Three-dimensional characterization of collagen remodeling in cell-seeded collagen scaffolds via polarization second harmonic generation

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Abstract: In this study, we use non-linear imaging microscopy to characterize the structural properties of porous collagen-GAG scaffolds (CGS) seeded with human umbilical vein endothelial cells (HUVECs), as well as human mesenchymal stem cells (hMSCs), a co-culture previously reported to form vessel-like structures inside CGS. The evolution of the resulting tissue construct was monitored over 10 days via simultaneous two- and three-photon excited fluorescence microscopy. Time-lapsed 2- and 3-photon excited fluorescence imaging was utilized to monitor the temporal evolution of the vascular-like structures up to 100 µm inside the scaffold up to 10 days post-seeding. 3D polarization-dependent second harmonic generation (PSHG) was utilized to monitor collagen-based scaffold remodeling and determine collagen fibril orientation up to 200 µm inside the scaffold. We demonstrate that polarization-dependent second harmonic generation can provide a novel way to quantify the reorganization of the collagen architecture in CGS simultaneously with key biomechanical interactions between seeded cells and CGS that regulate the formation of vessel-like structures inside 3D tissue constructs. A comparison between samples at different days in vitro revealed that gradually, the scaffolds developed an orthogonal net-like architecture, previously found in real skin.

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1. Introduction

Collagen is the most abundant component of the extracellular matrix (ECM), the acellular three-dimensional network of biopolymers that surrounds cells in tissues, providing structural support, cell stimuli via receptor-mediated binding and organizing functional tissues and organs [1–5]. ECM mechanical and chemical characteristics regulate many major cellular phenotypes including cell differentiation, proliferation, contraction and structural organization, which define key biological processes such as morphogenesis and wound healing. In many tissues, collagen type I constitutes up to 90% of ECM protein content [6]. Collagen chemical and mechanical attributes critically define ECM properties, which in turn, determine the structure and function of
the corresponding organ [7–10]. Therefore, the ability to characterize and control key biochemical and biomechanical properties of collagen is crucial in multiple medical and research applications. Due to the major role of collagen in multiple organs, a variety of collagen-based biomaterials have been fabricated, acting as ECM analogs. Collagen-based scaffolds [11–19] and hydrogels [20–23] are widely used to grow cells for a diverse set of applications, including in-vitro tissue models and grafts that can induce regeneration in injured organs. Much like the situation observed in tissue ECM, the structure, chemical composition and biomechanical properties of collagen-based biomaterials determine their ability to support and regulate the cells of interest in vitro or in vivo [12].

Among the many types of collagen-based biomaterials reported in the literature, this study focuses on porous collagen-GAG scaffolds (CGS). CGS are fabricated by lyophilizing suspensions of microfibrillar collagen I, resulting in a dry highly-porous sponge-like biomaterial [24]. CGS are important biomaterials due to their established clinical applications in induced skin regeneration [15]. Previous research has demonstrated that the regenerative ability of CGS depends strongly on several physicochemical properties (pore structure, mean pore diameter, cross-linking density) [13], which in turn regulate interactions of CGS with seeded cells.

Despite the importance of cell-matrix interactions, they are notoriously hard to quantify, in particular interactions related to their surrounding matrix. Established methods can quantify only the average macroscopic properties of cell-free or cell-seeded biomaterials. Quantifying the state of a cell-seeded biomaterial at the cellular level remains a challenging task. Existing methods include atomic force microscopy, optical trapping, bead-based rheology and 3D imaging of attached particles [25,26].

As briefly described above, when regarding collagen-based biomaterials, the term “characterization” is used for the determination of a number of their properties that define their interaction with cellular cultures. Attributes like fiber orientation and dispersion [8,10,16,19], strain [27], pore size and chemical composition [13] are found to have major influence on the cell properties. This means that control can be acquired over these properties if we are able to characterize scaffolds in detail and fine-tune their attributes accordingly.

Although established macroscopic properties of scaffolds are useful for biomaterial characterization, in this work we utilize state-of-the art imaging to contribute towards better quantification of CGS seeded with live cells at the microscopic level. Several imaging techniques, including confocal microscopy [6,28–31], second harmonic generation (SHG) microscopy [28,32–50] and scanning electron microscopy (SEM) [51] have been utilized for collagen scaffold characterization. In this work, we focus on SHG imaging, as it can image banded collagen samples without staining [52], it does not photobleach, it provides intrinsic z-sectioning and is suitable for deep tissue imaging [33]. Thus, SHG microscopy is considered nowadays as the gold standard for collagen type I characterization.

Collagen SHG originates in the organized structure of collagen fibrils and the non-centrosymmetric nature of collagen molecules. The intensity of collagen SHG emission depends on the relative orientation of fibrils with respect to the polarization of the incident light. Polarization-dependent SHG (PSHG) exploits this dependency in order to provide information on the orientation, the anisotropy parameter of collagen fibrils within the focal volume [33,35–37,39,44,45,52–57], thereby providing novel means to quantify the structure and strain in collagen fibrils found in tissues or biomaterials.

