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for Diagnosis & Regeneration in Thoracic Diseases & Applied Immunology

3D printed microchannel networks to direct vascularisation during endochondral bone repair

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Endochondral Ossification

- Bone formation during **fetal developement** of the mammalian skeletal system, **bone lenght growth** and **fracture healing (Callus formation)**
- continuous cell division of chondrocytes is accompanied by secretion of extracellular matrix
- temporary cartilaginous templates become vascularised and transformed into bone → OPCs recruitment
- chondrogenically primed MSCs secrete morphogenic proteins such as VEGF, BMP-2, SDF-1, and PDGF

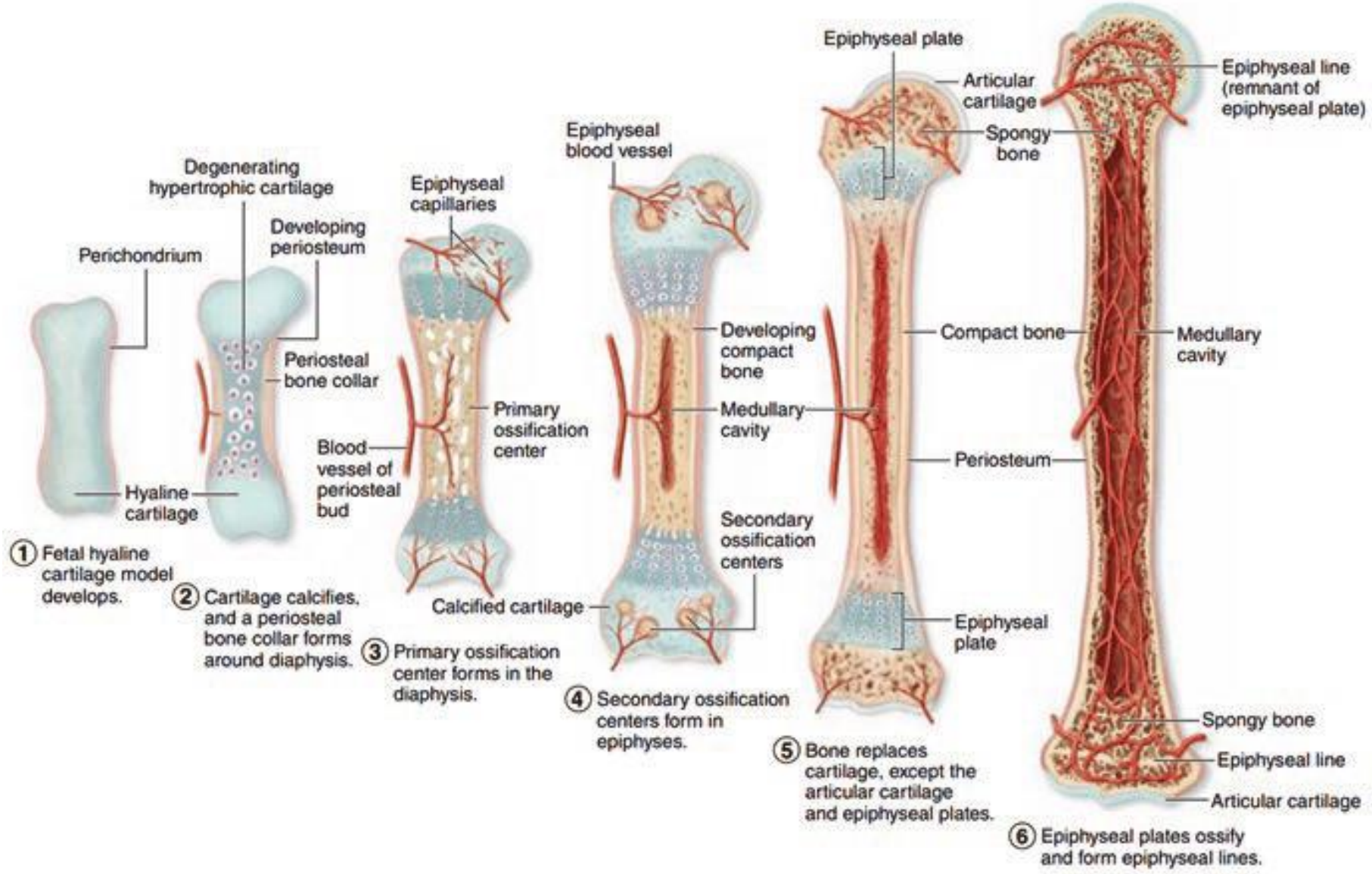
Ossification sites

Primary Ossification

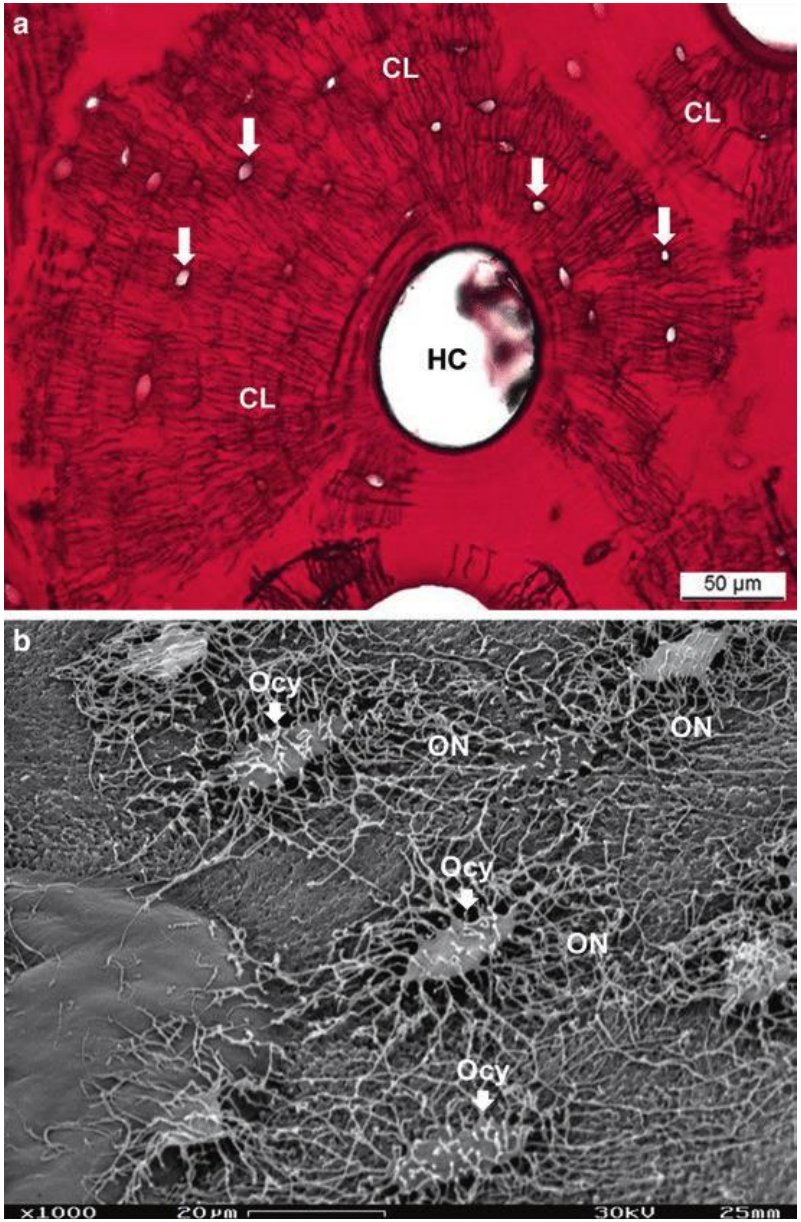
- Primary center of ossification is the diaphysis
- perichondrium becomes the periosteum
- Collar formation by osteoblast osteiod secrtetion
- Calcification (collagen, proteoglycan secretion ↓ alkaline phosphatase↑)

