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A miniaturized, optically accessible bioreactor for systematic 3D tissue engineering research

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Abstract Perfusion bioreactors are widely used in tissue engineering and pharmaceutical research to provide reliable models of tissue growth under controlled conditions. Destructive assays are not able to follow the evolution of the growing tissue on the same construct, so it is necessary to adopt non-destructive analysis. We have developed a miniaturized, optically accessible bioreactor for interstitial perfusion of 3D cell-seeded scaffolds. The scaffold adopted was optically transparent, with highly defined architecture. Computational fluid dynamics (CFD) analysis was useful to predict the flow behavior in the bioreactor scaffold chamber (that was laminar flow, Re=0.179, with mean velocity equal to 100 microns/s). Moreover, experimental characterization of the bioreactor performance gave that the maximum allowable pressure was 0.06 MPa and allowable flow rate up to 25 ml/min. A method, to estimate quantitatively and non destructively the cell proliferation (from 15 to 43 thousand cells) and tissue growth (from 2% to 43%) during culture time, was introduced and validated. An end point viability test was performed to check the experimental set-up overall suitability for cell culture with successful results. Morphological analysis was performed at the end time point to show the complex tridimensional pattern of the biological tissue growth. Our system, characterized by controlled conditions in a wide range of allowable flow rate and pressure, permits to systematically study the influence of several parameters on engineered tissue growth, using viable staining and a standard fluorescence microscope.

Keywords 3D cell culture · Tissue growth · Live imaging · Bioreactor · Interstitial perfusion · Microfluidic

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1 Introduction

Bioreactors for tissue engineering are traditionally intended for the production of implantable functional grafts but they are also able to provide reliable models to investigate the tissue growth under controlled conditions (Martin et al. 2004). An important application of these systems is in basic research, to increase the knowledge about the tissue growth principles and, potentially, for pharmaceutical research (e.g. drug screening) as substitutes of animals models (Elliott and Yuan 2010).

Perfusion bioreactors are widely used, because they are able to increase the mass transport of nutrients and oxygen and the waste removal from the tissue culture environment. A particular type of perfusion, called interstitial (or direct) perfusion, is considered to be an important stimulus in the mechanobiology of several three-dimensional (3D) tissues, because hydrodynamic shear stresses may positively affect the cells viability, differentiation and protein production (Gomes et al. 2003; Pedersen and Swartz 2005; Raimondi et al. 2008). In these systems, classical destructive assays performed on engineered tissue, like enzymatic digestion followed by DNA or protein analysis, are not able to follow the evolution of the tissue sample at different time points during culture time. Optical microscopy is considered the most widespread non-destructive analysis techniques. Fluorescence microscopy in particular, thanks to viable staining or transfection, is used to visualize cells nuclei, cytoplasm and specific proteins of the extracellular matrix (Chalfie et al.

1994; Krahn et al. 2006; Lydon et al. 1980). In the aim to use viable staining for non-destructively monitoring the tissue growth, previous authors have used a "window" opened on the culture chamber for bioreactor optical accessibility (Stephens et al. 2007; Vickerman et al. 2008; Witte et al. 2011). Pioneer works of Moore and Kleis (2008) and Powers et al. (2002) and, at the best of our knowledge, the ultimate frontier of miniaturized bioreactors for mechanobiology study, represented by complex mechanobioreactors for perfusion coupled with direct mechanical stimulation (Paten et al. 2011; Kensah et al. 2011) have shown the great evolution of miniaturized bioreactor for tissue engineering research occurred in the last 10-year period. The goal of this work was to develop a method for systematic research on *in vitro* 3D tissue growth. We designed and validated a miniaturized, optically accessible bioreactor for interstitial perfusion. We also defined and validated a suitable method for quantitatively estimate the cell proliferation and the tissue growth, on the basis of viable imaging obtained from a standard fluorescence microscopy. Taking advantage of a proper designed scaffold, it was possible to gain better results from systematic *in vitro* 3D tissue growth research. To fit with the aim of this work, the scaffolds were designed with a stable and regular architecture, they were made using a reproducible technology for rapid prototyping (i.e. fused deposition modeling) and were optically transparent and accessible to a standard phase contrast/fluorescence microscope. The scaffolds were able to provide the proper environment for cells adhesion and tissue growth but they were used also as a frame of reference to evaluate quantitatively the cells proliferation and the production of extracellular matrix. Conceptually, the bioreactor described in this work is newly redesigned from an earlier bioreactor (Raimondi et al. 2010). Since the overall scaffold dimensions are in the order of few millimeters, the bioreactor scaffolds chambers and the perfusion channels features weren't pushed to extremes of the microfluidic scale, however, microfluidic design principles were employed (Kim et al. 2007).

2 Materials and methods

2.1 Bioreactor development and validation

2.1.1 Bioreactor features

The bioreactor design was based on three main features:

I. to provide the best optical accessibility of the growing tridimensional tissue, using a standard inverted fluorescence microscope;
II. to allow the culture of three independent samples;
III. to be user-friendly.

