systems and measurement techniques.

The effect of different mechanical loading conditions on the collagen organization in engineered tissues has been studied using elastic scattering spectroscopy,[25] microscopic elliptical polarimetry,[42] and small light scattering.[15] In simple geometries of cell populated collagen gels, it was demonstrated that uniaxial constraints induced anisotropic collagen orientation,[8,25] whereas biaxial constraining resulted in isotropic orientations.[41] Comparable results were found by Cox *et al.* in engineered cardiovascular tissues based on cell-seeded biodegradable scaffolds.[9,10] In collagen based tissue-engineered heart valves, the application of specific mechanical constraints led to commissural alignment of the collagen fibers.[29] However, the measurement techniques used in these studies only monitor global collagen orientation, and do not provide three-dimensional information throughout the sample. Although the importance of a detailed description of collagen fiber orientation in the mechanics of load-bearing structures is widely recognized, 3D collagen fiber orientations have not yet been quantified and studied in response to mechanical loading in engineered tissues.

In this study a novel method for the quantification of collagen orientation, consisting of a combination of vital multiphoton imaging and mathematical algorithms, is applied to engineered tissues to study the effects of mechanical conditioning on the evolution of collagen orientation in time as a function of tissue depth. Cell-seeded biodegradable rectangular scaffolds were constrained or intermittently strained in longitudinal direction. Intermittent straining regimes were demonstrated to be favorable in terms of cell proliferation,[2,45] matrix production,[7] collagen cross-link density,[4] and mechanical properties,[34] as compared to continuous straining. In addition, intermittent straining was shown to result in stronger tissues in shorter culture periods in engineered heart valve tissue.[35] Apart from this, we hypothesize that the improved mechanical properties of the engineered tissues, when exposed to intermittent loading during culture, is also attributed to an improved and accelerated (anisotropic) collagen organization. Therefore, the present study extends the analyses of these engineered tissues by quantifying the collagen fiber orientation from three-dimensional multiphoton images of the collagen organization after 2, 3, and 4 weeks of culture of both the constrained and intermittently strained tissues.

MATERIALS AND METHODS

Tissue Culture and Mechanical Conditioning

The methods of scaffold preparation, cell culture, seeding, and mechanical conditioning have been described previously.[35] In brief, human saphenous vein myofibroblasts (p7) were seeded using fibrin as a cell carrier onto rectangular strips ($35 \times 5 \times 1$ mm) of non-woven polyglycolic acid scaffolds, coated with a thin layer of poly-4-hydroxybutyrate.[20] In the longitudinal direction, the scaffolds were attached to the flexible membranes of 6-well plates (Flexcell Int., Hillsborough, NC) at the outer 5 mm. After 1 week of culture under these constrained conditions, the engineered tissues were split into two groups (Fig. 1). One group ($n = 6$) served as constrained control, whereas the other group ($n = 6$) was subjected to intermittent uniaxial dynamic straining (4%, 3 h on/off, 1 Hz) using a modified version of a Flexercell FX-4000T straining device[5] (Flexcell Int., Hillsborough, NC). After 2, 3, and 4 weeks of culture the collagen fiber orientation was visualized.

Visualization of Collagen Orientation

To visualize the collagen fiber orientation, samples were labeled by 15 μM Cell Tracker Blue CMAC
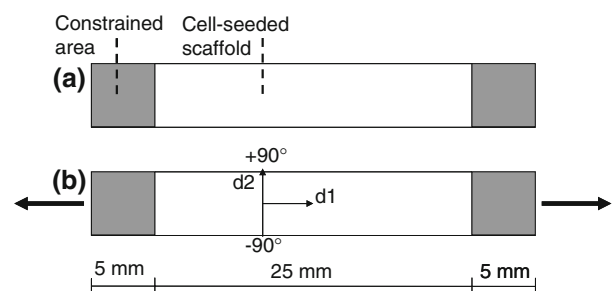

FIGURE 1. Top view of rectangular engineered constructs. The strips were longitudinally constrained at the outer 5 mm at both ends using silicone glue. Samples were either constrained (a) or intermittently loaded (b) in longitudinal direction (d1). Direction d2 represents the direction perpendicular to the long axis. Positive (+90°) and negative (−90°) directions of d2 are indicated.