Secondary Ossification

- Secondary center of ossification → epiphysis
- Cartilage between primary and secondary ossification center → **epiphyseal plate**
- Epiphyseal line – point of union between the two ossification centers
- Cartilage removed by bone



adapted from - JA Gasser et al. Bone Physiology and Biology. Bone Toxicology 09/2017



a) decalcified human femur

b) EM image of osteocytes

adapted from - JA Gasser et al. Bone Physiology and Biology. Bone Toxicology 09/2017

Materials and Methods

Isolation and expansion of MSCs

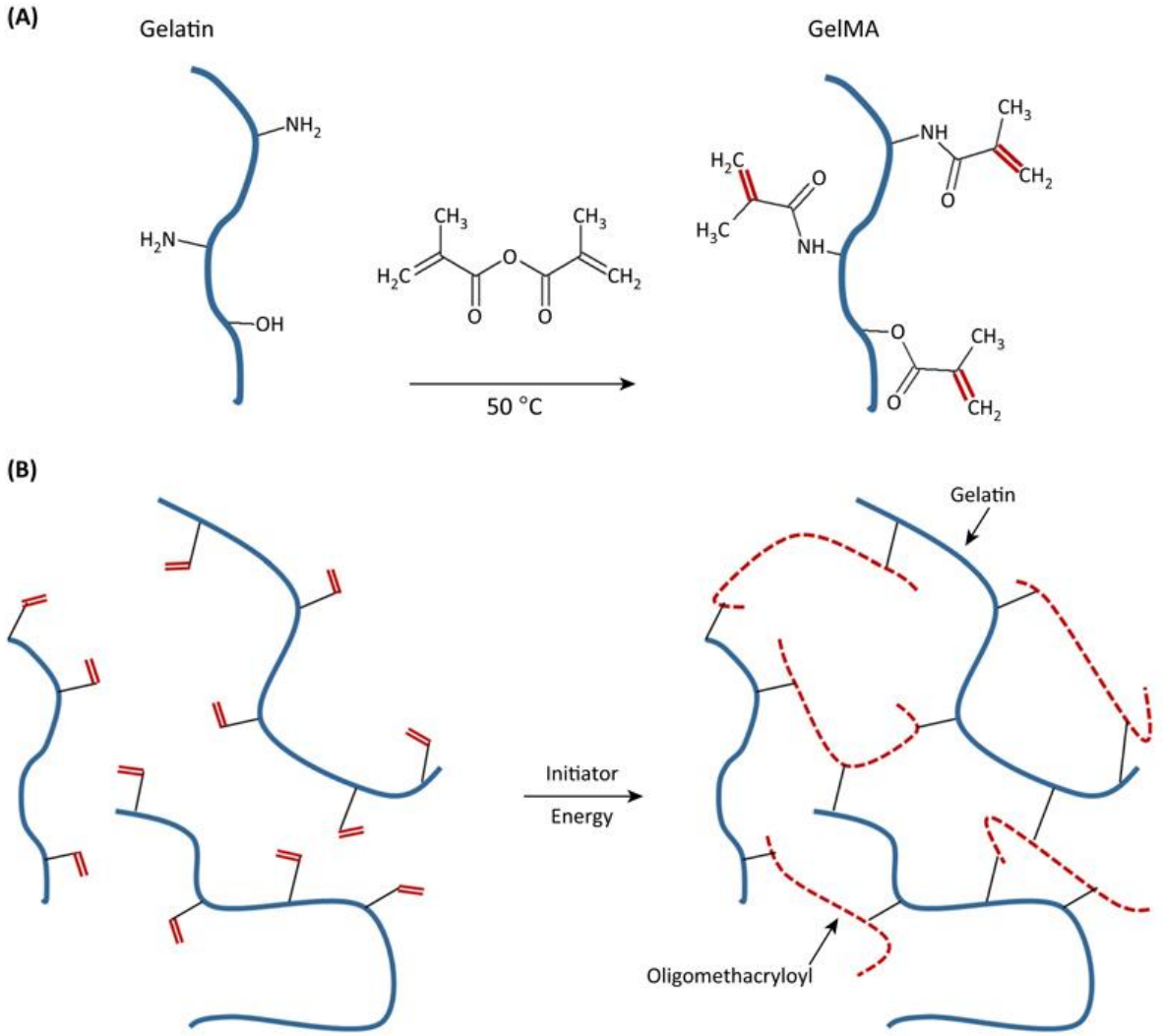
- BM – derived – MSCs isolated from femoral shaft of 6 week old Fisher Male rats
- Expanded in high – glucose Dulbecco´s modified Eagle´s medium GlutaMAX (hgDMEM)
- Supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2.5mg/ml amphotericin B at 5% pO_2
- Colony formation → MSCs were trypsinized, counted and seeded at a density of 5000 cell/cm^2 in 500 cm^2 triple flasks
- Again in supplemetnation medium + 5 ng/ml human fibroblastic growth factor-2

GelMA

- Gelatin Methacryoloyl (GelMA) is a hydrogel suitable for biomedical applications
- Posesses ECM properties - cell-attaching and MMP responsive peptide motifs

  → proliferation and spreading of cells in GelMA based scaffolds
- When exposed to light irradiation it crosslinks

  → form hydrogels with tunable mechanical properties



A) GelMA formation

B) GelMA chains form polymers

Gelatin and polymethacryoloyl Network formation

Adapted from - BJ Klotz et al. Gelatin-Methacryloyl Hydrogels: Towards Biofabrication-Based Tissue Repair. Publshed in Trends Biotechnology 05/2016

GelMA synthesis

- synthesized by reaction of porcine type A gelatin with methacrylic anhydride at 50˚C for 4h

- Methacrylic anhydride (1ml MA/gram gelatin) was added to a 10% solution of gelatin in PBS under constant stirring
  → methacrylation of approximately 75%

- functionalized polymer was dialyzed against distilled water for 7 days at 40˚C

  → dried and frozen at -20˚C until usage

In vitro culture conditions

- Culture conditiones defined as culture in a chondrogenic medium (CDM)
- Consists of **hgDMEM GlutaMAX** supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 100 mg/ml sodium pyruvate, 40 mg/ml L-proline, 50 mg/ml L-ascorbic acid-2-phosphate, 4.7 mg/ml linoleic acid, 1.5mg/ml bovine serum albumine, 1x insuline- transferrineselenium, 100 nM dexamethasone ,2.5 mg/ml amphotericin B, 500 ng/ml of recombinant human **bone morphogenetic protein 2 (BMP-2)** and 10 ng/ml of **human transforming growth factor-b3 (TGF-b3)**

- The constructs were primed for 2 weeks at 5% pO_2
  → followed by 2 weeks at 20% pO2 before implantation

3D printing system

- All 3D-printing was performed using a 3D Discovery system (Regen Hu, Switzerland)
- Pluronic F127 was dissolved at 40 wt% in deionized ultrapure water by mixing at 4˚C overnight - **sacrificial pluronic ink** -
- ink was loaded into a syringe → centrifuged to remove any air bubbles
- using a 30G needle → printed at 18˚C and an extrusion pressure of 0.5 MPa.
- cell density of 20 million BM – derived - MSCs/ml of hydrogel

Microchanneled cartilage templates

- free-standing network of micropillars was 3D-printed onto a glass slide using a pluronic ink (40 wt %)
- pluronic circle (4 mm inner-Ø) was printed around the micropillars
- cell-laden GelMA solution (10 wt% + irgacure 0.05 wt %), containing MSCs (20 million/ ml), was pipetted around the micropillars
- cross-linked using UV light for 25 min
- The constructs were next placed in CDM media at 37˚C for 3 h

