Image-based characterization of 3D collagen networks and the effect of embedded cells

Vanesa Olivares\textsuperscript{1,2}, Mar Cóndor\textsuperscript{1,2,3}, Cristina Del Amo\textsuperscript{1,2}, Jesús Asín\textsuperscript{4}, Carlos Borau\textsuperscript{1,2,4}, José Manuel García-Aznar\textsuperscript{1,2}

\textsuperscript{1}Multiscale in Mechanical and Biological Engineering (Department of Mechanical Engineering), University of Zaragoza, Zaragoza, Spain. 
\textsuperscript{2}Aragon Institute of Engineering Research, University of Zaragoza, Zaragoza, Spain. 
\textsuperscript{3}Biomechanics section, Department of Mechanical Engineering, KU Leuven, Belgium. \textsuperscript{4}Department of Statistical Methods, University of Zaragoza, Zaragoza, Spain. 
\textsuperscript{5}Defense University Center of Zaragoza, Spain.

Keywords: collagen gel, scaffold morphology, microstructure, 3D reconstruction, extracellular matrix, confocal microscopy.

Abstract

Collagen microstructure is closely related to the mechanical properties of tissues and affects cell migration through the extracellular matrix. To study these structures, three-dimensional (3D) \textit{in vitro} collagen-based gels are often used, attempting to mimic the natural environment of cells. Some key parameters of the microstructure of these gels are fiber orientation, fiber length or pore size, which define the mechanical properties of the network and therefore condition cell behaviour. In the present study, an automated tool to reconstruct 3D collagen networks is used to extract the aforementioned parameters of gels of different collagen concentration and how their microstructure is affected by the presence of cells. Two different experiments are presented to test the functionality of the method: first, collagen gels are embedded within a microfluidic device and collagen fibers are imaged by using confocal fluorescence microscopy; secondly, collagen gels are directly polymerized in a cell culture dish and collagen fibers are imaged by confocal reflection microscopy. Finally, we investigate and compare the collagen microstructure far from and in the vicinities of MDA-MB 231 cells, finding that cell activity during migration was able to strongly modify the orientation of the collagen fibers and the porosity-related values.

1. Introduction

Cells live in biological tissues, surrounded by a medium commonly known as the extracellular matrix (ECM). This ECM is an important structural entity mainly composed of different collagens, elastin, glycoproteins, proteoglycans and...
glycosaminoglycans (Wu et al., 2003). Collagen, in particular type I, is the main
structural component of the ECM (Alberts et al., 2002; Di Lullo et al., 2002) of tissues
and organs; therefore, knowing its internal micro-structure is critical to understand the
macroscopic mechanical behaviour and cell response during growth, migration or
differentiation (Adams & Watt, 1993; Aumailley & Gayraud, 1998; Berthiaume et al.,
1996; Fitton et al., 1998; Ranucci et al., 2000). Collagen-based gels are currently used
in many in vitro experiments which attempt to imitate the ECM. They consist of a
network of individual fibers, interconnected and surrounded by an aqueous medium,
forming a viscoelastic material (Moreno-Arotzena et al., 2015). Some macroscopic
properties of the tissues such as permeability or mechanical stiffness depend on the
architectural characteristics of the network (Doyle et al., 2015; Gardel, 2004; Petrie &
Yamada, 2012; Roeder et al., 2002) so their proper measurement is fundamental.
Collagen networks are commonly characterized using rheology (Arevalo et al., 2010;
Moreno-Arotzena et al., 2015; Piechocka et al., 2011) or computational models (Lee et
al., 2014; Sharma et al., 2016; Stein et al., 2011). However, those methods are limited
because none of them give information about the internal microstructure, which is
known to be affected by several diseases such as fibrosis or cancer, which may affect
the organization of the fibers and consequently, cell behaviour (Cox & Erler, 2011;
Mehlen & Puisieux, 2006).
Currently, there are many studies in two-dimensions (2D) about how the ECM affects
cellular behaviour (Discher et al., 2005; Kraning-Rush et al., 2012; Streuli, 1999).
However, in vivo, the 3D ECM presents a more complex environment which leads to a
wide variety of changes in cells in terms of their morphology, adhesion, migration or
biological response (Del Amo et al., 2018; Pedersen & Swartz, 2005). Consequently,
using in vitro 3D experiments reflects more realistically cell response in tissues and they
are becoming crucial research tools. However, although the study of 3D environments is
better to mimic in vivo conditions, the analysis becomes more complex. In any case, the
number of studies about the ECM and its 3D properties (mainly focused on collagen
networks) has been increasing in the recent years. I fact, several algorithms have been
developed for reconstructing 3D biopolymer networks from image stacks of fibered-
based gels. Normally, these algorithms need to perform two consecutive steps to
achieve the 3D reconstruction: i) binarization and ii) skeletonization (Bouix et al., 2005;
Bredfeldt et al., 2014; Ma & Sonka, 1996; Pudney, 1998; Stein et al., 2008; Wang &
Basu, 2007; Wu et al., 2003). Binarization stands for converting the image stack into a
binary matrix where voxels with value 0 are considered liquid phase and voxels with
value 1 are considered solid phase (fibers). To that end, different methods were
proposed, and most of them rely on thresholding combined with more complex
techniques (Wu et al., 2003). After binarization, the central line of each fiber, called
skeleton, is obtained. This skeleton is a thin version of the 3D shape that is equidistant
to its boundaries (medial axis), therefore emphasizing geometrical and topological
properties of the shape which is in this case the fiber network. There exist also other
types of algorithms to perform the 3D reconstruction, mainly based on template

