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## New tools for non-invasive exploration of collagen network in cartilaginous tissue-engineered substitute

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**Abstract.** In tissue engineering approaches, the quality of substitutes is a key element to determine its ability to treat cartilage defects. However, in clinical practice, the evaluation of tissue-engineered cartilage substitute quality is not possible due to the invasiveness of the standard procedure, which is to date histology. The aim of this work was to validate a new innovative system performed from two-photon excitation laser adapted to an optical microscope to evaluate at macroscopic scale the collagen network in cartilage tissue-engineered substitutes in confrontation with gold standard histologic techniques or immunohistochemistry to visualize type II collagen. This system permitted to differentiate the quality of collagen network between ITS and TGF- $\beta$ 1 treatments. Multiscale large field imaging combined to multimodality approaches (SHG-TCSPC) at macroscopical scale represent an innovative and non-invasive technique to monitor the quality of collagen network in cartilage tissue-engineered substitutes before in vivo implantation.

**Keywords:** cartilage, tissue-engineered substitute, second harmonic generation, mesenchymal stem cells.

## 1. Introduction

Tissue-engineering (TE) is an emerging field, whose goal is to replace damaged tissues with artificial tissues constructs grown *in vitro* to mimic the composition and properties of the cartilage in a controlled laboratory environment by delivering the appropriate cells, biomaterials and signaling factors to diseased or damaged areas. The quality of *in vitro* tissue-engineered substitute is a key element to determine the success of the cartilaginous graft *in vivo* [1]. The differences in remodeling outcome using implanted engineered cartilage in an animal osteochondral defect model depend on the degree of cartilage maturity *in vitro* [2]. The collagen network inside TE-substitute is one crucial criteria of quality control. However, current evaluation methods are destructive and reduce the amount of graft material finally available. Conventional optical techniques, like histology (Sirius red) and fluorescence (specific antibody), remained a reference for structural characterization.

Unfortunately, their invasive character leads to development and validation of non-invasive techniques to detect collagen molecules based on near-infrared illumination as two-photon excitation (TPE) and Second Harmonic Generation (SHG). Combined with multiphoton fluorescence excitation, SHG microscopy has been demonstrated to be effective in numerous label-free tissue imaging applications without any exogenous dye, and without damaging the collagen proteins to observe non-centrosymmetric molecular assemblies, such as collagen fibers discrimination [3] and collagen architecture in cartilage [4].

Recently, this approach using SHG imaging constitutes a diagnostic application for detecting the initial symptom of OA, including degeneration of the cartilage matrix and pathological changes in other connective tissues [5]. Moreover, as SHG is based on a light scattering and not on fluorescence, photo-bleaching and toxicity are significantly reduced. This multimodality technique could be considered as a precise tool for biodiagnosis to detect in safety conditions collagen synthesized by cells seeded in scaffolds in presence of chondrogenic growth factors [6].

In this work, we showed for the first time that multiscale large field imaging (macroscopy) combined to multimodality approaches (SHG-TCSPC) at macroscopical scale could be an innovative and non-invasive technique to monitor the quality of collagen network in cartilage TE substitutes before *in vivo* implantation. TE substitutes were compared to empty scaffolds and MSC-seeded sponges without growth factor (TGF $\beta$ 1).

## 2. Materials and methods

### 2.1. Preparation of cartilaginous TE substitute

Bone marrows were obtained in informed patients who underwent total hip replacement surgery in collaboration with the “Service de Chirurgie Orthopédique at Traumatologique” and “Unité de Thérapie Cellulaire et Tissus” of Nancy University Hospital. Mesenchymal stem cells (MSCs) from bone marrow were plated at 37°C at 5% CO<sub>2</sub> with Dulbecco’s modified Eagle Medium (DMEM-LG, 31885, Gibco) supplemented with 10% of fetal bovine serum (FBS, Sigma), 1 ng/ml bFGF (R&D), glutamine and penicillin-streptomycin. During the last passage before seeding in biomaterials (passage 3), MSCs were cultured with DMEM 4.5 g/ml supplemented with sodium pyruvate (110µg/ml), bFGF (1ng/ml) penicillin-streptomycin, Proline (P), L-Ascorbic acid-2-Phosphate (A) and Dexamethasone (D). MSCs were seeded in collagen sponges composed of type I and III collagens from bovine dermis (Symatase) at 5x10<sup>5</sup> cells/sponge cultured in two different medium supplements: ITS 1% (non-chondrogenic medium) or ITS 1% + TGF-β1 (10 ng/ml) (chondrogenic medium).

