PROCEEDINGS OF SPIE

SPIEDigitalLibrary.org/conference-proceedings-of-spie

A miniaturized chip for 3D optical imaging of tissue regeneration in vivo

Claudio Conci, Emanuela Jacchetti, Laura Sironi, Lorenzo Gentili, Giulio Cerullo, et al.

Claudio Conci, Emanuela Jacchetti, Laura Sironi, Lorenzo Gentili, Giulio Cerullo, Rebeca Martinez, Roberto Osellame, Mario Marini, Margaux Bouzin, Maddalena Collini, Laura D'Alfonso, Elmina Kabouraki, Maria Farsari, Anthi Ranella, Nikos Kehagias, Giuseppe Chirico, Manuela T. Raimondi, "A miniaturized chip for 3D optical imaging of tissue regeneration in vivo," Proc. SPIE 12144, Biomedical Spectroscopy, Microscopy, and Imaging II, 121440D (27 May 2022); doi: 10.1117/12.2629824

SPIE.

Event: SPIE Photonics Europe, 2022, Strasbourg, France

A miniaturized chip for 3D optical imaging of tissue regeneration in vivo

Claudio Conci[a], Emanuela Jacchetti[a], Laura Sironi[b], Lorenzo Gentili[a], Giulio Cerullo[a,c], Rebeca Martinez[c], Roberto Osellame[c], Mario Marini[b], Margaux Bouzin[b], Maddalena Collini[b], Laura D'Alfonso[b], Elmina Kabouraki[d], Maria Farsari[d], Anthi Ranella[d], Nikos Kehagias[e], Giuseppe Chirico[b*] , Manuela T. Raimondi[a]

[a]Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, Piazza L. da Vinci 32, 20133 Milan, Italy; [b]Department of Physics, Università di Milano-Bicocca, Piazza della Scienza 3, 20126, Milan, Italy; [c]Institute for Photonics and Nanotechnologies (IFN)-, CNR and Department of Physics, Politecnico di Milano, Piazza L. da Vinci 32, 20133 Milan, Italy; [d]Foundation for Research and Technology-Hellas (IESL-FORTH), Crete, Heraklion, Greece; [e]Nanotypos, Thessaloniki, Greece

ABSTRACT

The current protocols for biocompatibility assessment of biomaterials, based on histopathology, require the sacrifice of a huge number of laboratory animals with an unsustainable ethical burden and remarkable cost. Intravital microscopy techniques can be used to study implantation outcomes in real time though with limited capabilities of quantification in longitudinal studies, mainly restricted by the light penetration and the spatial resolution in deep tissues. We present the outline and first tests of a novel chip which aims to enable longitudinal studies of the reaction to the biomaterial implant. The chip is composed of a regular reference microstructure fabricated via two-photon polymerization in the SZ2080 resist. The geometrical design and the planar raster spacing largely determine the mechanical and spectroscopic features of the microstructures. The development, *in-vitro* characterization and *in vivo* validation of the Microatlas is performed in living chicken embryos by fluorescence microscopy 3 and 4 days after the implant; the quantification of cell infiltration inside the Microatlas demonstrates its potential as novel scaffold for tissue regeneration.

Keywords: 3D-microstructured scaffolds, two-photon polymerization, elasto-mechanics, in vivo implant, ex ovo implant, two-photon imaging, confocal microscopy, intravital imaging windows.

