sections of about 17.6 µm and 17.8 µm respectively and a major ellipse axis for both ZY and ZX sections of about 15.6 µm and 15.8 µm respectively. While Minimum method gave the lowest estimations of the rectangle height (and the Feret's diameter) for both ZY and ZX sections of about 14.7 µm and 15.5 µm respectively and a major ellipse axis for both ZY and ZX sections of about 12.9 µm and 13.6 µm respectively.

ZX Maximum Intensity Projection

Thresholding effect on the shape
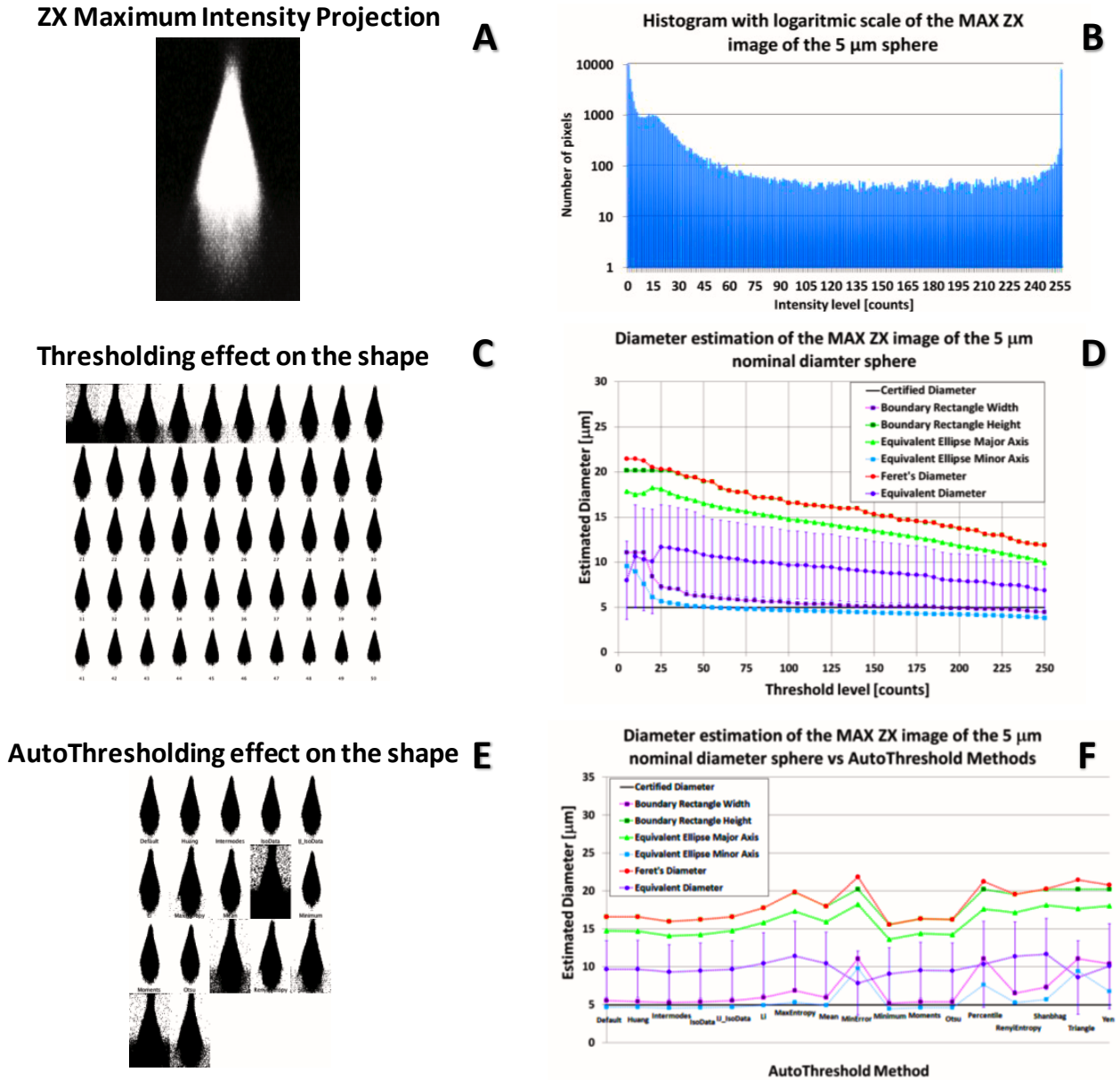
AutoThresholding effect on the shape

Fig. 4.26 Summary of the results of the image processing of the maximum intensity projection ZX section along the Y-axis for the 5 µm nominal sphere. (A) Processed image. (B) Histogram of the processed image. (C) Thresholding effect on the shape of the object applying increasing thresholding levels. (D) Diameter estimation using the chosen estimator in function of the thresholding level. (E) Binary images created applying all the ImageJ autothresholding methods. (F) Diameter estimation using the chosen estimator in function of the autothresholding methods used.

Default, Huang, Intermodes, IsoData, IJ_IsoData, Moments and Otsu methods led to an estimation of the rectangle height (and the Feret's diameter) for both ZY and ZX sections of about 16.5 µm and a major ellipse axis for both ZY and ZX sections of about 14.7 µm.

Default, Huang, Intermodes, IsoData, IJ_IsoData, Moments, Otsu and RenyiEntropy methods led to a good approximation of the certified diameter using the minor ellipse axis estimator.

Conclusion

The uncertainty sources in the dimensional measurements with CARS microscopy are due to three main aspects: the statistical dispersion of the particles population, the optical process linked to the non-resonant background and mainly to the optical characteristics of the lenses system and the image processing influenced by the method used to estimate the spheres diameter as for example the choice of the threshold value to create binary images.

In this characterization process the statistical dispersion of the particle population had a minor influence seen that the uncertainty related to the certified diameters of the reference polystyrene spheres was much lower than theoretical optical resolution of the microscope.

The axial resolution is limited to the employed objective depth of focus that is always greater than its XY plane resolution. Both change heavily with the objectives numerical aperture. The resulting voxel has an elongated not-spherical shape along the Z-axis causing an artefact that enlarges fictitiously the sample axial size. The measured intensity is related to the convolution between the voxel and the sample.

Manual measurements of the spheres size have been proposed together with automatic measurements approach, developing a specific algorithm to extract the diameter measure from the acquired images.

It has been done a comparison between the results obtained using the developed algorithm and those obtained with other ImageJ Analyze Particles tool estimators.

It has been analysed the influence of the choice of the threshold level and of the image quality on the measures, using images with different intensity characteristics that have been processed employing a linear variation of the threshold levels and the ImageJ autothresholding methods.

Part of this work has been presented as a poster in the microCARS2010 international workshop [142] and as a deliverable of the Regenmed project.

5. Realization of biological experiments using multimodal CARS-SHG-TPEF microscopy

The multimodal CARS-SHG-TPEF developed at the INRIM's laboratories has been successfully used to image biological samples in the frame of the research in the regenerative medicine.

The first biological experiment done using this microscope exploited the TPEF technique to image fixed stained cells for a study conducted in the laboratory by C. Prawettongsopon about the cell adhesion on different substrates namely: uncoated glass, fibronectin and polylysine. In this experiment the cells have been stained with three fluorophores to track the DNA (blue), the actin (red) and the integrin (green) to study their localization and shape inside at different times on the different coatings (Fig. 5.1). In this work have been obtained interesting results regarding the modification of the cells shape, the cell spread rate, the actin cytoskeleton structures and the integrin structures. In this study we saw that fibronectin coating affected cell adhesion more than polylysine and non-coated surfaces in terms of actin-integrin remodelling as well as spreading rate.
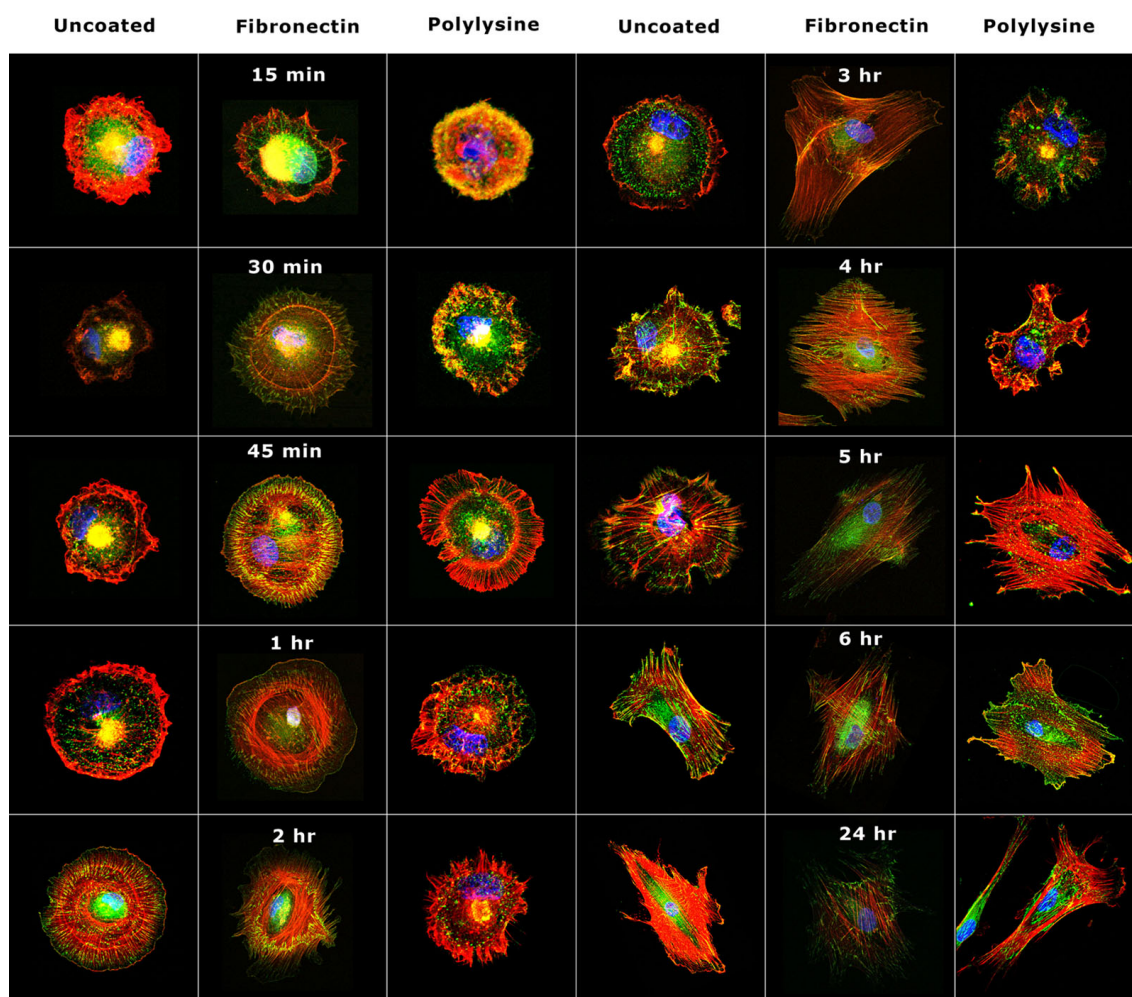

Fig. 5.1 3D imaging Z-projection of cells stained to track the DNA (blue), the actin (red) and the integrin (green) in adhesion on the different coatings measured at different times.