### 2.2. Multiscale and multimodality imaging

*Immunofluorescence.* Collagen II produced by MSCs embedded in scaffold was marked by indirect immunostaining. The primary antibodies were a type II anti-collagen with the suitable secondary antibody, rabbit polyclonal antibody associated to Alexa 488<sup>TM</sup>. After 15 min washing with PBS, scaffolds were fixed with PFA 4% during 20 min and then deposited on glass slides 2 hours for observation at room temperature.

*Two-photon excitation in macroscopy (TPE-Ma).* For Two Photon Excitation system, a femtosecond oscillator (Mira 900F, Coherent), pumped with a solid laser (Verdi 8 W, Coherent) was used to generate some ultra-short infrared pulses (120 fs, 76 MHz) at  $\lambda=800$  nm. An EOM (Electro Optical Modulator) enabled to control the power of excitation. With a scan head specially delivered for NLO, we injected this multiphoton source into a microscope (MacroFluo<sup>TM</sup> Z6 APO A, Leica Microsystems Mannheim, Germany) to realize the first prototype of Two-Photon Excitation Microscope (TPE-Ma). The laser beam was focalized to the samples through an dry objective showing x5 magnification (NA = 0.5) and infrared beam analyzer (Imaging French Network, RTmfm, MRCT, CNRS) used to calibrate optical alignment.

*TCSPC-SHG imaging and F-SHG ratio.* Decay times were measured in the backward direction by a time-correlated single photon counting method (SPC-730 TCSPC Imaging module, Becker & Hickl, Berlin) coupled to scan-head of CLSM TCS SP2-AOBS (Leica

Microsystems). A signal coming from the scan controller of the microscope enabled to trace decay curves for each pixel of an image (128\*128). The signal has to be deconvoluted with calculated instrumental response function and analyzed for fitting with SPC Image software (Becker & Hickl, Berlin). The time slope of the decay is usually a sum of different exponential decays and an index (F-SHG ratio) was described as the ratio between the long lifetime (extrinsic fluorescence relative to Alexa488<sup>TM</sup> labeling) and very fast lifetime (SHG due to collagen) which is obtained by two exponential components fitting. Images were coded with a color LUT according to F/SHG index values.

*Second Harmonic Generation in macroscopy.* For the two photon excitation system, a femtosecond oscillator (Mira 900F from 600 to 1100 nm, Coherent), pumped with a solid laser (Verdi 8 W, Coherent) was used to generate some ultra-short infrared pulses (120 fs, 76 MHz) at  $\lambda = 800$  nm. An EOM (Electro Optical Modulator LM 0202 P 5 W – n°21181, EO-Crystal KD\*P 3x3 mm<sup>2</sup>, 5W, 660-1050 nm, transmission 90.1%, ratio 1/254, gain 57%) enabled controlling the excitation power (2.5 nJ per pulse at 76 MHz). With a scan head specially dedicated to NLO, CFS-DM6000 (Leica Microsystems Mannheim, Germany) the multiphoton source was injected into a macro-zoom (MacroFluo<sup>TM</sup> Z6 APO A, Leica Microsystems Mannheim, Germany) to realize the first prototype of SHG-Microscope [7]. The second harmonic signal was collected in the reflected light channel (Non Descanned PMT hybrid detector, gain 500) through a x5 dry objective (NA 0.5), at half the excitation wavelength ( $\lambda/2=400$  nm). All image acquisition procedures were performed at an excitation wavelength of 800 nm, with the pinhole fully opened (8.41 AU); a band-pass filter enabled selecting the detection spectral range (400/25 nm center wavelength/bandwidth), and rejecting the infrared excitation beam (BM690). The image control parameters were defined for gold standard (scaffold) or for experiments (ITS, TGF- $\beta$ 1) obtained for 10 accumulations and stored as z-stack of 3 image sequences (3,84  $\mu$ m along the z-axis). The acquired image size with a macro zoom of 47,1 fold was 512 x 512 pixels for a scan field of 210.62  $\mu$ m<sup>2</sup>, 8 bits in depth, at 100 Hz for each experiment. Simple image processing, i.e. three-dimensional (3D), rendering was performed using the LAS AF ver. 2.7 software package (Leica).