The vast majority of reported PSHG applications quantified a single plane, usually on the sample surface. In studies like Reiser et al. [49] and Golaraei et al. [53], the local out-of-plane orientation of collagen fibrils has been determined by 2D polarization-dependent SHG, providing a 3D representation of collagen fibers of the sample surfaces. Few studies, including Gusachenko et al. [39] and Pendleton et al. [48] carried out 3D PSHG measurements across a thick sample.
To our knowledge, 3D PSHG characterization has not been applied to study CGS or other kinds of collagen-based biomaterials despite their widespread utilization. CGS can emit intense SHG signal, compared to hydrogels, because CGSs are made of microfibrillar collagen I, that is collagen molecules organized in partially-swollen fibrils [13]. The ability to probe the PSHG signature of collagen in CGS offers several unique opportunities to study CGS and cell-CGS interactions at high spatial resolution in 3D. Firstly, PSHG can reveal the 3D nature of collagen fibril organization in CGS struts, a structural information relevant to cell adhesion to CGS and multiple relevant phenotypes (migration, contraction, polarity) [8,10,16,19,55]. Secondly, PSHG can probe the state of a CGS by estimating the SHG fibril anisotropy parameter, a parameter inversely analogous to helical pitch angle [35]. Thirdly, due to the large penetration depth and intrinsic z-sectioning of SHG, PSHG can describe the spatial variation of collagen fibril properties (structure, strain) over a large imaging volume that goes deep inside the scaffold. Finally, by studying cell-seeded CGS at different time points, PSHG can quantify collagen remodeling (collagen synthesis and degradation, alterations in collagen fibril characteristics) inside a CGS in vitro.

This work utilizes simultaneous 3p- and 2p- excited fluorescence imaging and pixel-wise PSHG [58] to characterize collagen structure (fibril orientation) and strain state (fibril anisotropy parameter) alterations during the formation of vessel-like structures by a co-culture of human umbilical vein endothelial cells (HUVECs) and mesenchymal stem cells (MSCs) inside porous collagen-GAG scaffolds (CGS) over 10 days in vitro (DIV). Results demonstrate that PSHG can complement ordinary multiphoton cell imaging and provide novel quantification on cell-induced collagen remodeling inside CGS at high resolution and over a large imaging volume.

Comparison of the distribution of collagen orientation between the control (unseeded) CGS and the cell-seeded CGS, we found that the collagen in cell-seeded scaffold was reorganized by cells and created an orthogonal net-like architecture. This orthogonal net-like collagen structural organization has previously seen in skin [59]. The changes in the 3D remodelled collagen scaffold architecture were dependent on the DIV.

2. Methods

2.1. Scaffold fabrication

Porous collagen-GAG (CG) scaffolds were fabricated as described previously [12]. In summary, microfibrillar collagen I suspension supplemented with 0.44 mg/ml chondroitin-6-sulfate was dissolved in 50 mM acetic acid and homogenized at 4°C to prevent collagen denaturation. The suspension was degassed at room temperature and freeze-dried (−40°C freezing temperature, −35°C) to produce a porous CG scaffold sheet, which was crosslinked by dehydrothermal treatment (105°C, 50 mTorr, 24 h). Cylindrical CG scaffold samples (3 mm diameter, 2.5 mm height) were cut using a biopsy punch and utilized for cell culture and imaging.

2.2. Cell culture

Human endothelial cells isolated from the umbilical cord (HUVECs) were cultured in EGM-2 (Lonza) culture medium composed of basic culture medium (EBM-2, CC-3156) supplemented with EGM-2 SingleQuots (CC-4176). Cell culture plates were coated with type 1 collagen (20 µg/ml collagen I in 0.02M acetic acid 30 minutes at 37°C). hMSCs were supplied by Lonza (Cat. No PT-2501) and were cultured in MSCGM medium (Lonza) composed of the basic medium (MSCBM, PT-3238) supplemented with MSCGM SingleQuots. Culture medium was changed every 3 days and cells were dissociated with 0.05% trypsin solution when they reached 90% confluency. Cells were incubated at 37°C with 5% CO2 and used until passage 5.
2.3. Cell seeding in collagen-GAG scaffolds

Cylindrical CGS samples (3 mm diameter, 2.5 mm height) were placed on a 12-well plate and sterilized by UV exposure for 20 min. A drop of 200,000 HUVECs in 10 µl EGM-2 medium was placed on each CGS side (400k HUVEC per CGS sample total). The seeded CGS sample was incubated for 1 hour at 37°C to let cells adhere to the scaffold before adding 1 ml EGM-2 per well and transfer in a cell incubator. After 72h incubation (3 DIV), the culture medium was removed and 50,000 hMSCs suspended in 10 µl EGM-2 medium were placed on each CGS side. After 1 hour incubation, 1ml EGM-2 was added, followed by a 7-day incubation (10 DIV total). Cell medium was replaced with every 3 days.

2.4. Fluorescent imaging

Calcein AM (Thermo Fisher Scientific C1430) was used for live cell imaging. Samples were stained at 3, 5, 7 and 10 DIV and were observed using 2-photon excitation fluorescence (2PEF). The staining process involves removing the culture medium, PBS wash, incubating the cells in serum free culture medium containing 1µM Calcein AM at 37°C, removing the dye solution, two PBS washes in the dark and finally addition of culture medium.