To fit the first feature only optically transparent materials (glass and transparent polystyrene) were employed in the bioreactor light pathway. Moreover, the scaffold and the glass components thicknesses were limited to increase imaging quality. The bioreactor core was a custom made polystyrene scaffold manufactured by 3D Biotek (Fig. 1e). It was made from polystyrene treated for cells culture, the same material of disposable cells culture flasks. Fuse deposition modeling (FDM) was used to obtain scaffolds with an highly defined architecture, stability and repeatability. The scaffold fibers were 100 μm in diameter with a pore size of 300 μm and stacked in an offset cross-hatch geometry (four flats, each one was shifted 150 μm in respect of the adjacent flat). The overall scaffold dimensions are 6 mm×3 mm×0.4 mm. The scaffolds holder was made from polydimethylsiloxane (PDMS Sylgard 184, DowCorning), a biocompatible elastomer, via replica molding using a computer numerically controlled (CNC) micromachined polycarbonate mold. Three independent chambers were obtained on the same PDMS sheet and the chambers dimensions were suited for scaffolds interstitial perfusion. The scaffold chamber design was refined to have a fully developed flow with an homogeneous velocity at the scaffold inlet. The channels that fed the scaffold chambers were CNC machined directly on the surface of a standard (76 mm×26 mm) microscope glass slide, using a diamond coated ball mill (1 mm diameter, Proxxon). Three inlet and three outlet channels were used to perfuse the scaffold chambers, without interfering with the bioreactor optical accessibility. Each channel had a rounded cross section characterized by a maximum depth of 0.2 mm a maximum width of 0.8 mm and a channel length of 7 mm. Each channel was connected with a stainless steel coupler, that was cut from G20 hypodermic needles (Artsana S.p.A.). Each coupler was able to pass from the top to the bottom of the glass slide thickness, thanks to CNC machined holes. The glass slide-couplers connections were sealed with heat resistant silicon rubber (Saratoga). The scaffolds holder was permanently bonded to the channeled glass slide through air plasma surface activation. The bottom surface of the scaffolds holder was reversible sealed with a square (20 mm×20 mm) microscope cover-glass, properly locked by the magnetic holders. Summarizing, the chamber assembly requires of only three bioreactor pieces. Indeed the bioreactor was composed by a glass slide, with machined channels, that was permanently bonded to the scaffolds holder and to the perfusion couplers (first piece); moreover a reversible sealing was obtained through a microscope cover glass (second piece) locked by the magnetic holders (third piece). Each scaffold was placed in an independent chamber and it was fed by independent channels, so the second feature was reached too. The magnetic holders

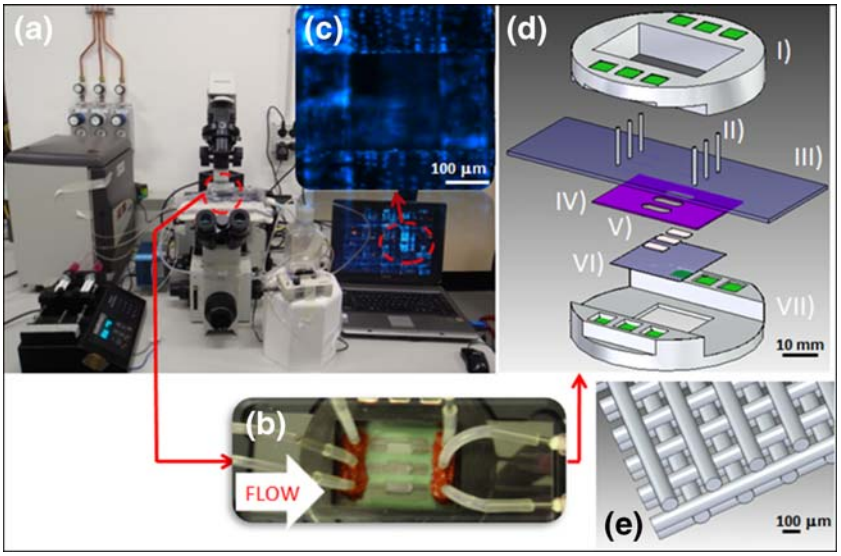

Fig. 1 (**a**) Experimental setup composed of: a syringe pump whose flow rate was set to 7.2×10^{-3} ml/min, that was able to move the medium through a gas permeable tube system placed inside a small cell culture incubator (37°C, 5% CO_2). After heat and gas exchange, the medium fed the cell-seeded scaffolds, placed inside the bioreactor and it was collected in separate reservoirs placed inside the incubator. (**b**) Bioreactor detail showing the three independent chambers. (**c**) Live imaging showing cell nuclei stained in blue with DAPI (SIGMA). (**d**) Bioreactor components are shown in exploded view, in a top-down order. I) & VII) Magnetic holders used to lock the reversible sealing of the scaffolds perfusion chambers. II) G20 stainless steel couplers used to connect the bioreactor to the perfusion circuit. III) Standard microscope glass slide used as channels bulk, the fluidic channels were obtained on the bottom face through CNC machining and they were connected to the couplers through CNC machined holes. IV) Scaffolds chambers made of a 0.4 mm thick PDMS sheet. V) Custom made polystyrene scaffold with overall dimensions 6 mm length, 3 mm width, 0.4 mm thick. VI) Standard 20 mm square microscope cover-glass used for reversible sealing. (**e**) Scaffolds fibers were 100 µm in diameter, the wheelbase between two fibers on the same plane was 300 µm and the scaffolds flats were 150 µm shifted each other for a better visibility

were designed to fit with the microscope stage and they contributed to reach the third feature because, without screws or other mechanical fasteners, the bioreactor was easily assembled by the users under sterile conditions.

2.1.2 Fluid dynamics analysis

The scaffold chamber design was evaluated by computational fluid dynamics (CFD) using the code Comsol Multiphysics 3.5a, (Comsol). We computed the velocity field inside one empty scaffold chamber and the Reynolds number at the chamber inlet. The numerical values of the parameters used in the simulation were:

- culture medium dynamic viscosity at 37°C, μ: $8.1\cdot10^{-4}$ Pa · s (Raimondi et al. 2002);
- culture medium density at 37°C, ρ: $1\cdot10^{3}$ kg/m^3;
- inlet velocity (inside the couplers), v: $2.39\cdot10^{-4}$ m/s;
- no viscous force at the outlet.

The fluid velocity vector, v, was determined using the Stokes equation in stationary conditions:

$$0 = -\nabla p + \mu\nabla^2 v + f \tag{1}$$

where μ is the dynamic viscosity of the culture medium, p is the fluid pressure, and f is the mass force vector.

The Reynolds number was determined from the fluid velocity vector (v) using the follow equation:

$$\mathrm{Re} = \frac{\rho v L}{\mu} \tag{2}$$

Where ρ is the fluid density and L the hydraulic diameter.