This is the post-print version of the following article: Olivares, V., Cóndor, M., Del Amo, C., Asín, J., Borau, C., & García-Aznar, J. M. (2019). Image-based Characterization of 3D Collagen Networks and the Effect of Embedded Cells. Microscopy and Microanalysis, 1-11. doi:10.1017/S1431927619014570, which has been published in final form here.
matching (Krauss et al., 2012; Lebbink et al., 2007; Rigort et al., 2012). These methods obtain characteristics from specific parts of the image. Then, the Euclidean distance between the characteristic points and the original image is used to determine whether the voxels belong to the liquid or the solid phase.

In this work, we performed two different types of experiments to study and characterize changes on the microstructure of collagen networks under different conditions. First, collagen gels are embedded within a microfluidic device and collagen fibers are imaged by using confocal fluorescence microscopy (CFM). Secondly, collagen gels are directly polymerized in a cell culture dish and collagen fibers are imaged by using confocal reflection microscopy (CRM). To perform the 3D reconstruction of the collagen gels, an existing algorithm based on binarization and skeletonization was used (Bredfeldt et al., 2014).

To quantify the morphological differences of the fibered networks we propose different structural parameters such as fiber orientation, fiber connectivity, pore size, porosity, fiber length and fiber radius. In particular, we use this method to explore and characterize the morphological differences under different collagen concentrations and to explore the collagen network structure far from and in the vicinity of tumor cells (MDA-MB 23).

2. Materials and Methods

The methodology relies on the 3D reconstruction of collagen networks through a series of cross-sectional images of the sample. Different parameters are estimated from the reconstructed 3D network, namely fiber orientation, fiber connectivity, pore size, porosity, fiber length and fiber radius. The methodology is valid for analysing both fluorescently labelled and unlabelled gels, which are the most extended techniques for imaging the fibers of collagen networks.

2.1. Preparation of collagen gels

Two types of experiments were employed to test the multi-functionality of the methodology. First, collagen gels were confined within microfluidic devices. In this case, gels were fluorescently labelled to analyse them with fluorescence confocal microscopy. Secondly, collagen gels were plated in cell culture dishes. In this second case, collagen gels were not fluorescently labelled and were analysed by means of confocal reflection microscopy. These two methods are currently the most used for the study of collagen microstructures in 3D in biological laboratories(Chung et al., 2012; Cóndor et al., 2017; Del Amo et al., 2018; Kueng et al., 1989; Leclerc et al., 2003; Sung et al., 2009).

2.1.1. Collagen gels embedded/confined in microfluidics devices
To obtain fluorescent images of collagen gels, a fraction of the Collagen type I stock solution was labelled with DQ-Collagen™ at 4°C, according to the manufacturer’s protocol.

Three different final collagen concentrations were prepared: 2mg/ml, 2.5mg/ml and 4mg/ml using a different collagen stock for each case (3.81mg/ml, 8.56mg/ml and 9.82mg/ml respectively). The reagents used were common in all three cases: 10 µl DPBS-10x supplemented with calcium and magnesium (Lonza), 10 µl of 25 µg/ml DQ-Collagen™ type I from bovine skin fluorescein conjugate (Thermo Fisher), 15 µl cell culture media FGM-2 BulletKit (Lonza), 0.5M NAOH to adjust the pH to 7.4 and cell culture-grade water (Lonza) to adjust the final volume of the gels to 100µl. After preparation, collagen solution was pipetted within the microfluidic devices as shown in Fig. 1A and polymerized inside humid chambers at 37 °C, 95% relative humidity and 5% CO₂ for 30min. Hydrogels were hydrated and incubated overnight at cell culture conditions. For more information about hydrogels preparation and the fabrication of microfluidic devices, see (Del Amo et al., 2018).

2.1.2. Collagen gels plated in cell culture dishes

In the second set of experiments, we prepared unlabelled 1.2 mg/ml collagen type I hydrogels. To that end, we mixed 1.2 ml of rat tail collagen (Collagen R, 2mg/ml, Matrix Bioscience), 1.2 ml Bovine skin collagen (Collagen G, 4mg/ml, Matrix Bioscience), 270 µl NaHCO₃ (23mg/ml), 270 µl 10x DMEM (Biochrom) and 43 µl of NaOH (1M) to adjust the pH to 10. The solution is then diluted with 3 ml of a mixture of 1 volume part of NaHCO₃ (23mg/ml), 1 part of 10x DMEM and 8 parts of distilled H₂O. 2 ml of the final collagen solution was pipetted in a 35 mm Petri dish (see Fig. 1B) and polymerized in a tissue culture incubator at 37°C, 95% relative humidity and 5% CO₂ for 1 hour. After polymerization, 2 ml of complete cell culture medium was added to prevent dehydration of collagen gels. For more information about the critical parameters and troubleshooting for generating collagen gels, see (Cóndor et al., 2017).

These gels were used for the characterization of matrix structure around cells. To that end, MDA-MB 231 cells were carefully mixed with the final collagen solution before...
gel polymerization at a ratio of 15000 cells/ml and incubated for 12 h (37°C, 95% relative humidity and 5% CO2) before imaging.