1. INTRODUCTION

The observation of the immunological response[1] to the implant of biomaterials in longitudinal studies in-vivo would help in renovating the statistical approach in evaluating of the biocompatibility of a medical device allowing to reduce the biological variance of the response in different animals. Key requirements for this goal are the possibility for the tissue to expand in 3D and to be efficiently vascularized when embedded into the surrounding host tissue. Fibrotic capsule formation and inflammation[2] together with the evaluation of the presence of immune cells (macrophages, polymorphonuclear cells, giant cell)[3] should be evaluated up to a few months after the implant[4]. For this purpose, histopathological inspections[5] are still largely based on *ex vivo* standard staining (such as hematoxylin & eosin, Masson trichrome, Alcian Blue, Oil red-O, etc) of tissue sections. These protocols are based on semi-quantitative scoring systems[6] standardized in the ISO 10993 norms[4]. New technological developments in the field of the biomaterial reaction assessment should aim at real intravital inspections[7] of the reaction to implants[8] and at a reduction of the number of laboratory animal used for testing. We suggest and start proving here that intravital microscopy (IVM) techniques[8] can help in this directions. In fact, by means of IVM we can quantify the subcutaneous neo-vascularization rate and the reaction to a foreign body[9] by observing a single animal at multiple time-points, without the need of their sacrifice or repeated surgeries[7]. Transparent observation chambers have been fabricated and implanted directly into the animal[10]. However, the implant is quite invasive and during the first two weeks after implantation the reaction due to the surgical procedure itself may be hardly distinguishable from the reaction elicited by the implant. In addition, observation window do not allow a micro-metric repositioning of the microscope

Biomedical Spectroscopy, Microscopy, and Imaging II, edited by Jürgen Popp, Csilla Gergely, Proc. of SPIE Vol. 12144, 121440D · © 2022 SPIE
0277-786X · doi: 10.1117/12.2629824

objective at each time point, hindering a direct quantification of the long-term reaction, required by the ISO/EN 10993-6 directive for the quantification of a biomaterial response.These limitations have been already overcome by means of micrometric scaffolds that, placed inside window chambers allows to confine the area to study[7,9]. A scaffold-based device fabricated by means of melt electrospinning writing of calcium phosphate-coated medical grade poly(ε - caprolactone) (mPCL-CaP) in a porous honeycomb geometry has been used for in-vivo studies by Dondossola et al.[10]. The same limitation affects the work of Lee et al.[9] used an inverted colloidal crystal hydrogel scaffold to mimic decellularized bone with sub-millimetric resolution. However, none of these scaffolds provides a pore size of the order of the single cell or a specific tracking geometry with a well-defined system of coordinates that would allow to perform repeated and prolonged IVM analyses. Moreover, these devices do not offer a truly 3D cellular environment.[11] In this paper we introduce an innovative and miniaturized imaging window for intravital nonlinear microscopy, the Microatlas, implantable sub-cute and inspectable without the need of a percutaneous accesses. The Microatlas is fabricated by 2-photon polymerization (2PP)[11, 12] of the SZ2080 acrylic photoresist[13]. The elasto-mechanical and optical properties are characterized. We also identified a set of parameters (writing power, step sizes etc.) controllable during 2PP fabrication, that allow us to optimize the Microatlas in terms of both autofluorescence and mechanical properties (Young's modulus). The dependence of the elasto-mechanical properties of the fabricated structures on the fabrication parameters was evaluated by microindentation experiments and the autofluorescence of the Microatlas was characterized by two-photon microscopy. Finally, we implanted the Microatlas in live chick embryos *ex ovo* and we imaged the devices under confocal and two-photon microscopy, to quantify features of cell repopulation within the device.

2. MATERIALS AND METHODS

Fabrication of the Microatlas. Four spacers are fabricated at the corners of a square with side = 1500 μm to avoid stresses acting directly onto the Microatlas grids. Four Microatlas were realized in the central portion of the glass coverslip as a set of crossing thin lines ("skeleton") and a matrix of thicker vertical columns ("pillars") that provide the mechanical stability of the structure. A cone and planar landmarks were also fabricated on the glass slide as a vertical and horizontal positioning references, respectively. The Microatlas unit cell size was here 20 μm and its overall size was 500 x 500 μm. Five levels were polymerized in the semisolid resin, for a total height of the 3D lattice of 100 μm. Further details on the fabrication and the structure elasticity can be found elsewhere.[14]

