Fabrication of multi-well chips for spheroid cultures and implantable constructs through rapid prototyping techniques

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Multi-well chips and constructs prototyping

Abstract

Three-dimensional culture models, such as cell spheroids, are widely used in basic and translational research. Since handling a high number of cell spheroids is more complex and time consuming than handling monolayer cultures, we exploited laser ablation and replica molding to fabricate polydimethylsiloxane (PDMS) multi-well chips for the generation and culture of multiple cell spheroids. Articular chondrocytes (ACs) were used to validate the multi-well PDMS chips. Multi-well spheroids were comparable or superior to standard spheroids in terms of chondrogenic differentiation. Moreover, the use of multi-well chips significantly reduced the operation time for cell seeding and medium refresh.

Using a similar approach, clinical-grade fibrin was used to generate implantable multi-well constructs, allowing differential cell seeding in the construct structure and in the wells. Multi-well fibrin constructs with high cell density regions were compared with constructs where ACs were homogeneously distributed. Expression of chondrogenic genes was increased after 7 days *in vitro* in both types of constructs, with multi-well constructs showing higher *SOX9* and *ACAN* levels than standard constructs. Through differential cell staining, we demonstrated that high cell density regions can be precisely generated in multi-well constructs and that they are still detectable after 5 weeks *in vivo*.

In conclusion, multi-well chips for the generation and culture of multiple cell spheroids can be fabricated by low-cost rapid prototyping techniques. Furthermore, these techniques can be used to generate implantable constructs with defined architecture and spatially controlled cell distribution, allowing the *in vitro* and *in vivo* investigation of cell interactions in a 3D environment.

1 Introduction

Three-dimensional (3D) cultures are widely used as *in vitro* models for cell culture. Compared with monolayer cultures, 3D models better mimic the *in vivo* tissue features, thus providing a physiologically relevant environment (Abbott 2003). Among different types of 3D models developed in the last years, multiple cell aggregates or spheroids have been widely used in tumor (Burdett et al. 2010) and tissue engineering (Fennema et al. 2013) research areas. The screening of multiple drugs or culture conditions through 3D cell spheroids requires a relevant number of experimental replicates with cells from different donors, thus making the use of this 3D model more complex and time-consuming compared to conventional monolayer cultures. 3D spheroids, whereby specific extracellular matrix is produced by aggregated cells, also represent the living "building blocks" in organ printing, which aims at the layer-by-layer biofabrication of 3D functional tissues and organs (Boland et al. 2003; Jakab et al. 2004; Mironov et al. 2009).

The need to generate cell spheroids in a high throughput fashion for cancer research and for basic and translational tissue engineering studies has encouraged the development of multi-well devices as an alternative to standard methods for the formation and culture of multiple 3D cell spheroids (Babur et al. 2013; de Ridder et al. 2000; Moreira Teixeira et al. 2012; Ratnayaka et al. 2013). Indeed, these devices provide a relevant tool to implement 3D spheroid models, reducing the time required for routine operations and minimizing differences between experimental replicates.

Rapid prototyping techniques represent an exceptional tool to generate easy-handling platforms for 3D cell culture. However, existing lithography-based approaches are often expensive and time consuming (Whitesides et al. 2001). Indeed, current approaches based on lithographic fabrication require cleanroom facilities where any minor change in the design involves increasing costs related to the fabrication process. Laser fabrication techniques offer an alternative to lithography-based approaches for rapid prototyping (Becker and Gartner 2008) and have been recently exploited for the fabrication of micro-wells in order to minimize fabrication complexity (Napolitano et al. 2007; Piraino et al. 2012; Selimovic et al. 2011).

Another key element for developing innovative 3D models is the possibility of generating scaffolds with a controlled micro- and/or macro-scale architecture , directing cell distribution and/or cell orientation by topographical cues (Bae et al. 2014; Gerberich and Bhatia 2013). Implantable multi-well scaffolds have recently been proposed for the *in vivo* evaluation of the influence of different extracellular matrix components on the differentiation of cells seeded within the wells (Higuera et al. 2013), demonstrating the great potentiality of this approach.

In this study we exploited laser ablation and replica molding to fabricate multi-well polydimethylsiloxane (PDMS) chips for the culture of multiple cell spheroids, using human articular chondrocytes (ACs) for their validation. ACs are widely investigated in the field of cartilage tissue engineering. Their re-differentiation, necessary to re-acquire their original phenotype after *in vitro* expansion (Giannoni and Cancedda 2006; Giannoni et al. 2005; Schulze-Tanzil 2009), is traditionally performed in 3D conditions (Bernstein et al. 2009; Dehne et al. 2010; Jakob et al. 2001; Zhang et al. 2004), indicating that this cell population is a suitable cell source for the validation of platforms for 3D spheroid formation and culture.

The same rapid prototyping techniques were used to biofabricate implantable fibrin multi-well constructs, allowing differential cell seeding into the construct structure and into the wells. We used a clinical-grade fibrin glue (Ahmed et al. 2008; Ahmed and Hincke 2010; Colombini et al. 2014a; Scotti et al. 2010) to validate our biofabrication process and through differential cell staining we verified the feasibility of precise cell seeding in different regions of the multi-well construct. As a proof of concept, we biofabricated fibrin constructs whereby human ACs were homogeneously dispersed or where different cell density regions were generated. Then, we evaluated cell behavior in standard and multi-well fibrin constructs after 1 and 7 days of *in vitro* culture and after 5 weeks of subcutaneous implantation in nude mice.

Materials and Methods

Cell isolation and expansion

Samples of articular cartilage were harvested from patients affected by osteoarthritis undergoing total hip replacement (5 males and 5 females, mean age 64±8 years), with patients' informed written consent and with the approval of the Institutional Review Board. ACs were isolated from cartilage by enzymatic digestion using 0.15% type II collagenase (Worthington Biochemical Co) for 22 h at 37°C, according to a previously established protocol (Lagana et al. 2014; Lopa et al. 2013). The cells were plated at a density of 10^4 cells/cm^2 and cultured in expansion medium. Expansion medium was composed as follows: High Glucose Dulbecco's Modified Eagle's Medium (HG-DMEM, Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Lonza), 1% HEPES (Gibco), 1% Sodium pyruvate (Gibco), 1% Penicillin/Streptomycin/Glutamine (Gibco), 1 ng/mL Transforming Growth Factor ß-1 (TGF-ß1, Peprotech) and 5 ng/mL Fibroblast Growth Factor 2 (FGF-2, Peprotech). At subconfluence, cells were detached by 0.05% trypsin/0.53 mM EDTA (Gibco) and plated at 5×10^3 cells/cm^2 for further expansion. At passage 2, cells were frozen and stored in liquid nitrogen. When needed, cells were thawed and expanded for one additional passage. To limit the expansion phase required to obtain a suitable number of cells, cells from different donors were pooled together to perform the experiments.

Multi-well PDMS chips

Fabrication of PMMA templates and multi-well PDMS chips

Drawings of PMMA master templates were made by *AutoCAD*® software (©*Autodesk, Inc.*) and processed by *Corel*® *DRAW*™ software (©*Corel Corporation*, 2002). Master templates were obtained by carbon dioxide (CO_2) laser ablation of a polymethylmethacrylate (PMMA) substrate (Fig. 1a, step I) in single hole drilling mode, as previously described (Piraino et al. 2012; Selimovic et al. 2011). The laser power was set at 22.5 W, the write speed at 0.08 mm/s, and z = 17.2 mm (VLS2.30, VersaLASER). Substrate removal through ablation produced features with a Gaussian-profile. A PDMS mixture (Sylgard® 184, Dow Corning) was prepared at a 10:1 ratio (prepolymer/curing agent), degassed, poured on the PMMA master template, and baked at 70°C for

2h (Fig. 1a, step II). Once polymerized, the PDMS was carefully peeled off the PMMA master template and cleaned with 70% EtOH. After rinsing with ddH$_2$O, PDMS multi-well chips were sterilized by autoclave.

Two different configurations of PDMS multi-well chips were designed to host 19 wells in a single chip. Features of the two multi-well chip configurations, named Chip A and Chip B, and their 3D renderings are reported respectively in Fig. 1b and 1c.