2.2. Confocal microscopy imaging

For imaging labelled collagen gel, a z-stack of consecutive cross-section images of the sample was taken with a Nikon D-Eclipse C1 confocal microscope equipped with a 40X oil objective. The vertical distance between planes \( d_z \) (considering z-direction perpendicular to the focal plane) was 0.5 \( \mu \text{m} \) and the pixel size \( d_{xy} \) was 0.31 \( \mu \text{m} \), resulting in a voxel size of 0.31 x 0.31 x 0.5 \( \mu \text{m} \). 61 cross-sections of the sample were imaged for each case of study. The total volume analysed was therefore a cube of 317.44 x 317.44 x 30.5 \( \mu \text{m} \). An example of a cross-section is shown in Fig. 2A.

In the second set of experiments, for imaging unlabelled collagen gels the biopolymer network fibers were imaged using an upright confocal reflection microscopy (CRM) with a 20x dip-in water-immersion objective with NA 1.0. An image z-stack was recorded representing the deformed state of the cell embedded within the collagen gel. The stack was composed by 512 x 512 pixels images and a total of 500 cross-sections. The voxel size in this case is 0.72 x 0.72 x 0.74 \( \mu \text{m} \). An example of a cross-section is shown in Fig. 2B.

Figure 2. A) Cross-section of a fluorescence labelled collagen gel imaged with CM (Nikon D-Eclipse C1 equipped with a 40X oil objective). B) Cross-section of an unlabelled 1.2 mg/ml collagen gel imaged with CRM (20X dip-in water-immersion objective with NA 1.0).

2.3. Image processing

Based on a previous work (Stein et al., 2008), an automatic FIRE algorithm for extracting the structure/skeleton of a 3D biopolymer network from confocal images was used. The algorithm, was partially modified to enhance the computation of the Euclidean distance between fiber to non-fiber points by taking into account uneven voxel aspect ratios (that is when a voxel is not a perfect cube) following the methods described by Mishchenko. (Mishchenko, 2015) In this way, we reduce the inaccuracy.
due the unpaired z-xy resolutions. s. Fig. 3 shows the fiber tracing process in one fiber of our stacks.

Figure 3. Fiber tracing process with FIRE (Stein et al., 2008). First, nucleation points (NP) are found, being local maximum values of the distance transform function. After that, branches are traced extending from NPs. A set of Local Maximum Points (LMPs) is defined for each NP. The set of LMPs includes all local maximum on the surface of the box which has as centre the nucleation point and a size equal to the value of the distance transform function in that NP. The branches are lines that link the NP with each of the LMPs. A) Nucleation point (green) and local maximum points (red) calculated in the fiber tracing process. B) Branches expansion. C) 3D View of the fibers.

2.4. Fiber orientation evaluation

To determine the fiber orientation we have relied on Lang’s algorithm (Lang et al., 2013), where the inertia tensor of small random mass distributions along the analysed volume is estimated to obtain principal directions. In fact, we can compute the mass distribution of each individual fiber since we know exactly which voxels of the skeleton volume correspond to it. In this way improve the accuracy compared to selecting small random volumes through the gel, which may include in the computation voxels of different oriented fibers. Finally, by computing the unit direction vector of the easy axis of minimal inertia, we determine the orientation of each fiber in the 3D space.

To represent all fiber orientations in the 3D space, we use a 3D-rose function, developed in a previous work (Del Amo et al., 2018), which takes into account 13 directions (each of them separated 45º) classifying fibers according to their proximity to those directions (see Fig. 4). This function outputs a cone aligned in each one of the 13 directions, whose size depends on the amount (percentage) of fibers that are oriented in a certain direction. The diagram in Fig. 4 shows the different zones in which we have divided the sphere to represent the fiber orientation in the 3D space and its identification number.
This is the post-print version of the following article: Olivares, V., Cóndor, M., Del Amo, C., Asín, J., Borau, C., & García-Aznar, J. M. (2019). Image-based Characterization of 3D Collagen Networks and the Effect of Embedded Cells. Microscopy and Microanalysis, 1-11. doi: 10.1017/S1431927619014570, which has been published in final form here.

Figure 4. Representation of the fiber orientation distribution in the 3D space. A) 3D Sphere divided into 13 zones. Cross-section of the 3D diagram in: B) the XY-plane and C) the XZ-plane.

2.5. Porosity evaluation

To evaluate the steric hindrance imposed by the fibrillar network against cell migration, it is important to know how much available space cells have to advance through the ECM network. This empty space corresponds to the pores. Porosity (equation 1) is a direct measure that evaluates how compact a network is, ranging from 0 (representing a completely empty volume) to 1 (representing a completely solid volume).

\[
P = \frac{V_p}{V_T} = \frac{V_T - V_F}{V_T}
\]

where \( P \) is porosity, \( V_p \) is pore volume, \( V_T \) is the total volume and \( V_F \) is the fiber volume.

To compute the network porosity, the original stack of images must be used, since the skeletonized volume does not contain the fiber thickness. For that purpose, we used the FIRE algorithm (Bredfeldt et al., 2014) which represents each fiber as an independent cylinder, enabling the calculation of radius and length for each individual fiber. Consequently, the real volume of pores can be computed as the total volume analysed (npixelsX*npixelsY*zstacks) minus the fiber volume computed one (see equation 1).