At different days of vascular structure formation (3, 7, 10 DIV), immunocytochemistry was performed in order to assess the expression of a-SMA. Briefly, cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeablized with 5% Knockout Serum–1% BSA–0.1% Triton X-100 in PBS for 2h at room temperature. Afterwards, cells were incubated with primary mouse anti-a-SMA (1:200) (Sigma A5228) antibody overnight at 4°C, followed by incubation with the secondary Alexa-488-conjugated antibody (1:1000) for 1h at room temperature. Cell cytoskeleton was stained with Phalloidin–iFluor 633 (1:1000 in PBS with 1% BSA) (Abcam ab176758) for 1 hour at room temperature. Nuclei were visualized with Hoechst 33342 (3µg/ml in PBS). Finally, image acquisition was performed using Confocal Microscopy (Leica SP8). In order to analyze the cellular migration inside the scaffold z-stacks were obtained. For 3 days in vitro culture (3 DIV) images were acquired for a depth of 50 µm with a step of 5 µm. For 7, 10 DIV images were acquired for a depth of 80 µm with a step of 5 µm.

Cell-seeded CGS were imaged either via confocal fluorescence microscopy (Leica SP8; IMBB-FORTH) or using a custom built multimodal nonlinear microscope described in the following section.

2.5. Imaging system: setup for PSHG measurements

Cell cultures were recorded via 2- and 3-photon excitation fluorescence (2- and 3-PEF). The scaffold collagen was simultaneously characterized by PSHG microscopy.

Experiments for PSHG and 2- and 3-PEF were conducted in a custom-built inverted laser-scanning non-linear microscope [60], illustrated in Fig. 1. The setup utilized as light source a diode-pumped Yb:KGW fs oscillator at 1028 nm (1.2 W, 70–90 fs, 76 MHz, Pharos-SP, Light Conversion, Vilnius, Lithuania). The direction of the laser linear polarization was automatically controlled by a zero-order half-wave retardation plate (QWPO-1030-10-2, CVI Laser) installed on a motorized rotation stage (M-060.DG, Physik Instrumente, Karlsruhe, Germany). The beam then passed through two silver-coated galvanometric mirrors (6215H, Cambridge Technology, Bedford, MA, USA) and arrived in an inverted Axio Observer Z1 microscope (Carl Zeiss, Jena, Germany) where it was expanded by a pair of achromatic doublet lenses and finally focused in the sample by an objective lens (Plan-Apochromat 20x /0.8NA, Carl Zeiss). The emission in the forward direction was collected by a high-NA condenser lens (achromatic-aplanatic, 1.4NA, Carl Zeiss). In the backwards direction, the emission was collected by the objective lens and separated from the incident laser via a short-pass dichroic mirror (FF700-SDi01, Semrock, Rochester, NY, USA), in a non-descanned geometry. The two galvanometric mirrors were utilized to raster-scan
the position of the laser focus on the sample along the x and y axes in order to acquire images along a specific focal plane.

**Fig. 1.** Schematic representation of the imaging system utilized. A $\lambda/2$ retarder plate controls the polarization of the incident laser beam. The beam is raster-scanned on the sample by a set of galvanometric mirrors and focused by an objective lens. The SHG signal is collected in the forward direction by a condenser lens. The emitted fluorescence signals of Hoechst and CalceinAM dyes are separated by a 509HP filter and collected in the backwards direction. The band pass filters utilized for detecting SHG, Hoechst and Calcein signals are 514/3 (green), 485/64 (blue) and 536/40 (red) respectively.

In order to carry out PSHG measurements, after the recording of a single image, the rotational stage of the half-wave plate is rotated by a step of 1°, a new image is recorded and the process is repeated until a 90° rotation is completed, i.e. a 180° rotation of the excitation linear polarization direction in steps of 2°. For our deep 3D P-SHG measurements, we first focus the beam on the surface of the scaffolds and after the 180° rotation is completed, the half-wave plate is reset on its initial orientation and we move the objective lens of the microscope in the z direction by a step of 2 μm, thus focusing the spot inside the scaffold. We then repeat the PSHG process and keep increasing the z value of the objective until a sufficiently deep volume of the scaffold is measured. If required, after the z-scan is finished, we can automatically move the sample stage in
steps in the x and y directions and repeat the above procedure. This way we are able to measure arbitrarily large scaffold volumes.