Chicken Embryo cultivation. Eggs were incubated until the chorioallantoic membrane (CAM) was sufficiently developed[15], then removed from the incubator and cracked using a sterile surgical scissor (110 cm Stainless steel, Biosigma, Italy), to gently cut the external eggshell. The eggshell was carefully opened and the inner content was poured into the P100 dish and kept at about 37˚C to reduce the thermal drift in a standard cell incubator (Galaxy 14S, New Brunswick - Eppendorf, USA). The culture parameters were: T = 38˚C, HR ~ 95%, carbon-dioxide partial pressure (pCO_2) 0.1% and oxygen partial pressure (pO_2) 20%.

Microatlas implantation. A sterile Microatlas[11] wetted in Pannett and Compton saline solution, was dripped from the excess of liquid and positioned upon the CAM membrane with the microgrid pointing toward the embryo surface,[16] storing back the Petri dish until the endpoint fixed at day 12 after implantation of the Microatlas.

Ex-vivo Fluorescence Imaging. The formalin fixed chicken embryos were dissected, the Microatlas comprising the surrounding tissue removed, positioned in a P35 Petri dish (Euroclone, Italy) and washed three times with fresh PBS solution. The non-ionic surfactant Triton X-100, 0.25% (Sigma Aldrich, USA) was used to permeabilize the cell membranes. After 15 minutes, the detergent solution was carefully removed, and the sample was washed three times in PBS. Then, cells nuclei staining was performed by means of the dye DRAQ-5.

Optical Microscopy. Confocal microscopy was performed on a A1R+ (Nikon, Japan) equipped with a water-immersion 40x LWD (1.15 NA) (ex vivo analysis) and an oil-immersion objective having a 60x magnification (1.4 NA) (bulk structures characterization). Multiplane 512x512 images (spacing along the optical axis = 0.33μm) were acquired with the highest NA objective (60x). Fluorescent spectral images were acquired by using the Nikon A1-DUS spectral detector unit. Two-Photon Emission and second harmonic generation Microscopy were conducted at the last implantation timepoint with a custom two-photon excitation microscope[17] in the range 690 nm < λ < 1020 nm on a BX51 Olympus upright optical microscope equipped with a 25x water-matched, (WD 2mm and NA 1.0, Olympus, Germany) objective[6]. Cells auto-fluorescence was detected mainly on the 535/50 nm channel. The second harmonic generation signal induced by excitation at 800 nm, was selected by means of the 400/40 nm filter. Some fluorescence signal from the Microatlas was detected also through the 535/50 nm channel.