Moreover, a qualitative fluid dynamic analysis was experimentally performed. Briefly, fluorescent microspheres (Fluo Spheres 1 µm, Invitrogen) were suspended in distilled water and the suspension was pumped, using a Phd 2000 syringe pump (Harvard apparatus), inside the scaffold chamber containing the polystyrene scaffold. The inlet flow rate used in the CFD simulation and in the experiments was used to perform the qualitative fluid dynamic analysis too. Using a fluorescence microscope (IX 70, Olympus), equipped with a CCD camera suitable for fluorescence imaging (OPTIKAM Pro Cool 3 CCD Cooled Camera, Optika), brightly fluorescence streamlines were captured by video acquisition at 3.3 MPixels, 10 frames/s.

2.1.3 Leakage test

The bioreactor capability to face pressure increase was tested with a static leakage test, similar to that used in a precedent work (Rasponi et al. 2010). Briefly, a precision

pressure regulator was used to provide pressure steps of 0.02 MPa to a distilled water reservoir that was connected to the inlet channel of the scaffold chamber. The three scaffold chambers were tested one by one, while the outputs channels were clamped. Each pressure step was maintained for three minutes. The maximum pressure value was measured when the first water drop started to come out from the tested chamber. The test was repeated three times for each scaffolds chamber. Anova was used to compare the results in the scaffold chambers, with differences considered to be significant if $p>0.05$.

2.1.4 Hydraulic characterization

To characterize the hydraulic resistance of the fluid pathway, a precision pressure regulator was used to provide pressure steps of 0.02 MPa, up to 0.1 MPa, to a distilled water reservoir connected to the inlet channel of the scaffold chamber. The chamber was filled with the same polystyrene scaffold used during cells culture experiments, in order to account for the scaffold hydraulic resistance. The scaffold chambers were tested one by one connecting the output channel to a reservoir, placed on a precision electronic balance (AP250D, Ohaus). Each pressure step was maintained for 30 s (perfusion time) then the inlet channel was clamped and a note for the weight reached was made. The flow rate was calculated from the measured weight and the perfusion time. The test was repeated three times for each input pressure value.

2.2 Cell culture

2.2.1 Cell culture protocols

Human osteosarcoma cells from an immortalized cell line (MG63, Sigma) were used for the bioreactor biological validation. The cells were cultivated with a medium composed by Minimum Essential Medium (MEM, Euro Clone) supplemented with 10% v/v Fetal Bovine Serum (FBS, Euro Clone), 1% v/v Non Essential Amino Acids 100X solution (NEAA, Euro Clone), 1% v/v Penicillin/Streptomicin 100X solution (Euro Clone), 1% v/v L-Glutamine 200 mM solution (Euro Clone). During a calibration cells-seeding experiment the following dynamic seeding protocol was defined to obtain a homogeneous cell distribution. Briefly, under sterile conditions, each scaffold was laid in a well of a 24 multiwell plate (Corning) and covered with a rich cell suspension (10^6 cells in 400 μl of medium). The multiwell plate was shaken, under 5% CO_2 at 37°C in an incubator, through a 3D multifunction rotator (PS-M3D, WWR), set as follows:

- orbital, OFF;
- reciprocal, mode=270°, time=01;
- vibro, mode=5°, time=2;
- cycle, mode=00, time=stop.

After 5 h the rotator was stopped and the seeded scaffolds stayed still overnight before being transferred in clean wells filled with 400 μl of medium.

The viable staining was made incubating the samples with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) at a concentration of 10 μg/ml in Phosphate Buffer Saline (PBS) for 90 min under 5% CO_2 and 37°C conditions, followed by a twice rinsing with PBS.

After cell staining, the seeded scaffolds were placed inside the bioreactor (steam sterilized, 24 min., 121°C, 1 bar), prefilled with the medium. Under sterile conditions, the bioreactor was laid in a disposable Petri dish with the perfusion chambers turned upside down with respect to their position during perfusion. The chambers and the channels were filled with medium using a 200 μl pipette (Biohit). Then the seeded scaffolds were laid using a sterile forceps and medium was used again to fill the void spaces. Carefully, avoiding air bubble formation, a 20 mm×20 mm cover glass was used to close reversibly the scaffolds chambers. The reversible sealing was then locked by the magnetic holders. The perfusion system, in which all tubes were steam sterilized and syringes were sterile and disposable, was prefilled with warm medium, avoiding air bubble formation, then the bioreactor was connected to the oxygenator tubes, to the disposable syringes and to the other components of the perfusion system (cfr. 2.2.2) using the drop merging technique (Kim et al. 2007).

2.2.2 Experimental set-up

The experimental setup (Fig. 1) was composed by a syringe pump (Harvard Apparatus) equipped with 30 ml Luer lock syringes (Artsana S.p.A.) filled with medium. The medium was perfused on single passage and was pumped with a flow rate of 7.2×10^{-3} ml/min inside a gas permeable tubing system (0.03″ID×0.065″ OD Silastic Tubing, ColeParmer). The oxygenator was laid inside a small cell culture incubator (Innova CO-14, Eppendorf) located close to the microscope. The medium, after heat and gas exchange in the incubator, was able to reach the bioreactor and perfuse the cell-seeded scaffolds. The bioreactor output channels were connected with separated reservoirs, placed slightly above the bioreactor level to avoid bubble formation. The reservoirs were equipped with 0.22 μm syringe filters (Corning) for air pressure equilibration without microbial contamination. An inverted, contrast phase and fluorescence microscope (IX 70, Olympus) equipped with an imaging system was used to complete the experimental set-up. The bioreactor shape was able to perfectly fit with the microscope glass slide

holder. A time lapse video acquisition (every 24 h) of the growing tissue was performed, during the whole culture time (5 days), both in phase contrast and in fluorescence, using a 4X and 10X objectives.