The first image experiments of biological samples using CARS microscopy had the aim to optimize the system in order to image live cells in a label-free way. At this purpose adenocarcinomic human alveolar basal epithelial cells (A549 cells) and hMSCs have been cultured on petri dishes at the I.N.Ri.M. laboratories by C. Divieto.

Cells have been imaged in forward direction using the resonant signal peaked around 2844 cm^{-1} from CH_2 stretch vibration in lipids present in the cellular membrane and in the liposomes [28,94]. Signal-idler excitation scheme has been chosen for the biological experiments imaging. In order to focus the excitation beams on living cells samples, a water immersion objective (LUMPLFLN 60XW NA=1 W.D.=2 mm, Olympus) fully compensated for both spherical and chromatic aberrations from the UV to the near infrared region, has been used. The water immersion objective has been cleaned and sterilized with a solution of 70% ethanol in water (v/v) before each imaging experiment.

The forward de-scanned CARS signal has been collected through an objective (UPLSAPO 20x objective NA=0.75 W.D.=0.6 mm, Olympus) and focused on the PMT (R3896, Hamamatsu) with a plano-convex lens with a focal length of 25 mm.

Bandpass filters centered at 716 nm with 43-nm bandwidth (FF01-716/43, Semrock), 480 nm with 20-nm bandwidth (BP470-490, Chroma Technology) all coupled with shortpass filters with 770nm of cut-off wavelength (FF01-770/SP, Semrock) are used before the detector to further block the residual excitation beams and transmit the CARS signal.
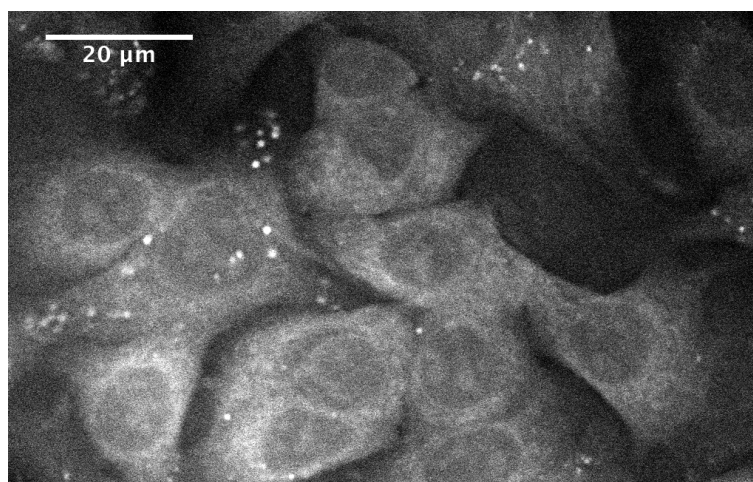

Fig. 5.2 CARS microscopy imaging of living A549 cells at 2844 cm^{-1}.

Imaging of A549 cells was done slicing the sample in three dimensions with a step in the Z-axis of about 0.5 μm between two adjacent slices. The overall Z scan range was chosen accordingly to the 3D extension of the cells in the petri dish (19 images). The XY pixel pitch was about 0.1 μm/pixel and the image size was adapted according to cells shape in the experiment. The overall 3D imaging lasted about 5 minutes and up to five images were acquired for each slice and adaptively averaged using a Kalman filter. Using the 3Dviewer plugin of ImageJ it has been done a three-dimensional reconstruction of the sample (Fig 5.3).
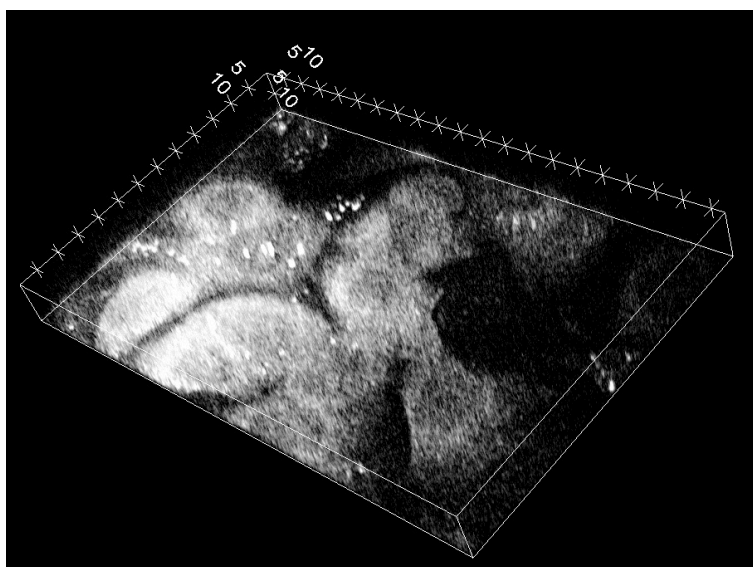
Fig. 5.3 3D reconstruction of living A549 cells imaged using CARS microscopy.

Also the imaging of hMSCs was done slicing the sample in three dimensions with a step in the Z-axis of about 0.5 µm between two adjacent slices. The overall Z scan range was chosen accordingly to the 3D extension of the cells in the petri dish (7 images). The XY pixel pitch was 0.164317 µm/pixel and the image size was adapted according to cells shape in the experiment. The overall 3D imaging lasted about 5 minutes and for each slice up to five images were acquired and adaptively averaged using a Kalman filter.
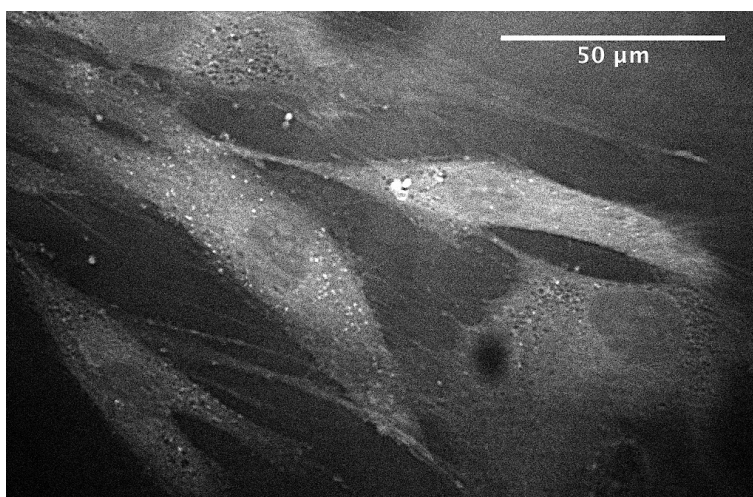

Fig. 5.4 CARS microscopy imaging of living hMSCs at 2845 cm^{-1}.

These images demonstrated the possibility to image in 3D live samples without any staining procedures using CARS microscopy technique. Since the OPO was tuned to image the CH_2 stretch resonance at about 2845 cm^{-1}, the images show rich lipids compartments such as the cell membranes and the internal liposomes responsible of the brighter spot in the images.

Multimodal CARS and SHG microscopy for label-free detection of collagen produced by hDFs in fibrin gel

Introduction

Tissue engineering and regenerative medicine are young research fields with the main goal to replace or repair damaged tissues and organs. The most established application field is the skin regeneration. Skin burns and diseases are the most common cause of skin damages and artificially produced dermis has been successfully implanted to replace the damaged tissue since the 1980s [95].

Type I collagen is the most abundant protein of skin providing, together with elastin, elastic properties, and with glycosaminoglycans, mechanical properties and is widely used as scaffold [96,97]. Collagen is synthesized by fibroblasts and in in vitro tests is a biomarker of fibroblasts functionality [98,99]. Autologous skin grafting is the main goal of the recent clinical research with the use of fibroblasts from the patient, seeded into a scaffold of fibrin gel and the implantation into the patient of the cells-scaffold construct [99]. The use of non-invasive techniques can allow an early detection of collagen production by human fibroblasts into scaffolds of fibrin gel in order to investigate this biological and natural process as marker of cell-scaffold interaction to translate the understandings into the clinic, extremely interested in the use of scaffold and autologous cells for tissues regeneration.

The more traditional methods for collagen detection such as the use of collagen specific antibodies and the tissue-specific dyes in Masson's Trichrome, or techniques that require more specialised operations namely matrix-assisted laser desorption/ionization (MALDI) time of flight (ToF) mass spectroscopy (MS) need a manipulation of the sample avoiding also collagen detection in live sample.