- Cooling of the pluronic network at 4°C for 5 minutes resulted in it´s sacrification

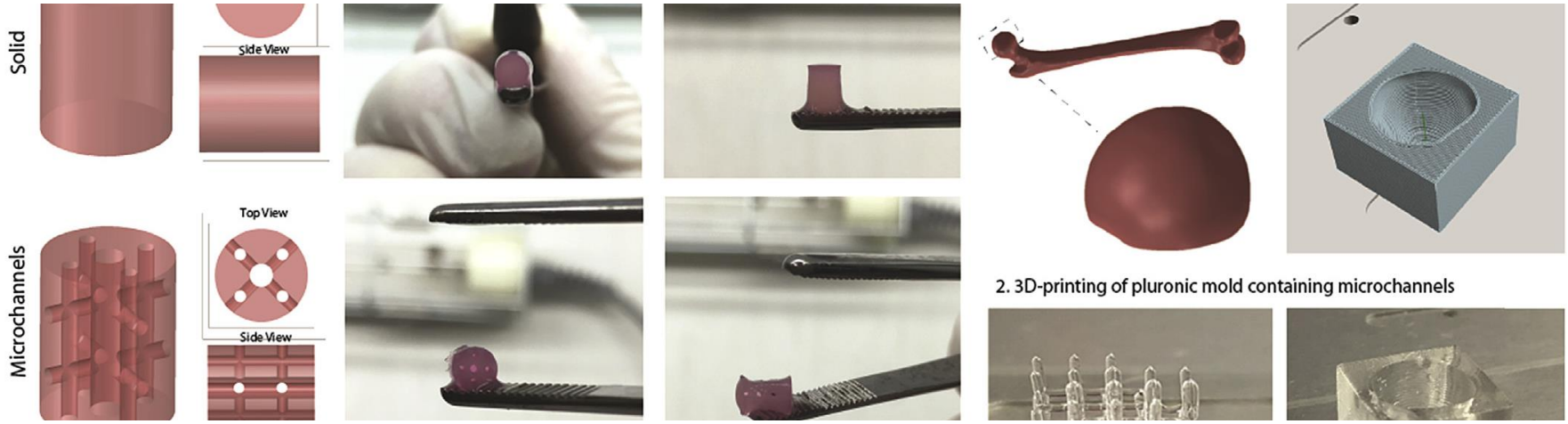
  → formation of an open network of interconnected microchannels inside the cylindrical hydrogel

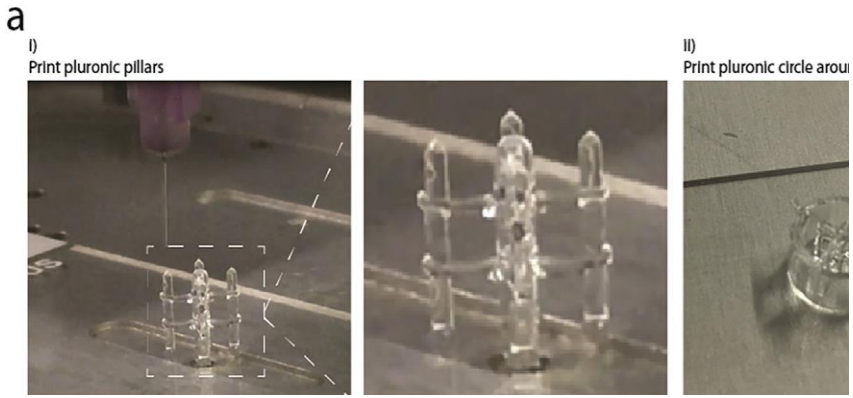
Live/dead confocal microscopy

- Using a LIVE/DEAD™ viability/cytotoxicity assay kit cell viability was assessed after 24h

- Constructs were washed in PBS → incubation in PBS containing 2 mM **calcein AM** (**green fluorescence** of **membrane for live cells**) and 4 mM **ethidium homodimer-1** (**red fluorescence** of **DNA** for **dead cells**)
- Sections were again washed in PBS, imaged with a Leica SP8 scanning confocal at 515nm and 615nm channels
- Analysis using Leica Application Suite X (LAS X) software

3D printing of microchanneled cartilage templates





Biochemical analysis

- Biochemical content of all hydrogels were analysed pre-implantation (after 4 weeks of in vitro cultivation)
- Priorly washed in PBS and frozen away at -20˚C
- Constructs were digested with papain (125 mg/ml) in 0.1 M sodium acetate, 5 mML-cysteine-HCl, 0.05 EDTA pH 6.0 at 60˚C and 10 rpm for 18 h
- amount of sulphated glycosaminoglycan (sGAG) was quantified using the dimethyl methylene blue dye-binding assay with a chondroitin sulphate standard.

Histological and immunohistochemical analysis

- Fixation in 4% formaldehyde → submerged in graded series of ethanol´s for dehydration → paraffine wax embedment → sections of 8µm affixed to microscope slides
- Post-implantation con-structs were decalcified in EDTA for 2 weeks
- H&E and safarin – O stainings for assesment of bone formation and sGAG content respectively
- Collagen types I, II and X were evaluated using a immunohistochemical standard precedure
- assayed for cellular expression of tartrate- resistant acid phosphatase (TRAP) using an acid phosphatase staining kit

Rat randomization

chondrogenical priming

- solid construct (Solid endo-chondral ossification (EO))
- channeled construct (Microchannels EO)

no chondrogenical priming

- No construct (EMPTY)
- BMP-2 loaded solid construct with no pre-culture (BMP-2)

Surgical procedure

- femoral defects (5mm, mid-diaphyseal) were created in immune-competent adult Fischer rats (>12 weeks old, one defect/animal)
- Femural shaft exposeure via sharp and blunt dissections → circumferential removal of the periostium.
- weight-bearing polyetheretherketone (PEEK) internal fixation plate was secured

  Defect created using an dental drill followed by thorough saline irrigation to remove bone debris

- Constructs were press-fit into the defect site

- The wounds were closed with sutures
- On scheduled explant retrieval, rats were sacrificed, CO_2 asphyxiation
- repaired femur, with the PEEK plate fixator intact, was carefully separated from the adjacent hip and knee joints for analysis
- repair tissue was harvested for analysis at 4 and 8 weeks post-implantation.