Cell spheroid formation

Sterile PDMS chips were placed in a 12 well multiwell plate and each PDMS chip was seeded with a total number of 2.85x10^6 cells by pipetting 1.5x10^5 cells in each single well (Fig. 1a, step III). The 12 well multiwell plate was then centrifuged for 5 min at 230g to favor spheroid formation. After 2 h, 2.85 mL of chondrogenic medium were gently added to each well (Fig. 1a, step IV). Control (Ctrl) spheroids were prepared in 1.5 ml screw-cap conical tubes. Briefly, 1.5x10^5 cells were suspended in 150 μL of chondrogenic medium, transferred to a tube, and centrifuged for 5 min at 230g. Chondrogenic medium was composed as follows: HG-DMEM supplemented with 0.029 mg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes, 1 mM sodium pyruvate, 1.25 mg/ml Human Serum Albumin (HSA, Sigma-Aldrich), 1% ITS+1 (containing 1.0 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin, 0.5 μg/ml sodium selenite, 50 mg/ml bovine serum albumin and 470 μg/ml linoleic acid, Sigma-Aldrich), 0.1 μM dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate and 10 ng/ml TGF-β1. Samples were cultured for 21 days (37°C, 5% CO$_2$). Medium was replaced twice per week.

Measurement of operation time for cell seeding and medium refresh

For spheroid culture in standard tubes and in multi-well PDMS chips, we measured the operation time required to perform cell seeding and medium refresh. Independent measures were obtained testing 3 operators with different skill levels in cell culture techniques.

Spheroid analysis

Evaluation of spheroid area

After 7, 14, and 21 days of culture in chondrogenic medium, spheroids in the multi-well chips were photographed to monitor the size variation. Spheroid area was determined by image analysis using ImageJ software.

Quantification of metabolic activity and DNA content

After 21 days of culture in chondrogenic medium, cell metabolic activity was evaluated by Alamar Blue assay. Briefly, spheroids were randomly harvested from different PDMS chips and from the tubes (control group), transferred to a 96 well multiwell plate, and incubated with 100 μL HG-DMEM supplemented with 10% Alamar Blue for 4 h at 37°C. After incubation, the supernatant was harvested and fluorescence was measured (540 nm-580 nm) using a Victor X3 Plate Reader (Perkin Elmer). The same spheroids were lysed for DNA quantification. After washing with Phosphate Buffered Saline (PBS), spheroids were digested (16 h, 60°C) in 500 μl of PBE buffer (100 mM Na_2HPO_4, 10 mM NaEDTA, pH 6.8) containing 1.75 mg/ml L-cystein (Sigma-Aldrich) and 14.2 U/ml papain (Worthington). DNA quantification was performed by CyQUANT Kit (Invitrogen), according to the manufacturer's instructions.

Histological and immunohistochemical analysis

For histological analysis, chondrocyte spheroids cultured for 21 days were fixed for 24 h in 10% neutral buffered formalin, embedded in paraffin and sectioned at 3 μm. Sections were stained with alcian blue (pH 2.5, Sigma-Aldrich) to detect glycosaminoglycans (GAGs). For immunohistochemistry, sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide in distilled water for 15 min. Mouse monoclonal anti-Collagen II antibody (SPM239, ab54236,Abcam) was applied at room temperature for 1 h. The sections were labeled by the avidin-biotin-peroxidase (ABC) procedure (Hsu et al. 1981) with a commercial immunoperoxidase kit (Vectastain Standard Elite; Vector Laboratories). The immunohistochemical reaction was developed with 3.3′ diaminobenzidine (DAB) for 5 min and sections were counterstained for 2 min with Mayer's hematoxylin.

Implantable multi-well fibrin constructs

Fabrication of PMMA master templates and PDMS counter-molds

PMMA master templates and the corresponding PDMS counter-molds were fabricated by CO_2 laser ablation (Fig. 2a, step I) and replica molding (Fig. 2a, step II), as previously described for the fabrication of multi-well PDMS chips.

Three different configurations of PDMS molds were designed to biofabricate fibrin constructs with multi-well bottom layers containing 19, 10, or 7 wells (Fig. 2b). Features of the three different PDMS mold configurations, named Mold X, Mold Y, and Mold Z, and their 3D renderings are reported respectively in Fig. 2b and 2c. Immediately prior to use, PDMS molds were plasma treated (Harrick Plasma Instruments, USA) for 8 minutes to reduce surface hydrophobicity.

Biofabrication of multi-well fibrin constructs

The two-component fibrin sealant Tisseel® (Baxter Biosurgery) was used to generate the fibrin constructs. The fibrinogen component contained 91 mg/mL human fibrinogen and 3000 KIU/mL aprotinin solution. The thrombin component was diluted to 5 U/mL in 40 mM of $CaCl_2$ at pH 7.0. Initially, fibrin constructs with three different configurations of the multi-well bottom layer were prepared according to the protocol depicted in Fig. 2a: (X) 19 wells, $\varnothing_{well}$ 1 mm, h_{well} 1.5 mm; (Y) 10 wells, $\varnothing_{well}$ 1.5 mm, h_{well} 1.5 mm; (Z) 7 wells, $\varnothing_{well}$ 2 mm, h_{well} 1.5 mm. For each construct, 1.15×10^6 cells were suspended in fibrinogen and thrombin mixed at a 1:1 ratio (cell density 15.4×10^6 cells/mL) and poured into the PDMS molds until polymerization (1 h, 37°C, Fig. 2a, step III). The bottom layer was then gently removed from the PDMS mold and transferred into a 12 well multiwell plate. Custom made PDMS rings ($\varnothing_{out}$ 10 mm, $\varnothing_{in}$ 8 mm, h 3 mm) were placed around the bottom layers. Then, different cell numbers were seeded in each well, depending on the construct configuration (Fig. 2a, step IV): (X) 1×10^5 cells in $\varnothing_{well}$ 1 mm; (Y) 2×10^5 cells in $\varnothing_{well}$ 1.5 mm; (Z) 4×10^5 cells in $\varnothing_{well}$ 2 mm. After 15 minutes, a fibrin top layer (Ø 8 mm, h 1 mm) was generated by suspending 8.5×10^5 cells with fibrinogen and thrombin mixed at a 1:1 ratio (cell density 15.4×10^6 cells/mL) and pouring the mixture over the multi-well bottom layer (Fig. 2a, step

V). In this phase, the PDMS ring was used to match the bottom layer shape and to avoid fibrin leaking. After polymerization, the PDMS ring was removed and constructs were cultured in 3 mL chondrogenic medium (Fig. 2a, step VI). After selecting the best bottom layer configuration based on the reproducibility of construct fabrication and cell seeding into the wells, we compared the multi-well fibrin constructs (10 wells, $\varnothing_{well}$ 1.5 mm, h_{well} 1.5 mm, $2x10^5$ cells/well) with a similar construct without wells. Both types of fibrin constructs ($\varnothing$ 8 mm, h 3 mm) were seeded with a total number of $4x10^6$ cells. Bottom and top layer of the multi-well fibrin constructs were prepared as aforementioned. To seed the wells of the constructs, a cell suspension with a density of 10^8 cells/mL was prepared. In each well, we added 2 µL of solution, corresponding to $2x10^5$ cells. To prepare standard fibrin constructs without wells, $4x10^6$ cells were suspended in fibrinogen and thrombin mixed at a 1:1 ratio (cell density $26.7x10^6$ cells/mL). The mixture was poured into the custom made PDMS rings and let polymerize for 1 h at 37^oC. After polymerization, all constructs were cultured in 3 mL chondrogenic medium.

Fibrin construct analysis

Evaluation of multi-well structure and cell distribution

The macroscopic features of the different bottom layers were evaluated by stereomicroscope (Olympus SXZ10). Cell distribution into the construct and feasibility of cell seeding into the wells were evaluated by fluorescence microscopy (Olympus IX71) using green- or red- cells stained either with the green fluorescent dye Vybrant DiO or with the red fluorescent dye Vybrant CM-Dil (both from Invitrogen) according to the manufacturer's instructions. Briefly, after trypsinization cells were suspended at a density of $1x10^6$ cells/mL in serum-free culture medium. Vybrant DiO or Vybrant CM-Dil was added to the cell suspension (5 µL/mL). Cells were incubated for 10 min at 37°C and then centrifuged (350*g*, 5 min). The supernatant was removed and the cells were washed twice in expansion medium prior to use.