In this doctorate thesis label-free combined CARS and SHG microscopy techniques have been used as powerful tool to follow the cells behaviour in cell-scaffold construct for regeneration of tissues. Imaging of histological section of hDFs seeded in fibrin gel scaffold and imaging of collagen produced by hDFs in a time course experiment at different culture days (0, 7, 21, 42) is performed. A study on the limit of collagen detection of the imaging system is reported using sample prepared with different collagen concentrations. The results show that also the small amount of collagen produced by hDFs after few hours of incubation in fibrin gel is detected. Co-localization of hDFs and detected collagen is also reported in function of the culture days.

Methods

In order to focus the excitation beams on the samples it was used an oil immersion objective (Olympus UPLASAPO 60XO; NA=1.35; W.D.=0.15 mm), fully compensate for both spherical and chromatic aberrations in the UV-VIS-NIR region. Forward de-scanned CARS and SHG signals have been collected through an Olympus UPLSAPO 20x objective; NA=0.75; W.D.=0.6 mm and focused on a PMT (model R3896, Hamamatsu) with a plane-convex lens with a focal length of 25 mm.

Cell membranes and rich lipidic structures were imaged using CARS looking for the CH_2 symmetric stretch Raman modes around 2844 cm^{-1}, tuning the pump and the Stokes beams to 924.1 nm and 1253.7 nm respectively and generating the CARS signal at a wavelength around 731.8 nm.

Collagen structures were imaged using SHG technique, tuning the OPO signal at 950 nm and detecting the corresponding halved wavelength at 475 nm.

Bandpass filters centered at 716 nm with 43-nm bandwidth (FF01-716/43, Semrock), 480 nm with 20-nm bandwidth (BP470-490, Chroma Technology) all coupled with shortpass filters with 770nm of cut-off wavelength (FF01-770/SP, Semrock) are used before the detector to further block the residual excitation beams and transmit the CARS and the SHG signals respectively.

CARS and SHG imaging are obtained on the same sample. The two measurements were performed sequentially and SHG images at low collagen concentrations were enhanced using ImageJ software.

The limit of collagen detection of the optical system was investigated using as reference material a set of five histological sections of fibrin gels spiked with various concentrations of rat tail collagen (1 mg/ml, 0.1 mg/ml, 0.05 mg/ml, 0.01 mg/ml and fibrin gel only) prepared by Dr. G. Morley at the LGC laboratories (Teddington, UK), by following the briefly described protocol.

The fibrin gels were made by mixing in a 8 well plate thrombin (25 µg/mL) with neutralized collagen (1 mg/mL) and finally adding fibrinogen (5 mg/mL). The solution was then allowed to set at room temperature, then the gels were dehydrated in a graded series of ethanol washes and embedded in a low-melting paraffin wax to be sectioned (sliced of 10 µm and 30 µm) by the use of a microtome. Finally the wax was removed and the samples were rehydrated.

Results and Discussion

Images of fibrin gel spiked with rat tail type I collagen were obtained using SHG microscopy, doing a single slice measurement with a XY pixel pitch ranging from 0.164541 to 0.333333 µm/pixel depending by the analysed sample. For each experiment up to 7 images were averaged using an adaptive Kalman filtering.
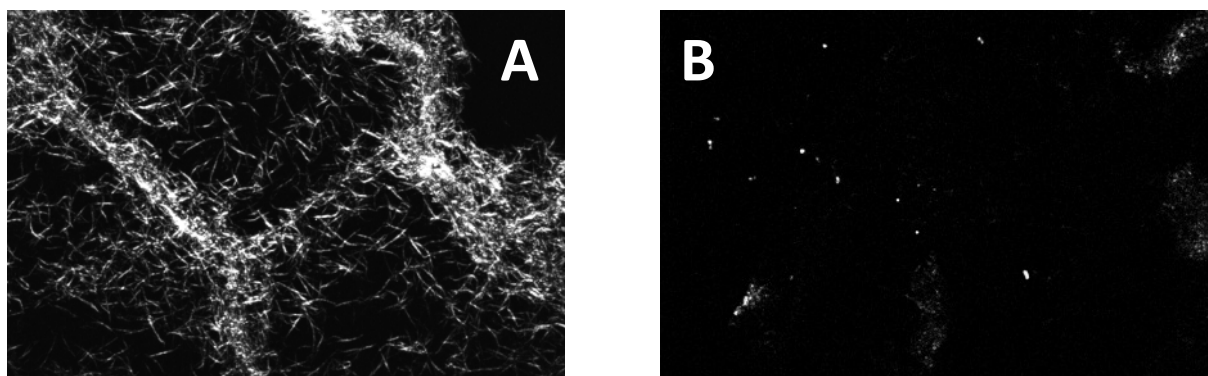

Fig. 5.5 Images of fibrin gel spiked with collagen (in white) at concentrations of 1 mg/ml (A) and of 0.1 mg/ml (B)

At collagen concentrations lower than 0.05 mg/mL the SHG signal starts to be weak and image processing is necessary to improve signal contrast. SHG signal strength of acquired images (Fig. 5.6A) is improved using ImageJ software doing a contrast enhancement (0.4% of saturate pixels with histogram equalization – Fig. 5.6B) followed by a Gaussian blurring (one pixel size – Fig. 5.6C) and a manual adjustment of the brightness/contrast levels (Fig. 5.6D).
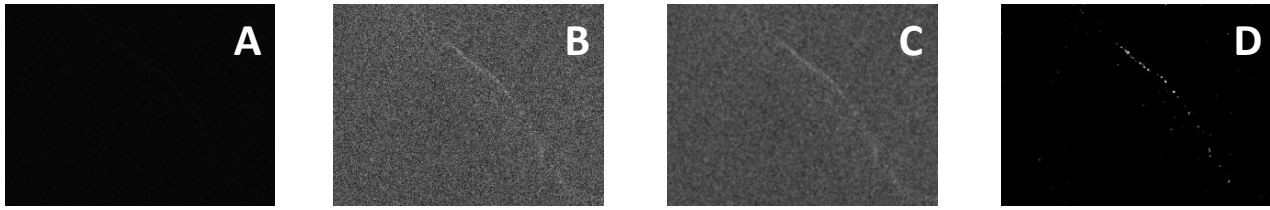
Fig. 5.6 SHG signal strength of acquired images (A) using contrast enhancement (B), Gaussian blurring (C) and manual adjustment of the brightness/contrast levels (D).

Using this method it is possible to localize also low concentrations of collagen pushing the limit of detection beyond the lowest collagen concentration spiked in the fibrin gel. Collagen traces (in white) detected using SHG technique in fibrin gel spiked with collagen at concentration of 0.05 mg/ml and 0.01 mg/ml are showed in Fig. 5.7A and Fig. 5.7B respectively. At low concentrations collagen tends to agglomerate in small spots with a size of some µm. In Fig. 5.7C is showed the sample with only fibrin gel and no collagen and no relevant signal was detected using SHG technique, confirming the absence of collagen in the sample.

Fig. 5.7 SHG images of collagen (in white) spiked in fibrin gel with at concentrations of 0.05 mg/ml (A) and of 0.01 mg/ml (B) and without collagen (C).

Sections of fibrin gel containing hDFs producing collagen, were prepared by LGC as described: fibrin gels were made by mixing in a 8 well plate thrombin (25 µg/ mL) with cell growth medium containing hDFs ($1x10^6$ cells in 315 µl of medium) and finally adding quickly fibrinogen (5 mg/mL). The solution was then allowed to set at room temperature and then the gels were fixed with 4% paraformaldayde (PFA). Then the gels were embedded in a low-melting paraffin wax and sectioned (sliced of 10 µm and 30 µm) with a microtome. Finally the wax was removed and the samples were re-hydrated.

Collagen produced by hDFs was detected and localized in a time-course experiment using several histological sections of cells fixed at different days in culture (day 0, 7, 21, 42).

Three-dimensional imaging was done slicing the sample with a step of about 800 nm in the Z-axis between two adjacent slices. The 3D extension of the cells in the fibrin gel determined the overall Z scan range. The XY pixel pitch was equal to 0.15875 µm/pixel for the measurement related to the day 0 and equal to 0.333333 µm/pixel for all the other measurements. The acquired image size was adapted to cells shape in each experiment. Each slice was obtained adaptively averaging with a Kalman filter up to seven images and the 3D imaging lasted about 5 minutes according to the Z scan range. Pixel dwell time was around 10 µs and the average power at the sample was about 25 mW for the pump signal and less than 10 mW for

Stokes signal. Cells were localized using CARS microscopy looking for cells rich lipidic structures in correspondence of the CH_2 symmetric stretch at 2844 cm^{-1}.

Collagen detection was done using SHG microscopy technique doubling the imaging experiment and keeping the same dimensional and temporal parameters of the related CARS imaging and average excitation power at the sample was about 20 mW.

At the end of the experiment a maximum intensity Z-projection image was extracted from the obtained slices, creating a single picture with all the interesting extension of the occupied cells volume.
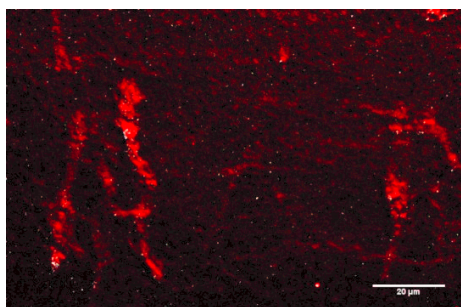

Fig. 5.8 Image of hDFs (in red) and collagen produced (in white) at culture day 0.