However, since fibers are not homogeneously distributed in the 3D space, further measurements (e.g. pore size) need to be performed in order to characterize this complex structures.

2.6. Pore size determination

Pore size of collagen networks governs their mechanical properties and influences the ability of cells to migrate through the ECM (Zaman et al., 2006). There is a critical pore size value below which the cells ability to migrate decreases dramatically (Wolf et al., 2013) so the determination of this parameter may have a great importance when dealing with cell migration-related assays. A mesh size, or pore size, is given by the 3D spacing.
of the fibers within the interstitial fluid, which can be obtained from microscopy imaging. We rely on the method introduced by Lang (Lang et al., 2013) in which the pore size can be determined using the nearest-obstacle distance (NOD). The distribution of NODs follows a Rayleigh distribution, regardless the network presents an isotropic or anisotropic topography. In CRM stacks, that Rayleigh distribution is scaled by a parameter that depends on a certain cut-off angle from which the most pronounced fibers are not visible and which is calculated from fiber orientations. For CFM images, there is no scaling factor of the distribution, so the value of pore size can be directly obtained.

In a 3D binarized matrix, \( r_{\text{NOD}} \) describes the Euclidean distance from a background point to the closest fiber point and represents the radius of the largest sphere that can be introduced into each of the analysed pores. To obtain the NOD distribution, \( p(r_{\text{NOD}}) \), we computed \( r_{\text{NOD}} \) at 100,000 random points (see S1 - supplementary material) of the stack. \( p(r_{\text{NOD}}) \) is then fitted by a Rayleigh distribution (Eq. 2) with a single parameter, \( r_{\text{mean}} \), which represents the mean value of \( r_{\text{NOD}} \).

\[
p(r_{\text{NOD}}) = \frac{r_{\text{NOD}}}{\sigma^2} e^{-\frac{r_{\text{NOD}}^2}{2\sigma^2}}
\]

where \( \sigma = \sqrt{\frac{2}{\pi}} r_{\text{mean}} \) is the most probable distance, i.e. the mode of the distribution, and therefore depends on the density of the network. If the network has a high fiber density, the distribution is narrower with a prominent peak, whereas if it has a low density, the peak shifts to the right and the distribution broadens. As \( r_{\text{mean}} \) is the only parameter needed to fit the Rayleigh distribution to the measured \( p(r_{\text{NOD}}) \), it is a robust measure for the average pore size and it can be calculated as shown in Eq. 3.

\[
r_{\text{mean}} = \frac{\sigma}{\sqrt{\frac{2}{\pi}}}
\]

Hence, \( r_{\text{mean}} \) is the radius of the biggest sphere that can be introduced into the pores without breaking through a fiber.

2.7. Fiber connectivity evaluation

Some mechanical properties of the networks, such as rigidity, highly depend on the internal connectivity and the interactions between the individual fibers (Shoulders & Raines, 2010). Thus, a detailed knowledge of fiber connectivity would help to correlate

This is the post-print version of the following article: Olivares, V., Cóndor, M., Del Amo, C., Asín, J., Borau, C., & García-Aznar, J. M. (2019). Image-based Characterization of 3D Collagen Networks and the Effect of Embedded Cells. Microscopy and Microanalysis, 1-11. doi: 10.1017/S1431927619014570, which has been published in final form here.
mechanical properties with the internal microstructure of the gels. In this work we quantify fiber connectivity as the number of fibers that are bonded at each crosspoint of the fibrillary network.

3. Results

3.1. Effect of collagen concentration on network architecture

To test the effect of the collagen concentration on the network architecture, we applied our method to a set of collagen gels polymerized at different monomer concentrations of 2 mg/ml, 2.5 mg/ml and 4 mg/ml (see Fig. 5A). An example slice of each collagen concentration is shown in Fig. 5A. The maximum intensity projection in Z direction of the 3D reconstruction is illustrated in Fig. 5B, defined as \( J(x, y) = \max_z(I(x, y, z)) \) where \( I \) refers to the image stack. Fig. 5C-D, show the resulted 3D reconstructions for each collagen gel obtained with Paraview and Matlab respectively.

This is the post-print version of the following article: Olivares, V., Cóndor, M., Del Amo, C., Asín, J., Borau, C., & García-Aznar, J. M. (2019). Image-based Characterization of 3D Collagen Networks and the Effect of Embedded Cells. Microscopy and Microanalysis, 1-11. doi: 10.1017/S1431927619014570, which has been published in final form here.
Per each monomer concentration we quantified the network porosity, pore size, average fiber length, number of fibers and average radius of fibers. Porosity and pore size are parameters related to the liquid phase of the collagen gels, while fiber length, fiber radius or number of fibers are related to the solid phase. Data distribution is presented in Fig. 6.

As expected, porosity decreases as the collagen concentration increases, since it is inversely related to the volume of fibers (see Fig. 6A). Porosity drops from values of 85.01 % for 2.5 mg/ml collagen gels, to 67.01 % for 4 mg/ml collagen gels. A Kruskal-Wallis test was done to check whether the median of any concentration was significantly different followed by post-hoc pairwise Mann-Whitney tests that revealed significant differences for the different collagen concentrations at 0.01 significance.
level. A statistical regression model has been adjusted for porosity values using a quadratic relationship with concentration (p value of 0.033 in the F-test for null hypothesis about not requiring the quadratic term, and a goodness-of-fit of $R^2=88.8\%$), and a 95% prediction interval for porosity values is obtained (see Fig. 7A). The estimated regression curve expresses a negative relationship, and the prediction interval shows that porosity values around 70% are expected for 4.0 mg/ml.

\[ \hat{Y}(X) = 108.3 - 16.38X + 1.65X^2 \]

Through this prediction interval, porosity values can be predicted for different collagen concentrations without performing the associated experiments, suggesting that porosity decreases quadratically with the collagen concentration according to the equation given above.