(CTB; Invitrogen, The Netherlands) and 3 μM CNA35-OG488 as specific vital fluorescent markers for cell cytoplasm and collagen,[26] respectively. CTB and CNA35-OG488 are excitable with multiphoton microscopy and exhibit broad spectra at 466 nm and 520 nm, respectively. An inverted Zeiss Axiovert 200 microscope (Carl Zeiss, Germany) coupled to an LSM 510 Meta (Carl Zeiss, Germany) laser scanning microscope was used to visualize cell and collagen organization. A chameleon ultra 140 fs pulsed Ti-Sapphire laser (Coherent, Santa Clara, CA) was tuned to 760 nm and two photomultiplier tube (PMT) detectors were defined as 435–485 nm for CTB and 500–530 nm for CNA-OG488. Two samples per group were scanned at three different locations: (1) the center point of the tissue, (2) 5 mm left to the center point, and (3) 5 mm right to the center point. At each location a stack of image slices was obtained. The thickness of one image slice was 1 μm and the maximum thickness of the stack was 100 μm. Separate images were obtained from each PMT and combined into single images.

Quantification of Collagen Orientation

The collagen fiber orientations were determined by analyzing the individual images of each image stack as described by Daniels et al.[11] In brief, coherence-enhancing diffusion (CED) was applied for denoising to improve the quality of structures in the image without destroying the boundaries of the fibers.[44] Using CED, smoothing occurs along, but not perpendicular, to the preferred orientation of the structures in the image. Subsequently, the local orientations of all collagen fibers were determined by calculating the principal curvature directions from the eigenvalues and the eigenvectors of the Hessian matrix (second

order structure).[40] As fibers appear at different widths, the second order derivatives were determined at a scale adaptive to the local width of the fiber. The optimal scale was determined with a contextual confidence measure.[31] At each location a stack of (in-plane) orientation histograms was obtained, representing the statistical distribution of local orientations in each image. To calculate the mean fiber angle and the dispersity of the distributions, circular statistics were required due to the periodicity of the fiber distributions.[23,41,48] Circular statistics handle periodic data by representing each angle as a unit vector oriented at that angle. To calculate the mean angle, the individual unit vectors are decomposed into vector components on which statistical operations, such as averaging, are permitted (Fig. 2). Histograms of collagen orientations were obtained from each multiphoton image, and of each the mean vector was calculated, representing a mean angle α and a mean vector length r. The mean vector length represents a measure for the dispersity of the fiber orientations. A vector length of 1 indicates no variation in fiber orientations (i.e., all fibers perfectly aligned) while a vector length of 0 indicates a random distribution of fiber orientations. Courses of mean angles and vector lengths as a function of tissue depth were obtained per image location. To generalize the results, the vector components at each depth were averaged over all samples per loading condition per time point. Subsequently, averaged courses of mean angles and vector lengths were obtained. It should be noted that when the dispersity of the collagen orientation distribution is large, the distribution becomes essentially random. This diminishes the meaning of the value of the mean angle. Hence, for clarity, orientation angles were not shown when mean vector lengths were consistently lower than 0.2.
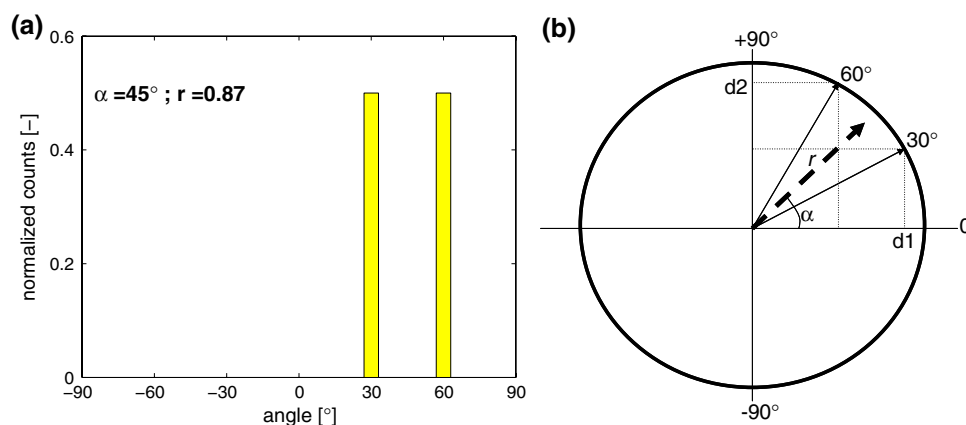