As it is showed in Fig. 5.8, on day 0 there is not an important amount of collagen (in white) produced by hDFs (in red). However, some small collagen traces are visible indicating an initial collagen production by hDFs already in the first hours of incubation inside the fibrin gel. The poor spreading of the SHG signal is with high consistency generated by collagen and native cell autofluorescence can be excluded using 950 nm as excitation wavelength for SHG.
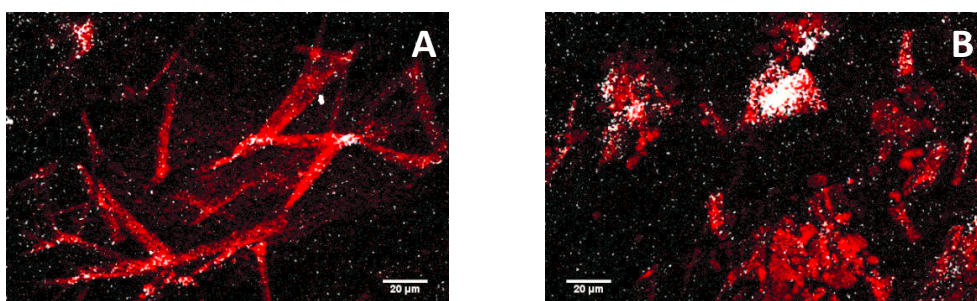

Fig. 5.9 Image of hDFs (in red) and collagen produced (in white) at culture day 7 (A) and culture day 21 (B).

On day 7 and day 21, as it can be seen in Fig. 5.9A and Fig. 5.9B respectively, hDFs (in red) produced a clearly localizable amount of collagen (in white). On day 21, collagen seems to be well organized on the whole surface of a group of cells.

In Fig. 5.10 is showed the image acquired with the hDFs on day 42. The cells (in red) produced a high amount of collagen (in white) that covers widely most of the cells surface.

In order to quantify the amount of collagen produced by the hDFs in culture and, at the same time, to evaluate the spatial localization of collagen in respect to the cell location, the co-localization percentage of the collagen surface over the cells surface was analysed. In this way it is possible to study the production of collagen in terms of spatial and temporal increase of its amount.

A specific ImageJ plug-in, written in Java language, was developed in order to analyse the co-localization percentages of all the samples investigated at different culture days, starting from binary images obtained using a manual threshold of hDFs (CARS) and collagen (SHG) acquired images.

The co-localization algorithm creates a new binary image (Fig. 5.11C) in which only the common surface belonging to both input images (Fig. 5.11A and 5.11B) are visualized.
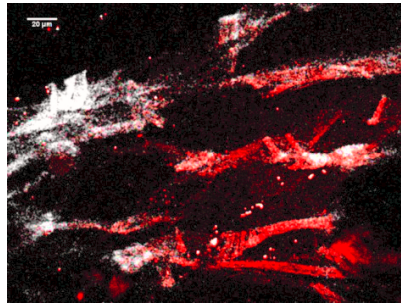

Fig. 5.10 Image of hDFs (in red) and collagen produced (in white) at culture day 42.

Then the percentage of the common surface (Fig. 5.11C) with respect to the total cells surface referred to the CARS binary image (Fig. 5.11A) is computed.
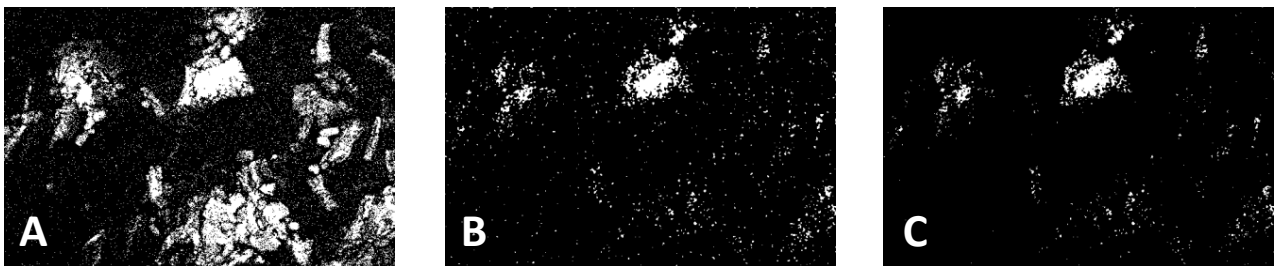

Fig. 5.11 Binary images of hDFs acquired with CARS technique (A), of collagen produced by hDFs acquired with SHG technique (B) and the colocalization image of hDFs and collagen (C).

As it could be expected, the co-localization percentage increased with the culture time (Fig. 5.12), indicating that collagen produced by the hDFs increased with time.
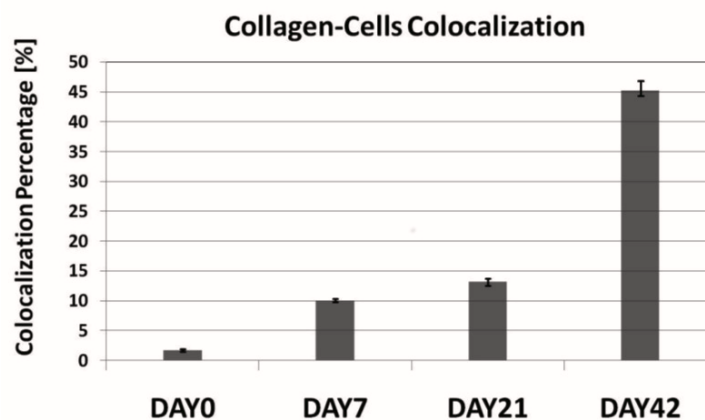

Fig. 5.12 Co-localization percentage of the collagen surface referred to hDFs surface at different culture days.

Conclusion

This work demonstrates that CARS - SHG combined technique is a powerful tool to follow label free cells behaviour, in this case the collagen production, by detecting and localizing in a 3D fibrin gel matrix both cells (i.e. hDFs) and cell-produced collagen.

The microscopy limit of detection for collagen has been investigated in the fibrin gel with spiked collagen at different concentrations and a limit down to 0.01 mg/ml was proved.

This low detection limit allowed testing the cells production of collagen in a very early cell culture (less than 24 hours).

In order to better understand the amount of collagen produced in relation with the cells surface, an analysis was conducted estimating the co-localization of the SHG (collagen) signal and the CARS (cells) signal with a specifically developed imageJ plug-in. With this method it was possible to determine that collagen co-localization percentage tends to increase with the culture time showing that on day 42 about the half of the entire cell surface was covered with collagen.

This work has been presented in the world conference on regenerative medicine 2011 (Leipzig, Germany) [143].

Acknowledgements

CARS and SHG microscopy to follow the collagen production in living human corneal fibroblasts and mesenchymal stem cells in fibrin gel 3D cultures

Introduction

In regenerative medicine, human mesenchymal stem cells (hMSCs) cultured on biomimetic scaffolds are induced to differentiate in order to facilitate regeneration of in vivo tissues and organs. In vitro experiments are necessary to monitor the interactions between scaffold and stem cells before implanting the cell-scaffold construct.

One of the first evidence of hMSCs differentiation is the formation of an extracellular matrix (ECM) where the collagen protein is its main component.

The biomimetic scaffolds offer to MSCs the native physiologic-like and three-dimensional (3D) environment that surrounds in vivo tissues and organs [100]. Recent studies showed that scaffold chemical composition, internal architecture and stiffness are parameters of influence for the behaviour and functions of the cells seeded on it [42,46,101]. This makes extremely important the study of interactions between scaffold and stem cells in vitro before implanting the cell-scaffold construct.

Stem cells are surrounded in their native environment by a 3D extracellular matrix (ECM) produced by fibroblasts. ECM is involved in regulating cell behaviour (e.g. survival and proliferation) and function (e.g. differentiation) and several proteins and other macromolecules compose it. Collagen is the main ECM component and the most abundant protein in mammals and it gives a mesh structure to the ECM [102,103]. In stem cell biology, it is well known that MSCs cultured in vitro can differentiate producing ECM when chemically or mechanically stimulated [104-107].

Collagen production represents one of the first steps of ECM formation [103] and it is normally assessed in vitro as an indicator of cell differentiation process [108-110]. Collagen shows a non-centrosymmetric ordered triple-helix structure with a very high level of crystallinity, which is a suitable condition for enabling second harmonic generation (SHG) process [111]. Stem cell behaviour can be studied through their morphology changes, thus their membrane image is a suitable tool for cell behaviour studies in interaction with scaffolds. CARS microscopy, providing chemical contrast from Raman active CH_2 symmetric stretching vibration wavenumber at around 2845 cm^{-1}, provides high contrast membranes morphology.

The dynamics of collagen production can be studied as a biomarker of cell differentiation with several techniques. However, conventional techniques are destructive and/or invasive, require manipulation of the cells, do not provide information about spatial distribution of the protein: gene expression analysis contemplates destructive procedure steps and loses the spatial distribution of the protein; western blot protein analysis needs to destroy samples and requires a consistent amount of protein; immunofluorescence techniques for protein analysis give information on the spatial distribution of the protein but samples need to be fixed and/or sectioned. Moreover, traditional techniques are unable to follow, in non-destructively short and long-term experiments, the dynamic distribution of collagen produced from the same sample.

A non-destructive and non-invasive procedure based on CARS and SHG combined microscopy, able to investigate the cell-scaffold interaction in terms of cell behaviour and functions over the time is discussed in this work. The aim of this work is to demonstrate that a combined CARS and SHG microscopy is an

adequate and optimal technique to follow on different time scales the collagen produced by cells cultured in a 3D scaffold, with a non-invasive, non-destructive and label free method.

This study aims to demonstrate the efficacy of the CARS-SHG combined technique to investigate, in a non-invasive and non-destructive experiment, the dynamics and the distribution of the collagen produced by living stem cells seeded in a 3D fibrin scaffold. The monitoring of stem cell differentiation within a scaffold in a non-destructive way will be an important advantage in regenerative medicine and tissue engineering field.

The collagen produced by living human corneal fibroblasts (hCFs) and human mesenchymal stem cells (hMSCs) seeded in a fibrin hydrogel matrix has been monitored over time.