Vascular µCT analysis and rat µCT

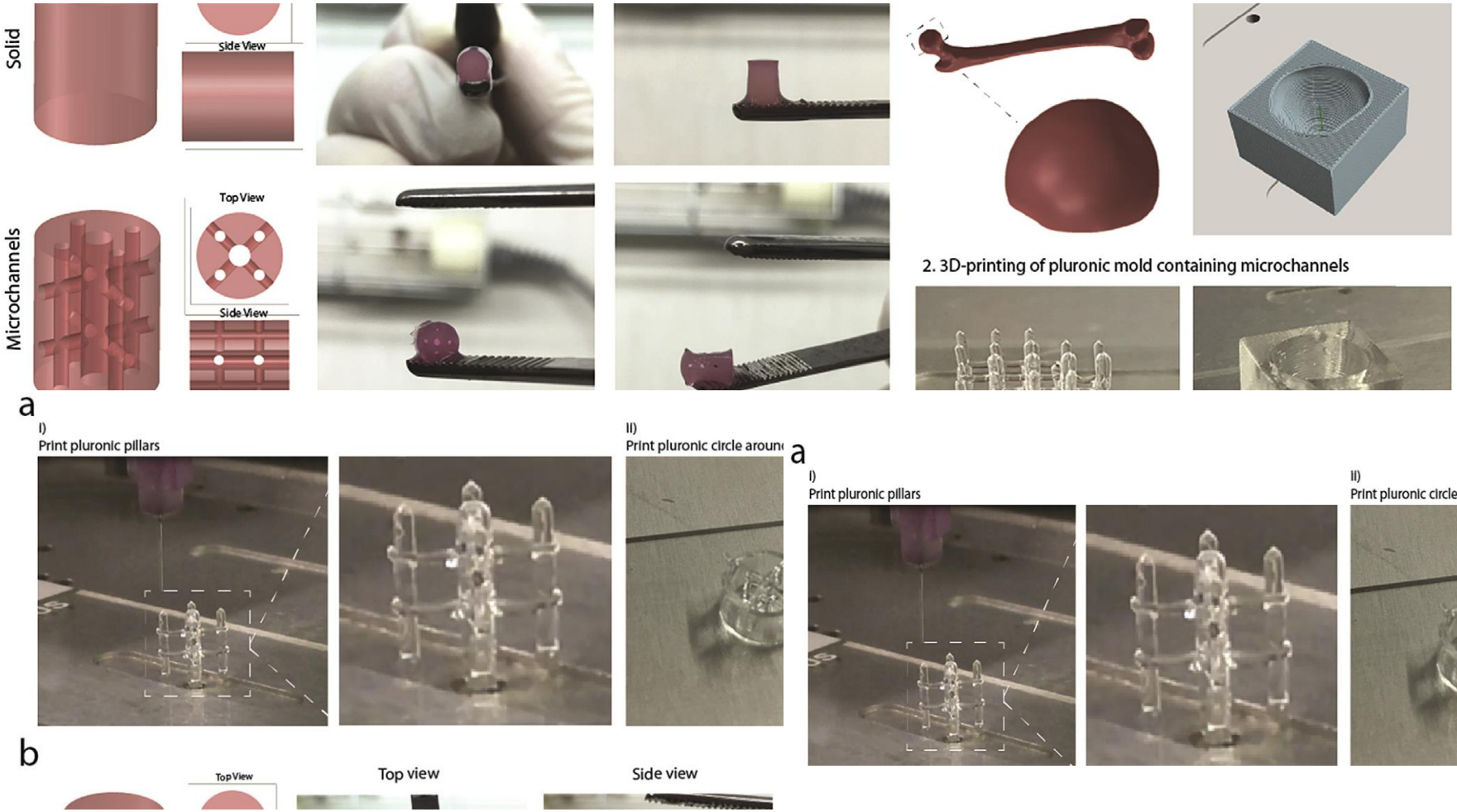
- Contrast-enhanced angiography was performed at week 4 to assess vascularisation within the bone defects
- After sacrification of the rats the vasculature was immediately perfused through the ascending aorta with sequential solutions of heparin (25 U/ml), formalin, PBS, and a barium-based contrast agent (delivered at 6 ml/min)
- Contrast agent : porcine type A gelatin (3 wt%), sodium chloride (0.9 wt%) and barium powder (40 wt%) – delivered at 3 ml/min
- 18G intravenous catheter was used to deliver the perfusion solutions into the rats left ventricle.

- limbs were excised and scanned using mCT with both bone and contrast agent present.
- For visualisation and quantification of the vascular volume alone, the excised limbs were next decalcified in EDTA (15 wt%, pH 7.4) for 2 weeks to remove the bone mineral content
- µCT scans were performed before and after decalcification allowing comparison and defining the defect VOI
- 3D evaluations was carried out on the segmented images to determine vascular volume and to reconstruct a 3D image
- vessel thickness, degree of anisotropy and connectivity were analysed using trabecular thickness analysis scripts provided by SCANCO

- Rats (n =9) were scanned at 2, 4, 6 and 8 weeks post-surgery to assess defect bridging and bone formation
- 3D evaluation was carried out on the segmented images to determine bone volume and density and to reconstruct a 3D image
- Bone volume and bone density in the defects was quantified by measuring the total quantity of mineral in the central 130 slices of the defect.
- For differentiation of regional differences three VOIs were created

Results

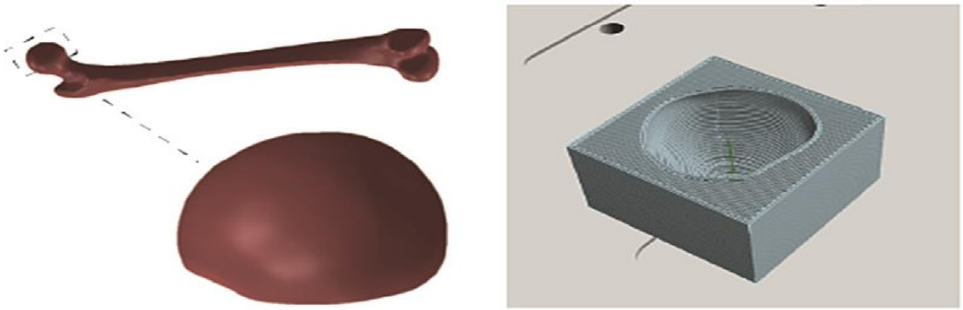
3D printing of microchanneled cartilage templates



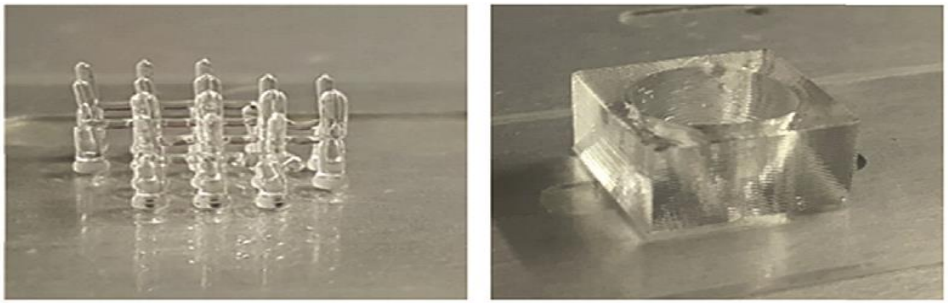
e

Anatomical bone printing

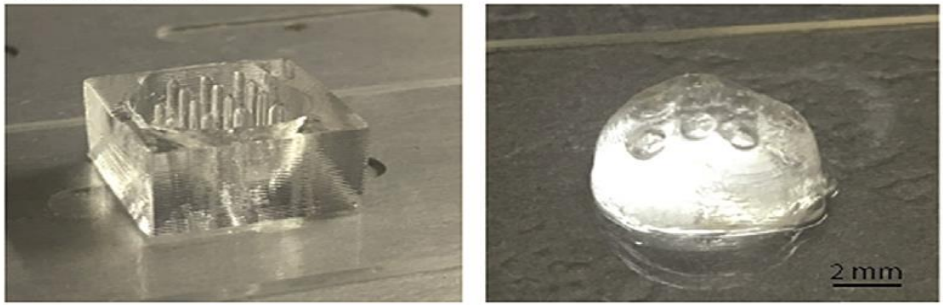
1. Isolation of humeral head and creation of negative mold