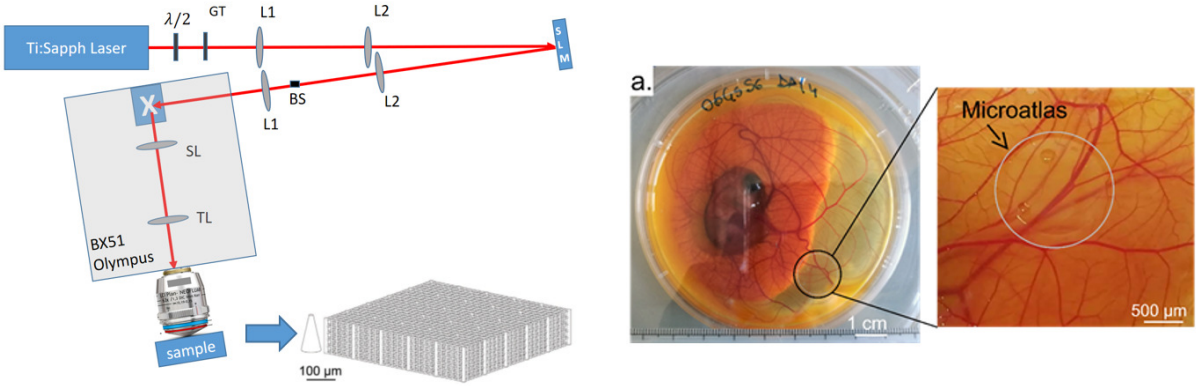

Figure 1. Schematics of the optical setup for the two-photon autofluorescence / second harmonic generation imaging. The source is a Ti:Sapph laser (MaiTai Deep-sea, Spectra Physics, CA), coupled to an Olympus, BX51 microscope (Olympus, J) equipped with a 20× XLUMPLFLN (Olympus, Japan) objective. The laser power is modulated by means of a half-wavelength plate ($\lambda/2$), a Glan-Thompson polarizer (GT). The laser is then coupled to the Spatial Light Modulator (SLM) through a telescope (L1, focal length= 125 mm; L2, focal length= 750mm). The zero order of the Spatial Light Modulator (SLM) is blocked by a beam stopper (BS). The beam is directed to the BX51 microscope galvo system (X in the schematics) and scanned at the entrance pupil of the objective through a third telescope composed of the scan lens (SL) and the tube lens (TL). A sketch of the Microatlas is shonw. Panel a: Chicken embryo 4 d after the implant, cultured in a Petri dish. The Microatlas device is highlighted by a black circle, implanted in the chorioallantoic membrane (CAM), close to a blood vessel bifurcation. The zoomed image of the CAM shows the edges of the Microatlas edges.

3. RESULTS.

3.1 2PP fabrication.

The Microatlas, shown schematically in Fig.1, was developed using as photoresist the biocompatible[18] SZ2080 acrylic resin. The microstructures show some fluorescence signal[19] that allows their imaging when implanted. The mechanical properties of the structures were previously reported[14] and indicate a compression Young module in the range $2 < E < 4$ GPa when polymerized with a voxel of $\cong 250\ nm$ in the transverse plane and $\cong 1\ \mu m$ in the longitudinal dimension. The device contains micro-scaffolds shaped as grids with a square cell 20 µm in side, comparable to the cell dimension. This allows long-time IVM observations, having the micro-scaffold as a reference. The structure was polymerized with in-plane raster pitch of $R = 0.25\ \mu$m and a writing speed of 3mm/s. Thicker columns, added every 100 µm along the XY plane to assure Microatlas stability, were written at 3 mm/s. The vertical writing step was Z =1.0 μm. The whole structure was written in about 200 minutes[14]. The fabrication times, at least for the Microatlas microgrid, could be further reduces by adopting a parallel writing protocol based on a spatial light modulator, as recently done by Raimondi et al.[20]. Other miniaturized imaging window have been reported in the literature[7,9,21,22]. Differently from these reported cases, our Microatlas platform carries a bioengineered interface between the material to be tested and the host and a microscopic autofluorescent scaffold system that guides tissue regeneration and acts as a 3D reference for IVM.

3.2 Implant procedure.

Microatlas can be advantageously used in a biologically relevant environment: we implanted it *ex ovo* using a modified protocol of the chorioallantoic membrane (CAM) assay at different time points. The CAM can be host foreign objects and it is optically accessible for microscopy analysis[23,24], allowing to carefully analyze blood vessels[25]. It has a fast angiogenic response, and it allows us to observe, in few days, a host response comparable to that obtainable in mice in weeks.

3.3 Fluorescence microscopy in ex-ovo embryos.

By means of two-photon, second harmonic generation (SHG) and confocal fluorescence microscopy microscopy we could study the host response to implantation of the Microatlas (Fig. 2). No infiltration occurred inside the Microatlas scaffolds until the third day after implant (Fig. 2). Confocal analyses indicate that the tissue density in the control regions outside the Microatlas increased by 1.8 ± 0.1 times from day 3 to day 4. The infiltration in the Microatlas was delayed by about 2