Fibrin hydrogel is largely used in regenerative medicine as a biodegradable, biocompatible and non-cytotoxic scaffold, capable of inducing both osteogenic and chondrogenic stem cell differentiation [112,113]. This scaffold is known to support and to stimulate, through its composition and stiffness, in absence of any external chemical and mechanical stimulus, stem cells differentiation with collagen production [114]. hCFs have been chosen as positive control for collagen production because they are normally devoted to produce collagen in vivo and they maintain this property also when cultured in vitro [115-117].

hMSCs have been chosen to investigate the fibrin hydrogel capability to induce stem cells collagen production without any other kind of stimuli. Multiphoton microscopy based on the combination of multiple nonlinear optical phenomena like CARS and SHG [118], allows deep tissue penetration [9] and high 3D spatial resolution [25] that are powerful requirements in analysing thick tissue sections at cellular level. CARS microscopy provides chemical contrast from Raman-active molecular vibrations and it is able to detect membranes and lipid droplet compartments in living cells, like fibroblasts [28,94] tuning the excitation sources to the CH_2 symmetric stretching vibration wavenumber at around 2845 cm^{-1}. SHG microscopy is based on a second order nonlinear optical process and it is sensitive to the molecular structures, enabling a strong imaging contrast for non-centrosymmetric molecular ordered structures such as collagen [112,119-123], microtubule arrays [124], skeletal muscle myosin [22,125], cell membranes [126], and also cellulose [23]. Recently it has been demonstrated that CARS and SHG techniques can be easily combined together in the same microscopy allowing multiple chemical contrasts [127-130].

Experimental

In order to focus the excitation beams on living cells samples, a water immersion objective (LUMPLFLN 60XW NA= 1 W.D. = 2mm, Olympus), fully compensate for both spherical and chromatic aberrations in the UV-VIS-NIR region. The water immersion objective was cleaned and sterilized with a solution of 70% ethanol in water (v/v) before each imaging experiment.

Forward de-scanned CARS and SHG signals have been collected through an Olympus UPLSAPO 20x objective; NA=0.75; W.D.=0.6 mm and focused on a PMT (model R3896, Hamamatsu) with a plane-convex lens with a focal length of 25 mm.

Cell membranes and rich lipidic structures were imaged using CARS looking for the CH_2 symmetric stretch Raman modes around 2844 cm^{-1}, tuning the pump and the Stokes beams to 924.1 nm and 1253.7 nm respectively and generating the CARS signal at a wavelength around 731.8 nm.

Collagen structures were imaged using SHG technique, tuning the OPO signal at 950 nm and detecting the corresponding halved wavelength at 475 nm.

Bandpass filters centred at 716 nm with 43-nm bandwidth (FF01-716/43, Semrock), 480 nm with 20-nm bandwidth (BP470-490, Chroma Technology) all coupled with shortpass filters with 770nm of cut-off wavelength (FF01-770/SP, Semrock) are used before the detector to further block the residual excitation beams and transmit the CARS and the SHG signals respectively.

In order to prevent sample damages and optimize the output signal the excitation beams were attenuated through a neutral density variable filter wheel (NDC-50C-4M, Thorlabs).

CARS and SHG imaging are obtained in sequence on the same sample and SHG images were processed using ImageJ software in order to enhance their contrast.

FluoView Olympus software was used to acquire the images; the acquisition speed was about 9 µs/pixel. SHG images were processed using ImageJ software to enhance their contrast; a detection limit enhancement (0.4% of saturate pixels with histogram equalization) followed by a Gaussian blurring (one pixel size) and a manual adjustment of the brightness/contrast levels were performed.

Coherent anti-Stokes Raman scattering and SHG imaging are obtained on the same sample in sequence for a total observation time ranging between 10 and 20 min. This time is negligible with respect to the characteristic time of the collagen production process and of the dynamic of a cell in the fibrin hydrogel, allowing assuming a simultaneous observation of the cell morphology and of the produced collagen distribution.

Coherent anti-Stokes Raman scattering microscopy at 2844 cm^{-1} was performed in three dimensions at Z-axis steps of about 800 nm. The overall Z scan range was chosen accordingly to the 3D extension of the cells in the fibrin hydro-gel. The XY pixel pitch was equal to 0.230178 mm/pixel for all the measurements, while the image size was adapted according to cells shape in each experiment. The overall 3D imaging lasted between 5 and 10 min according to the Z scan range, and for each Z -step up to nine images were acquired and adaptively averaged using a Kalman filter.

Pixel dwell time was 9.1 µs and the average power at the sample was about 25 mW for the pump signal and less than 10 mW for Stokes signal. The slices obtained for each experiment were used to create a maximum intensity Z-projection image to have in a single picture all the interesting extension of the cell in the measured volume.

Collagen detection was performed using SHG microscopy technique after CARS imaging, keeping the same dimensional and temporal parameters of the related CARS imaging. The average excitation power at the sample was about 20 mW.

A maximum intensity Z-projection was made also for SHG imaged slices, obtaining a single picture of the measured volume.

SHG Z-projected images were processed using ImageJ software contrast enhancement followed by a Gaussian blurring with one pixel size and a manual brightness/contrast adjustment. After these operations, the SHG intensity image became a quasi-binary image, holding all the information related to the collagen distribution in a clearer manner.

Cell selection and culture in fibrin gel scaffolds

Human mesenchymal stem cells were purchased from Lonza (Basel, Switzerland). They are bone marrow derived-hMSCs from a donor. They were expanded and maintained in a complete nondifferentiating growth medium (MSCBM, Lonza) supplemented with 10% fetal bovine serum (FBS), 2% L-glutamine, 0,1% antibiotics (gentamicin and amphotericin B) (Lonza).

Human corneal fibroblasts were kindly provided by Dr. Sizzano (Transplants Centre of the Regione Piemonte). hCFs were expanded, maintained and resuspended in Dulbecco's Modified Eagle Medium (DMEM) (Listarfish, Milano, Italy) containing 10% FBS, 1% L-Glutamine and 1% kanamycin. Cells were cultured until they reached 80–85% of confluency (cells surface per total area), then they were washed with 1x phosphate buffer saline (PBS), detached with 0.05% trypsin/0.53mM Ethylenediaminetetraacetic acid (EDTA), counted by means of a hemocytometer and suspended at 1×10^6 cells per 200 µL complete growth medium to be seeded within the scaffolds of fibrin gel. hMSCs were used at passage 8 and hCFs at passage 10 to prepare the fibrin gel and cells constructs.

Scaffolds of fibrin gel preparation

Fibrin gel scaffolds were prepared from fibrinogen and thrombin, both proteins involved in blood clotting. Fibrinogen (5 mg/mL) (Sigma, USA; Cat #.F8630) was reconstituted in PBS 1x and thrombin (25 µg/mL) (Sigma, USA; Cat #. T9549) was reconstituted in PBS 1x. 3D fibrin scaffolds containing cells were prepared in 35x10 mm cell culture dishes (CytoOne) by mixing in the following order: 325 µL of thrombin (25 µg/mL), 125 µL of medium, 200 µL of cell suspension, 1350 µL of fibrinogen (5 mg/mL). The components were allowed to polymerize undisturbed at room temperature for 5 min to obtain fibrin 3D scaffolds with thickness of about 4 mm. Then the scaffolds were covered with 2mL of cell culture medium and placed in an incubator for cell cultures under controlled conditions of temperature (37 °C) and CO2 (5%). Cells within the scaffolds were fed every 3–4 days by completely replacing the medium with fresh medium. Five samples were prepared for both cell types to be able to detect the collagen production at days 0, 7, 14, 21, 28 even in case of culture damage because of the measurement procedure.

Results and Discussion

Three-dimensional CARS imaging of living hCF and hMSC cells, cultured in a 4-mm-thick fibrin hydrogel scaffolds, was conducted in time-course experiment at different days in culture (day 0, 7, 14, 21, 28); in the same way the collagen produced by the same cells was detected and imaged using the second harmonic signal generated by the collagen itself.

For every measurement, cells to be imaged have been chosen in the thick scaffold, selecting those being in the fibrin matrix region with a mid confluence level (40% – 50%). A comparison between hCFs and hMSCs collagen production over time was performed, where hCFs represent the positive control for collagen production while the hMSCs allow a measurement of the fibrin hydrogel capability to induce stem cells collagen production without any other kind of stimuli. Even if submitted to subsequent measurements, cells in the fibrin gel were not contaminated, continuing to proliferate in culture. This is a further proof of using a non-invasive technique that does not induce any relevant influence on cell growth, thus allowing very interesting studies in unperturbed conditions.
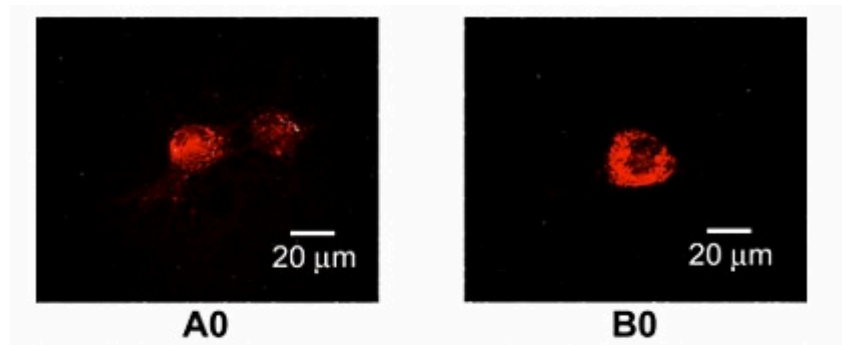
Fig. 5.13 Living human corneal fibroblast (A) and human mesenchymal stem cells (B) morphology in a 4-mm-thick fibrin hydrogel scaffolds (3D CARS imaging in red) and their collagen production (3D SHG imaging in white) at culture day 0.