2.6. Theory: collagen PSHG characteristic curves

In this work, we assume a well-established biophysical model, Eq. (1) \[33,34,36,45\], according to which the intensity of the emitted SHG signal, $I_{\text{SHG}}$, depends on the angle between the polarization of the incident light and the local fibril orientation. Collagen fibrils are modeled as cylindrical objects and fibril orientation is defined by the cylinder’s long axis, which is assumed to concur with the alignment of the supramolecular assembly \[33\]. Additionally, Kleinmman symmetry is assumed, resulting in SHG signal being described by the second order non-linear susceptibility tensor $\chi^{(2)}$, which under these symmetries has just two independent non-zero elements: $\chi_{zzz} = \chi_{xzx} = \chi_{yxz} = \chi_{xzy} = \chi_{zxy}$, where the $z$ coincides with the principal axis of the fibril cylinder. Moreover, we set the microscope coordinate system $(X, Y, Z)$ so that the incident light propagates along the $Z$ axis and its linear polarization lies on the $X - Y$ plane, at an angle $\theta_p$ relative to the $Y$ axis. Since all angles are measured relative to the lab coordinate system, it is the relative angle $(\theta_p - \theta_f)$, $\theta_f$ being the local fibril orientation angle, that modulates the SHG signal intensity.

$$I_{\text{SHG}}(\theta_p) = E\left(\sin^2(2(\theta_p - \theta_f)) + A \sin^2(\theta_p - \theta_f) + B \cos^2(\theta_p - \theta_f) \right)$$  \hspace{1cm} (1)

where $E$ is a multiplication factor depending on the excitation intensity, the tilt angle $\delta$ of the fibril cylinder relative to the $X - Y$ plane and the optical collection and signal detection characteristics, $A$ is the check parameter and $B$ the anisotropy parameter of collagen, given by

$$A = \frac{\chi_{zxy}}{\chi_{zzy}}$$ \hspace{1cm} (2)$$
$$B = \frac{\chi_{zzz} \sin^2(\delta) + (2 + A) \cos^2(\delta)}{\chi_{zzy}}$$ \hspace{1cm} (3)

In this model, information about the polarization dependence of the collagen SHG intensity lies in the four parameters $A$, $B$, $E$, and $\theta_f$. Here we specifically focus on the determination of the local fibril orientation, $\theta_f$ and the anisotropy parameter $B$, across the entire imaged volume of a CGS.

Instead of directly fitting our data to Eq. (1), which is time consuming, we follow the method of Fast Fourier Polarization SHG (FF-PSHG) analysis \[33,56\]. Using the trigonometric identities for $\cos(2\varphi)$, Eq. (1) can be expressed as a sum of cosines with different “frequency” components as

$$I_{\text{SHG}}(\theta_p) = E[a_0 + a_1 \cos(2(\theta_p - \theta_f)) + a_2 \cos(4(\theta_p - \theta_f))]$$ \hspace{1cm} (4)

with

$$a_0 = \frac{3}{8}(A^2 + B^2) + \frac{1}{4}AB + \frac{1}{2}$$ \hspace{1cm} (5)$$
$$a_1 = \frac{1}{2}(B^2 - A^2)$$ \hspace{1cm} (6)$$
$$a_2 = \frac{1}{8}(A - B)^2 - \frac{1}{2}$$ \hspace{1cm} (7)

where, the parameters $a_0$, $a_1$ and $a_2$, now provide the same information as $A$, $B$ and $C$.

In contrast to other studies that used FFT image processing to retrieve the angle of collagen fibers present in one image, we used a biophysical SHG model and performed FFT in the polarization axis of PSHG data \[33,56\] to rapidly extract the distribution of collagen fiber orientation and anisotropy parameter in z-stacks, where each z-plane is comprised of 45 images ($180^\circ$ rotation of polarization orientation in steps of $4^\circ$).
2.7. **PSHG data analysis: extraction of collagen biophysical information**

In order to draw out the important biophysical information regarding the collagen scaffold, we use a custom MatLab script. As mentioned above, a one-dimensional Fourier Transform (1D-FT) is applied to Eq. (4) with respect to $\theta_p$, which yields

$$\hat{I}_{SHG}(\Omega) = a_0 \delta(\Omega) + a_1 \exp(i2\theta_f) \delta(1 - \Omega) + a_2 \exp(i4\theta_f) \delta(2 - \Omega) + c.c. \quad (8)$$

where, $\delta(\alpha)$ is the Dirac function, FT of the cosine function and c.c. stands for complex conjugate. At any pixel, local fibril orientation is calculated as half the argument of the 1st exponent, for $\Omega = 1$, i.e.

$$\theta_f^{exp} = \frac{\arg(a_2 \exp[i2(\theta_p - \theta_f)])}{2} \quad (9)$$

Determination of $\theta_f$ via the phase of polarization spectral components drastically decreases calculation time and provides more robust estimates compared to direct fitting data to Eq. (1) [61], as the result does not depend on parameters $a_0$, $a_1$ and $a_2$. Subsequently, parameters $A$ and $B$ are calculated [56] by combining Eqs. (1) and (4) as

$$A^2 = a_0 - a_1 + a_2 \quad (10)$$

and

$$B^2 = a_0 + a_1 + a_2 \quad (11)$$

Furthermore, by conveniently setting $A = 1$ (Kleinmann symmetry holds) leads to $B$ normalization as

$$B = \sqrt{\frac{a_0 + a_1 + a_2}{a_0 - a_1 + a_2}} \quad (12)$$

The effect of birefringence polarization cross-talk and diattenuation in the anisotropy parameter $B$ has been studied earlier in tendon collagen type-I [62] (collagen type-I is the main constituent of our collagen scaffolds under study) and has been found that could introduce small changes in $B$. Nevertheless, the main goal of our study is the monitor of the remodeling of the collagen in cell-seeded scaffolds and not the calculation of the absolute value of $B$ parameter. Indeed, our findings suggest that in cell-seeded scaffold the collagen is reorganized in an orthogonal net-like architecture, also seen in skin collagen [59].