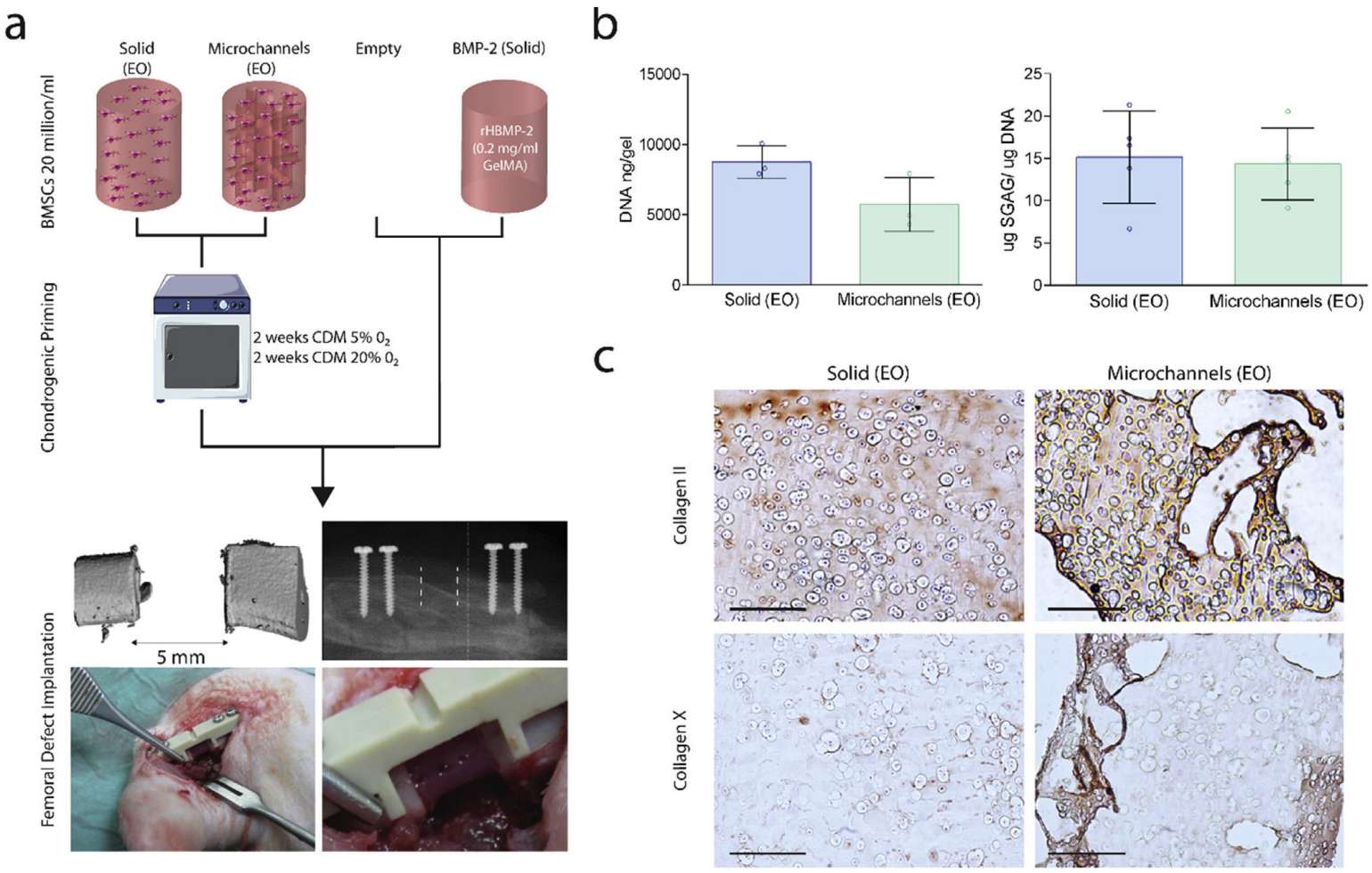


2. 3D-printing of pluronic mold containing microchannels

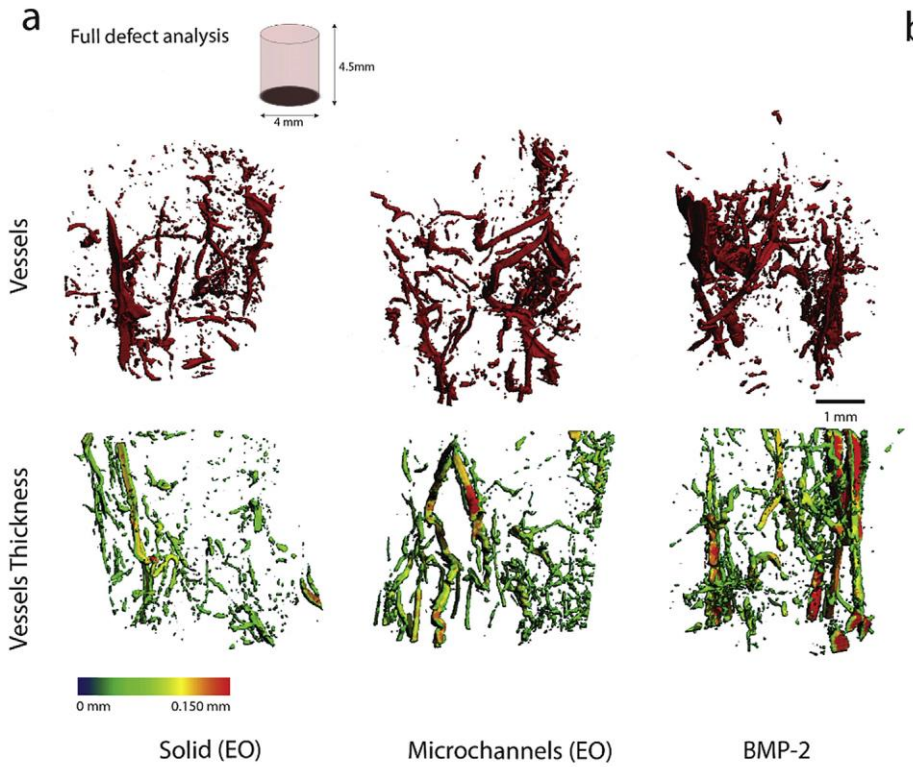


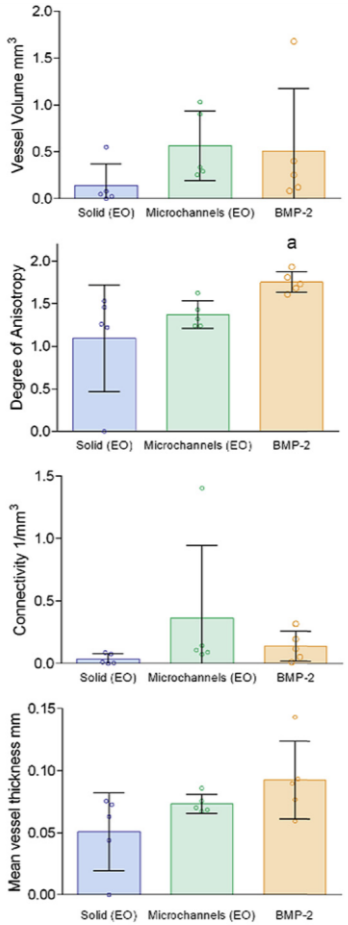
3. Casting of anatomically shaped humeral head containing microchannels