Histological analysis

After 7 days of *in vitro* culture, fibrin constructs cultured for 7 days were fixed for 24 h in 10% neutral buffered formalin, embedded in paraffin and sectioned at 3 μm. For morphological evaluation of fibrin glue constructs, sections were stained with hematoxylin and eosin (HE).

Quantification of DNA and GAGs content

After 7 days of *in vitro* culture, fibrin constructs were digested, as previously described for chondrocyte spheroids. DNA quantification was performed by CyQUANT Kit. The amount of GAGs was spectrophotometrically measured using 16 mg/mL dimethylmethylene blue (Sigma-Aldrich), with chondroitin sulphate as standard (absorbance 525 nm) (Lopa et al. 2014). GAGs content was normalized to the DNA content and expressed as μg GAGs/μg DNA.

Gene expression analysis

After 1 and 7 days of *in vitro* culture, fibrin constructs were harvested and stored in liquid nitrogen for RNA isolation and gene expression analysis. Total RNA was purified using RNeasy Mini kit (Qiagen) following sample disruption with TissueRuptor (Qiagen). RNA was quantified using a spectrophotometer (Nanodrop, Thermo Scientific) and reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Gene expression was evaluated by real time PCR (StepOne Plus, Life Technologies). 40 ng of cDNA were incubated with a PCR mixture including TaqMan® Universal PCR Master Mix and TaqMan® Assays-on-Demand™ Gene expression probes (Life Technologies) using the following assays: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Hs99999905_m1), SRY (Sex determining region Y)-box9 (*SOX9*, Hs00165814_m1), collagen type II alpha I (*COL2A1*, Hs00264051_m1), aggrecan (*ACAN*, Hs00153936_m1), and cartilage oligomeric matrix protein (*COMP*, Hs00164359_m1) The expression of the analyzed markers was normalized to *GAPDH* using the delta Ct method.

Subcutaneous implantation of fibrin constructs in nude mice

Fibrin constructs without or with wells were seeded with green- and red-stained cells and subcutaneously implanted in nude mice. The *in vivo* study was approved by the Mario Negri Institute for Pharmacological Research (IRFMN) Animal Care and Use Committee (IACUC).

Animals and their care were handled in compliance with institutional guidelines as defined in national (Law 116/92, Authorization n.19/2008-A issued March 6, 2008, Italian Ministry of Health) and international laws and policies (EEC Council Directive 86/609, OJ L 358. 1, December 12, 1987; Standards for the Care and Use of Laboratory Animals - UCLA, U.S. National Research Council, Statement of Compliance A5023-01, November 6, 1998).

Three 6-week-old female athymic mice were obtained from Harlan® and maintained in the Animal Care Facilities of the IRFMN, under specific pathogen-free conditions with food and water provided *ad libitum*. Animals were anesthetized by intraperitoneal injection of ketamine chloride (80 mg/kg, Imalgene, Merial) and medetomidine hydrochloride (1 mg/kg, Domitor, Pfizer). Surgical procedures were performed in sterile conditions under a laminar flow hood. Four subcutaneous pockets were created on the dorsum of each mouse by blunt dissection through cranial and caudal skin incisions. One fibrin construct was inserted in each pocket, then the skin was sutured with #4-0 Monocryl thread (Ethicon). After 5 weeks, mice were euthanized by CO_2 inhalation and constructs were explanted.

Explant analysis

Evaluation of cell distribution

After 5 weeks in vivo, the distribution of green- and red- stained cells in the explants was evaluated by fluorescent microscopy (Olympus IX71).

Quantification of GAGs content

Fibrin constructs cultured in vitro for 7 days were analyzed before and after *in vivo* subcutaneous implantation for 5 weeks. Samples were digested in PBE buffer and GAGs were quantified by dimethylmethylene blue assay, as aforementioned. GAGs content was normalized to the construct wet weight (mg).

Statistical analysis

All data are presented as mean±SD. Kolmogorov-Smirnov test was used to assess the normal data distribution (GraphPad Prism version 5.00 for Windows, GraphPad Software). For spheroid analysis, comparison among the three different experimental groups (control spheroids, spheroids in chip A, spheroids in chip B) was performed by One-Way ANOVA with Dunnett's post hoc-test. Mann-Whitney test (unpaired data) for non-normally distributed data was performed to compare different types of fibrin constructs at the same time point (i.e. w/ wells 1d vs. w/o wells 1d) and to compare the same type of fibrin construct at different time points (i.e. w/ wells 1d vs. w/ wells 7d).

Results

Multi-well PDMS chips support the generation and culture of chondrocyte spheroids

Multi-well chips were generated by PDMS replica molding on a laser-ablated PMMA master templates. Optical images of the fabricated multi-well PDMS chips in figure 1d show the fidelity of the multi-well arrays to the original design. As expected, the side view of the PDMS chips section revealed the conical shape of the wells (Fig. 1d, bottom panel).

Articular chondrocytes were used to validate the multi-well PDMS chips as devices for the formation and culture of 3D cell spheroids. The PDMS chips were designed to fit the wells of a 12 multiplate, allowing the simultaneous culture of several spheroids in different experimental conditions in the same plate, as shown in figure 3a, where PDMS chips immediately after cell seeding are depicted. The use of these devices significantly reduced the time required for cell seeding and medium refresh operations compared to standard spheroid culture, representing a technical amelioration in terms of work efficiency (Fig. 3b,c). The PDMS chips, thanks to the transparency of the material, also allowed us to monitor the samples through microscopy over the entire period of culture and to measure basic parameters, such as spheroid area. As shown in figure 3d, cell aggregates started to form already 24 hours after cell seeding and, after 21 days of culture, spheroids were well-formed in both PDMS chips. Spheroid area increased from 7 to 21 days of chondrogenic culture due to matrix deposition (Fig. 3e). At the end of culture, we compared the

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metabolic activity of spheroids cultured in standard tubes (Ctrl) and in PDMS chips. No significant

difference in metabolic activity was observed among the experimental groups (Fig. 3f). In the same

way, the DNA content and the metabolic activity normalized on DNA of spheroids cultured in

standard tubes and in PDMS chips did not differ, showing that spheroids cultured in PDMS chips

were at least as viable and active standard spheroids (Fig. 3g,h). Histological evaluation revealed

that GAGs deposition, which is a key marker of chondrogenic differentiation, was comparable in

Ctrl spheroids and in spheroids cultured in multi-well PDMS chips (Fig. 3i). Lacunar structures,

similar to those found in native hyaline cartilage, were observed in all the experimental groups.

Analysis of type II collagen deposition by immunohistochemistry displayed a more intense staining

in spheroids cultured in PDMS chips compared to standard spheroids, indicating the production of

highly specialized extracellular matrix in these samples (Fig. 3j).

**Implantable multi-well fibrin constructs with high cell density regions support the 3D culture
and re-differentiation of articular chondrocytes**

Multi-well fibrin glue constructs were prepared according to the scheme in Fig. 1b. The simplicity

of the biofabrication process allowed us to prepare 65 multi-well constructs in a single experimental

run. PMMA master templates and PDMS molds with the three different configurations (X, Y, Z)

were used to generate the corresponding bottom layers (Fig. 4a). We noticed that the configuration

with 10 wells ($\emptyset_{well}$ 1.5 mm) was the easiest to fabricate and handle. Indeed, the configuration with

larger wells ($\emptyset_{well}$ 2 mm) had a very thin outer border that did not allow for the easy removal of the

construct from the PDMS mold. The peripheral border of these constructs tended to break during

this operation (Fig. 4a, upper row), resulting in leaking of the seeded cells into the adjacent wells.

On the other hand, the configuration with smaller wells ($\emptyset_{well}$ 1 mm) was quite easy to manipulate

since stiffer, but small imperfections in the fibrin bottom layer resulted in a dramatic reduction of

the volume of the wells (Fig. 4a, upper row), which challenged the reproducibility of cell seeding.