3. **Results**

We first present our results from the simultaneous deep SHG and 2- and 3-PEF microscopy, where we observed the formation of tissue attached to the collagen fibers of the scaffold up to 100 µm, for increasing days in vitro (DIV) up to 10. In the second part, we proceed with the 3D PSHG characterization of the scaffolds, and we investigate if and how the collagen properties differ with depth. Finally, we discuss the evolution of the scaffold-cells interaction, observing how the structural properties of the scaffold change with DIV.

3.1. **Formation of live tissue in collagen-based scaffolds**

Three identical scaffolds were imaged upon 3, 7 and 10 DIV, together with a control scaffold without cells. We used a triple photomultiplier tube (PMT) based detectors setup, to measure simultaneously the signals coming from 2- and 3-PEF (backwards direction detection from a-SMA staining in the cytoskeleton and Hoechst in the nucleus, respectively) and SHG (forwards direction from collagen), as described in methods. Each time we started by imaging a $300 \times 300 \mu m^2$ plane and in total we went 100 µm deep inside the sample, moving the objective in the $z$-direction in steps of 2 µm, so the total volumes studied were $300 \times 300 \times 100 \mu m^3$. 
3.1.1. Validation of vessel-like structure formation

“Vessel-like” cell structures inside CGS were formed by co-culture of HUVECs and MSCs following previously published protocols [57,58]. Briefly, 400k human endothelia cells (HUVECs) were seeded per cylindrical CGS sample on day 0 followed by 50k MSCs three days later. Cell-seeded CGS samples were incubated on EGM2 medium for ten days. The formation of vessel-like structures with visible lumens inside CGS was visualized by immunostaining for α smooth muscle actin (αSMA), a marker of pericytes and phalloidin-Alexa633 (counterstains the actin-rich cytoskeleton of HUVECs and MSCs). Figure 2 shows representative fluorescence confocal images of vessel-like cell structures at 3, 7 and 10 DIV respectively. At 3 DIV, HUVECs have already formed vascular-like structures with visible lumens throughout the scaffold (Fig. 2(a)). The delayed addition of hMSCs promoted the stabilization of this network, as observed by imaging at 7 DIV, that revealed a uniform vessel-like network with well-established lumen structures (Fig. 2(b)) which remained stable for at least 10 DIV (Fig. 2(c)). As observed with both confocal and multiphoton imaging, HUVECs stained positive for α-SMA, a result also previously observed [50,58], thus pericytes generation was not observed.

3.1.2. Visualizing cell-CGS interaction via multimodal nonlinear imaging

CGS seeded with HUVECs and MSCs were fixed at 3, 7 and 10 DIV, immunostained for αSMA and phalloidin, and imaged using a custom nonlinear microscope (methods) equipped with three photomultiplier tube (PMT) detectors. The setup enabled simultaneous imaging of fluorescence signals arising from 2p- and 3p- excitation (αSMA staining, Hoechst nucleic acid counterstains) in the backward (epi) direction and SHG (from collagen) in the forward direction. For each sample, a $300 \times 300 \times 100 \, \mu m^3$ volume was acquired at 2 µm plane sampling.

![Fig. 2. Formation of vascular-like networks by HUVECs and hMSCs inside CGS. (a) vascular-like network formed by HUVECs at 3 DIV shows visible lumens. (b) Stable vascular-like structures at 7 DIV and (c) at 10 DIV indicate the supportive role of hMSCs to pre-formed HUVECs. Red: Phalloidin (cytoskeleton), green: α-SMA (pericytes) and blue: Hoechst (cell nuclei). Scale bars: 100 µm.](image)

Images of cell-seeded CGS samples at 3, 7 and 10 DIV reveal the time evolution of both the cellular component (HUVECs, MSCs) and the collagensous biomaterial component of the 3D cell construct, see Fig. 3(a-d). Cells gradually formed a relatively uniform distribution inside the pores of the scaffold (Fig. 3(b-d)) by attaching to collagen fibers. Examination of acquired SHG images over the period of 10 days show a significant alteration of collagen structure in the scaffold (Fig. 3(e-h)). Initially, the scaffold was composed almost entirely of approximately 3.2 µm thick collagen fibers (struts) that define clear and relatively big pores (the average pore diameter of cell-free scaffolds is approximately 95 µm). Collagen fiber morphology was altered already at 7 DIV (Fig. 3(c)), while at 10 DIV collagen signal was even...
Fig. 3. Evolution of cell-seeded CGS as visualized by multimodal nonlinear microscopy over a period of 10 days. Left: Non-linear imaging of CGS seeded with HUVECs and MSCs. (a) Cell-free (control) scaffold. (b, c, d) Cell-seeded CGS at 3 (before adding MSCs), 7 and 10 DIV. Red: a-SMA immunolabeling (cytoskeleton), green: SHG (collagen), blue: Hoechst (cell nuclei). Right: Summation of SHG images, over all polarizations and all z-planes, demonstrate alterations in CGS collagen architecture at increasing DIV (e-h).

more dispersed and pores were less clear (Fig. (3(d)). This observation is further supported by the maximum projections of the SHG channel over whole 100 µm imaging volume, Fig. 3(e-h). These observations suggest that the cell-seeded CGS is a dynamic system, where initially cells follow the scaffold structure to start forming vessel-like structures. In doing so, they interact with the scaffold, altering its morphology, which in turn drives cell structure formation.