2.3 Estimation of cell number through live imaging

2.3.1 Method

Fluorescence video imaging (Fig. 2) was used to estimate the cell number on the scaffold fibres at different culture times. DAPI stained cell nuclei were used for cell counting. Everyday a video of the same scaffold area was registered, varying the focal plane in order to visualize the cells on each scaffold layer. Several frames were extracted from these video and the cell nuclei (n) were counted on each frame thanks to the scaffold transparency. The total scaffold fiber length (L=222 mm) was compared with the whole scaffold fibers length considered during the cell count (l_c) so L/l_c ratio gave the coefficient we used to estimate the total cell number (N) as follows:

$$N = \frac{n \times L}{l_c} \tag{3}$$

2.3.2 Validation

An experiment was performed to validate the cell number estimation method described above. Three dynamically-seeded and DAPI-stained scaffolds were statically cultivated for 24 h in a multiwell plate. The cell number was estimated on each scaffold with the described method. The cells were then trypsinized using a 0.05% trypsin-0.02% EDTA solution (Euro Clone) and counted by Trypan Blue dye exclusion using a Burker chamber. A F-test was used to compare the results for the three scaffolds, with $p<0.05$ considered to be significant.

2.4 Estimation of tissue growth through live imaging

The estimation method adopted for the tissue growth is shown in Fig. 3. From phase contrast video imaging, extrapolated frames of the scaffold layers were used to define the scaffold pore space filled by the growing tissue. At each culture time, the distance (Fig. 3, dash line A) between the tissue edge and the corner of nine pores per frame were measured to calculate the percentage of pore occlusion. Due to the transparency, the high regularity (the hemi-diagonal of the pore, Fig. 3 line B, is constant) and the stability of the rapid-prototyped polystyrene scaffold, it was possible to use the A/B ratio as a live imaging estimation of tissue growth.

2.5 End point viability assay and tissue morphology

At the end of the culture period, a viability assay (LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells, Invitrogen) was performed, following the manufacturer protocol. Briefly, the cultivated construct was taken out

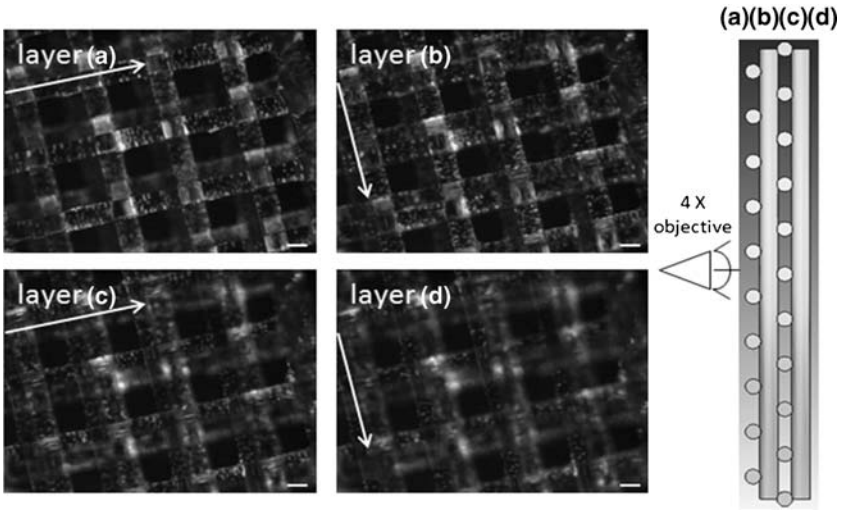

Fig. 2 Method adopted for the estimation of cell proliferation on live images acquired from the bioreactor. From fluorescence video imaging, the extrapolated frames were used to estimate the cell proliferation. Cell nuclei were stained with DAPI. Everyday a video of the same scaffold area was registered, varying the focal plane to visualize the cells on each scaffold layer. Arrows show the direction of the fibres used for cell count on the relevant layer. The cell nuclei (n) were counted thanks to the fibres transparency. The fibre length of the whole scaffold (L=222 mm) was divided by the fibre length used for cells count (l_c). The L/l_c ratio was used to estimate the total cell number (N) on the scaffold fibres as $N = n \times L/l_c$. The scale bar is 100 μm in all figures

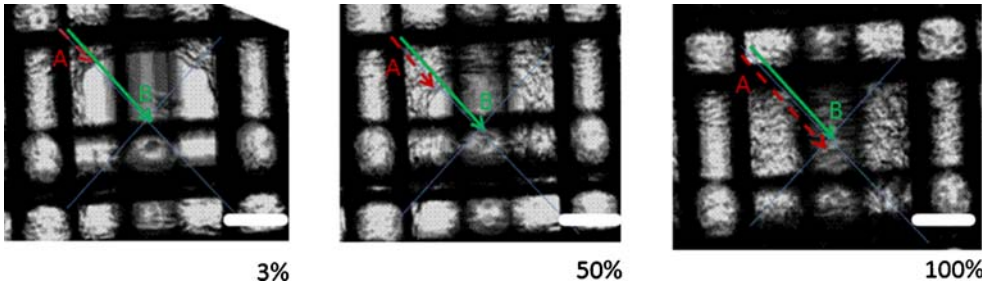

Fig. 3 Method adopted for the estimation of tissue growth on live images acquired from the bioreactor. From phase contrast (PhC) video images (4X and 10X objectives), the extrapolated frames of the scaffold layers were used to define the distance covered by the growing tissue. Briefly, the distance (*dashed line*, A) between the tissue edge and the corner of nine pores per frame were measured everyday during the entire culture time. Thanks to the transparency, the high regularity (the hemi-diagonal of the pore, B line, is constant) and the stability of the rapid prototyped polystyrene scaffold, it was possible to estimate the percentage of pore occlusion from the A/B ratio. The scale bar is 100 μm in all figures

from the bioreactor, placed in a multiwell plate and incubated for 30 min (5% CO_2 at 37°C) with a 2 μM calcein AM (Ex/Em=494/517 nm) and 4 μM EthD-1 (Ex/Em=528/617 nm) solution in PBS. The FITC (Olympus U-MWB2: EX 460–490 nm, DM 505 nm, EM 520 nm IF) and the TRITC (Olympus U-MWG: EX 510–550 nm, DM 570 nm, EM 590 nm) filters were used to visualize cells in the live/dead assay, the live cells stained green and the dead cells stained red.