The average pore size (the variable related to the liquid phase of the collagen gels) was also found to decrease with increasing collagen concentration, since the more volume of fibers the less empty hollows will remain. Nevertheless, the pore shape also plays a key role in the characterization of the network structure. Importantly, 2.5 mg/ml collagen gels presented a high variability due to their heterogeneity, which caused the median pore size to be actually higher (2.307 µm) than the obtained for 2 mg/ml collagens (1.978 µm). Kruskal-Wallis test confirmed that at least one of the concentration had a significantly different median than the others. In addition, the following Mann-Whitney test revealed not significant differences between 2 mg/ml and 2.5 mg/ml, and significant differences between 2.5 mg/ml and 4 mg/ml. Hence, a quadratic regression model of the transformed data with a 95% prediction interval was adjusted (p value of 0.000 associated to quadratic term and a goodness-of-fit of 82.54%) (Fig 7B). A Box-Cox transformation ($Y'=-1/Y^2$) was made to the pore size results, in order to verify the normality conditions necessary for correct inference in the regression model which is presented in Fig. 7B. The expression of fitted model shows a non-linear relationship that distinguishes that the pore size distribution for 4.0 mg/ml is located in a lower range compared to the other concentrations.

\[ \hat{Y}'(X) = -1.082 - 0.671X - 0.125X^2 \]
Figure 6. A) Porosity. The porosity is inversely related to the volume of fibers, consequently, it decreases as the collagen concentration increases. B) Average Pore Size. The average pore size was also found to decrease with increasing collagen concentration. C) Number of fibers per µm³. The number of fibers per µm³ increases with the concentration, but not significantly differences can be found between 2 and 2.5 mg/ml collagens. D) Average fiber length. This boxplot shows that average fiber length increases in denser collagen gels. E) Average fiber radius. Not significant differences were found for the radius of the fibers. F) Connectivity of the fibers. The connectivity is the number of fibers connected to the same cross-link. 2 mg/ml gels have the highest percentage of low connectivity (≤ 5 fibers/cross-link). On the other hand, for higher connectivity, the denser matrices (4 mg/ml) curve is always above the others concentrations. In all cases, Kruskal-Wallis followed by post-hoc pairwise Mann-Whitney tests were performed to reveal significant differences in the parameters for the different collagen concentrations: *p ≤ 0.05, **p ≤ 0.01, n.s., not significant (p > 0.05). n = 27 in 2 and 4 mg/ml experiments and n = 18 in 2.5 mg/ml experiments.

Regarding the variables related to the solid phase (number of fibers, fiber length and radius), no regression models could be properly adjusted to smoothly reflect the dependence on collagen concentration. Nevertheless, Kruskal-Wallis and post-hoc pairwise Mann-Whitney tests were carried out, confirming significant differences between 2.5 mg/ml and 4 mg/ml gels for all variables except for the fiber radius, in which no significant differences were found (Fig 6C-D).
Figure 7. Regression models for porosity and pore size as a function of collagen concentration. A) Regression model for porosity. A statistical regression model has been adjusted for the porosity parameter with a 95% prediction interval (p value of 0.033 and a goodness-of-fit (R2) of 88.8%). Through this interval, porosity values can be predicted for different concentrations without performing the associated experiments, suggesting that porosity decreases quadratically with the collagen concentration according to the given equation. B) Regression model for the average pore size. A quadratic regression model of the transformed data with a 95% prediction interval was adjusted (p value of 0.000 and a goodness-of-fit of 82.54%). A Box-Cox transformation was made to the pore size results to improve the correlation between pore size and concentration, improving the presented regression model.

The number of fibers increases with the collagen concentration, so this will directly affect the mechanical properties of the gel. In the same way, the fibers are longer in denser collagen gels (a mean value of 4.083 µm in 4mg/ml compared to 3.794 µm in 2 mg/ml). It is worth noting that the measured fiber lengths in 2.5 mg/ml gels had a great dispersion, although the mean (4.142 µm) was similar to that of 4 mg/ml gels (Fig 6D). The average fiber radius, ranged from 0.963 µm to 1.327 µm in all conditions, not presenting significant differences in any of the cases.

In sum, for higher collagen concentrations we obtain more and larger fibers with similar radius, although the transition from 2 to 4 mg/ml is not smooth, as suggested by our disperse data from 2.5 mg/ml gels. This may be due to the conditions in which gels polymerize, that lead to more heterogeneous networks. The type of chemical bond that collagen presents, generates some internal stresses during the polymerization that make fibers only join lengthways and not widthways, which could explain the shift occurring at intermediate concentrations.