Cell embedding in the bottom layer resulted in a homogeneous cell distribution (Fig. 4b, upper row,

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1   green-stained cells) and cell seeding in the multi-wells proved also to be effective (Fig. 4b, middle

2   and lower row). Based on the aforementioned considerations about simplicity and reproducibility of

3   fabrication, we selected the configuration (Y) with 10 wells ($\varnothing_{well}$ 1.5 mm, h_{well} 1.5 mm) for the

4   prosecution of the study. The central section of the bottom layer shows the cell deposition on the

5   bottom side of each well (Fig. 4c), demonstrating that red-stained cells were confined into the wells

6   and that we were able to generate regions with different cell density in a single construct. The multi-

7   well constructs were completed by adding the top layer, as shown in figure 4d, where the multi-well

8   array in the finalized scaffold can be observed.

9   In figure 5a, macroscopic pictures of fibrin constructs without and with wells show that the pattern

10   of multi-wells was still visible under the top layer (right image). In fibrin constructs without wells,

11   cells were homogeneously distributed throughout the entire scaffold (Fig. 5b,c, left image), whereas

12   in multi-well constructs single wells were clearly visible and hematoxylin/eosin staining showed

13   that a cell multi-layer was present in the wells (Fig. 5b,c, right image), demonstrating once again

14   that different cell density areas were generated in the construct. As revealed by histological

15   analysis, poor matrix deposition and construct remodeling occurred after 7 days of *in vitro* culture

16   (Fig. 5c), probably due to the high concentration of fibrinogen used in our constructs as well as to

17   the short period of *in vitro* culture.

18   After 7 days of culture in chondrogenic medium, the same number of cells was found in fibrin

19   constructs without and with wells (Fig. 5d) and no significant difference in terms of GAGs content

20   per cell between the two types of fibrin constructs was observed (Fig. 5e). Gene expression analysis

21   performed after 1 and 7 days of culture in chondrogenic medium revealed that both types of fibrin

22   constructs well supported chondrocyte re-differentiation. Indeed, we observed that levels of

23   chondrogenic markers such as *SOX9*, *COL2A1*, *ACAN*, and *COMP* increased from day 1 to day 7,

24   with significant differences between time points for all the analyzed markers with the exception of

25   *SOX9* (Fig. 5f-i). Higher transcriptional levels of all the tested markers were measured in ACs

cultured in fibrin constructs with high cell density regions for 7 days, albeit increases with respect to constructs without wells were not significantly different (Fig. 5f-i).

Finally, we evaluated the *in vivo* behavior of ACs in fibrin constructs without or with wells through subcutaneous implantation in nude mice. After 5 weeks of implantation, construct size was dramatically reduced. The pictures reported in Fig. 6a represent the same constructs imaged before and after *in vivo* implantation with the same microscope magnification, clearly showing the diameter reduction from 8 mm to 1.5-2 mm. Surprisingly, despite the reduction in construct size, the multi-well structure was maintained, as shown in the post-implantation pictures where the high cell density areas corresponding to the wells are still visible. GAGs content was evaluated in the fibrin constructs cultured *in vitro* for 7 days (pre-implantation) and in the explants harvested after 5 weeks of subcutaneous implantation (post-implantation). The content of GAGs normalized on construct wet weight was found to be significantly increased in both types of fibrin constructs after 5 weeks *in vivo,* with no significant differences between the two types of fibrin constructs at any time point (Fig. 6b).

Discussion

The primary purpose of this study was to generate multi-well PDMS chips supporting the formation and culture of cell spheroids and to biofabricate a multi-well fibrin construct through low-cost and easily accessible rapid prototyping techniques.

Compared to standard protocols, the use of the PDMS multi-well chips significantly reduced the operation time for cell seeding and medium refresh, which are highly time-consuming factors when preparing several experimental replicates and working with many experimental groups. The possibility to culture simultaneously a maximum of 228 cell spheroids in a single 12 well multiwell plate also reduced the space required for the culture of such a high number of samples in the CO_2 incubator. Furthermore, the optical transparency of the PDMS chips allowed us to monitor the

process of spheroid formation and the changes in dimension and shape occurring during culture. The high hydrophobicity of PDMS (Berthier et al. 2012), related to the low cell adhesion on this material, led to the formation of well-defined chondrocyte spheroids in both the tested configurations. The comparison between spheroids generated and cultured in PDMS chips with spheroids in polypropylene tubes did not show any significant difference in terms of cell proliferation and metabolic activity. On the contrary, while deposition of GAGs was similar in the different experimental groups, type-II collagen was more present in spheroids cultured in the PDMS chips compared to standard spheroids. These results indicate that the multi-well devices were able to support very well both cell viability and chondrogenic differentiation, thus representing an appealing option for 3D cell culture. A similar approach has been used in a very recent study to produce and culture chondrocyte micro-pellets (166 cells/pellet) by Babur and co-workers (Babur et al. 2013). In their study, the PDMS insert surface had to be chemically modified to avoid cell spreading all over the well. By contrast, in our devices the higher ratio between the number of plated cells and the surface available for cell growth overcame the need for any chemical surface treatment, since centrifugation was sufficient to trigger spheroid formation in the wells. Rapid prototyping of PDMS devices hold great potential for studies requiring the indirect co-culture of different cell types, as previously reported for hepatocytes and Kupffer cells (Zinchenko and Coger 2005). In our system, the possibility to precisely seed each well with a specific cell population, together with the easiness of spheroid harvesting from selected wells, allows to evaluate the effect of paracrine factors in each cell type, representing a complementary alternative to the trans-well system where cells are traditionally cultured in monolayer. A major limitation in the use of the presented multi-well devices may be represented by the high hydrophobicity and porosity of PDMS which may cause the nonspecific absorption of small molecules and hence bias the biological outcomes in cell culture applications (Toepke and Beebe 2006). In our multi-well system, to maintain stable cell culture conditions and to reduce the effects of nonspecific absorption into PDMS, we performed systematic replacement of culture medium. Indeed, we did not observe any

negative effect on the re-differentiation of ACs cultured in multi-well PDMS chips compared to spheroids cultured in standard polypropylene tubes. However, since in the last years a number of surface treatments have been developed to prevent nonspecific absorption of molecules, such as sol-gel chemistry (Gomez-Sjoberg et al. 2010) or pre-treatment with n-dodecyl-β-D-maltoside (Huang et al. 2005), or polyethylene glycol and N-acetyl-L-cysteine (Harris et al. 2011) we envision in the future to pre-treat our PDMS devices to minimize possible bias in studies evaluating the paracrine interaction between multiple cell types. Besides applications in basic research investigating the mutual influence between different cell types, multi-well PDMS chips can be used to produce a high number of mini-tissue spheroids, which represent the building blocks in organ printing approaches (Boland et al. 2003; Jakab et al. 2004; Mironov et al. 2009). Indeed, we found that spheroids were viable and metabolically active after a relatively long culture period and histological evaluation showed that cells were able to produce specific extracellular matrix. Finally, considering the field of cartilage tissue engineering, the low-cost multi-well PDMS chips described here could be exploited to generate a high number of autologous 3D chondral spheroids to be used in the treatment of chondral defects, as proposed by a recent clinical trial (http://clinicaltrials.gov/, NCT01222559).

Here, to generate cell spheroids we exploited low-cost rapid prototyping techniques to fabricate multi-well PDMS chips and we took advantage of centrifugal force to promote cell assembly into 3D spheroids. More complex systems can also be implemented. For example, cell spheroids can be generated within microfluidic devices combining the actions of fluid flow and gravity force, whereby cell trapping can be controlled modulating flow rate, cell density and seeding time (Fu et al. 2014; Patra et al. 2013). Alternatively, multiple cell spheroids can be generated using hanging drop plates which rely on gravity-enforced cell assembly (Cavnar et al. 2014; Tung et al. 2011). This method has been recently combined with the bioprinting approach to generate uniform-sized embryoid body replacing manual pipetting with the automated printing of single cells and exploiting the gravity force to induce cell assembly (Xu et al. 2011). All these methods represent

have advantages and disadvantages and require a preliminary optimization phase, which is more or less extensive depending on the easiness of the system. Anyhow, the growing body of literature in this field is a clear indication of the wide number of applications whereby cell spheroids can be used both as 3D models or as tissue building blocks.