days. However, we found that the cellular density in the Microatlas at day 3 and 4 from implantation was 4.4± 0.4 times more than for untreated samples. The cell nuclei of cells found inside the Microatlas scaffolds showed a shape of an oblate ellipsoid probably ascribed to neutrophils as they have more deformable lobulated nuclei[26]. Our observation (SHG microscopy, Fig. 2) indicate a reduced formation of a fibrotic capsule around the scaffolds, probably because 3D scaffolds may elicit the production of collagen I inside each pore, and therefore there is a reduced tendency of formation of a fibrotic capsule, a clear marker of inflammatory response.[2] As a matter of fact, SZ2080 is more biocompatible than other hybrid organo-metallic polymers[27] in vitro and in-vivo, as can be judged from preclinical tests reported[18] on SZ2080 microstructures implanted in the weight-bearing area of the medial femoral condyle in rabbits for up to 6 months. In those studies, the microstructures did not provoke a marked foreign body reaction nor infiltration of any inflammatory type cells, i.e. leukocytes and macrophages, could be detected at least from a morphological analysis. Type-X collagen, a marker for fibrogenesis, was not detected in the implants, thus indicating limited or no fibrogenesis. However, further studies directed toward the direct evaluation of macrophages phonotype transition from M1 to M2[28] should be performed.

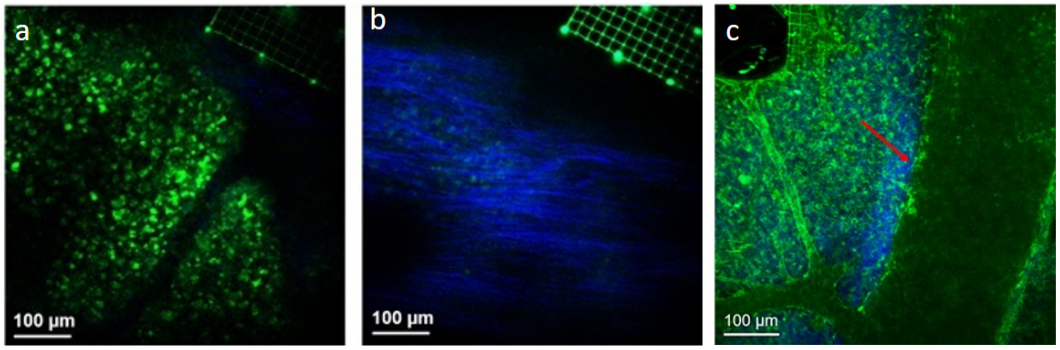

Figure 2. Two-Photon Emission Microscopy image of a label free chick embryo. The embryo was implanted at day 8 and fixed in formalin after 48hrs of implant grafting (panels a and b) or after 15 days (panel c). In green tissue auto-fluorescence signal, while in blue the SHG, which defines the collagen I. Starting from the glass substrate (panel a), and moving inside the embryo (panel b) we experienced new tissue generation (green signal), surrounding a Microatlas side, and collagen fibers. No tissue inside the grid, due to an insufficient grafting time, can be detected at 48 hrs implant. At later implant times (panel c), highly vascularized tissue can be detected around blood vessels. The scaffold (still visible in the upper part) collapses due to the big vessel growth (red arrow).

4. CONCLUSIONS.

Microatlas is a microstructure encompassing a mm^2 area that is promising in terms of tissue engineering and of the possibility of in vivo optical inspection of the regenerated tissue. The Microatlas is colonized by the host tissue, appears to be vascularized and does not seem to elicit massive fibrotic reaction. Due to its autofluorescence under two-photon excitation, the Microatlas allows the exact repositioning of the sample in the optical microscope field of view for long lasting longitudinal studies. It also constitutes a regular micro-structure that could be used as beacon for optical aberrations corrections[14]. We are currently exploiting these unique features to devise implantable micro-devices for the test of the inflammatory reaction to biomaterials in laboratory animals.

ACKNOWLEDGMENTS.