A cultivated scaffold was prepared for the tissue morphology evaluation through Scanning Electron Microscopy (SEM). The sample was fixed with 1.5% glutaraldeide/0.1 M sodium cacodylate solution in PBS for two hours then rinsed with 0.1 M sodium cacodylate solution and dehydrated with steps from 20% to 100% v/v ethanol solutions in distilled water. After dehydration, the sample was sputtered with gold, SEM images of the cultivated construct surface were acquired in high vacuum mode at 17.5 kV, magnification 40 X, using a EVO50EP system (Zeiss).

3 Results

3.1 Bioreactor characterization

The results of CFD simulations and qualitative fluid dynamic analysis are resumed in Fig. 4. Using the same flow rate adopted for the tissue growth experiments, the flow was fully developed inside the scaffold chamber, with a flat profile and average velocity equal to 100 μm/s. At the beginning of the scaffold chamber region, the maximum value of the Reynolds number was equal to 0.179, indicative of a laminar flow. The experimental fluorescence particle video imaging showed a homogeneous fan-shaped distribution of the fluorescence microspheres from the inlet to the beginning of the scaffold region and homogeneous distribution of path lines inside the scaffold fibres net, coherently with the predicted velocity pattern.

Results of the fluidic tests are shown in Fig. 5. The bioreactor reversible sealing was able to resist up to 0.08 MPa of hydrostatic pressure, as shown by the static leakage test

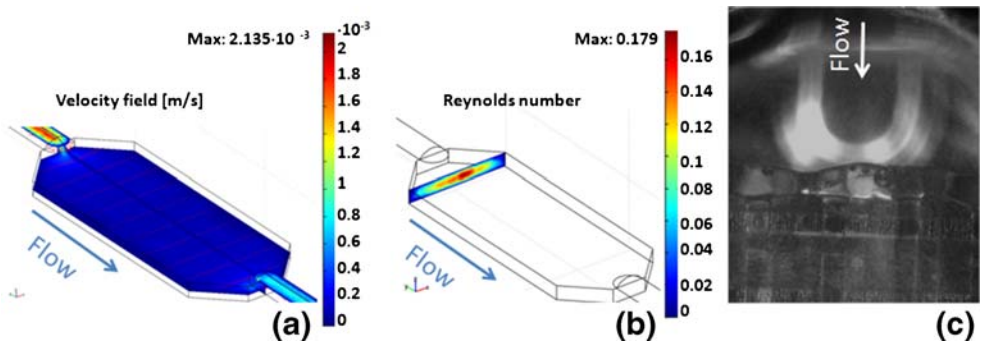

Fig. 4 Results of the CFD simulations and fluorescence particle imaging of the bioreactor culture chamber. (**a**) The flow was fully developed with an homogeneous flat velocity profile inside the scaffold chamber equal to 100 μm/s. (**b**) At the inlet of the scaffold chamber, the maximum value of the Reynolds number was equal to 0.179 indicative of a laminar flow. The inlet flow rate, necessary to lose the laminar flow state at the beginning of the scaffold chamber, was estimated to be 72 ml/min (10^4 times the flow rate used). (**c**) The results of the CFD model were in qualitative agreement with those of the experimental particle imaging characterization

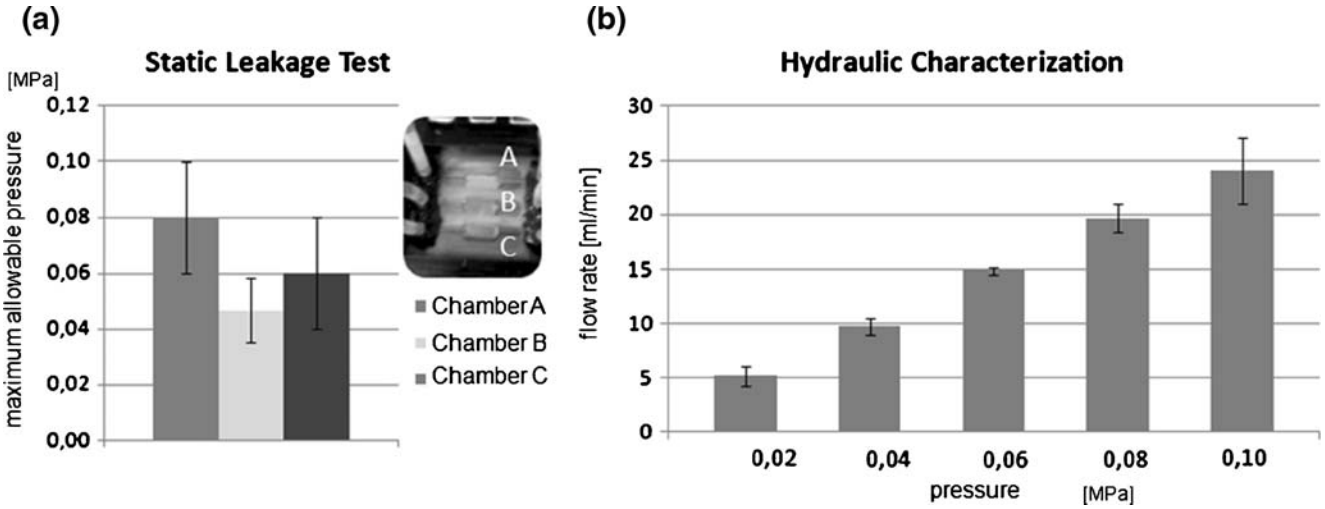

Fig. 5 Results of the hydraulic characterization of the bioreactor. (**a**) Results of the static leakage experiment. A precision pressure regulator was used to provide pressure steps of 0.02 MPa to a distilled water reservoir connected to the inlet channel of the scaffold chamber. The chambers were tested one by one, while their output channels were clamped. Each pressure step was maintained for three minutes. (**b**) Results of the hydraulic characterization experiment. A precision pressure regulator was used to provide pressure steps of 0.02 MPa up to 0.1 MPa to a water reservoir connected to the inlet channel of the scaffold chamber filled with the polystyrene scaffold. The scaffold chambers were tested one by one, while their output channel was connected to a reservoir that was placed on a precision electronic balance. Each pressure step was maintained for 30 s of perfusion, and the inlet was clamped and the weight reached was noted. The flow rate was calculated from the measured weight and the perfusion time. The device was able to provide a flow rate several times greater than the flow rate used for the cell experiments, without visible leakages

(Fig. 5a). Even if the middle chamber was observed to resist less than the others, statistically significant differences among the three bioreactor chambers was not present.