In the second part of the study, we applied the same rapid prototyping techniques to produce a multi-well implantable fibrin construct. Considering the field of cartilage tissue engineering, we suggest that a similar configuration can be used to generate high cell density areas within the construct, leading to the formation of "chondrogenic centers" able to promote the differentiation of low cell density regions. Indeed, it has been shown that cell density affects the capability of chondrocytes to produce specific matrix and that a high cell density improves cartilaginous matrix deposition in 3D scaffolds (Mauck et al. 2003; Talukdar et al. 2011). Since cartilage biopsies harvestable from patients are limited in size and allow the retrieval of a limited number of chondrocytes, an extensive *in vitro* expansion phase is required to obtain enough cells to seed a 3D scaffold with a high cell density, which implies chondrocyte de-differentiation (Giannoni and Cancedda 2006; Giannoni et al. 2005; Schulze-Tanzil 2009; Steinert et al. 2007). The possibility of limiting the high cell density to some construct regions, acting as "chondrogenic centers", implies a reduction in the number of cells required to seed the construct, and subsequently a shorter expansion phase and a lower impact of cell de-differentiation. Similar approaches have been proposed by other groups that have used chondrocytes aggregates of different dimensions seeded either in a collagen porous scaffold (Wolf et al. 2008) or embedded in a hydrogel (Moreira Teixeira et al. 2012). Here, we did not generate the chondrocyte aggregate before, but high cell density centers were represented by confluent cell multilayers within the wells. We found that 3D culture in fibrin constructs supported the chondrogenic re-differentiation of articular chondrocytes, as demonstrated by gene expression analysis over time. Furthermore, we found that the presence of high cell density regions in the fibrin constructs improved the transcriptional expression of chondrogenic markers such as *SOX9* and *ACAN*, which encode respectively for a transcriptional

factor modulating the chondrogenic cascade and for one of the major components of cartilaginous extracellular matrix, supporting the hypothesis that high cell density regions may act as chondrogenic centers; however, the short period of *in vitro* culture did not allow us to appreciate possible differences in terms of extracellular matrix deposition.

Remarkably, the construct configurations proposed here also offer the flexibility to be seeded with pre-formed cell aggregates where desired, being the dimensions of the wells compatible with those of chondrocyte spheroids obtained through standard techniques (1.5×10^5-5×10^5 cells/spheroid). In this case, the use of multi-well constructs described here would allow the controlled distribution of cell aggregates which cannot be achieved through a randomized seeding.

As hypothesized for the PDMS chips, the multi-well construct produced in this study, can be used for the co-culture of different cell types that is usually performed by more standard 3D models such as spheroids (Giovannini et al. 2010; Lopa et al. 2013; Wu et al. 2012) and random cell dispersion in 3D scaffolds (Hildner et al. 2009; Sabatino et al. 2012). The controlled distribution of mesenchymal stem cells and bovine chondrocytes in an implantable multi-well scaffold has been performed in a recent study screening the effect of several extracellular matrix components on the chondrogenesis of MSCs co-cultured with bovine chondrocytes (Higuera et al. 2013). Differently from this screening platform where a polymeric scaffold is used and cells are seeded uniquely into the wells (Higuera et al. 2013), in our fibrin multi-well construct cells can be seeded both into the construct structure and in the wells, further allowing to evaluate the interactions among multiple cell types distributed in different construct regions.

Bioprinting techniques could be exploited as an alternative to the biofabrication approach employed here to control cell distribution in 3D fibrin constructs, as reported in a recent study where human endothelial cells and fibrin have been used as bio-ink for microvasculature construction (Cui and Boland 2009). Similarly to the biofabrication method that we have used in the present study, the bioprinting approach would be suitable for the generation of constructs seeded with multiple cell types (Kolesky et al. 2014; Schuurman et al. 2011). As demonstrated by several studies employing

bioprinting to generate complex 3D structures, this approach allows a precise control over construct configuration and cell distribution (Murphy and Atala 2014). However, differently from the easy biofabrication method that we have presented here, the implementation of bioprinting would require an extensive preliminary phase to establish the optimal concentration of fibrinogen, thrombin, $CaCl_2$ as well as the polymerization time to obtain the desired configuration and grant proper cell distribution and viability, representing a highly time-consuming phase in the biofabrication process. Regarding the behavior of fibrin constructs *in vivo*, we observed a dramatic size reduction of the implants after 5 weeks of subcutaneous implantation. This result has been previously reported in several short-term *in vivo* studies (Colombini et al. 2014b; Hwang et al. 2013; Peretti et al. 2000) and can be explained by two major features of fibrin: its tendency to shrink and its fast degradation rate (Ahmed et al. 2008; Meinhart et al. 1999; Wolbank et al. 2014). Thanks to fluorescent cell staining, we verified that the implant size reduction did not alter cell distribution. Indeed dispersed red-stained cells and green-stained spot corresponding to the high cell density regions were clearly visible before and after subcutaneous implantation. On the other hand, when fibrin constructs were cultured *in vitro* for 7 days we observed a poor construct remodeling, meaning that the multi-well structure was well preserved and that cells were not able to deposit a relevant amount of extracellular matrix. These observations can be explained by the short period of in vitro culture applied, sufficient to reveal an increase in the transcriptional expression of chondrogenic genes, but not a relevant deposition of matrix proteins, and by the high concentration of fibrinogen used in our fibrin glue formulation, further stabilized by the presence of aprotinin (Meinhart et al. 1999). Hence, since it has been shown that lower concentrations of fibrinogen are compatible with the biofabrication of patterned scaffolds (Koroleva et al. 2012), we envision to tune this parameter in future *in vitro* studies in order to favor the interaction of multiple cell types seeded in different construct regions as well as extracellular matrix deposition.

Conclusions

We have presented a cost-effective method exploitable for the fabrication of platforms for the culture of multiple cell spheroids and for scaffold prototyping. Remarkably, the proposed fabrication approach is simple and exploits economic tools, as commercial design software and desktop laser, which do not require dedicated facilities and are accessible to most research labs. These features prompting the application of this approach in basic and translational research, whereby cell spheroids and 3D constructs represent key models in drug screening studies as well as the building blocks for translational tissue engineering approaches.

Disclosure

Authors have no competing interests to declare

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1   representative well of chip A and chip B at different time points after cell seeding (4 hours, 24

2   hours, 21 days, scale bar 1 mm). (**e**) Average area of spheroids cultured in Chip A and Chip B over

3   time (n=54, data obtained from 3 independent chips both for configuration A and B). (**f-h**)

4   Metabolic activity, DNA content, and metabolic activity normalized on DNA measured in spheroids

5   cultured for 21 days in standard conditions (n=9) or in PDMS chips (n=9, 9 samples harvested from

6   3 independent chips both for configuration A and B). (**i-j**) Histological evaluation of GAGs and

7   type II collagen deposition in spheroids cultured for 21 days in standard conditions and in PDMS

8   chips (scale bars 200 μm).

9

10   **Figure 4. Fabrication of multi-well fibrin constructs and evaluation of cell distribution.** (**a**)

11   Design and stereomicroscope micrographs showing the three different bottom layer configurations

12   (scale bars 1 mm). (**b**) Micrographs showing cell distribution in the multi-well fibrin constructs.

13   Green-stained cells are embedded into the bottom layer, whereas red-stained cells are seeded into

14   the wells (scale bar 1 mm). (**c**) Micrographs showing the central section of a cell-seeded fibrin

15   constructs ($\varnothing_{well}$ 1.5 mm, h 1.5 mm, phase contrast and fluorescence microscopy, scale bar 1 mm).

16   (**d**) Micrographs showing the multi-well pattern in the construct after the top layer polymerization.

17   Green-stained cells are embedded into bottom and top layer and red-stained cells are seeded into the

18   wells (scale bar 1 mm).