To confirm this, we studied the connectivity of the networks (evaluated as the number of fibers connected to the same cross-link). Fig. 6F shows the connectivity for three different gels (repetitions) of each concentration. The X-axis shows the number of fibers emanating from a cross-link and the Y-axis gives the percentage of the total number of cross-links that have that amount of fibers coming out from it. Note that our connectivity parameter starts at 3, because by considering the fibers as bound cylinders, connectivity equal to 2 means two consecutive segments (i.e., the same fiber) and connectivity equal to 1 makes no sense (floating fragments or artefacts). An additional analysis was performed to check the correct functionality of this study (see S2-supplementary material). From these results, we can conclude that 2 mg/ml gels have
the highest percentage of connectivity in the lower zone (3-5 fibers per cross-link), or in
other words, the lower connectivity. 2.5 mg/ml and 4 mg/ml gels appear to be quite
similar in terms of connectivity. These matrices present an overall percentage of about
15% in the low connectivity range (<=5 fibers/cross-link). However, the maximum peak
for 2.5 mg/ml is the same of 2 mg/ml gels (4 fibers per cross-link) whereas the
maximum peak for 4 mg/ml is found around 8 fibers per cross-link. From this point, the
percentage of greater connectivities falls, but always maintaining the curve above the
other concentrations. In other words, 4 mg/ml gels have more overall more number of
fibers connected to each cross-link point compared to 2 and 2.5 mg/ml gels.

In sum, our results suggest that not all the studied parameters can be controlled just by
the collagen concentration specified at the experiment, nor they vary linearly with it.
Hence, it is interesting to study the relationships that exist between these internal
parameters. A Pearson correlation test was carried out to study the correlations between
them. The results are shown in table 1.

<table>
<thead>
<tr>
<th>Pore Size (µm)</th>
<th>Porosity</th>
<th>Pore Size (µm)</th>
<th>Num. Fibers/ µm³</th>
<th>Av. Fiber</th>
<th>Length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.596</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Num. Fibers/ µm³</td>
<td>-0.664</td>
<td>-0.977</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Av. Fiber length (µm)</td>
<td>-0.524</td>
<td>0.196</td>
<td>0.999</td>
<td>-0.143</td>
<td>0.232</td>
</tr>
<tr>
<td>Av. Fiber radius (µm)</td>
<td>-0.153</td>
<td>0.646</td>
<td>0.000</td>
<td>-0.583</td>
<td>0.808</td>
</tr>
</tbody>
</table>

Table 1. Correlation matrix of the network morphology parameters. First row of each cell shows the Pearson
correlation value and the second row, the p value associated to the hypothesis that the correlation is null. There was
a strong positive correlation between porosity and pore size. On the other hand, the number of fibers had a negative
correlation with both porosity and pore size. Interestingly, the average fiber length was also related with the liquid
phase parameters. In fact, we found a negative correlation between fiber length and porosity, but a positive one
between fiber length and pore size. Finally, the radius of the fibers was positively correlated with the pore size and
the fiber length but negatively with the number of fibers per µm³.

According to the results, almost all parameters are interrelated. There was a strong
positive correlation between the two variables regarding pores (0.596) so that a higher
porosity is related to a larger pore size. On the other hand, as could be expected, the
number of fibers had a negative correlation with both porosity (-0.664) and pore size (-
0.977). In fact, such correlation coefficient so close to -1 is showing that the number of
fibers and the pore size could be considered two aspects of the same information.
Interestingly, the average fiber length was also related with the liquid phase parameters.
In fact, we found a negative correlation between fiber length and porosity (-0.524), but a
not significant one between fiber length and pore size. That is, longer fibers tend to
form networks with lower porosity but larger pore sizes. Finally, the radius of the fibers

This is the post-print version of the following article: Olivares, V., Cóndor, M., Del Amo, C., Asín, J., Borau, C., &
Cells. Microscopy and Microanalysis, 1-11. doi: 10.1017/S1431927619014570, which has been published in
final form here.
was correlated with the pore size (0.646), the number of fibers per \(\mu m^3\) (-0.583) and their length (0.808).

### 3.2. Effect of cellular proximity on network architecture

We next applied the method for studying the collagen network structure in the cell vicinities. During migration, cells adapt their shape and generate forces, producing deformations in the surrounding ECM, thus leading to a change in the structure of the surrounding ECM. To quantify that, a region of interest around the cell (ROI) was manually selected (blue rectangle showed in Fig. 8A) and extended through several z-slices (also manually selected) in which the cell is present, conforming the cell volume for analysis (\(V_{cell}\)). Importantly, a smaller volume centered on the cell location (red rectangle showed in Fig. 8A) is removed from \(V_{cell}\) to avoid distorting the computation of porosity and pore size (since the cell body appears as a hole in the collagen images).

Finally, a cubical domain of the same size as \(V_{cell}\) is selected randomly from some part of the network away from the cell. The volume of the cell-free zone was obtained by taking a cube of sides equal to the cubic root of the total volume chosen around the cell.

The 3D reconstruction of both cubes (near cell and far from the cell) was then carried out and one example of this reconstruction is shown in Fig. 8B-C respectively.

### Figure 8. 3D reconstruction of collagen fibers in the vicinities of the cell. A) Analysis area that contains the cell. B) 3D reconstruction of the collagen fibers in the vicinities of the cell. C) 3D reconstruction of the collagen fiber in a cell-free zone. Different colors are used for better visualization of the fibers.