### 3. Results

#### 3.1. Two-photon excitation in macroscopy

On the figure 1 Part 1, three different scaffolds were observed by Multiphoton Excitation Macroscopy (TPE-Ma) in fluorescence at 488 nm excitation after Alexa488<sup>TM</sup> fluorescence indirect labeling for type II collagen. Native sponge without cells (A: SWC) presented just the basal autofluorescence of native collagen fibers of sponge. With no chondrogenic medium

(B: ITS), the labeling inside sponge corresponded to autofluorescence of cells and scaffold. In contrary, a strong signal of type II collagen was observed in ECM synthesized by MSCs inside the scaffold with chondrogenic medium (C: TGF- $\beta$ 1) after 28 days of culture.

### **3.2. F-SHG index obtained by TCSPC analysis**

For MSCs seeded in 3D scaffolds, challenge was to distinguish changes in collagen providing from fluorescence (F) signal and from Second Harmonic Generation (SHG) signal. The change in F-SHG index was monitored according to medium culture (supplemented or not) at 28 days and confirmed identity of type II neo-synthesized fiber collagen in scaffold and enhanced expression in the presence of TGF- $\beta$ 1. The results revealed a precise range of F-SHG index (from 2 to 8), distributed among 4 defined areas (I, II, III, IV) in relation with cellular behavior and metabolism (Figure 1 Part 2). The range detection of Alexa488<sup>TM</sup> fluorophore coupled to Mab against type II collagen (Mab col II) was restricted in part I (from 2 to 2.3).

Then, part II (from 2.3 to 3.3) concerned more specifically global autofluorescence (AF) coming from scaffold (type I collagen) and cells. SHG signal from the scaffold (with cells seeded or not) was strictly confined in area III with a range from 3.3 to 4.4. Finally, SHG signal relative to synthesized collagen by MSCs was significantly well distributed in the last range IV (from 4.4 to 8). For figure 1, for MSCs cultured in the presence of TGF- $\beta$ 1 or not (ITS), type II collagen has been specifically detected by immunolabeling in part A. However, coll II-SHG signal appeared only in the presence of TGF- $\beta$ 1 in part D, showing the abundant extracellular collagen synthesis and as an indication on organized and structured collagen fibers. This result indicated that a change in the collagen conformation and its ability to be detected as a harmonophore by TCSPC-SHG imaging. The part B was also informative as an indication on the cellular density in scaffold and proliferation and its effect on global autofluorescence.

### **3.3. SHG-imaging in macroscopy**

Direct observation of three different scaffolds by TCSPC-SHG macroscopy for the detection of collagen as a harmonophore is presented on figure 1 Part 3. In our three different culture conditions (SWC, ITS and TGF- $\beta$ 1), the SHG signal is detected only in sponges treated with TGF- $\beta$ 1 in comparison with sponges treated with ITS characterized by absence of type II collagen synthesis.

#### **4. Discussion**

In cartilage bioengineering, early detection according to degree of organization of collagen network could be a crucial step in the differentiation process of autologous cells seeded in a biomaterial. The amount of produced matrix and especially the synthesis of type II collagen and otherwise standardization of preclinical step of setting-up of one biomaterial is a crucial step.