At culture day 0 both hCFs (Fig. 5.13-A0) and hMSCs (Fig. 5.13-B0) did not show relevant SHG signal (white) since the cells (red) need some time in order to produce collagen and ECM. hCFs show a slight initial collagen formation, indicating that the technique has a very low limit of detection and it is able to detect collagen production after only few hours of culture. This result also indicates that using for SHG detection a sufficient narrow band optical filter and the excitation wavelength 950 nm, the eventual two-photon autofluorescence generated from natively fluorescent compounds inside the cells (like NAD(P)H or oxidized flavoproteins) is not an issue. The spurious white spots in the images outside the cells come from SHG signal enhancement process noise.
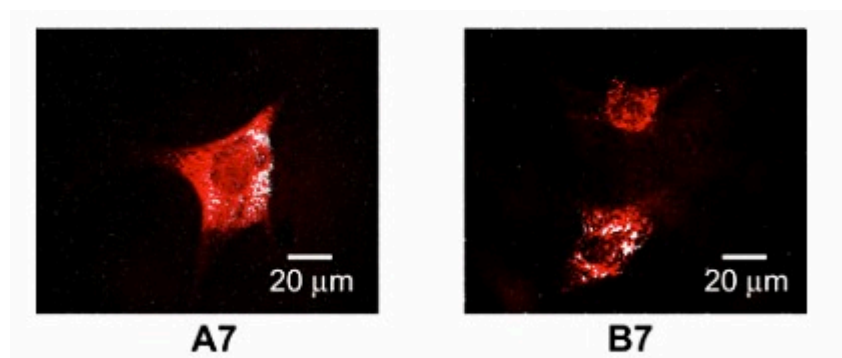

Fig. 5.14 Living human corneal fibroblast (A) and human mesenchymal stem cells (B) morphology in a 4-mm-thick fibrin hydrogel scaffolds (3D CARS imaging in red) and their collagen production (3D SHG imaging in white) at culture day 7.

At day 7 both hCFs (Fig. 5.14-A7) and hMSCs (Fig. 5.14-B7) show relevant SHG signal arising from collagen (white) produced by the cells (red). In hCFs sample collagen produced by the cells is clearly localized. In hMSCs sample some cells begin to produce collagen indicating an initial differentiation process with the formation of the ECM. This demonstrates that SHG microscopy can detect small amounts of collagen produced in a short period of time by living cells without fixing and staining the sample. Collagen production observed in hMSCs sample demonstrates the ability of this technique to discriminate the initial differentiation step in living stem cells.
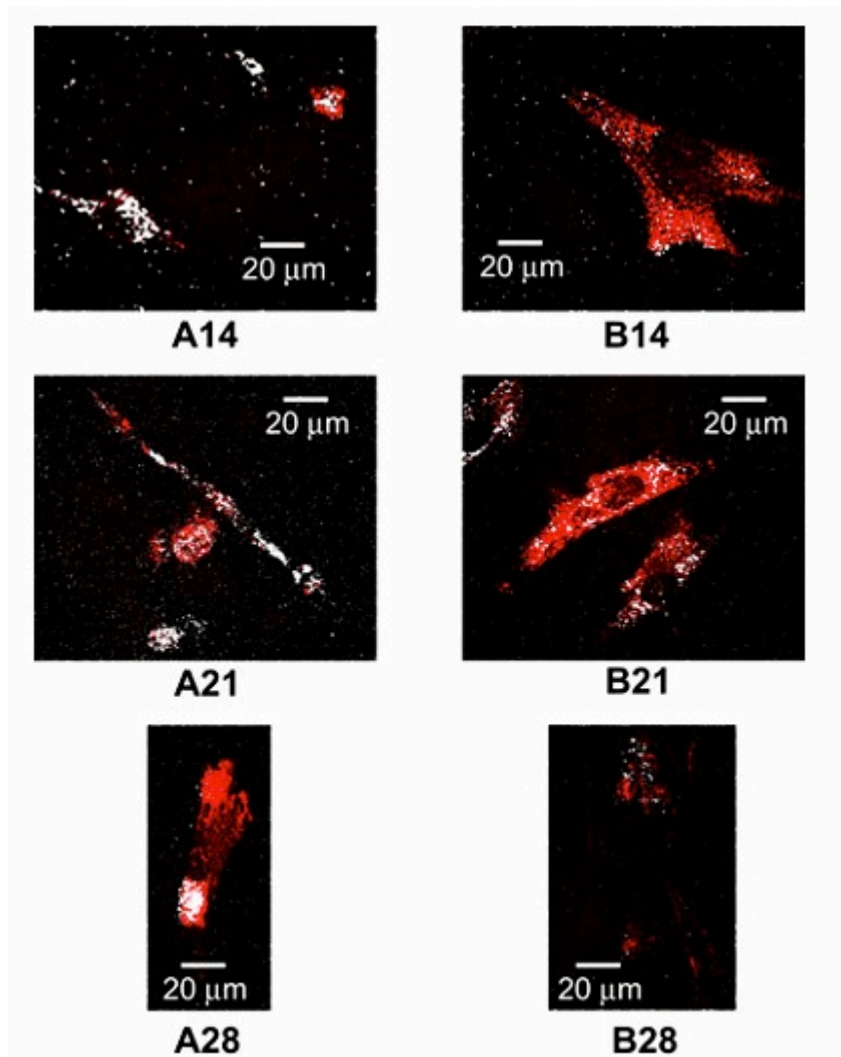
Fig. 5.15 Living human corneal fibroblast (A) and human mesenchymal stem cells (B) morphology in a 4-mm-thick fibrin hydrogel scaffolds (3D CARS imaging in red) and their collagen production (3D SHG imaging in white) at different days in culture (day 14, 21, 28).

The results obtained demonstrate that CARS and SHG microscopy is able to investigate non-invasively the stem cells differentiation induced by the scaffold, confirming that fibrin scaffold properties can induce stem cells differentiation. However, since collagen production is the first step of chondrogenic, fibroblastic and osteogenic differentiation processes [108,117], aspecific collagen detection does not allow determining what type of differentiation process is occurring.

Collagen was detected also at days 14, 21 and 28 for both hCFs (Fig. 5.15-A14, 5.15-A21 and 5.15-A28) and hMSCs (Fig. 5.15-B14, 5.15-B21 and 5.15-B28). In this experiment variations on the distribution of collagen produced by cells at different culture days is not biologically relevant, because images acquired on different analysed samples, are not related to the same cell or sample region. Cells in the fibrin gel are living and proliferating and continue to duplicate their number over the time in culture also after imaging. This is a further proof that laser power used for imaging is safe and does not induce any relevant influence on cell growth.

It is important to stress that, as it is shown in Fig. 5.15-B14, 5.15-B21 and 5.15-B28, hMSCs produced collagen also at days 14, 21 and 28, confirming that fibrin gel scaffold induced hMSCs to produce collagen and hence ECM.

In the majority of the acquired images, collagen produced by hCFs and hMSCs is not localized in correspondence of the nuclear region (the darker round shape region generally situated in the centre of the cells). This is a further confirmation that the collagen protein generates the SHG signal, because proteins are produced in the cytoplasm of the cell and not in the nucleus.

Conclusion

This work demonstrated the multimodal CARS and SHG microscopy as a powerful non-invasive label free technique to follow the collagen production in living cell 3D cultures. Its ability to image the cell morphology and the produced collagen distribution on the same sample at the same time, on a long term (4 weeks) experiment allowed to obtain important information about the cell-scaffold interaction and the ECM production. The very low limit reached in detecting collagen has permitted to map even the small amount of collagen produced by the cells in few hours of culture. This demonstrates multimodal CARS and SHG microscopy as a novel method to follow cells collagen production and cells differentiation process in both short and long term experiments.

As collagen production is considered a biomarker for ECM production and also a signal of initial stem cells differentiation, the study conducted on mesenchymal stem cell in 3D cultures confirmed the fibrin gel scaffold induces that differentiation stimulus.

The results of this work open new perspectives for tissue engineering and regenerative medicine to investigate rapidly and in a non-invasive way living cell cultures, enabling a better understanding of the interaction between cells and scaffolds and to detect early ECM formation, in the initial stages of the stem cells fibroblastic, chondrogenic and osteogenic differentiation processes. This technique offers advantages to characterize and to evaluate the performance of the cell-ECM/scaffold constructs before implanting them into the donor in pre-clinical and clinical applications of regenerative medicine.

This work has been published [144] and presented in the European conference on nonlinear optics spectroscopy ECONOS and CARS workshop 2011 (Twente, Netherlands) [145], as well as the world conference on regenerative medicine 2011 (Leipzig, Germany) [146] and the workshop on applications of coherent Raman scattering microscopy 2012 (Exeter, United Kingdom) [147].

Acknowledgements

NLO multimodal microscopy as a novel tool for scaffold characterization in 3D cell culture.

Introduction

In regenerative medicine and in tissue repair engineering, imaging and characterization of biomimetic materials such as scaffolds are of wide interest. In this field, it is important to understand the interactions between cells and scaffold in three-dimensional biological systems, since in most tissues, cells are surrounded by a tissue specific extracellular matrix (ECM) forming a structured microenvironment that allows cells to grow in specific high ordered assembly [131].

Scaffold characteristics like chemical composition, topographic features, and mechanical properties influence cells growing and tissue quality and several studies have been conducted in order to investigate these interactions [39-50]. The high complexity of this subject demands a diversified approach that makes use of several investigation techniques such as imaging at micro and nano scales, RNA profiles expression assays, protein analysis, etc. Moreover, scaffolds can be characterized using different measurement techniques. For instance, in scaffold morphological characterization, usually properties related to pore-solid distributions are measured, evaluating size, geometry and interconnectivity of the pores and the void-space ratio. Generally these properties are evaluated through imaging techniques [51] based on x-ray micro-tomography (micro CT) [52-54], scanning electron microscopy (SEM) [55,56] and through permeation of fluids [57-59], or atomic force microscopy (AFM) when nanostructure properties of the scaffold surface are analyzed [60]. Characterization of scaffolds from its biological behavior is also relevant [61] and usually it is done evaluating the cytotoxicity, the cell adhesion, proliferation, differentiation, mobility and migration, using several techniques like optical microscopy, laser scanning confocal microscopy, fluorescence, absorbance, gene expression, etc [62-66].

The micro CT technique is widely used for scaffold characterization and although it has important advantages like high spatial resolution together with high material penetration, which allow three-dimensional samples reconstruction, it makes use of an x-ray source that could be harmful for living samples. SEM makes use of an electron beam that does not allow analyzing living samples.