In Fig. 9, we show the statistical distribution of the main results (porosity, pore size, fiber length, fiber radius and ratio of cross-links) obtained from the image-based analysis. To test the significance of these results, a t-test of paired data was carried out. Our hypothesis was that cells are able to adhere to the surrounding fibers to migrate, exerting forces that stretch, deform and orient the fibrillar network.
Figure 9. Collagen structure in the vicinities of the cells. A) Porosity. A lower porosity was found in the proximity of the cell. B) Average Pore Size. Although not very relevant in terms of value, statistically significant differences were found between pore size near and far from the cell. C) Average Fiber Length. Near the cell the average fiber length was significantly higher than far from the cell. As could be expected, cells stretch and lengthen the fibers surrounding them. D) Average Fiber Radius. Higher fiber radius were found in the vicinities of the cell. E) Fiber connectivity: Near the cell, the connectivity of the network is clearly higher compared to far away volumes, meaning that fibers are linked in many more points due to the concentration of collagen produced by the cell contraction. F) Tentative schema of fibers and pore distribution near and far from the cell that explain the obtained results (red: fibers, blue: pores, yellow: cell). On the top, oriented fibers in the vicinities of the cell. On the bottom, random oriented fibers. To obtain the significance, t-test of paired data was done. For all the boxplots: *p ≤ 0.05, **p ≤ 0.01, n.s., not significant(p > 0.05). n = 9 in all cases.

The results show that the density of fibers in the vicinities of the cell increases, as reflected by a significantly lower porosity median value (62.801 %) compared to the rest of the network (81.394 %) (see Fig. 9A). Regarding the pore size, we expected smaller pore size values in the vicinities of the cell, since the porosity is overall smaller (Fig. 9A). However, we found a slight increase of the pore size in the vicinities of the cell (2.63 µm) compared to regions far from the cell (2.52 µm, see Fig. 9B), which, although not very relevant in terms of value, was statistically significant. This result, apparently counterintuitive, could be explained by studying the fibers orientation. Near the cell, fibers are strongly oriented in certain preferential directions (see Fig. 9F and Fig. 10B) so the pores in this case are thinner but longer, resulting in an increase of the average pore size of the collagen network in these areas. On the other hand, far away from the cell, fibers are randomly oriented and pores are more homogeneous (see Fig. 9F and Fig. 10A).

The average fiber length in the proximities of the cell was significantly higher than far from the cell (average difference of 1.8128 µm) which agrees with the idea that cells stretch fibers in a mechanosensing process (Doyle et al., 2017; Tondon & Kaunas, 2014) deforming and lengthening them. Cells also orient the fibers in certain preferred
directions which, as stated before, makes the pores longer and more uniform in shape
and size, and maybe facilitating the migration. We also observe that the fiber radius is
significantly higher in the vicinity of the cells (Fig. 9D).

Next, we explored the fiber connectivity. Fig. 9E shows the connectivity curves for both
studied cases: near and far from the cell. Each curve is the average of 9 different
experiments. Overall, connectivity of collagen networks in regions far from the cell is
higher than regions near the cell for low connectivity (about 5). However, for greater
connectivity (from 10 to 20 fibers/cross-link) the curve corresponding to zones near the
cell is always above, meaning that crowded connections of fibers are frequent in the
vicinity of the cell probably due to network contraction, whereas in far away regions,
this high connectivity is non-existing.

Finally, we investigated the fiber orientation by quantifying the distribution of fiber
angles in the 3D space by means of 3D rose graphs. Results confirmed that fibers away
from the cell were randomly oriented (Fig. 10A), with some alignment in z probably
due to gravity effects during polymerization, whereas fibers in the vicinities of the cell
were primarily oriented with the xy-plane and in particular with the x-direction, (Fig.
10B). Fig. 10C shows the measured preferential fiber directions superimposed to the
cell position in a central plane of the z-stack.

Figure 10. Collagen fiber orientation in regions: A) far from the cell, where fibers are randomly oriented in the 3D
space, and B) in the vicinities of the cell, where fibers are oriented in some preferential directions. C) Principal
orientations of the fibers in the XY-plane centered in the cell body and superimposed to the confocal reflection image
of the collagen fibers. In the 3D rose, size and color (from yellow to red) of the cylinder represents the percentage of
fibers aligned in each direction.

4. Discussion

Collagen gels are heterogeneous structures that vary enormously according to the
conditions of processing and polymerization. This feature makes collagen gels a
difficult structure to study due to the large number of factors involved in its process of
elaboration. However, since they are crucial to understand how cell migration is
regulated by extracellular matrix, different techniques are being developed for the study
of these complex structures (Anguiano et al., 2017; Krauss et al., 2012; Lang et al.,

This is the post-print version of the following article: Olivares, V., Cóndor, M., Del Amo, C., Asín, J., Borau, C., &
Cells. Microscopy and Microanalysis, 1-11. doi: 10.1017/S1431927619014570, which has been published in
final form here.
A very promising tool, for instance, is the virtual 3D reconstruction of these structures from microscopy images. It is important for these tools to be traceable and robust under different conditions. The tool used in this work allows us to analyse gels in microfluidic devices and in Nunc dishes.