3.1.3. Evolution of cell migration inside the scaffold

Having acquired images up to 100 µm deep inside the CGS samples at 2 µm steps, we reconstructed 3D volume representations of acquired images using the 3D Viewer plugin of ImageJ, as shown in Fig. 4(a). Since we were interested in investigating cell penetration into the scaffold, we present these cross sections for 3, 5, 7 and 10 DIV (Fig. 4(b-e)), where we show live cells, stained with Calcein (blue), from a single sample, studied at different DIV. As previously, green is used for the SHG signal.

Figure 4(f) presents histograms of the area fraction occupied by cells for each DIV, while in Fig. 4(g) we plotted their respective z-axis center of mass (in µm), which represents the center of the culture, used as a means to quantify how deep the center of the culture is located. In Fig. 4(f), we observe the gradual cell penetration deeper into the scaffold, from about 50 µm deep at 3 DIV to at least 100 µm deep at 10 DIV.

Additionally, we performed diameter analysis for all layers in the z-stacks of all samples. In Fig. 4(h) we plotted collagen fiber diameter, averaged across the entire volume imaged, as a function of DIV. CGS fiber diameter were measured using the DiameterJ (version 1–017) [63] plugin of ImageJ (version 1.48v). Fibers in control cell-free CGS samples have diameter 3.2 ± 0.4 µm, while in the cell- seeded CGSs fiber diameter shows a drop to 2.6 ± 0.4 µm, 2.5 ± 0.4 µm, 2.7 ± 0.4 µm and 2.5 ± 0.4 µm at 3, 5, 7 and 10 DIV, respectively. We compared the average diameters for all z-planes of all samples, by performing a t-test between all the diameters of samples at 3, 5, 7 and 10 DIV and the diameters of the control sample, at the same depth. We found significant statistical differences between all seed samples and the control (p<0.05,
Fig. 4. Migration of HUVEC and MSCs inside CGS samples over 10 days. $300 \times 300 \times 100$ $\mu m^3$ volumes were imaged in $2 \mu m$ z-steps using a 3-PMT setup. The resulting channels were visualized using the 3D Viewer ImageJ plugin. Top: 3D volume reconstruction of CGS hosting cells at 7 DIV (a). Bottom: Maximum intensity projections of 3D images on the y-z plane at 3 (b), 5 (c), 7 (d) and 10 (e) DIV. Blue: CalceinAM (live cell tracker). Green: SHG (Scaffold collagen). The surface of the CGS is located at the bottom of each picture. The vertical axis of each image is parallel to the sample’s z-axis. Distributions of imaged cells as a percentage of the total imaged area for each DIV (f) and their respective z-axis center of mass (g) reveal the progress of cellular penetration per DIV. At 10 DIV, cells were present at least $100 \mu m$ deep inside CGS. Average collagen fiber diameters plotted against DIV (h) show a decrease of the diameter in the cell-seeded scaffolds.
p5DIV < 0.05, p7DIV < 0.05 and p10DIV < 0.05, for 3, 5, 7 and 10 DIV, respectively). This clearly indicates that the fiber diameters have decreased in the seeded samples at all DIV. Please, refer to the chapter “S.3 Volume statistics” in the supplementary material for a more detailed analysis of these 3D volume measurements.

3.2. 3D characterization of CGS collagen by 3D PSHG

PSHG images of unfixed cell-seeded CGS were collected for 91 different incident (0–180° at 2° step) linear polarization directions at 12 z planes (5 µm steps, up to 55 µm deep inside the sample). In cell-free control CGS samples, PSHG measurements were conducted up to 200 µm deep. PSHG measurements were carried out in the forward direction, using a silver mirror (to avoid the effect of polarization scrambling by dichroic mirrors), a high-numerical aperture condenser for efficient SHG collection and a single PMT (Supplement 1 S.1).

During our experiments we noted that, the majority of SHG signal from dry CGS were detected in the backward direction, while the majority of SHG signal from hydrated CGS were detected in the forward direction, providing completely different images in the backwards and forwards detection geometries (see Supplement 1 S.2). This behavior of SHG emitted by CGS is completely different from the SHG emission of tissue collagen fibrils, where the forward/backwards signal provides quantification of the collagen fibril diameter, as seen in [64]. Thus, the fact that the forward and backwards images where completely different in hydrated (and dry) CGS did not allow us e.g. the determination of the collagen fiber diameter by measuring the forwards/backwards signals like in [64].