FIGURE 2. Example of circular statistics. A circular distribution is represented in a histogram (a) and depicted as unit vectors with corresponding angles of 30 and 60 degrees in a unit circle (b). The mean vector (dashed line) is calculated by averaging the decomposed sine and cosine vector components of the individual vectors. α represents the mean angle and r the length of the mean vector. A vector length of 1 indicates no variation in fiber orientations, while a vector length of 0 indicates a random orientation of fiber orientations.

Statistics were performed to determine the effect of mechanical loading on the alignment of the collagen fibers, as described previously.[41] In short, alignment in the longitudinal (d1) direction was tested for significance at each imaging depth in each set of samples per time point and loading condition by performing one-sample t-tests against a reference value of 0 on the cosine components of the mean vectors. A p-value lower than 0.05 was considered statistically significant.

RESULTS

Quantification Algorithm

A representative example of the application of the orientation algorithm is shown in Fig. 3. In brief, to each multiphoton image of cells and collagen fibers (Fig. 3a), coherence enhanced diffusion is applied. This enhances the collagen fiber structures (Fig. 3b).

Next, the principle curvature directions are calculated per pixel (Fig. 3c). Subsequently, the orientation analysis program generates a histogram of the orientations (Fig. 3d), from which the mean angle and vector length are calculated using circular statistics.

Spatial Organization of Collagen Fibers

Representative pictures of constrained and intermittently strained samples at 15 and 50 μm depth after 2 weeks of culture are depicted in Fig. 4. Collagen fibers were more randomly distributed in the constrained samples (Figs. 4a and 4b), as compared to the intermittently strained samples (Figs. 4c and 4d), as is depicted in the corresponding histograms. No preferred orientations were found in the constrained samples ($r = 0.13$ at 15 μm and $r = 0.03$ at 50 μm), whereas intermittent loading resulted in fiber distributions with more distinct fiber orientations ($r = 0.42$ at 15 μm and $r = 0.37$ at 50 μm). The courses of the angle and vector length as a function of depth of the
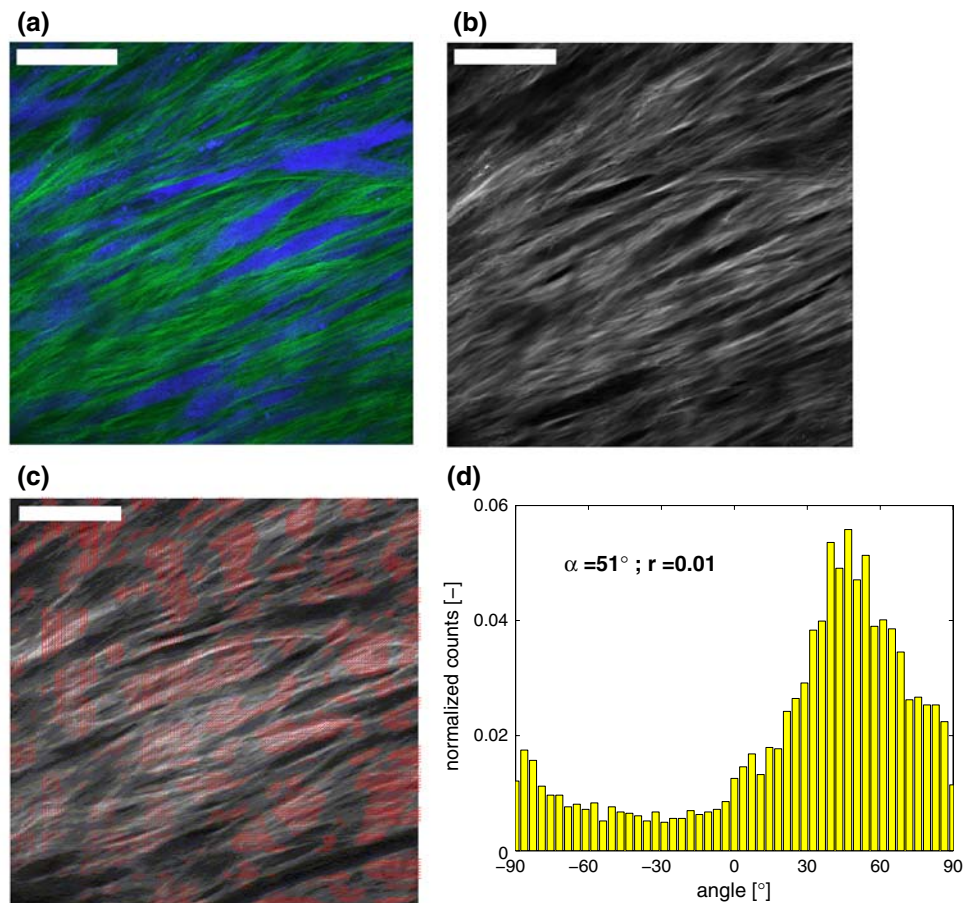