19

20   **Figure 5. Comparison between fibrin constructs without and with wells.** (**a**) Representative

21   pictures of fibrin constructs w/o wells and w/ wells (scale bar 2 mm). (**b**) Micrographs showing cell

22   distribution in fibrin constructs w/o wells and w/ wells (scale bar 2 mm). (**c**) Hematoxylin/eosin

23   staining of construct sections. The border of the well is indicated by a dotted line and cells

24   distributed along the lower border of the well are indicated by arrows (scale bar 200 μm). (**d-e**)

25   DNA content and GAGs content normalized on DNA measured in fibrin constructs w/ and w/o

26   wells after 7 days of *in vitro* culture (n=3). (**f-i**) Transcriptional levels of *SOX9*, *COL2A1*, *ACAN*,

1 and *COMP* evaluated in fibrin constructs w/ and w/o wells after 1 and 7 days of *in vitro* culture

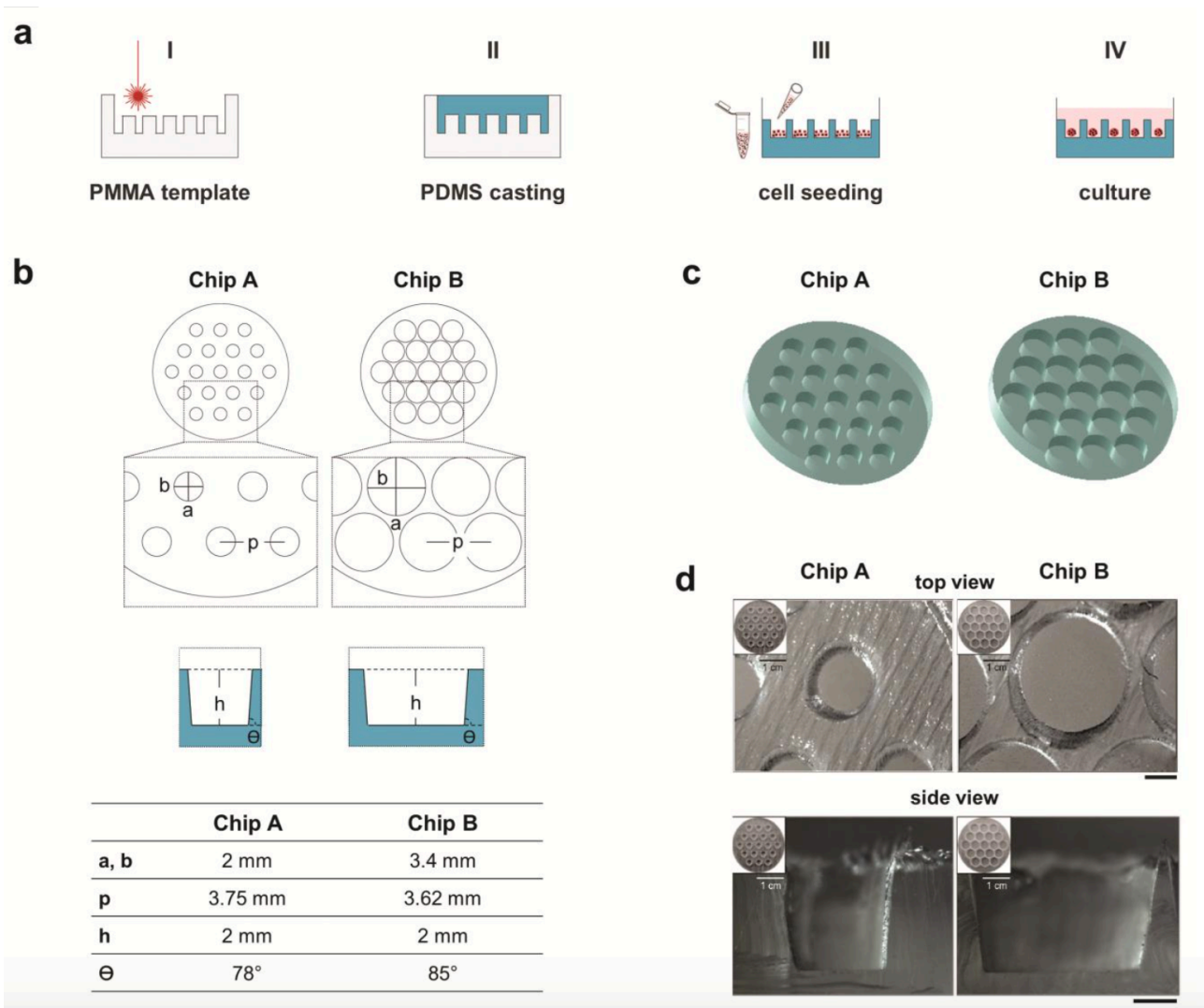2 (n=4). Gene expression data are normalized on GAPDH using the delta Ct (dCt) method.

3

4 **Figure 6. Cell distribution and GAGs content after *in vivo* implantation in nude mice.** (**a**)

5 Micrographs showing fibrin constructs w/o and w/ wells seeded with red and/or green stained cells
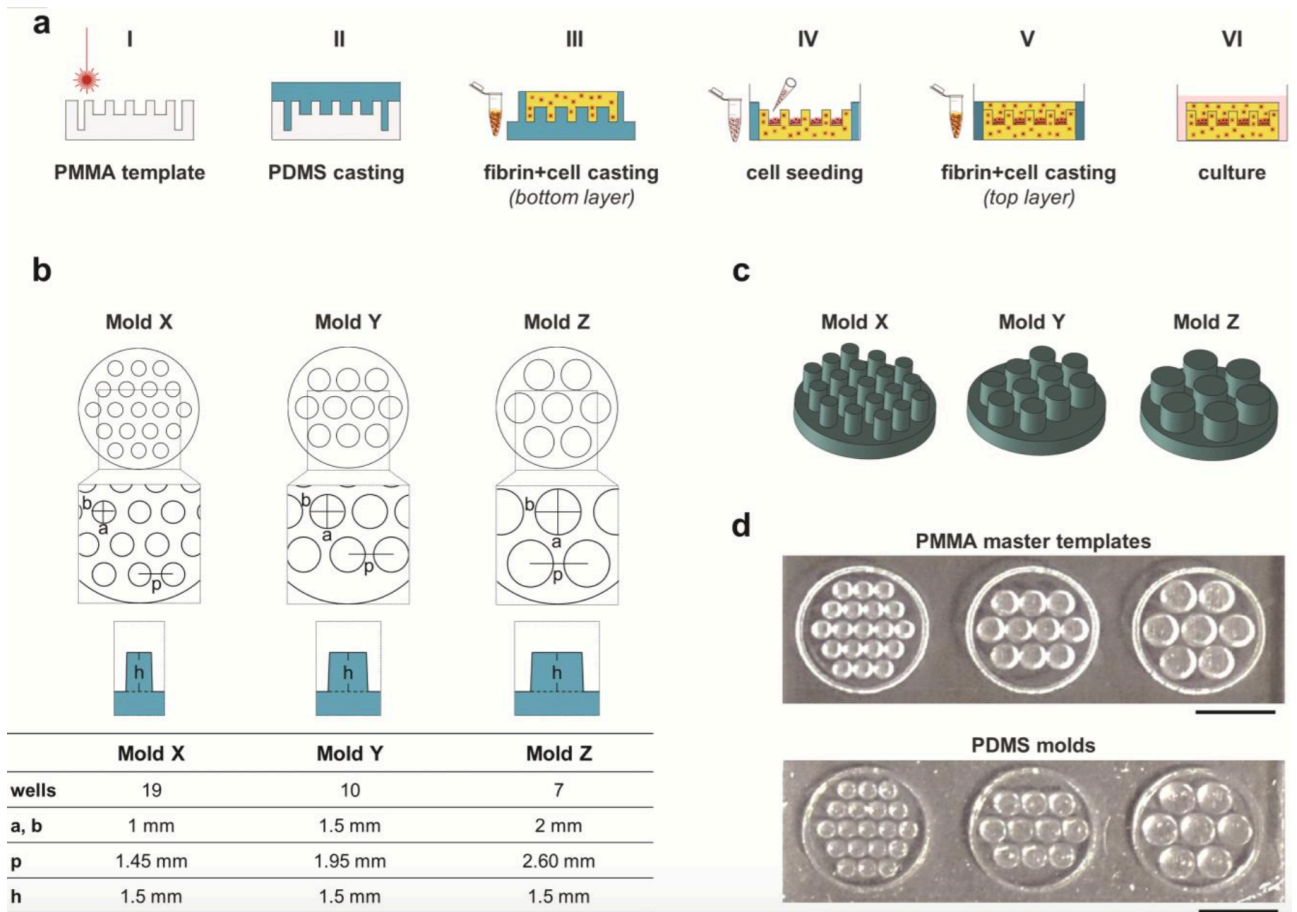
6 prior and after subcutaneous implantation in nude mice (scale bar 1 mm). (**b**) GAGs content

7 normalized on construct wet weight measured in fibrin constructs w/o and w/ wells prior and after

8 subcutaneous implantation.