This work has been funded under the project IN2SIGHT that has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 964481

REFERENCES

[1] Menger, M. D., Lehr, H. A. "Scope and perspectives of intravital microscopy--bridge over from in vitro to in vivo", Immunol. Today, 14, 519 (1993).

[2] Mariani, E., Lisignoli, G., Borzì, R. M.,L. Pulsatelli. “Biomaterials: Foreign Bodies or Tuners for the Immune Response?”, Int. J. Mol. Sci. 20, 636 (2019).
[3] S. Franz, S. Rammelt, D. Scharnweber, J. C. Simon. “Immune responses to implants”, Biomaterials, 32, 6692 (2011).
[4] ISO/EN10993-6, “Biological evaluation of medical devices — Part 6: Tests for local effects after implantation”, (2007).
[5] Mulrane, L. Rexhepaj, E., Penney, S., Callanan, J. J., Gallagher, W. M. “Automated image analysis in histopathology: a valuable tool in medical diagnostics”, Expert Rev. Mol. Diagn. 8, 707 (2008).
[6] Cohen J.,“Guided bone regeneration for dental implants”, Bone Jt. Surg. 41A, 152 (1959).
[7] Dondossola E., Holzapfel B. M., Alexander S., Filippini S., Hutmacher D. W., Friedl P., “Examination of the foreign body response to biomaterials by nonlinear intravital microscopy”, Nat. Biomed. Eng. 1, 0007 (2016).
[8] Prunier C., Chen N., Ritsma L., Vrisekoop N., “Procedures and applications of long-term intravital microscopy” Methods 128, 52 (2017).
[9] Lee J., Li M., Milwid J., Dunham J., Vinegoni C., Gorbatov R., Iwamoto Y., Wang F., Shen K., Hatfield K., Enger M., Shafiee S., McCormack E., Ebert B. L., Weissleder R., Yarmush M. L., Parekkadan B, “Implantable microenvironments to attract hematopoietic stem/cancer cells”, Proc. Natl. Acad. Sci. U.S.A 109, 19638 (2012).
[10] Dondossola E., Friedl P, “Host responses to implants revealed by intravital microscopy”, Nat. Rev. Mater. 7, 6 (2021).
[11] Raimondi, M. T., Eaton, S. M., Laganà, M., Aprile, V., Nava, M. M., Cerullo, G., Osellame, R., “3D structural niches engineered via two-photon laser polymerization promote stem cell homing”. Acta Biomater. 9, 4579 (2013).
[12] Zeynali, A., Marini, M., Chirico, G., Bouzin, M., Borzenkov, M., Sironi, L., D’Alfonso, L., Pallavicini, P., Cassina, V., Mantegazza, F., Granucci, F., Marongiu, L., Polli, D., De la Cadena, A., Collini, M., “Multiphoton Fabrication of Proteinaceous Nanocomposite Microstructures with Photothermal Activity in the Infrared”, Adv. Optical Mater. 8, 2000584 (2020).
[13] Ovsianikov, A., Viertl, J., Chichkov, B., Oubaha, M., MacCraith, B., Sakellari, I., Giakoumaki, A., Gray, D., Vamvakaki, M., Farsari, M., Fotakis, C., “Ultra-Low Shrinkage Hybrid Photosensitive Material for Two-Photon Polymerization Microfabrication”, ACS Nano 2, 2257 (2008).
[14] Conci, C., Jacchetti, E., Sironi, L., Gentili, L., Cerullo, G., Osellame, R., Chirico, G., Raimondi, M.T., "A Miniaturized Imaging Window to Quantify Intravital Tissue Regeneration within a 3D Microscaffold in Longitudinal Studies" Adv. Optical Mater. 2101103 (2022)
[15] Nguyen, M., Shing, Y., Folkman, J., “Quantitation of Angiogenesis and Antiangiogenesis in the Chick Embryo Chorioallantoic Membrane”, Microvasc Res. 47, 31-40 (1994).
[16] Zwadlo-Klarwasser, G., Görlitz, K., Hafemann, B., Klee, D., Klosterhalfen, B. “The chorioallantoic membrane of the chick embryo as a simple model for the study of the angiogenic and inflammatory response to biomaterials”, J. Mat. Sci: Mat. Med. 12, 195 (2001).
[17] Caccia, M., Sironi, L., Collini, M., Chirico, G., Zanoni, I., Granucci, F. “Image filtering for two-photon deep imaging of lymph nodes”, Eur. Biophys. J. 37, 979 (2008).
[18] Mačiulaitis, J., Deveikytė, M., Rekštytė, S., Bratchikov, M., Darinskas, A., Šimbelytė, A., Daunoras, G., Laurinavičienė, A., Laurinavičius, A., Gudas, R., Malinauskas, M., Mačiulaitis, R. “Preclinical study of SZ2080 material 3D microstructured scaffolds for cartilage tissue engineering made by femtosecond direct laser writing lithography”, Biofabrication, 7, 015015 (2015).
[19] Raimondi, M. T., Nava, M. M., Eaton, S. M., Bernasconi, A., Vishnubhatla, K. C., Cerullo, G., Osellame, R. “Optimization of Femtosecond Laser Polymerized Structural Niches to Control Mesenchymal Stromal Cell Fate in Culture”, Micromachines 5, 341 (2014).
[20] Zandrini, T., Shan, O., Parodi, V., Cerullo, G., Raimondi, M. T., Osellame, R. “Multi-foci laser microfabrication of 3D polymeric scaffolds for stem cell expansion in regenerative medicine”, Sci. Rep. 9, 1 (2019).
[21] Bhagavatula, S., Thompson, D., Ahn S. W., Upadhyaya, K., Lammers, A., Deans, K., Dominas, C., Ferland, B., Valvo, V., Liu, G., Jonas, O. “A Miniaturized Platform for Multiplexed Drug Response Imaging in Live Tumors”, Cancers 13, 653 (2021).
[22] Liu, G., Valvo, V., Ahn, S. W., Thompson, D., Deans, K., Kang, J. W., Bhagavatula, S., Dominas, C., Jonas, O. “A Two-Photon Microimaging-Microdevice System for Four-Dimensional Imaging of Local Drug Delivery in Tissues”, Int. J. Mol. Sci. 22, 11752 (2021).
[23] Deryugina, E. I., Quigley, J. P. “Chick embryo chorioallantoic membrane models to quantify angiogenesis induced by inflammatory and tumor cells or purified effector molecules”, Methods Enzymol. 444, 21 (2008).