FIGURE 3. Different steps of the orientation analysis. On the multiphoton image (a) coherence enhanced diffusion is applied (b). Using the Hessian matrix the principal curvature directions are calculated (c) which results in a histogram of orientations (d). Scale bar represents 50 μm.
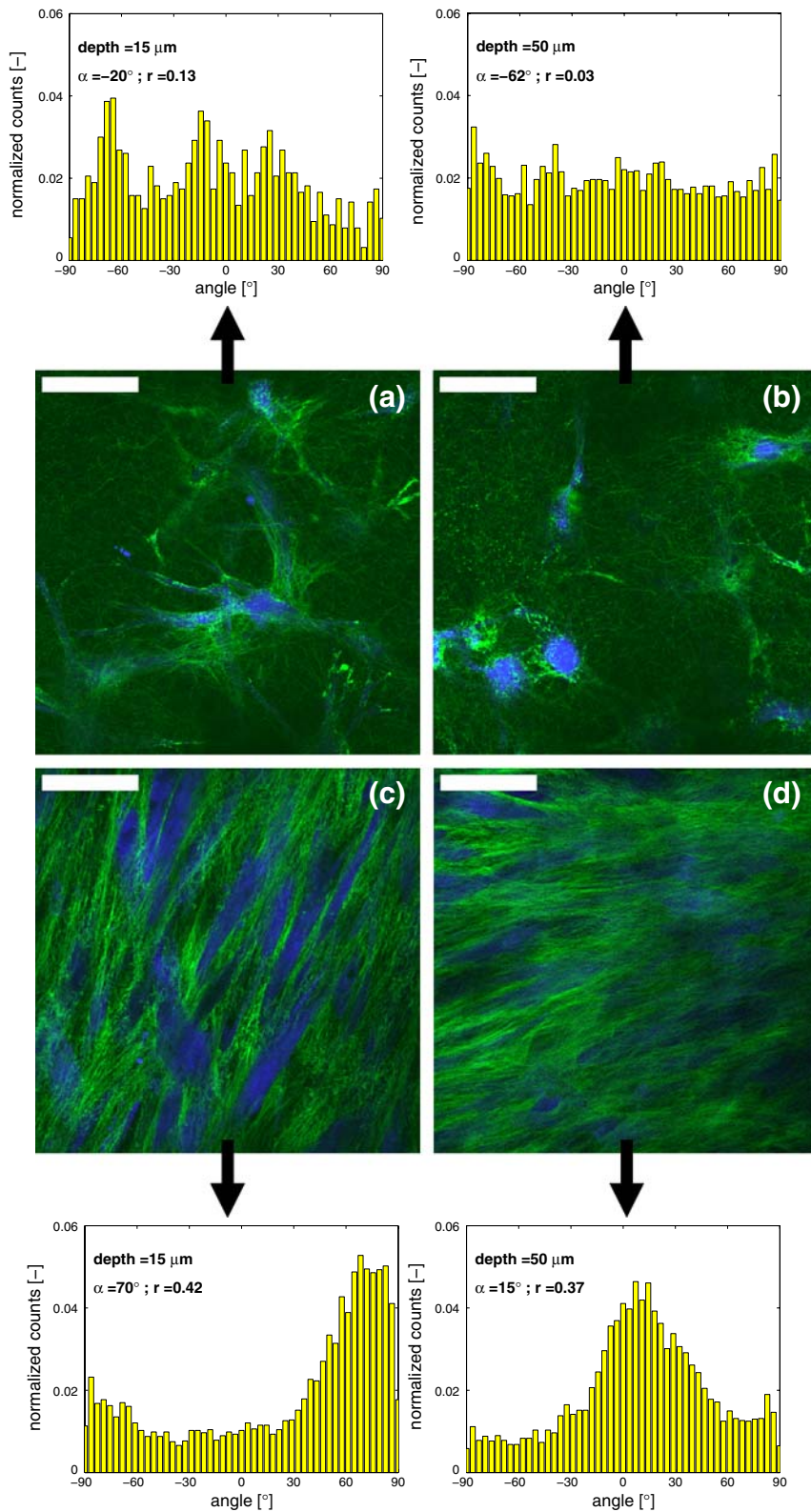
FIGURE 4. Representative multiphoton images of cells (*blue*) and collagen (*green*) organization in constrained (a, b) and inter-mittently strained (c, d) samples after 2 weeks at 15 (a, c) and 50 μm (b, d) imaging depths with the corresponding histograms of collagen orientations at 15 and 50 μm depth. Straining direction was from left to right. Scale bars represent 50 μm.