Figure 5(a-d) show representative PSHG measurements acquired in a random location inside a cell-free control scaffold at four different incident light polarization orientations (polarization direction is indicated by white arrows). Data demonstrate that measured SHG intensity depended on the relative orientation of incident laser polarization with respect to the collagen fiber. Figure 5(e) shows in detail the modulation of the measured SHG intensity due to the change in the polarization orientation for two regions of interest (ROIs). Figure 5(f) shows the corresponding polar graph, where the measured SHG intensity of both ROIs is normalized between 0 (minimum SHG intensity) and 1 (maximum SHG intensity). In agreement with theory, SHG is maximized when incident laser polarization lies parallel to the collagen fiber axis [50,61].

3.2.1. Fibrillar orientation and anisotropy parameter by 3D PSHG

In order to get further insight on collagen remodeling during the 10-day culture of HUVECs and MSCs inside CGS samples, we utilized Fourier Transform analysis (methods) to calculate pixel-by-pixel the angle $\theta^{exp}_f$ and the anisotropy parameter $B_\theta$ for each xy-plane in our 3D PSHG measurements. Figure 6(a-c,d, f) presents representative results for a single plane of a cell-free control CGS sample. Figure 6(a) shows the average SHG intensity across all polarization orientations. Figure 6(b) and 6(c) show the calculated pixel-wise values of collagen fibril orientation $\theta^{exp}_f$ (in degrees) and anisotropy parameter $B_\theta^{exp}$ estimates shown in Fig. 6(b) show very good agreement with the macroscopic direction of collagen fibers. Figure 6(e) present the histogram of $\theta^{exp}_f$ distribution corresponding to Fig. 6(b). Results show that in the particular imaging location shown in Fig. 6(a-c) collagen fibers were not completely randomly aligned as there is a single peak of $\theta^{exp}_f$ distribution. Figure 6(f) presents the histogram of $\gamma$ distribution corresponding to Fig. 6(c). Results show that in cell-free scaffolds the estimated anisotropy parameter distribution lies within 1.2 and 2 and has a single peak around $\gamma = 1.6$. These values agree within previously published PSHG estimates of collagen structures type I, where 1 ≤ $\gamma$ ≤ 2 [57] while, Fig. 6(d) and 6(f) show the same histograms concerning the control sample.
3.2.2. Evolution of scaffold characteristics by comparison of the sample on different DIV

Having performed the same 3D PSHG characterization on scaffolds at different DIV, we observed how scaffold properties change as cells grow and interact with them. On these samples we went 55 µm deep in 5 µm steps. On the top-right of Fig. 6 we observe from the representative distributions of the fibril orientations that, although at the control sample (Fig. 6(d)) the fibrils are relatively aligned (note that 90° and −90° refer to the same fibril orientation), as the DIV increase the cells reorganize the collagen, leading to a structure composed of fibers crossing at an almost right angle (90°) at 7 DIV (Fig. 6(e)).

Furthermore, comparing the anisotropy parameter histograms of the control sample (Fig. 6(f)) and the sample at 7 DIV (Fig. 6(g)) we observe a shift towards smaller anisotropy values. In Fig. 6(h) we have plotted the average anisotropy parameter across the entire volumes of the scaffolds that we measured, as a function of the DIV. The error bars represent standard error of the mean anisotropy across each sample. In accordance with our previous observation, we detect a decrease of the anisotropy parameter from a value of 1.65 ± 0.01 for the control scaffold, to 1.41 ± 0.01 for the sample at 10 DIV. This change in anisotropy compliments our previous observation, where we suggested that as the cells grow, they interact with the scaffold, altering its structural characteristics.

Interestingly, we observe that while initially CGS collagen fiber orientation is distributed around a major orientation, as can be seen in Fig. 6(e), the orientation of collagen fibers of cell-seeded CGS at 7 DIV are distributed around two major orientations, with a 90° relative angle difference between them. This orthogonal net-like collagen structure has previously been reported in real skin [59].

In order to achieve 3D characterization of CGS, we performed PSHG measurements in multiple z-stacks and analyzed the data by Fast Fourier Transform. For each layer we obtained a distribution of anisotropy parameters. Figure 7 shows PSHG analysis results for a control ce-free CGS (blue), and a cell-seeded CGS at 10 DIV (orange), as a function of depth inside the scaffold, were we observe that the anisotropy of each sample remains constant.
Fig. 6. Integrated pixel-by-pixel intensity over all incident light polarization orientations (a). Two pieces of information retrieved by PSHG are shown in color scale, local fibril orientation in (b) and anisotropy parameter in (c). Histograms of local fibril orientation and anisotropy parameter. We observe that the initial alignment of collagen fibrils of the control sample (d) is altered towards a relative angle about 90° at 7 DIV (e), while the mean anisotropy parameter drops from 1.65 ± 0.01 for the control sample (f) to 1.41 ± 0.01 at 10 DIV (g). Average anisotropy plotted against DIV (h). Error bars represent the standard error of the mean anisotropy for each DIV. A continuous decrease in anisotropy is observed.