In this experiment is proposed the NLO multimodal microscopy, based on coherent anti-Stokes Raman scattering (CARS), two-photon excitation fluorescence (TPEF) and second harmonic generation (SHG) modalities, as an optimum way to characterize the scaffolds in the morphological and biological aspects. Multimodal CARS/SHG/TPEF microscopy, allowing the dynamic analysis of living samples in liquid media with high three-dimensional spatial resolution, is a strong tool to understand the interactions between cells and scaffold in complex *in vitro* experiments in a complete manner [9,25,118].

CARS microscopy has been used to characterize the morphology of two different artificial polymeric systems functionalized as bioactive scaffolds able to guide cardiac tissue formation from dissociated stem cells, both in air and in a culture medium and in presence of growing stem cells. The first scaffold, labeled as K-BC2000 (produced by the group of G. Ciardelli at Polytechnic in Torino, Italy) is a polyurethane based on 1,4-butane diisocyanate (BDI), polycaprolactonediol (MW 2000), and Ethyl L-lysine dihydrochloride(K) [133], the second one, labeled as PHBHV-gel (produced by the group of C. Cristallini at Institute for Composite and Biomedical Materials, National Research Council, Pisa, Italy) is a microstructured scaffold based on Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric) acid with gelatin from porcine skin [134]. SHG microscopy was used to morphologically characterize a collagen-based scaffold. Collagen based scaffolds are widely used for regeneration of skin [135] and also of other tissue such as for example bone [136], articular cartilage [137], cardiac valves [138] and mammary stromal tissue [139].

By imaging the same sample in the same conditions with CARS and SEM and with CARS and simultaneously TPEF, a quantitative comparison between the different techniques was possible and measurement artifacts were studied. Using chemical contrast from CARS, one of the scaffold (K-BC2000) was also characterized immersed in water, obtaining a three dimensional reconstruction of the scaffold itself and of the surrounding water; this permitted to highlight some interesting measurement artifacts. With the aim of demonstrating the ability of NLO multimodal microscopy to characterize the dynamic interaction between scaffolds and living cells, the PHBHV-gel scaffold seeded with human mesenchymal stem cells (hMSCs) was imaged for two consecutive days in a time-lapse experiment. The scaffold was imaged using CARS and the hMSCs, stained with calcein AM, were imaged simultaneously using TPEF. A 3D movie of the dynamic process was reconstructed.

Experimental

The signal-idler excitation scheme was chosen for this experiment because longer wavelengths resulted in deeper penetration of the samples. The OPO signal wave excited both SHG and TPEF processes with a higher efficiency then the idler.

For all the results provided in this experiment CARS and SHG signals were measured in forward detection, while TPEF signal was measured in epi-detection.

Polymeric scaffold structures were imaged using CARS looking for the methyl group stretching Raman modes around 2920 cm^{-1}, tuning the pump and the Stokes beams to 920.9 nm and 1259.5 nm respectively and generating the CARS signal at a wavelength around 735.7 nm.

The same pump beam with wavelength of about 920.9 nm was used to excite also cyanine3 fluorophore in TPEF microscopy imaging.

Collagen structures were imaged using SHG technique, tuning the OPO signal at 950 nm and detecting the corresponding halved wavelength at 475 nm.

Bandpass filters centred at 716 nm with 43-nm bandwidth (FF01-716/43, Semrock), 480 nm with 20-nm bandwidth (BP470-490, Chroma Technology) all coupled with shortpass filters with 770nm of cut-off wavelength (FF01-770/SP, Semrock) are used before the detector to further block the residual excitation beams and transmit the CARS and the SHG signals respectively.

A stepping motor moving the focusing objective provides the Z depth scanning. In the different experimental conditions several objective lenses were used to focus the excitation beams on the samples (Olympus UPLSAPO 20x NA=0.75, W.D.=0.6 mm, for dry samples; Olympus LUMPLFLN 60XW NA=1, W.D.=2mm, alternatively LUMPLFLN 40XW NA=0.8, W.D.=3.3mm, in aqueous solution), or to collect CARS and SHG forward signals (Olympus UPLSAPO 20x objective NA=0.75, W.D.=0.6 mm, alternatively Olympus UPLSAPO 10x objective NA=0.4, W.D.=3.1 mm). A neutral density variable filter wheel (NDC-50C-4M, Thorlabs) regulated the excitation beams powers in order to prevent sample damages.

A scanning electron microscope Hitachi TM3000 was used to image a piece of K-BC2000 scaffold to compare the results with those obtained using CARS microscopy. This type of scanning electron microscope allows to image samples without a specific preparation such as metallic coating for non-conductive samples due to observation under variable vacuum pressure. The sample of K-BC2000 was placed on the sample

holder inside the observation chamber of the microscope and it was imaged using a voltage of 5 KV and a low magnification (60x).

A mini-incubator system was designed, realized in-house and integrated under the microscope to maintain in sterile conditions and minimize perturbations during long time experiments. This mini-incubator accommodates a standard Petri dish of about 60 mm of diameter (Cat No.: 628160, Greiner), and maintains a temperature of about 37 °C. Stable temperature is obtained by an electric control using the free heat exchange between the chamber and the laboratory temperature (controlled at 20 ± 0.1 °C over the days) as a controlling parameter. A thermal calibration of the incubator to maintain the culture medium temperature at about 37 °C over the days, has been done before performing time-lapse experiments with living samples. Moreover, the focusing water immersion objective lens was cleaned and sterilized with a solution 70% ethanol in water (v/v) to avoid sample contamination. As a further solution to minimize sample contamination and culture medium evaporation, the objective lens and the incubator Petri dish holder were sealed by mean of a thin sterile lattice tube and a couple of o-rings. However, an average evaporation of culture medium of about 100 µl per hour was measured. An intravenous cannula, piercing the lattice, during the time-lapse experiment, was used to periodically add culture medium together with calcein AM to compensate medium evaporation and calcein AM bleaching.

Mesenchymal stem cells (hMSCs; Lonza) were cultured in cell culture flasks with cell culture medium (DMEM from Sigma Aldrich with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin) until they reach the 80% of confluence. Cells were detached from the culture flasks using a trypsin/EDTA solution (Invitrogen) and counted using a hemocytometer. 10^5 cells were fluorescently stained with Calcein AM (Invitrogen), a vital staining specific for living cells only: the cell suspension (in cell culture medium) were centrifuged at 1000 g for 5 minutes in a centrifuge tube to obtain a pellet of cells and 500 µl of PBS containing calcein AM 4µM was used to re-suspend the pellet. After 10 minutes at 37 °C the cell suspension (in PBS/Calcein AM), stained cells were washed in PBS to eliminate the excess of Calcein AM. The pellet of stained hMSCs has been re-suspended in 100 µl of cell culture medium and this volume (a drop) was seeded on the top surface of the scaffold PHBHV. The scaffold, with the drop containing cells, was incubated in a Petri dish at 37 °C and 5% CO_2 for 1 hour to allow the cells in the drop to invade the scaffold. Then, the scaffold with cells was attached to a Petri dish to clamp the sample during the imaging. To this purpose it was used the fibrin gel made by mixing thrombin (12,5 µg/ml) and fibrinogen (5 mg/ml) at 1:2 ratio. This solution is liquid at 4°C but gradually became gel at room temperature. A small drop (10 µl) of fibrin gel is added on a dry Petri dish and was placed quickly on the fibrin gel drop. After 5 minutes at 37 °C the scaffold is glued to the Petri dish. Then, 10 ml of cell culture medium was added to the Petri dish and incubated at 37 °C and 5% CO_2 over night to allow cells to adhere to the scaffold and to migrate into the scaffold internal structure.

Results and discussion

CARS microscopy imaging was compared with SEM imaging (Hitachi TM3000) acquiring the same portion of K-BC2000 scaffold using the two techniques, to validate qualitatively the matching of the two results. CARS microscopy was also compared with TPEF microscopy by simultaneously imaging a slab of K-BC2000 scaffold stained with cyanine3 fluorophore.

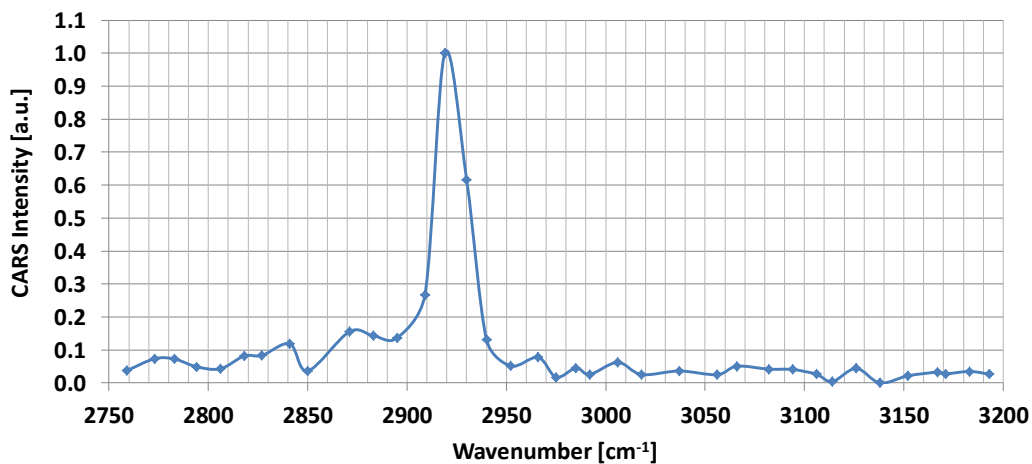## CARS measured spectrum of PHBHV/gel scaffold

Fig. 5.16 Measured CARS spectrum of PHBHV/gel scaffold.