same samples are shown in Fig. 5. No alignment was observed in the constrained samples (Fig. 5a), except for the first few slices. On the contrary, higher values of the vector length were found throughout the imaging stack of the intermittently strained samples (Fig. 5b), indicating a higher degree of alignment. Interestingly, the orientation of the collagen fibers in the intermittently strained samples shifted from an almost perpendicular orientation at the surface to an orientation into the direction of straining deeper into the tissues.

Temporal Evolution of Collagen Fiber Orientation

Figure 6 shows representative pictures of constrained and intermittently strained samples at an imaging depth of 50 μm after 2, 3, and 4 weeks of culture. Cells and collagen fibers were randomly distributed in the constrained samples after 2 (Fig. 6a) and 3 (Fig. 6b) weeks of culture. After 4 weeks, some alignment of the collagen fibers in the constrained direction was observed (Fig. 6c), comparable to the alignment which was present after 2 weeks in the intermittently strained samples (Fig. 6d). Collagen and cellular alignment in the direction of straining was also observed after 3 (Fig. 6e) and 4 (Fig. 6f) weeks of culture.

Spatio-temporal Evolution of Collagen Fiber Orientation

The quantified collagen fiber orientations were averaged using circular statistics for all imaging depths per loading condition per time point. The courses of the resulting orientation angles and vector lengths (as a measure for dispersity) as a function of the imaging depth are depicted in Fig. 7. Low values for the vector length (<0.2) were found in the constrained samples after 2 and 3 weeks, except for the first few slices, indicating a random orientation of the collagen fibers (Fig. 7a). Interestingly, after 4 weeks, the vector lengths became higher (>0.2) and a change in orientation from 70 degrees at the surface to an orientation along the longitudinal sample direction (d1) in deeper tissue layers was found. Significant alignment ($p < 0.05$) in the d1 direction was found at imaging depths ranging from 35 to 40 μm. Similar courses of orientation angles were found in the intermittently strained samples for all time points (Fig. 7b). Here, the orientation of the collagen fibers shifted from an oblique orientation (70–80 degrees) at the surface to a parallel orientation along d1 at 50 μm deeper in the samples. The lowest and most stable dispersities were found in the intermittently loaded samples after 4 weeks of culture. Significant alignment ($p < 0.05$) in the direction of straining was found at an imaging depth of 30 μm and deeper.