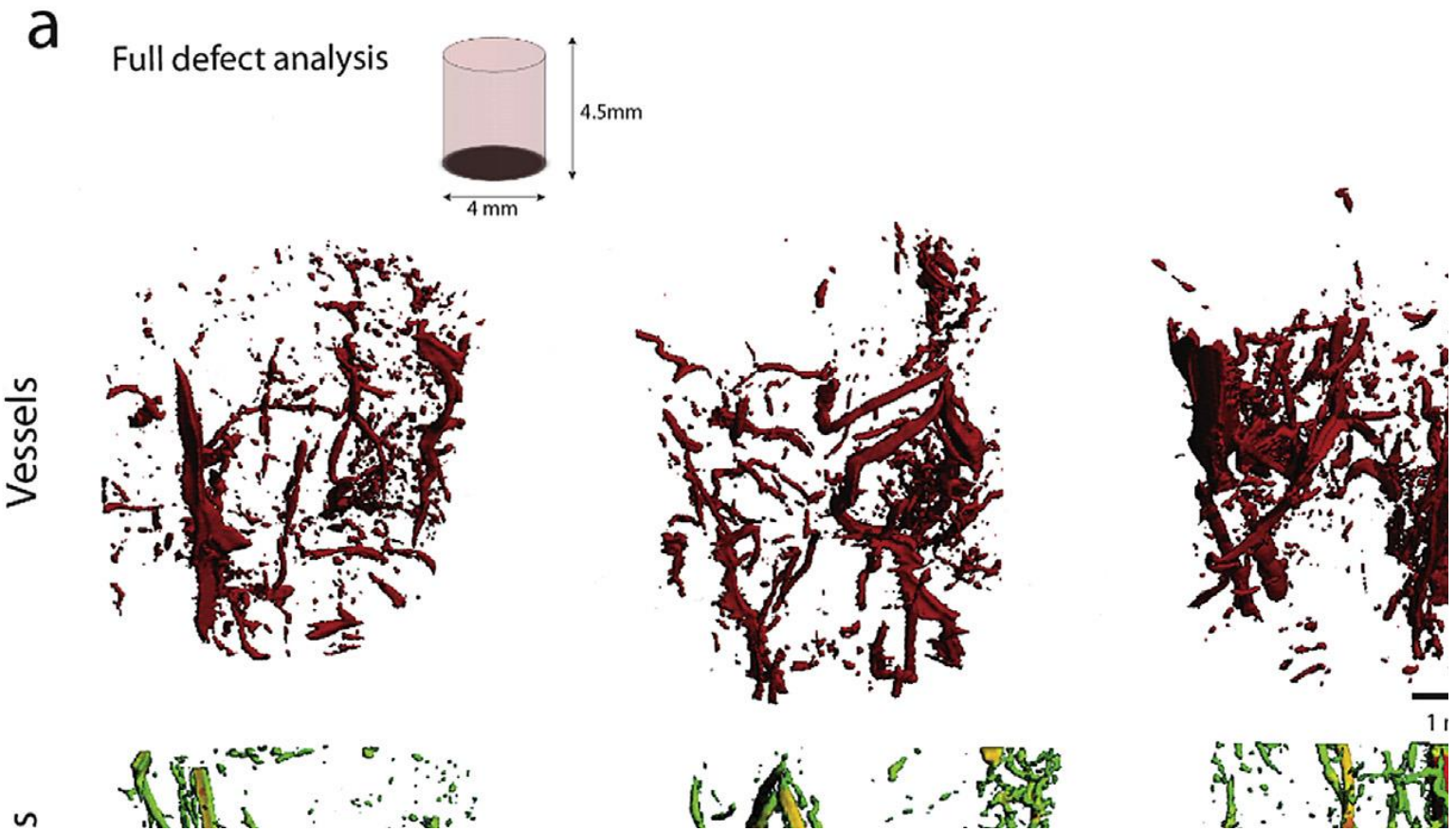



a
Solid (EO)
Microchannels (EO)
Empty
BMP-2 (Solid)
BMSCs 20 million/ml
rHBMP-2 (0.2 mg/ml GelMA)
Chondrogenic Priming
2 weeks CDM 5% 0_2
2 weeks CDM 20% 0_2
Femoral Defect Implantation
5 mm
b
DNA ng/gel
15000
10000
5000
0
Solid (EO)
Microchannels (EO)
ug SGAG/ ug DNA
25
20
15
10
5
0
Solid (EO)
Microchannels (EO)
c
Solid (EO)
Microchannels (EO)
Collagen II
Collagen X

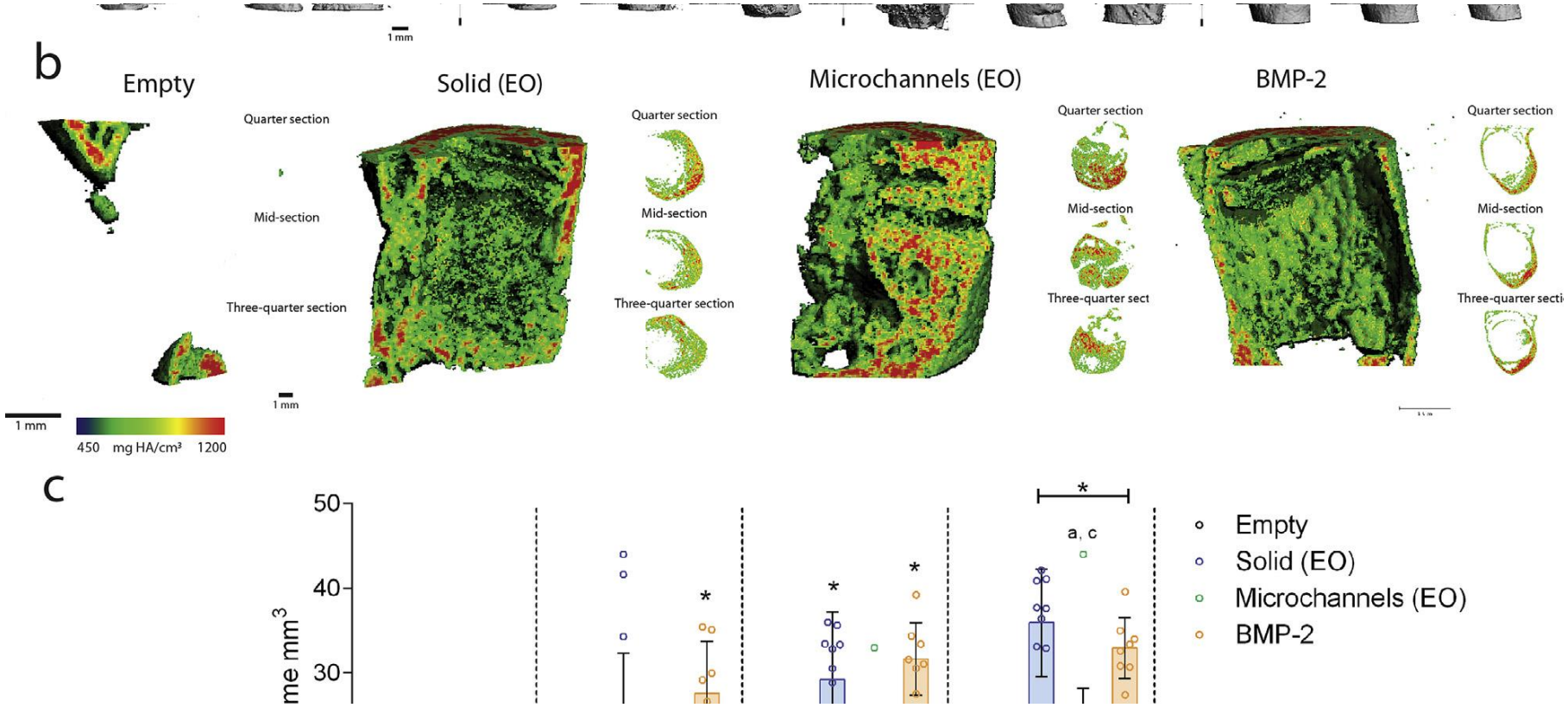
µCT angiography to assess vascular network formation