Figure 1

a

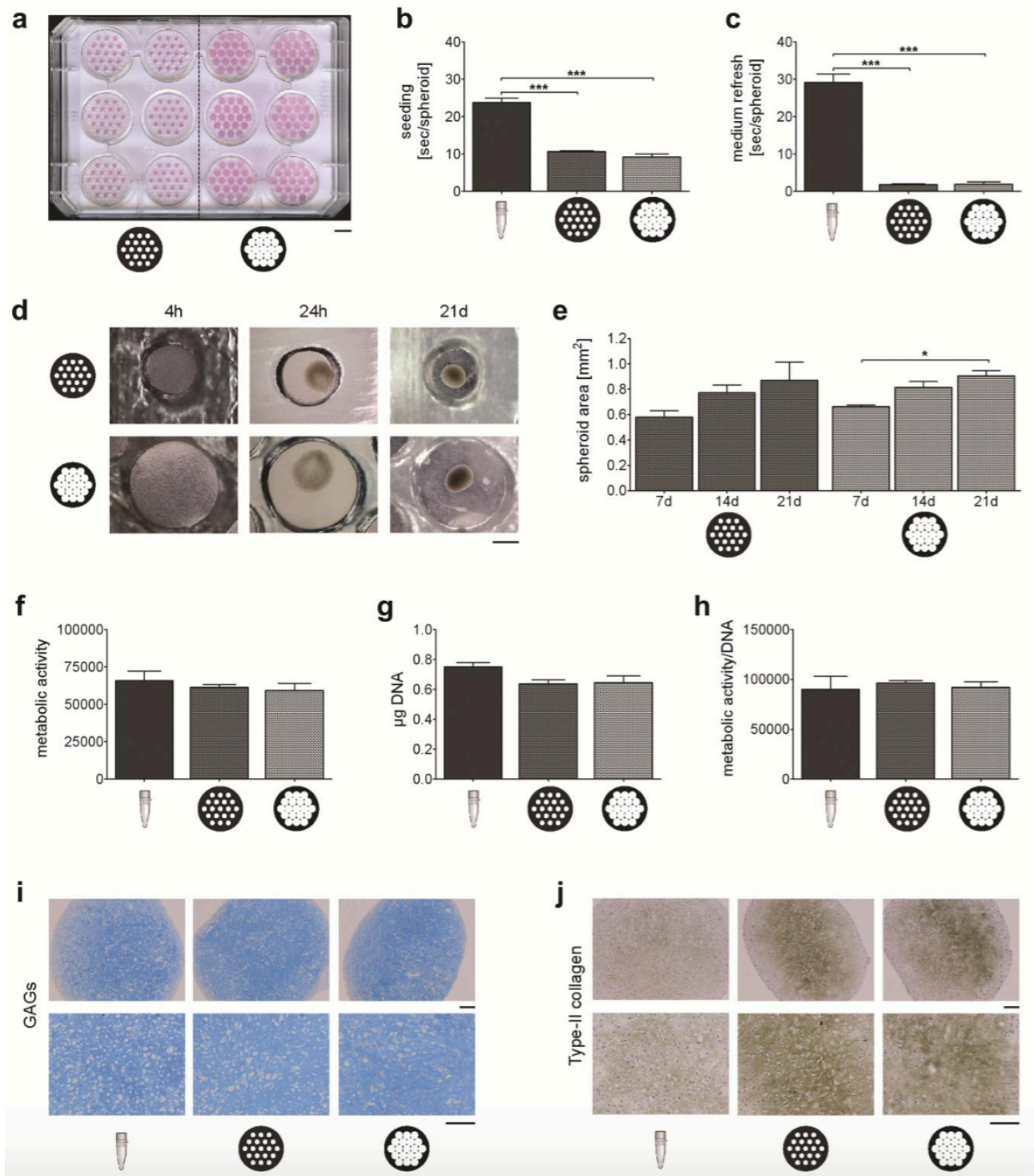| I	II	III	IV	V	VI
PMMA template	PDMS casting	fibrin+cell casting *(bottom layer)*	cell seeding	fibrin+cell casting *(top layer)*	culture

b

Mold X　　Mold Y　　Mold Z

	Mold X	Mold Y	Mold Z
wells	19	10	7
a, b	1 mm	1.5 mm	2 mm
p	1.45 mm	1.95 mm	2.60 mm
h	1.5 mm	1.5 mm	1.5 mm