The first experiments analysed in this work, were collagen fibers in microfluidic devices which allows mimicking in vivo conditions in a better way than traditional 2D cultures. In these experiments different concentrations of collagen have been compared to see how it affects the micro-architecture. We have observed that there are some parameters such as pore size or porosity that can be quantitatively described with a mathematical function depending on the collagen concentration. However, due to the heterogeneity of the image-based data, we were not able to fully adjust a model to describe fiber length and fiber radius as a function of collagen concentration. On the other hand, connectivity is higher as the collagen concentration increases, but we weren't able to find a correlation either. Nevertheless, from all these results, we can observe that we do not find significant differences in none of these structural properties at 2 and 2.5 mg/ml, but these are statistically significant for collagen gels of 4 mg/ml.

In the other kind of experiments in which cells were cultured in dishes, we have also analysed cells and collagen architecture. In these probes, the collagen microstructure was compared near and far from the cell. Our hypothesis was that cells cling to the collagen fibers that are around it and make a force on them to produce migration. In the obtained results we have seen that the concentration of fibers around the cell is greater than far from the cell since the porosity decreases. Therefore, there is an agglomeration of fibers around the cell when it is migrating. In addition, the cell pulls the fibers lengthening their length in comparison with the rest of the individual fibers that define the gel. Also, near the cell, the fibers are interconnected in cross-points. Maybe these cross-points are the points where the cell join the fibers. In terms of orientation, the cell is able to orientate the fibers with its forces in the main direction of contraction. In conclusion, the cell to migrate joins many fibers around it, exerting a force that stretches and orients the fibers and thus, achieves its movement.

Finally, with all these results and the methods used, structural properties of the collagen fibers could be studied in different experiments and conditions. These results together with mechanical experiments, will allow researchers to have the collagen microstructures characterized in a complete way.

5. Conclusions

With the different analysed cases, we have seen that by varying the concentration some parameters of the microstructure of the collagen gels could be controlled. The greatest differences in microstructure have been found between 2 mg/ml and 4 mg/ml collagen concentration gels. In previous mechanical studies (Valero et al., 2018) where the mechanical properties of these collagen-based were quantified by rheology, we observe that these two concentrations also have a higher difference in terms of mechanical
response (shear modulus vs shear strain) than the gels of 2 and 2.5 mg/ml. In fact, storage shear modulus of collagen hydrogels are respectively (40.12 ± 3.29; 62.14 ± 4.87; 121.03 ± 9.94 Pa) (Valero et al., 2018) for collagen concentration (2, 2.5 and 4 mg/ml). Therefore, it can be concluded that mechanical properties are directly related to some of the parameters of the internal microstructure such as porosity and number of fibers or length of fibers.

In addition, we have been able to evaluate that the presence of cells quantitatively modify the matrix by accumulating a greater volume of fibers around it, lengthening the fibers and orienting them in preferential directions. This fact could explain the local rigidization that ECM suffers in the surrounding of cells as have been recently evaluated in different experimental works (Cóndor et al., 2019; Han et al., 2018).

Taken together, the methods here presented could be used to estimate the matrix mechanical properties of collagen-based gels from image-based analysis without having to perform the mechanical tests. In fact, this work could suppose a first step forward this direction.

6. Acknowledgments

We acknowledge support by the European Research Council (ERC) through project INSILICO-CELL (ERC-2012-StG 306571) and its proof of concept IMAGO (ERC-PoC-2016-737543), the Spanish Ministry of Economy and Competitiveness through projects DPI2015-64221-C2-1-R and MTM2017-83812-P; and the Government of Aragon (T50_17R, E46_17R). The authors would like to thank Professor Ben Fabry for his valuable help with the pore size algorithm and for the images used that were taken in his lab.
This is the post-print version of the following article: Olivares, V., Cóndor, M., Del Amo, C., Asín, J., Borau, C., & García-Aznar, J. M. (2019). Image-based Characterization of 3D Collagen Networks and the Effect of Embedded Cells. Microscopy and Microanalysis, 1-11. doi: 10.1017/S1431927619014570, which has been published in final form here.
This is the post-print version of the following article: Olivares, V., Cóndor, M., Del Amo, C., Asín, J., Borau, C., & García-Aznar, J. M. (2019). Image-based Characterization of 3D Collagen Networks and the Effect of Embedded Cells. Microscopy and Microanalysis, 1-11. doi: 10.1017/S1431927619014570, which has been published in final form here.
Supplementary material

S1 – Optimum number of random points to obtain the NOD distribution.

To determine which was the optimum number of random points for the calculation of the NODs distribution, we did a sensitivity analysis. Sup. Fig. 1 shows the computed pore size and the execution time versus the number of random points. As can be observed, the pore size stabilizes at about 50,000 random points with a reasonable computation time. In fact, we finally used 100,000 random points for safety.

Supplementary figure 1. Variations of the pore size and runtime with the number of random points.

S2 – Connectivity check by adding the crosslinking enzyme transglutaminase 2 (TG2).

We have analysed other images obtained in our laboratory for a concentration of 4mg/ml with and without the enzyme TG2. Transglutaminase are a group of enzymes that can modify some protein functionalities, the most important being the ability to cross link the peptides or proteins, which strengthen the matrix (Chau et al., 2005). To test this, we quantified the connectivity of a collagen with and without TG2. It can be observed in Sup. Fig. 2 that low connectivities, the curve of cross-linked collagen matrix is below the control collagen curve. However, for high connectivity, this is...
inverted and the collagen curve with TG2 is above the collagen curve. This means that collagen with transglutaminase presents a higher connectivity than without.

Supplementary figure 2. Connectivity for a non cross-linked and a cross-linked matrix of collagen.