In this work, we showed that SHG signal specific to collagen network is a non-invasive technology to monitor the quality of collagen network of TE substitutes before in vivo graft, in comparison with classical techniques as immunofluorescence using a specific anti-collagen type II antibody to localize specifically this synthesized protein. The major disadvantage of use antibodies was their cost, their limited penetration in tissue and the introduction of an exogenous dye. So, they are almost exclusively used on fixed sections and absolutely not on a precious biological sample reserved for graft in patients. The interest and the difficulty of this approach were to work on a biomaterial with collagen. The collagen sponges, already used in hospital in other applications, shows to be a biomaterial of choice for the tissue repair of the cartilage being given the very good preliminary results already obtained in vitro and in vivo (in comparison with a biomaterial with alginate for example, easier to study).

It will thus be necessary to consider for quite used techniques the obtained signal corresponding to the sponge it even and the signal corresponding to the collagen synthesized by cells. In conclusion, we have demonstrated with regard to the conventional techniques that SHG at the macroscopically scale is an innovative technique to evaluate noninvasively collagen network of cartilage TE substitute. This technique could be used in a clinical step to screen easily and rapidly a large number of TE substitutes in a same time without exogenous probe and without alteration of substitute to select the better for the graft.

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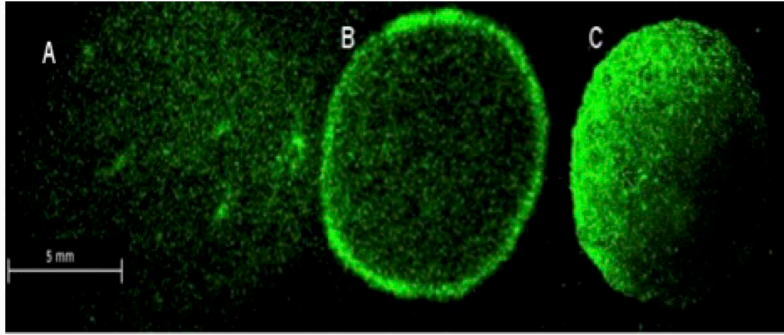
**Figure 1. Study of 3 different scaffolds after 28 days of culture:** sponge without cell (A), MSC-seeded sponge cultured with ITS (B), MSC-seeded sponge cultured with TGF- $\beta$ 1 (C).

**Part 1.** Direct observation of scaffolds by Multiphoton Excitation Macroscopy (TPE-Ma) in fluorescence at 488nm excitation after Alexa488<sup>TM</sup> fluorescence indirect labeling for type II collagen.

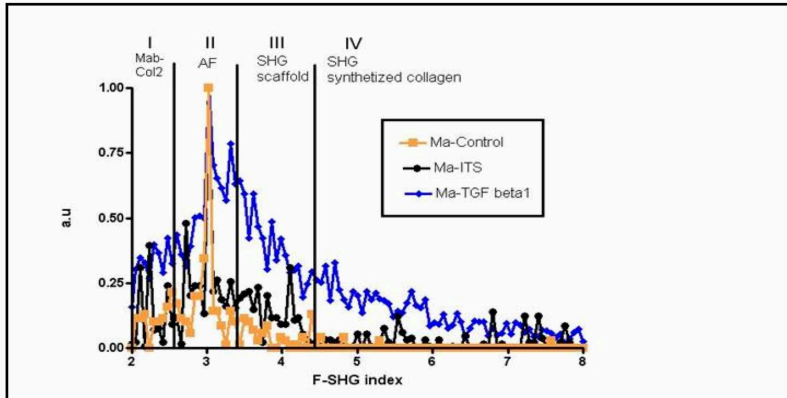
**Part 2.** Samples were analyzed in TCSPC-SHG macroscopy to extract F-SHG index, after immunolabelled with Mab directed against type II collagen. Distribution of F-SHG index is defined in four defined areas (segments I, II, III and IV) respectively in close relation with detection of type II collagen only (Mab-Col2), global autofluorescence (AF), SHG scaffold and SHG synthesized collagen.

**Part 3.** Direct observation of MSC-seeded scaffolds by SHG-Macroscopy (original figure: <http://link.springer.com/article/10.1186/s40634-015-0026-0#copyrightInformation>, © Cucchiarini et al.; licensee Springer. 2015, no changes were made).

**Part 1:**



**Part 2:**



**Part 3:**