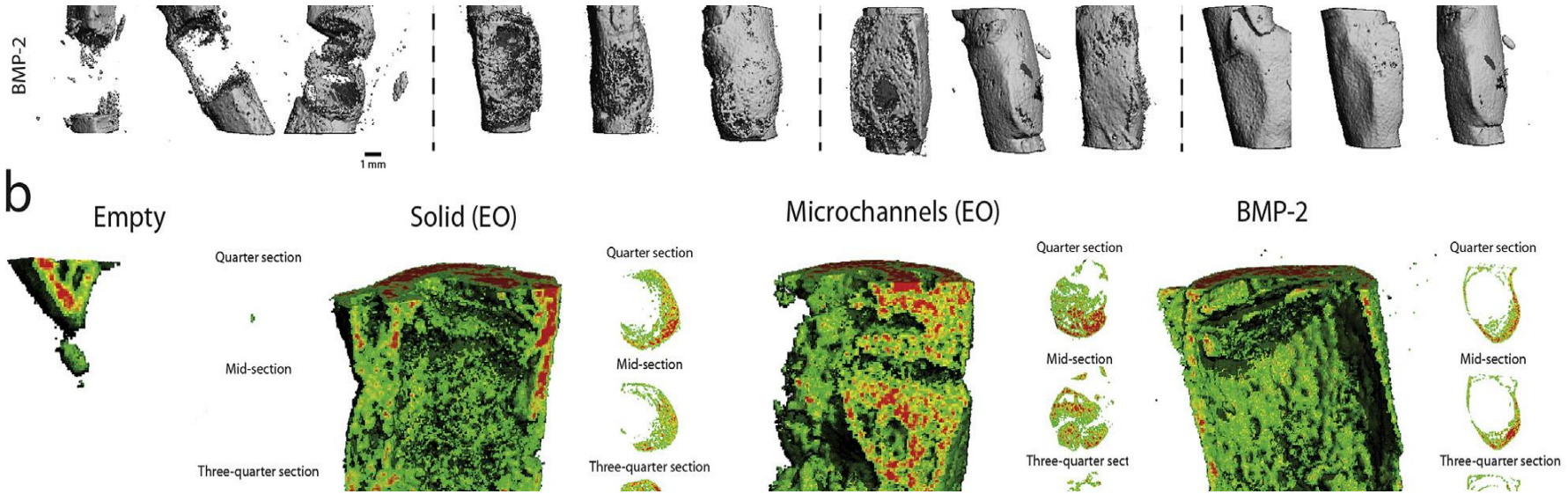


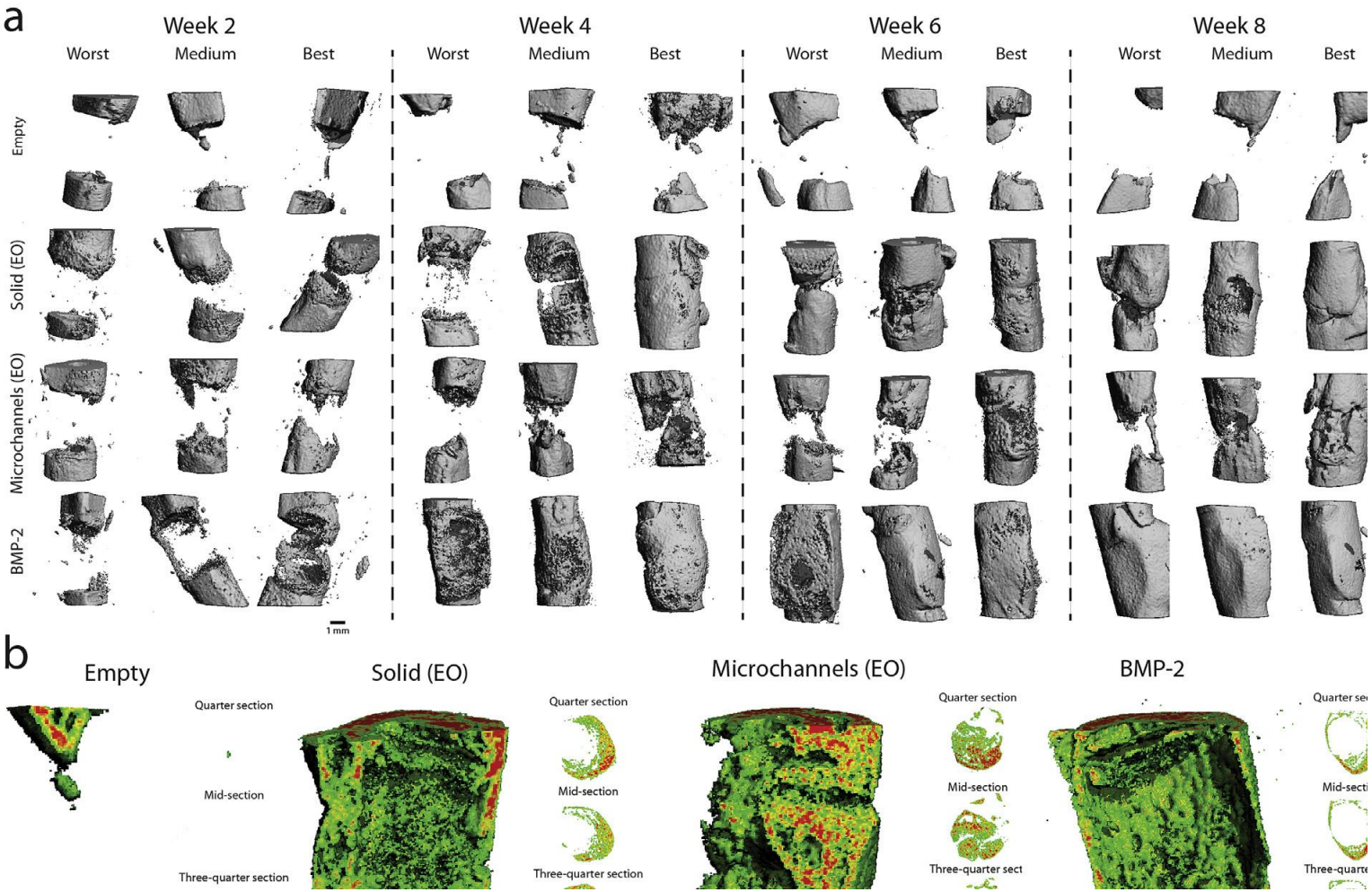

a
Full defect analysis
4.5mm
4 mm
Vessels

In vivo µCT analysis of bone volume and bone density

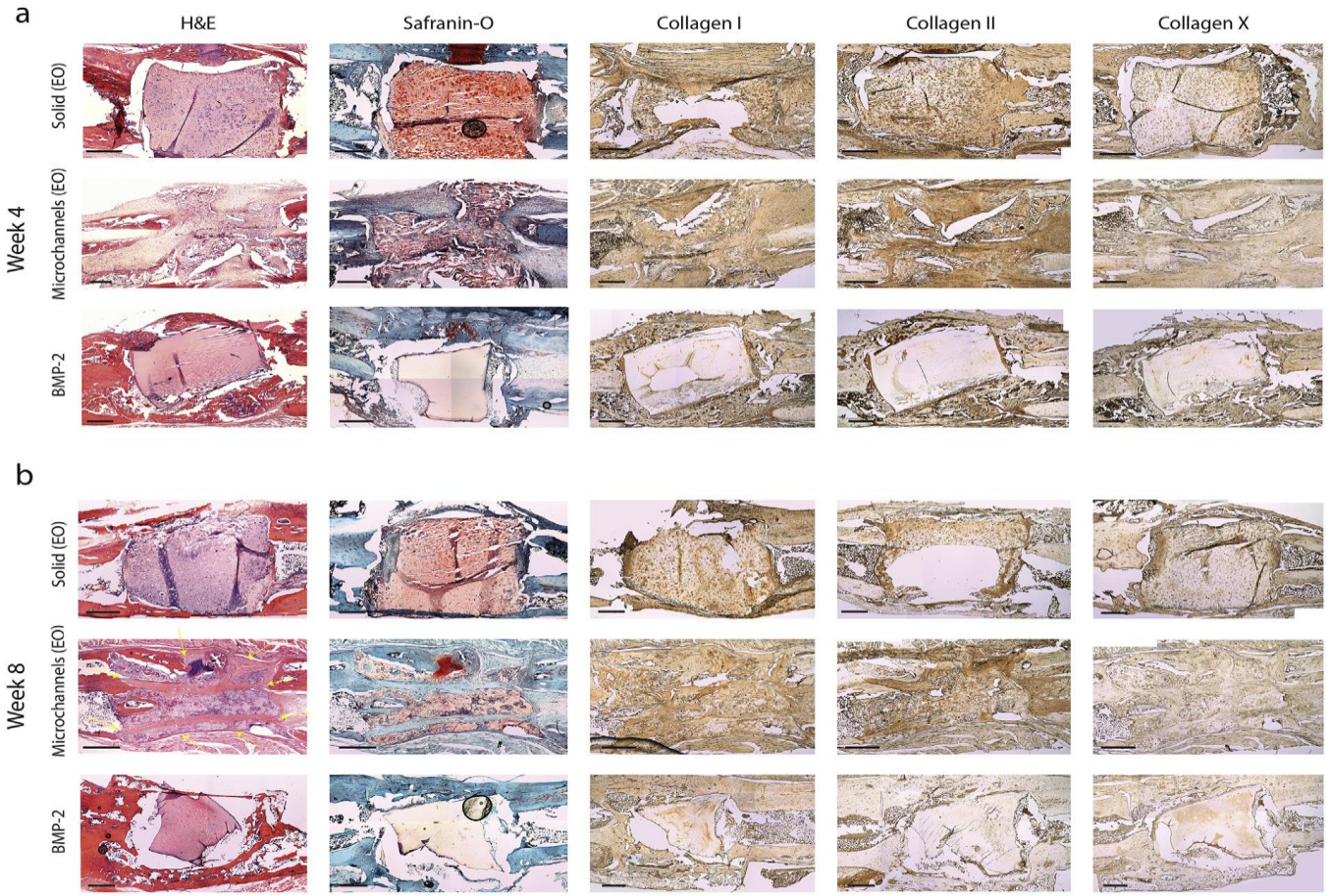


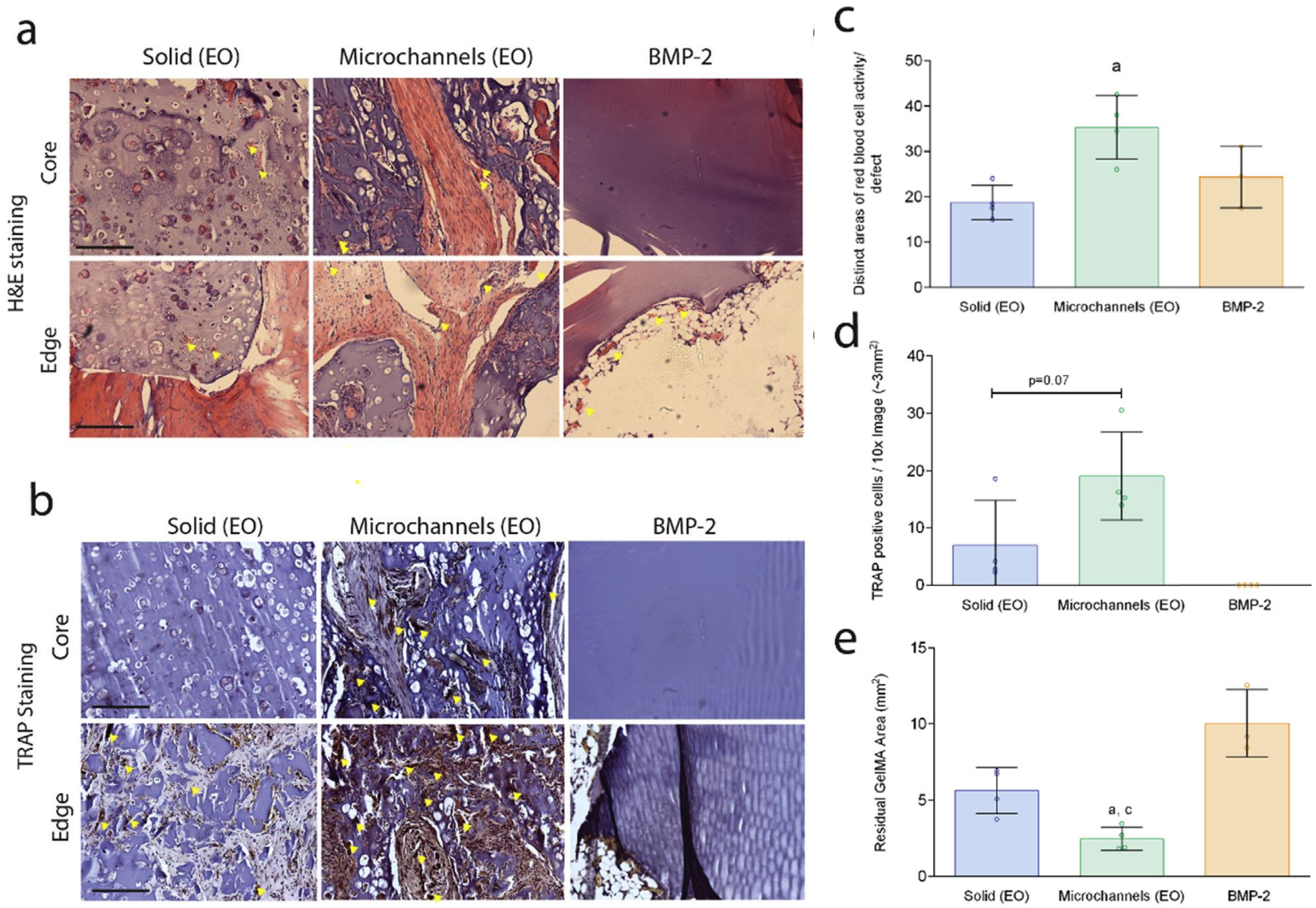
Densiometry




a
Week 2
Week 4
Week 6
Week 8
Worst
Medium
Best
Empty
Solid (EO)
Microchannels (EO)
BMP-2
1 mm
b
Empty
Solid (EO)
Microchannels (EO)
BMP-2
Quarter section
Mid-section
Three-quarter section

Histological and immunohistochemical analysis of implant vascularisation and endochondral bone formation




a
Solid (EO)
Microchannels (EO)
BMP-2
H&E staining
Core
Edge
b
Solid (EO)
Microchannels (EO)
BMP-2
TRAP Staining
Core
Edge
c
Distinct areas of red blood cell activity/ defect
50
40
30
20
10
0
a
Solid (EO)
Microchannels (EO)
BMP-2
d
TRAP positive cells / 10x Image (~3mm²)
40
30
20
10
0
p=0.07
Solid (EO)
Microchannels (EO)
BMP-2
e
Residual GelMA Area (mm²)
15
10
5
0
a, c
Solid (EO)
Microchannels (EO)
BMP-2

Results

- Incorporation of microchannels **did not** enhance overall levels of bone formation
- However, improved core defect vascularization after 4 weeks
- lower levels of heterotopic bone formation observed for the microchanneled cartilage templates
- higher number of host osteoclast/immune cells had invaded the cartilage template via the microchannel network
  → correlated with **higher levels of vascularisation** and increased degradation of the GelMA hydrogel

Discussion/Conclusion

- these results confirm the potential of endochondral bone tissue engineering

- demonstrate that 3D-printed microchannels offer a promising approach for guiding vascularisation and implant remodelling during endochondral bone repair.

- Ectopic bone formation is lower and vascularization is higher in the microchannel network.
- Limited by lower bone density and MSCs density in the solid phase of the construct

- Number of challenges before it could be adopted in a clinical setting
- Require patients MSCs, expansion, template formation for large bone defect.
- Bears high costs and regulatory challenges