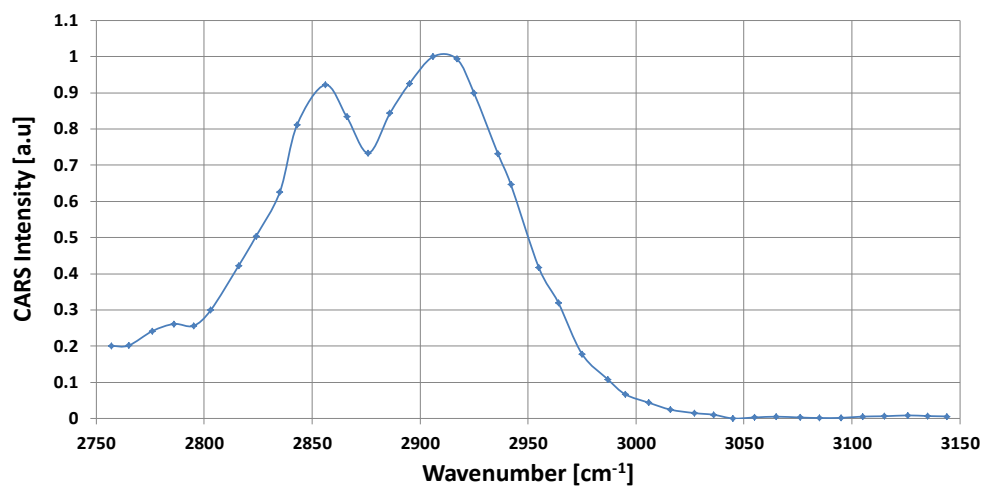## CARS measured spectrum of K-BC2000 scaffold

Fig. 5.17 Measured CARS spectrum of K-BC2000 scaffold.

The CARS spectrum of the two scaffolds was detected and recorded in the wavelength range between 2750 cm^{-1} and 3150 cm^{-1}. For both scaffolds a consistent CARS peak was found around 2920 cm^{-1}. This wavelength was used to image the two structures.

CARS three-dimensional reconstruction of a dry fragment of K-BC2000 architecture was done using a focusing 20x objective lens and a 10x collecting lens. A region with a size of about 1414x1414x350 μm^3 was measured doing a Z stack of 141 images of 1024x1024 pixels with a Z step of 2.5 μm. The stack of images was imported on ImageJ and after setting up the voxel size parameters it was possible to evaluate the 3D scaffold structure using 3D viewer plug-ins (Fig. 5.18).
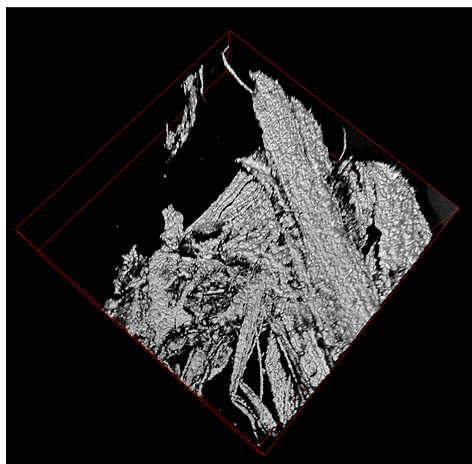
Fig. 5.18 Three dimensional view of K-BC2000 scaffold imaged using CARS microscopy.

The same scaffold fragment was analyzed using SEM. A comparison between CARS and SEM images was done: using the maximum projection intensity process of ImageJ in which the stack of collected CARS images is reduced in a single image and overlapped with the image obtained by SEM technique (Fig. 5.19). It can be observed that the two images match almost perfectly and some details related to the material domains appear enhanced in CARS image with respect to SEM image. Moreover CARS microscopy has the capability to investigate the 3D structure of the material, while SEM technique gives information mostly related to the sample surface.
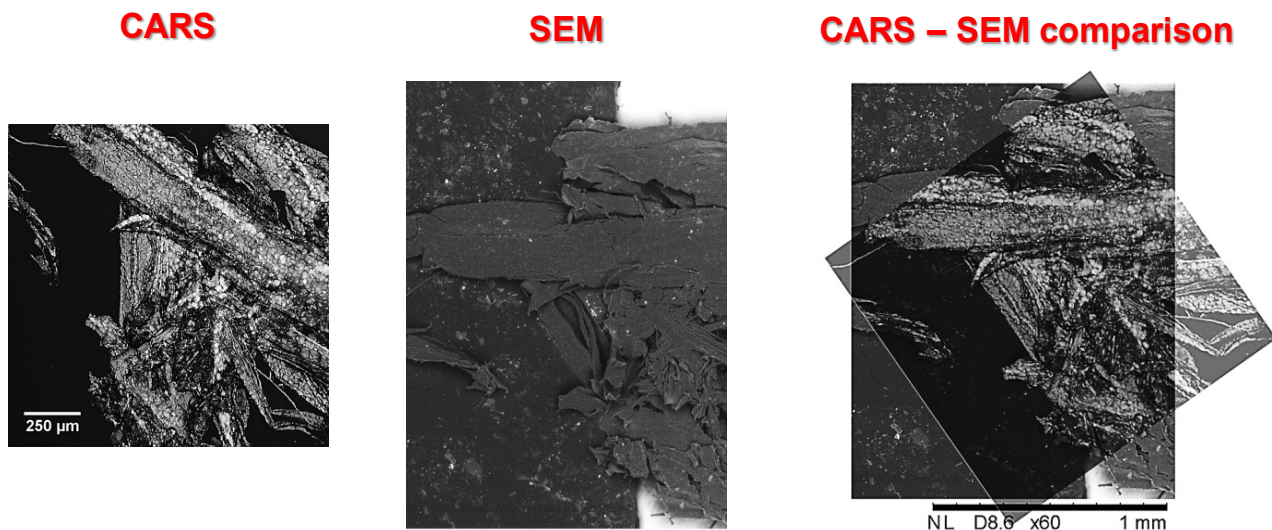

Fig. 5.19 Images of K-BC2000 scaffold acquired with CARS and SEM microscopy and overlap of the two images.

In this experiment some common shape descriptors have been used to characterize scaffolds from a morphological point of view. The parameters are: the *Pore Circularity* (a value between 0 and 1 indicating a thin long shape when approaches 0 and a perfect circle when approaches 1), the *Pore Roundness* (a value between 0 and 1 indicating an elongated shape when approaches 0 and a perfect circle when approaches 1), the *Equivalent Diameter* (the diameter that describes the circle that has the same pore area), the *Average Porosity* (the ratio between the total pores area and the total scaffold area) and the *Void Space Ratio* (the ratio between the total pores volume and the total scaffold volume).

$$Circ = 4\pi \frac{A}{P^2} \tag{43}$$

$$Round = 4\frac{A}{\pi(Major_{axis})^2} \tag{44}$$

ImageJ software has been used to extract these parameters from the scaffolds images acquired through multimodal CARS/SHG/TPEF microscopy. A binary process has been conducted on the images using the Otsu's thresholding method [74] and all the parameters excluding the Void Space Ratio have been extracted through the Analyze Particles routine of ImageJ. The binary process allows discriminating and localizing the scaffold among the whole sample. A special plug-in for ImageJ was developed in Java language, in order to compute the Void Space Ratio that is the ratio between the total pores volume and the total scaffold hindrance volume. Starting from the Z stack of binary images, the voxel was considered entirely occupied when scaffold appeared in the related pixels of the two adjacent images, half occupied (half voxel volume) when scaffold appeared on only one pixel of two adjacent images, empty when no scaffold appeared in the pixels of the two adjacent images. Later it is computed the sum of all the voxels where the scaffold appeared and since the interest is to determine the percentage of void space with respect to the total scaffold hindrance volume, the computed sum was subtracted to the total scaffold hindrance volume and the obtained value was then divided by the total scaffold hindrance volume. In the specific case since the analyzed images represent portion of the scaffold, the total scaffold hindrance volume coincides with the total sample volume. The *Equivalent Diameter* has been computed using a Matlab routine starting from the pores area extracted with ImageJ Analyze Particles tool and its average, mode, minimum and maximum values together with the average pores number per acquired slice have been reported.
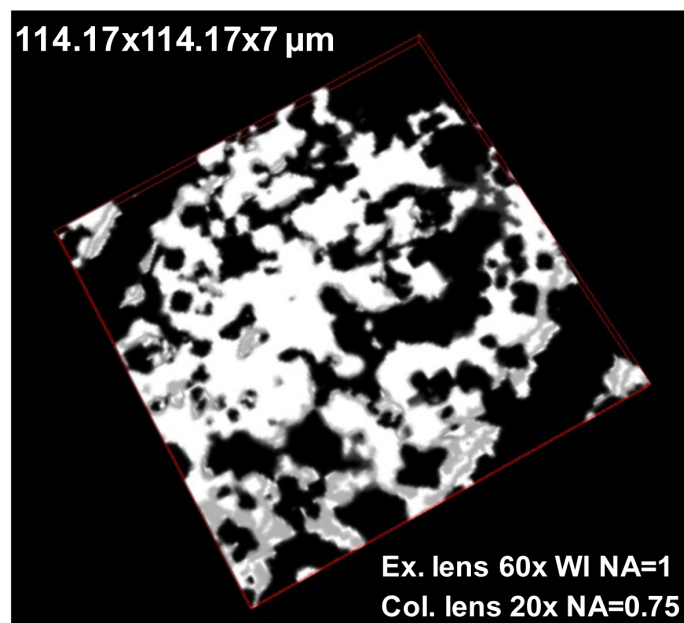

Fig. 5.20 Three dimensional view of a thin slab of PHBHV-gel scaffold imaged using CARS microscopy.

CARS three-dimensional reconstruction of PHBHV-gel scaffold architecture when immersed in culture medium was done. A water immersion 60x objective (LUMPLFLN 60XW NA=1, W.D.=2mm, Olympus) was used to focus excitation sources on the sample, while a 20x objective (UPLSAPO 20x objective NA=0.75, W.D.=0.6 mm, Olympus) was used to collect CARS signal arising from the scaffold in forward detection. A thin slab of the acquired scaffold with a volume size of about 114x114x7 μm^3 (Fig. 5.20) has been processed