The hydraulic characterization (Fig. 5b) showed a maximum flow rate up to 25 ml/min, achievable without leakages, when the bioreactor chamber was fed with the maximum pressure (0.10 MPa) deliverable by the precision pressure regulator.

3.2 Bioreactor use for 3D tissue growth study

The dynamic seeding protocol gave good results in term of the homogeneity of cells distribution, in that nuclei were equally distanced each other on the whole scaffolds fibers surface (Fig. 2).

The results of the experiment performed to validate the cell number estimation method are shown in Fig. 6. A slight underestimation was seen, without statistically significative differences between the count on live images and the cell count after Trypsinization.

It was possible to estimate the cell expansion (Fig. 7a) and the tissue growth (Fig. 7b), non destructively, during culture time. From 0 to 24 h a reduction of cells in the scaffold occurred (from 15 to 8 thousand of cells). Cell expansion was evident (from 8 to 42 thousand of cells) in the period from 24 to 62 h of perfusion then from 62 to 96 it was reduced. Tissue growth under perfusion rapidly increased, from 2% to 40%, in the culture period from 0 to 48 h and then it stabilized.

Results of construct characterization at the end of culture are gathered in Fig. 8. The viability assay (Fig. 8a) showed a diffused bright green fluorescence, indicating live cells filling the scaffold at the end of the culture period (5 days). SEM imaging (Fig. 8b) show details of the complexity of the tridimensional tissue. Several connections, made by the growing tissue, were visible among the scaffold layers. These connections were hardly visible with fluorescence and phase contrast imaging. Moreover, SEM imaging showed the circular shape of pore occlusion pattern, that was also evident in live fluorescence and phase contrast imaging.

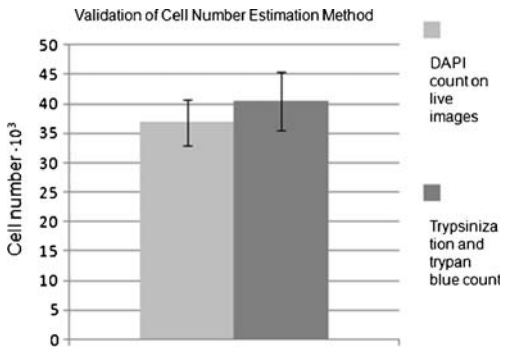

Fig. 6 Validation of live cell number estimation method. No statistically significant difference was noted, comparing the cell number estimated from fluorescent live imaging versus from trypsinization followed by counting

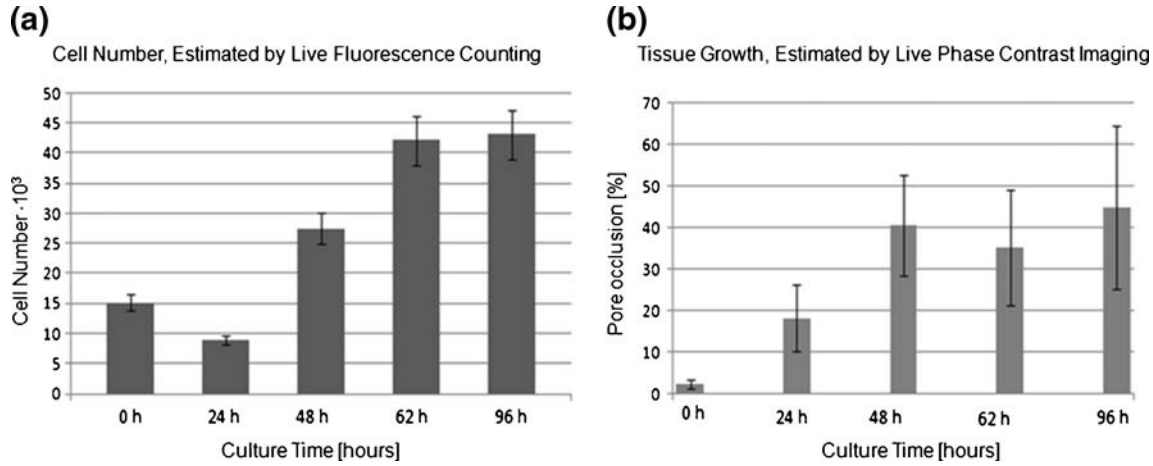

Fig. 7 Results of cell number and tissue growth estimation on live images acquired from the bioreactor culture. (**a**) From 0 to 24 h proliferation is reduced. Cell expansion was more evident from 24 to 62 h of perfusion. From 62 to 96 h the cells expansion was reduced. (**b**) Tissue growth rapidly increases in the culture period from 0 to 48 h and then stabilizes

4 Discussion

A specific purpose of this work was to produce a miniaturized, optically accessible bioreactor suitable for systematic research on 3D tissue growth under interstitial perfusion. The core of the system was a 3D custom made polystyrene scaffold supporting tissue growth. The small thickness (0.4 mm) and the transparency of the scaffold made live observation of cell proliferation and of tissue growth possible, in every scaffold layer, with a standard reversed contrast phase/fluorescence microscope. Using a well defined flow regime in the interstitial perfusion bioreactor, the regular scaffold architecture, repeatability and geometrical stability gave a controlled environment for systematic *in vitro* 3D tissue growth studies. Indeed, the scaffold architecture was used as reference frame for the tissue growth estimation like in Sun et al. (2009). The previous work cited (Sun et al. 2009) is based on static culture of non transparent nickel grid scaffolds so, differently from our work, a live imaging of the growing tissue, under controlled perfusion conditions, was impossible in the whole scaffold thickness using a standard fluorescence microscope. At the best of our knowledge, differently from similar devices (Leclerc et al. 2003; Stephens et al. 2007; Vickerman et al. 2008; Witte et al. 2011), the bioreactor described here was implemented for parallel interstitial perfusion because three scaffolds can be independently cultured inside the same miniaturized bioreactor, under the same environmental conditions, at the same time, increasing the data obtainable from a single experiment. Moreover, a method for quantitatively estimate the cell proliferation and tissue growth from live imaging was here adopted.