c

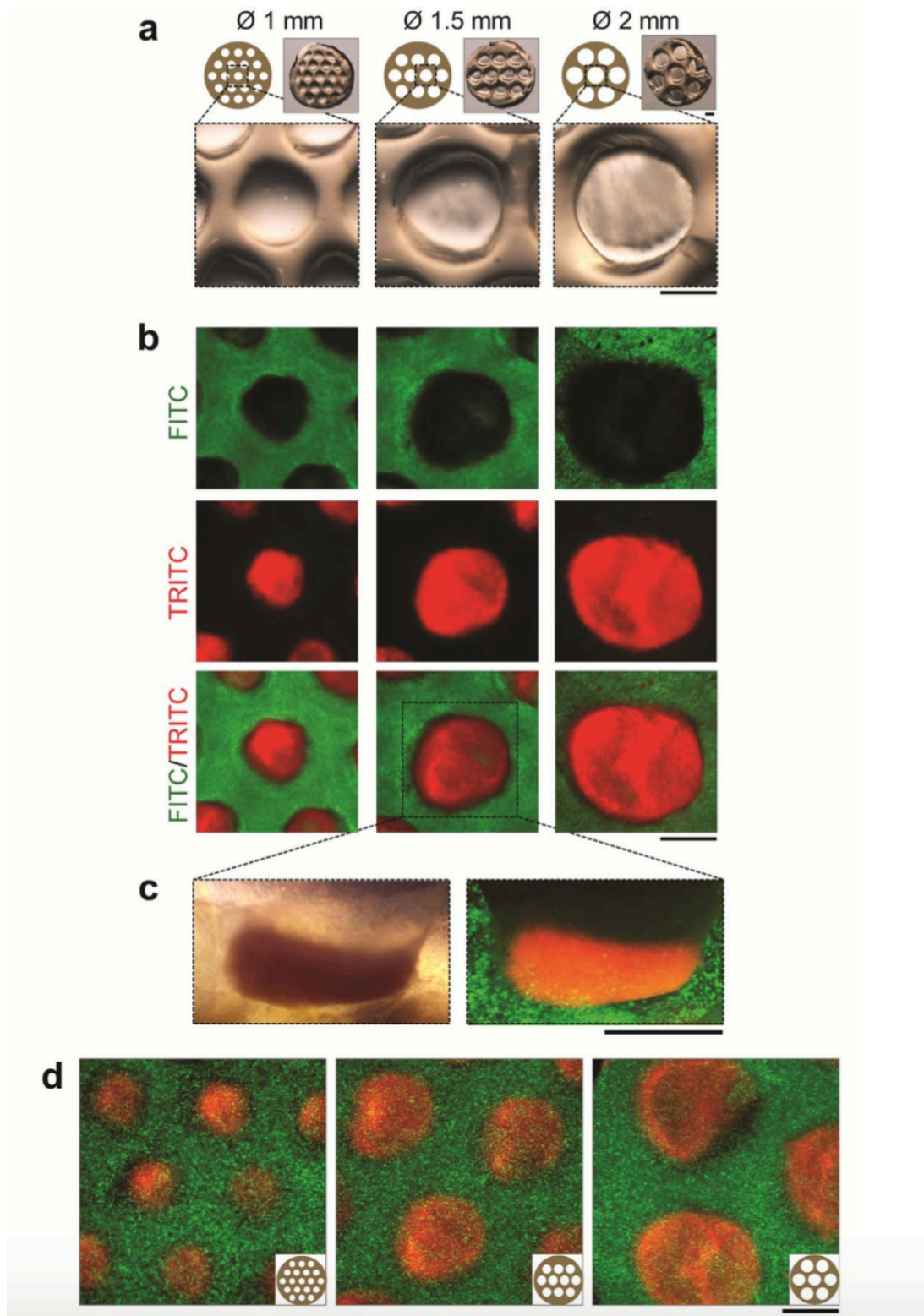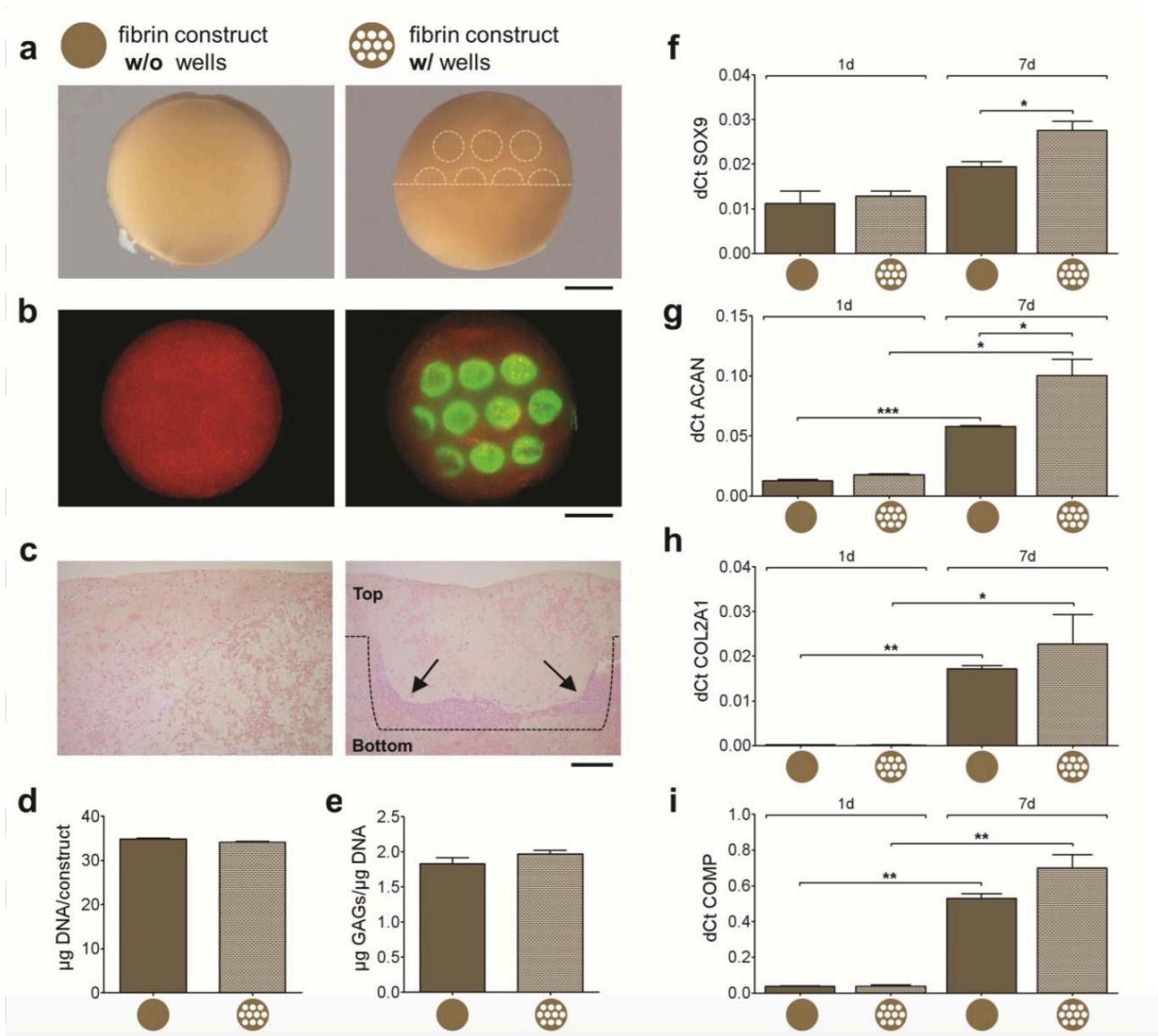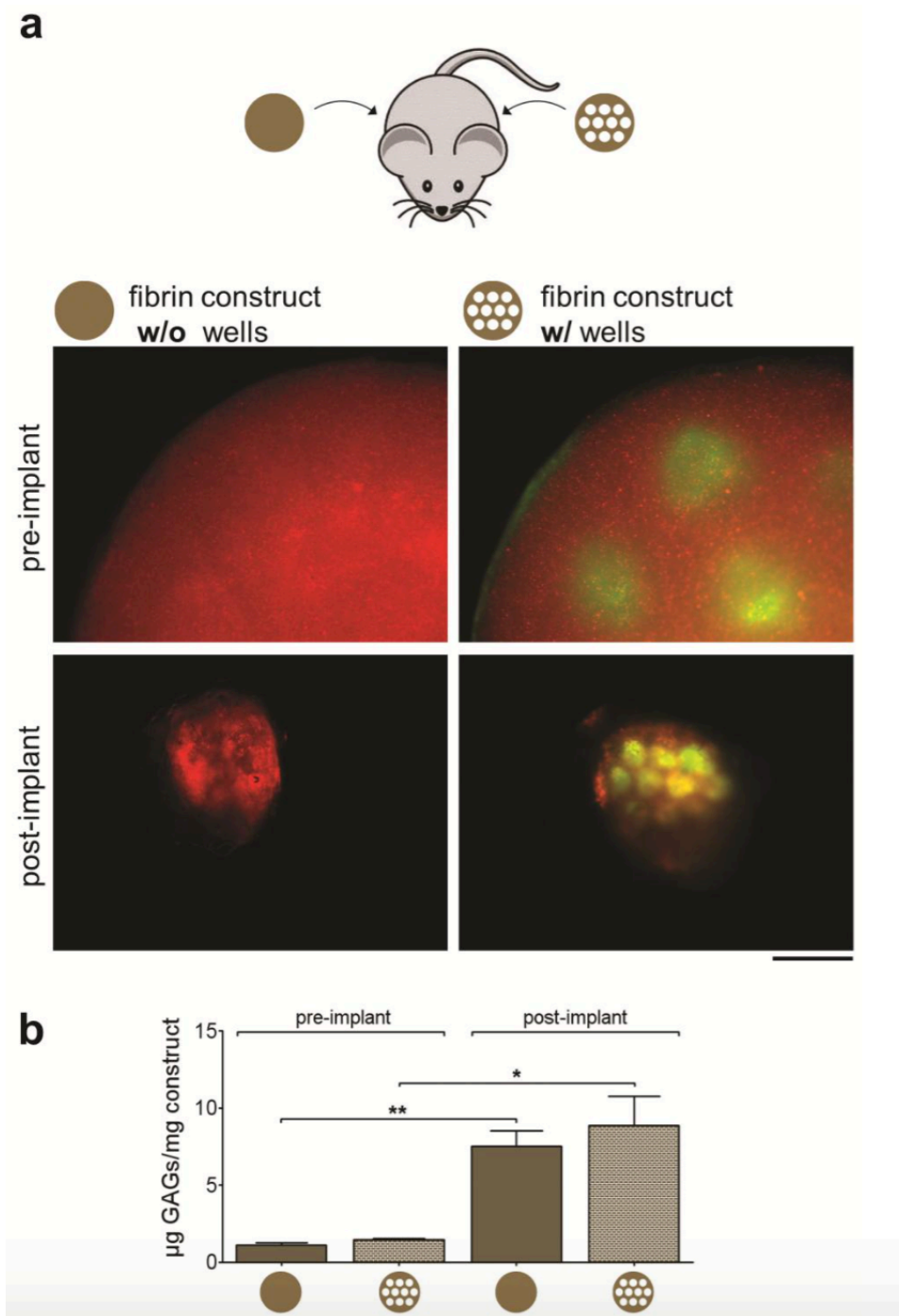Mold X　　Mold Y　　Mold Z

d

PMMA master templates

PDMS molds

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6