[24] Schomann, T., Qunneis, F., Widera, D., Kaltschmidt, C., Kaltschmidt, B. “Improved Method for Ex Ovo-Cultivation of Developing Chicken Embryos for Human Stem Cell Xenografts”, Stem Cells Int. 2013, 960958 (2013).
[25] Ribatti, D. “Chick embryo chorioallantoic membrane as a useful tool to study angiogenesis”. Int. Rev. Cell Mol. Biol. 270, 181 (2008).
[26] Dahl, K. N., Ribeiro, A. J. S., Lammerding, J. “Nuclear shape, mechanics, and mechanotransduction”. Circ. Res. 102, 1307 (2008).
[27] Malinauskas, M., Baltriukiene, D., Kraniauskas, A., Danilevicius, P., Jarasiene, R., Sirmenis, R., Zukauskas, A., Balciunas, E., Purlys, V., Gadonas, R., Bukelskiene, V., Sirvydis, V., Piskarskas, A. “In vitro and in vivo biocompatibility study on laser 3D microstructurable polymers”. Appl. Phys. A 108, 751 (2012).
[28] Witherel, C. E., Abebayehu, D., Barker, T. H., Spiller, K. L. “Macrophage and Fibroblast Interactions in Biomaterial-Mediated Fibrosis”. Adv. Healthcare Mater. 8, 1801451 (2019).