Steam sterilization is considered a low cost, efficient and widely spread method to prevent contaminations of biomedical and surgical devices (Rutala and Weber 1999). The bioreactor components in contact with cells were made of glass, PDMS and stainless steel, all able to resist to ethanol 70% v/v solution and to several steam sterilization cycles. The magnetic holders (which are not in contact with the cells) were designed to minimize the overall dimensions, so that the bioreactor here presented could be used with the majority of inverted microscopes without any

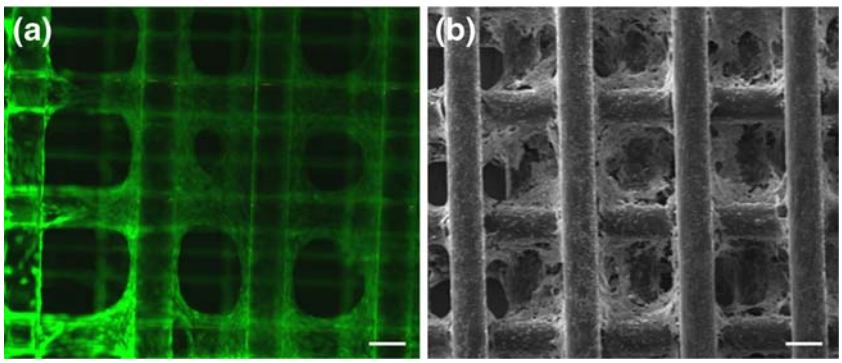

Fig. 8 (**a**) Results of destructive construct characterization at the end of culture. Viability test, in which live cells stain green and dead cells stain red, showing the majority of cells viable. (**b**) SEM images showing the scaffold pores filled with the engineered tissue. The scale bar is 100 μm in all figures

modification. Furthermore, the bioreactor was designed to be used also with a long distance objective up to 40 X (LCACHN 40X, working distance 2.2 mm, Olympus) which can be moved up to 1.5 mm close to the cover-glass surface, but considering the scale of observation the 4X and 10X objectives were used only.

Even though the CFD analysis performed was limited to the scaffold chamber, without considering the scaffold presence its results were significant to understand the flow behavior which was predicted to be laminar, fully developed and with homogeneous velocity at the scaffold inlet. Moreover, the qualitative fluorescence particle imaging, performed with the scaffold placed in the chamber, showed a well distributed flow through the scaffolds fibers (Fig. 4). The flow rate, set in both CFD simulations and cells experiments, generated a velocity at the scaffold inlet equal to 100 μm/s and, thanks to its similarity with the scaffold architecture adopted in a previous work (Raimondi et al. 2010), we have estimated an expected velocity magnitude in the range from 0 to 370 μm/s and a shear stress levels ranging from 0 to 21 mPa inside the scaffold fibers net, all conditions proved to be compatible with long time cell culture.

Experimental fluidic tests (Fig. 5) showed the bioreactor ability to face pressure and flow rate several times over the conditions used in the actual tissue growth experiments. These results indicated some bioreactor flexibility. Indeed, it could be possible to perform cell culture experiments with different flow conditions or to test tissue growth using different medium/drugs in different channels. Another possibility could be to test tissue growth under different shear stress regimes, by changing the flow rate or alternatively using culture media of different viscosity (Sikavitsas et al. 2003).

The cells homogeneous distribution, obtained during the dynamical seeding, made live imaging estimation of the tissue growth easier, because the variability among scaffolds regions was lowered. Concerning the method adopted for the cells number estimation, based on live imaging, it was considered reliable because it gave a result comparable with respect to the most widely diffused destructive method (Fig. 6). Increased cell proliferation (Fig. 7a) was observed after an initial decrease, probably due to the detachment of weakly adhered cells caused by the hydrodynamic shear stress. Our bioreactor allowed to quantify cell expansion during a culture time equal to 5 days. Through live imaging, it was possible to quantitatively estimate tissue growth too (Fig. 7b). A circumferential occlusion pattern of the scaffold pores was observed (Fig. 8), in agreement with previous observations (Engelmayr et al. 2005; Hutmacher and Singh 2008).

A widely diffused viability test, performed at the end of the culture period, proved that the bioreactor and the experimental set up adopted can be successfully used for engineered tissue perfused culture experiments.

5 Conclusion

The main challenge of this work was to design and validate a miniaturized, optically accessible perfusion bioreactor for systematic *in vitro* 3D tissue engineering research. The bioreactor described here was characterized by an optical accessibility up to 40X (using a long distance objective) even if, for reasons related to the scaffold dimensions, only the 4X and 10X objectives were used here. CFD simulations and qualitative fluid dynamic analysis showed a laminar fluidic environment in the bioreactor scaffold chamber. The bioreactor was able to face pressure and flow rate increments several times higher than those used in conventional tissue growth experiments. Triplicate could be retrieved from a single experiment thanks to the possibility to simultaneously cultivate three independent scaffolds using the same device, under identical environmental conditions. These results indicated some bioreactor flexibility and our system allows to study the influence of several parameters on engineered tissue growth.