We then compared the average anisotropy parameter values for all z-planes of all seeded samples with the respective anisotropy values of the control sample, by performing a t-test between the average z-plane anisotropy of all seeded samples and the control, at the same depth, for each z-plane up to 50 µm. We found significant statistical differences between all seeded samples and the control (p<0.05, p<0.05, p<0.05 and p<0.05, for 3, 5, 7 and 10 DIV, respectively). This clearly indicates that the B-parameter decreases in the seeded samples at all DIV.
4. Discussion and conclusions

This study utilizes a custom multimodal nonlinear microscope to perform simultaneous deep 3D PSHG and multiphoton imaging of cell-matrix interactions during the formation and stabilization of vascular-like networks inside CG scaffolds. We utilized a previously-published protocol for generating vascular-like networks by HUVECs supported by hMSCs [65]. Multiphoton microscopy visualization of vascular-like networks replicated previous findings about the positive effects of MSCs on network stabilization. Indeed, 10 days after initial HUVEC seeding, HUVECs and hMSCs infiltrated and formed vascular-like networks consisting of lumen structures at least 100 µm deep from the CGS surface.

SHG and PSHG microscopy revealed that over the 10-day period when vascular-like networks remained stable there was significant matrix remodeling throughout the cell-seeded CGS. Our study exploited the unique ability of CGS to emit SHG since collagen molecules in CGS are organized in partially-swollen fibrils (microfibrillar collagen) [13]. In contrast, SHG cannot equally well visualize collagen hydrogels, the most widely-utilized type of collagen biomaterials, since randomly-oriented tropocollagen molecules cannot emit SHG. We found that hydrated CGS provide robust SHG in the forward direction, which can be utilized to efficiently and reliably perform PSHG calculations (Fig. 5). SHG imaging revealed that over a period of 10 days there were significant alterations in the structure of CGS collagen struts and fibers, which became thinner and more randomly oriented in space (Fig. 3).

In order to get further insight on collagen remodeling during vascular-like network formation, we utilized time-lapsed 3D PSHG at 0, 3, 7 and 10 days after the initial HUVEC seeding. The acquired PSHG signatures enabled the reliable estimation of collagen fiber direction and collagen SHG anisotropy parameter via an efficient FFT-based method in a pixel-wise manner over large imaging volumes. Our findings demonstrate significant alterations in the SHG anisotropy parameter B, which progressively decreases from 1.65 ± 0.01 (cell-free CGS) to 1.41 ± 0.01 (cell-seeded CGS, 10 DIV). Measured values of collagen SHG anisotropy parameter obtained from CGS samples agree with previous PSHG measurements (1.4 to 2) obtained from different kinds of collagen samples [50,61,62]. As seen in [50], the anisotropy parameter B has smaller values roughly between 1.4 and 1.6 for fiber diameters greater than 2 µm, while collagen fibers below 0.5 µm they exhibit higher values, around 2. Nevertheless, because images of CGS detected
in the forward direction were different from the image detected in the backward direction (for both dry and hydrated CGS), we were not able to perform such forward/backwards measurements. However, using simple image analysis we measured the diameter of collagen fibers in CGS, all of which were larger than 2 μm. The measured microscopic SHG anisotropy parameter of collagen is analogous to the individual collagen molecules. Therefore, the observed decrease in anisotropy parameter (Fig. 6) corresponds to an increase in collagen helical pitch, which suggests that collagen molecules in cell-seeded CGS are longer compared to collagen molecules in cell-free CGS. The corresponding increased length in CGS collagen estimated by PSHG agrees with previous observations of significant macroscopic contraction of cell-seeded CGS [15]. However, while previous studies on cell-mediated contraction of CGS relied on macroscopic measurements (CGS dimensions, macroscopic resulting force) or utilized low-throughput methods [26], here, for the first time, we utilize PSHG to monitor the microscopic state in cell-seeded CGS at μm resolution, deep inside CGS. Furthermore, our data suggest clear structural cell-mediated remodeling of collagen fibers in CGS scaffolds since at increased DIV, collagen fibers acquired an orthogonal net-like architecture, like the one reported for collagen in skin [59].

In summary, we demonstrate that PSHG can be utilized to study the kinetics of cell-induced collagen remodeling in cell-seeded CGS at unprecedented temporal and spatial sampling and over large acquisition volumes. We propose that PSHG microscopy of in vitro tissue CGS models provide novel means to study cell effects on their surrounding collagen microenvironment. Such elementary cell-matrix interactions are involved in important biological phenomena including blood vessel formation and stabilization, wound healing and scar formation, and tumor initiation, fibrosis, progression and metastasis [7,18]. Finally, PSHG microscopy of in vitro tissue CGS models provide novel tools for detailed biomechanical studies at the cellular and subcellular level, paving new directions towards the quantifying the dynamic nature of cell-biomaterial interactions.

Funding. European Commission (820677, Project IQONIC (Grant Agreement 820677)).

Acknowledgements. The research work was supported by the Hellenic Foundation for Research and Innovation (HFRI) under the HFRI PhD Fellowship grant (Fellowship Number: 621).

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

See Supplement 1 for supporting content.

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