DISCUSSION

Mimicking native biomechanical behavior is a major challenge when tissue engineering cardiovascular substitutes. The anisotropic mechanical behavior of these load-bearing tissues depends on the tissue's architecture and its collagen fiber organization in particular. To optimize collagen organization and alignment, and hence improve the mechanical properties of engineered tissues, *in vitro* mechanical conditioning strategies are crucial. Although the importance of collagen orientation in the determination of anisotropic mechanical properties has been
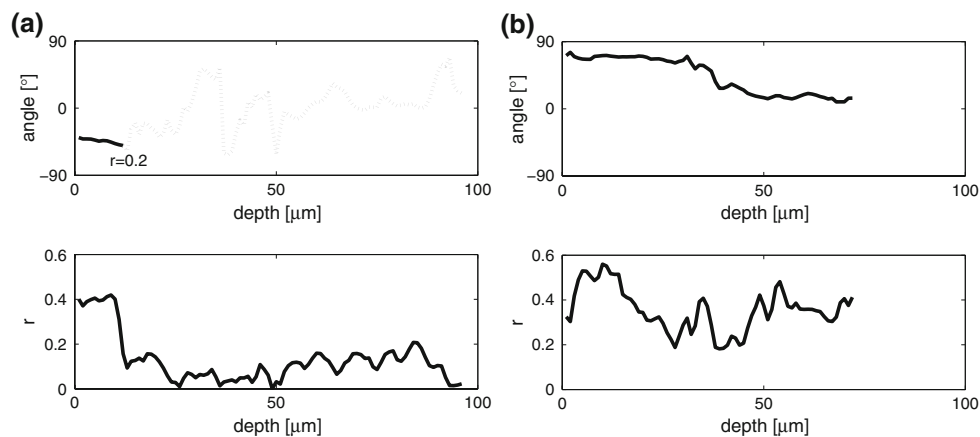