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References

M. Chalfie, Y. Tu, G. Euskirchen, W.W. Ward, D.C. Prasher, Green fluorescent protein as a marker for gene-expression. Science (1994). doi:10.1126/science.8303295

G.C. Engelmayr Jr., D. Papworth Glenn, C. Watkins Simon, J.E. Mayer Jr., S. Sacks Michael, Guidance of engineered tissue collagen orientation by large-scale scaffold microstructures. Journal of Biomechanics (2005). doi:10.1016/j.jbiomech.2005.05.020

M.E. Gomes, I. Sikavitsas Vassilios, Esfandiar Behravesh, L. Reis Rui, G. Mikos Antonios, Effect of flow perfusion on the osteogenic differentiation of bone marrow stromal cells cultured on starch-based three-dimensional scaffolds. Journal of Biomedical Materials Research (2003). doi:10.1002/jbm.a.10075

D. Hutmacher, H. Singh, Computational fluid dynamics for improved bioreactor design and 3D culture. Trends in Biotechnology (2008). doi:10.1016/j.tibtech.2007.11.012

G. Kensah, I. Gruh, J. Viering, H. Schumann, J. Dahlmann, H. Meyer, D. Skvorc, A. Bar, P. Akhyari, A. Heisterkamp, A. Haverich, U. Martin, A novel miniaturized multimodal bioreactor for continuous *in situ* assessment of bioartificial cardiac tissue during stimulation and maturation. Tissue Engineering (2011). doi:10.1089/ten.tec.2010.0405

L. Kim, Y.-C. Toh, J. Voldman, H. Yu, A practical guide to microfluidic perfusion culture of adherent mammalian cells. Lab on a Chip (2007). doi:10.1039/b704602b

K.N. Krahn, V.C. Bouten Carlijn, S. van Tuijl, M.A.M.J. van Zandvoort, M. Merkx, Fluorescently labeled collagen binding proteins allow

specific visualization of collagen in tissues and live cell culture. Analytical Biochemistry (2006). doi:10.1016/j.ab.2006.01.013

E. Leclerc, Y. Sakai, T. Fujii, Cell culture in 3-dimensional microfluidic structure of PDMS (polydimethylsiloxane). Biomedical Microdevices (2003). doi:10.1023/A:1024583026925

M.J. Lydon, K.D. Keeler, D. Thomas, Vital DNA staining and cell sorting by flow microfluorometry. Journal of Cellular Physiology (1980). doi:10.1002/jcp.1041020208

I. Martin, D. Wendt, M. Heberer, The role of bioreactors in tissue engineering. Trends in Biotechnology (2004). doi:10.1016/j.tibtech.2003.12.001

S.K. Moore, S.J. Kleis, Characterization of a novel miniature cell culture device. Acta Astronautica (2008). doi:10.1016/j.actaastro.2008.01.027

N.T. Elliott, F. Yuan, A review of three-dimensional *in vitro* tissue models for drug discovery and transport studies. Journal of Pharmaceutical Sciences (2010). doi:10.1002/jps.22257

J.A. Paten, R. Zareian, N. Saeidi, S.A. Melotti, J.W. Ruberti, Design and performance of an optically accessible, low-volume, mechanobioreactor for long-term study of living constructs. Tissue Engineering (2011). doi:10.1089/ten.tec.2010.0642

J.A. Pedersen, M.A. Swartz, Mechanobiology in the third dimension. Annals of Biomedical Engineering (2005). doi:10.1007/s10439-005-8159-4

M.J. Powers, K. Domansky, M.R. Kaazempur-Mofrad, A. Kalezi, A. Capitano, A. Upadhyaya, P. Kurzawski, K.E. Wack, D.B. Stolz, R. Kamm, L.G. Griffith, A microfabricated array bioreactor for perfused 3D liver culture. Biotechnology and Bioengineering (2002). doi:10.1002/bit.10143

M.T. Raimondi, F. Boschetti, L. Falcone, G.B. Fiore, A. Remuzzi, E. Marinoni, M. Marazzi, R. Pietrabissa, Mechanobiology of engineered cartilage cultured under a quantified fluid-dynamic environment. Biomechanics and Modeling in Mechanobiology (2002). doi:10.1007/s10237-002-0007-y

M.T. Raimondi, G. Candiani, M. Cabras, M. Cioffi, K. Laganà, M. Moretti, R. Pietrabissa, Engineered cartilage constructs subject to very low regimens of interstitial perfusion. Biorheology (2008). doi:10.3233/BIR-2008-0490

M. T. Raimondi, D. T. Bridgen, M. Laganà, B. Tonnarelli, M. Cioffi, F. Boschetti, D. Wendt, In Methods in bioengineering 3D Tissue Engineering. Ed. By Berthiaume F and. Morgan Js (MIB), Yarmush ML, Langer RS (Eds) (Artech House, Boston, 2010), P. 237 ISBN: 978-1-59693-458-0.

M. Rasponi, F. Piraino, N. Sadr, M. Lagana', A. Redaelli, M. Moretti, Reliable magnetic reversible assembly of complex microfluidic devices: fabrication, characterization, and biological validation. Microfluid Nanofluid (2010). doi:10.1007/s10404-010-0738-5

W.A. Rutala, D.J. Weber, Infection control: the role of disinfection and sterilization. Journal of Hospital Infection (1999). doi:10.1016/S0195-6701(99)90065-8

V.I. Sikavitsas, G.N. Bancroft, H.L. Holtorf, J.A. Jansen, A.G. Mikos, Mineralized matrix deposition by marrow stromal osteoblasts in 3D perfusion culture increases with increasing fluid shear forces. PNAS (2003). doi:10.1073/pnas.2434367100

J.S. Stephens, J.A. Cooper, F.R. Phelan Jr., J.P. Dunkers, Perfusion flow bioreactor for 3D *in situ* imaging: Investigating cell/biomaterials interactions. Biotechnology and Bioengineering (2007). doi:10.1002/bit.21252

T. Sun, R. Smallwood, S. Mac Neil, Development of a mini 3D cell culture system using well defined nickel grids for the investigation of cell scaffold interactions. Journal of Materials Science Materials in Medicine (2009). doi:10.1007/s10856-009-3703-7

V. Vickerman, J. Blundo, S. Chung, R. Kamm, Design, fabrication and implementation of a novel multi-parameter control microfluidic platform for three-dimensional cell culture and real-time imaging. Lab on a Chip (2008). doi:10.1039/b802395f

H. Witte, M. Stubenrauch, U. Frober, R. Fischer, D. Voges, M. Hoffmann, Integration of 3-D cell cultures in fluidic microsystems for biological screenings. Engineering in Life Sciences (2011). doi:10.1002/elsc.201000045