FIGURE 5. Courses of the orientation angle and vector length as a function of the imaging depth for constrained (a) and intermittently strained (b) samples after 2 weeks of culture. These figures correspond to the samples of which 2 images are depicted in Fig. 4. Note that the meaning of the mean angle diminishes for low mean vector lengths. Therefore, when the mean vector length is consistently lower than 0.2, the orientation angle is shown by a dashed line.
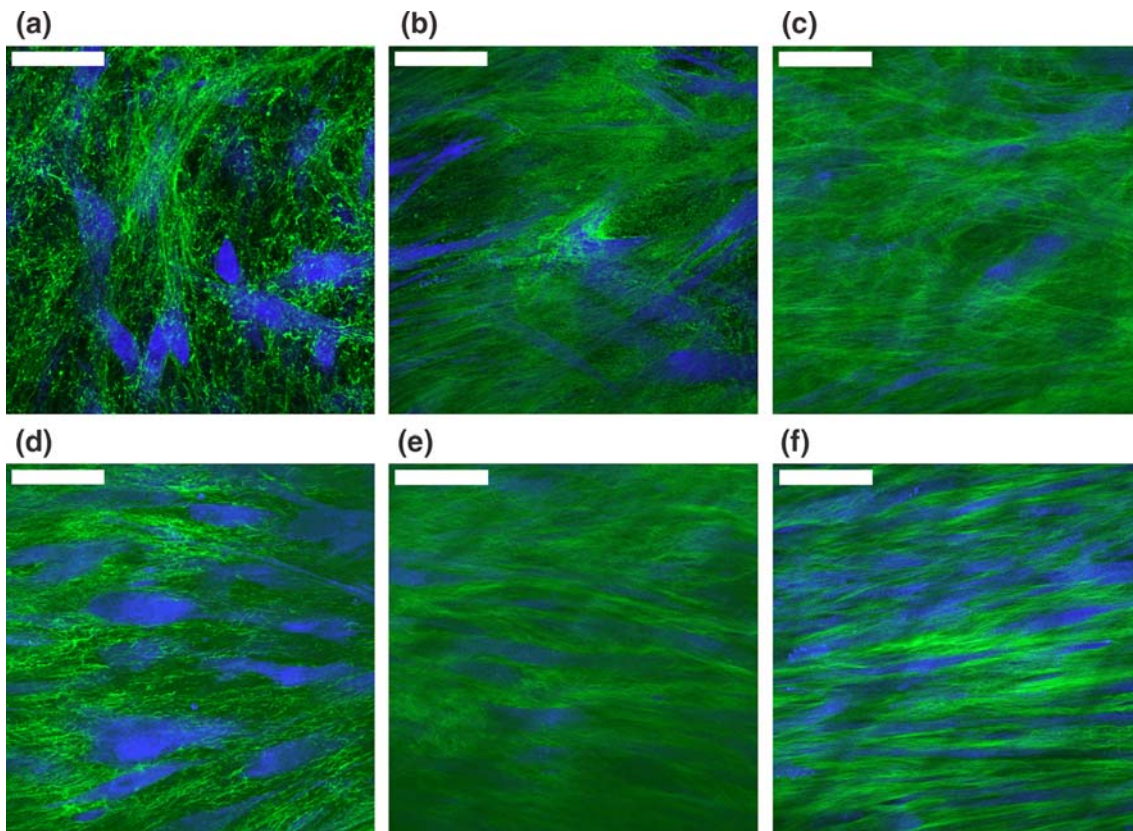
FIGURE 6. Representative multiphoton images of cells (*blue*) and collagen fibers (*green*) in constrained (a–c) and intermittently strained (d–f) samples at a imaging depth of 50 μm after 2 (a, d), 3 (b, e), and 4 (c, f) weeks of culture. Straining direction was from left to right. Alignment of cells and collagen fibers developed in time and was enhanced by intermittent straining. Scale bars represent 50 μm.
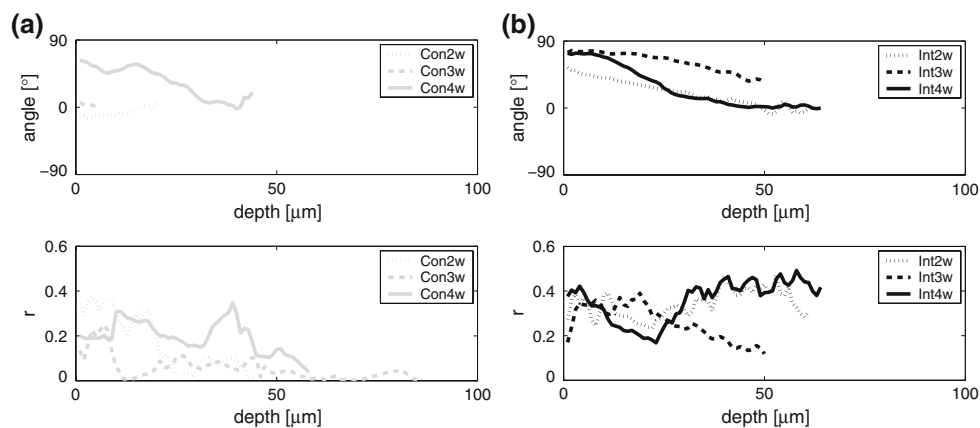

FIGURE 7. Orientation angle (α) and vector lengths (r) as a function of the penetration depth into the tissue for constrained (a) and intermittently loaded (b) samples after 2, 3, and 4 weeks (w) of culture. For clarity, orientation angles at low mean length vectors (<0.2) are not shown. In the constrained samples, alignment of the fibers and a shift in orientation were obtained after 4 weeks of culture. Similar results were found in the intermittently strained samples at all time points.

widely recognized, the effect of mechanical conditioning on the temporal development of 3D collagen fiber orientation has not yet been quantified in engineered tissues. In this study a new method to quantify collagen orientation was used to investigate the temporal

effects of intermittent dynamic straining on the collagen orientation in engineered cardiovascular tissues.

The collagen fibers were stained with a vital collagen specific probe and visualized with multiphoton laser scanning microscopy to obtain detailed images of the

collagen structure as a function of depth within the tissue. Due to the penetration depth of the laser, however, this technique is limited to the top 100 μm of the tissue. Multiphoton microscopy produces large three-dimensional datasets. Due to the size, and the complexity of the collagen network an automated method for the observation and extraction of quantitative orientation information provides a faster, more objective and more accurate way to analyze the data compared to analysis by hand. Wu *et al.*[46,47] previously designed an automated analysis algorithm to extract quantitative structural information of collagen in bovine collagen gels. Collagen fibers were visualized using confocal laser scanning microscopy and the orientation, length, and diameter of individual fibers were quantified. In contrast to those datasets, the multiphoton images generated in the present study contain complex collagen networks, making it unfeasible to extract and analyze single fibers. Therefore, similar to other studies,[14,23,24] the orientation analysis was based on the spatial context of the images using methods from differential geometry, based on an eigen-analysis of the second order Hessian matrix. The principal curvatures, calculated at multiple scales, are used in adaptive denoising of the datasets and turned out to be robust measures for the local fiber shape and orientation.[11,40]

Subsequently, this collagen fiber quantification method was used to study the effect of intermittent dynamic straining on temporal variations in collagen orientation in engineered cardiovascular tissues. It has been demonstrated that factors, such as tissue compaction, contact guidance, and aspect ratio of the scaffold may influence collagen orientation.[8,15,29,36] Since these factors were assumed to be identical in both strained and constrained samples, their interference with the effect of mechanical conditioning is considered to be minimized.

In the constrained samples, a random organization of cells and collagen fibers was observed after 2 and 3 weeks of culture. However, after 4 weeks, constraining led to alignment of the collagen fibers in the constrained direction. This can be explained by the generation of internal strains in this direction due to tissue compaction.[29] Interestingly, in the intermittent dynamically strained samples, collagen alignment was already present after 2 weeks of culture and continued to improve up to 4 weeks. By this time, the lowest and most stable fiber dispersities were seen, indicating a highly aligned collagen structure. Significant alignment ($p < 0.05$) in the straining direction was found at an imaging depth of 30 μm and deeper. Thus, intermittent dynamic loading can be used to accelerate and improve anisotropic collagen organization in engineered tissue, thereby reducing tissue culture times.

It is likely that the improved alignment of collagen will result in improved (anisotropic) mechanical properties. In a parallel experiment with similar culture and conditioning regimes, it was shown that the mechanical properties in the straining direction (d1) of intermittently strained samples were indeed higher as compared to constrained samples.[35] Scaffolds of different geometries would be required to study the effect of uniaxial straining on the mechanical properties in different directions. Furthermore, using square or circular scaffold geometries, the developed quantification method could be used to study the influence of biaxial loading protocols that closely resemble the *in vivo* environment of cardiovascular tissues.

In addition to the assessment of temporal development, collagen orientation was quantified as a function of depth into the tissue. Analyses revealed a shift from a perpendicular or an oblique orientation at the surface to an orientation in the direction of straining deeper into the tissue. The orientation of the cells was observed to change in a similar way. This co-alignment of cells and collagen fibers corresponds to previous findings, where it was demonstrated that collagen orientation was reflected by cell orientation.[17,43] Interestingly, the oblique or perpendicular orientation of the cells in the superficial layer of our tissue-engineered constructs corresponds well to that observed for monolayers of strained cells,[12,19,21,30] where cells 'tend' to 'avoid' strains. We therefore assume that the superficial cells in our 3D tissues behave like a monolayer or cell coverage on top of the construct. Similar to monolayers, relatively low amounts of collagen are present in this superficial layer, which might explain the comparable orientation results. On the contrary, cells entrapped in three-dimensional environments are known to align to the strain direction.[13,18,37] This corresponds to the orientation of cells below the surface layer, where a three-dimensional collagen network collagen is present. Regarding the collagen fibers below the top 100 μm that was analyzed, we hypothesize that the collagen fibers are mainly aligned in the longitudinal direction due to constraining and dynamic straining in this direction, providing the presence of sufficient oxygen and nutrients for cellular survival and collagen synthesis.

In conclusion, a novel method to quantify collagen orientations in tissues was applied to quantify the effect of intermittent straining over time in engineered cardiovascular tissues. Intermittent loading resulted in improved collagen alignment in the direction of straining in shorter culture periods, compared to static, constrained loading. Both the method and the results are important to create and monitor load-bearing tissue-engineered substitutes with an organized anisotropic collagen network.

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