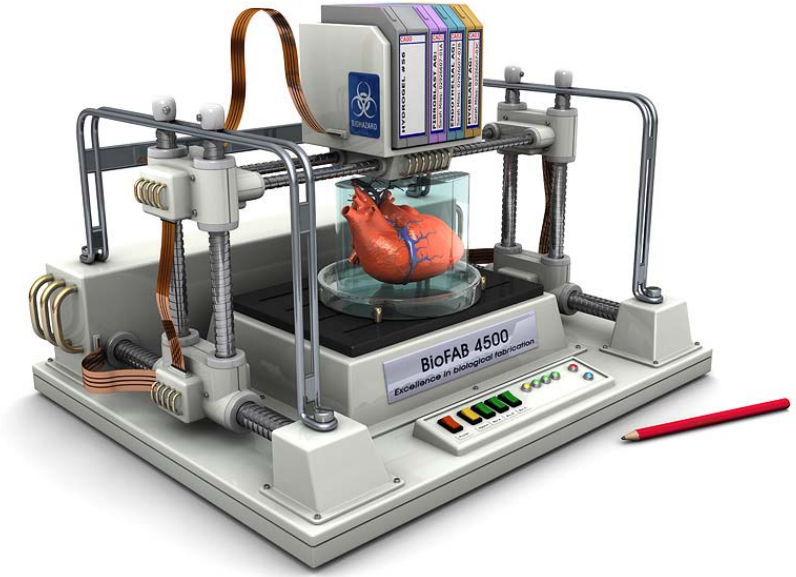
Print it out! – Latest achievements in bioprinting



Regina Reimann

Content

1. Introduction and Definitions

1. Latest achievements
   - Interweaving functional electronic components with biollogical tissue: Mannoor et al., 2013
   - Printing of vascularized, heterogeneous cell-laden tissue: Kolesky et al., 2014
   - Printing natural extracellular matrix: Pai et al., 2014

2. Summary and Conclusion

History of printing

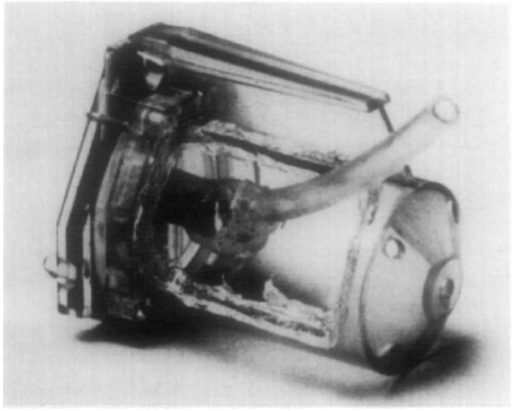
Part of a series on the
History of printing

Woodblock printing	200 CE
Movable type	1040
Printing press	1453
Etching	c. 1515
Mezzotint	1642
Aquatint	1772
Lithography	1796
Chromolithography	1837
Rotary press	1843
Hectograph	1869
Offset printing	1875
Hot metal typesetting	1884
Mimeograph	1886
Photostat and Rectigraph	1907
Screen printing	1910
Spirit duplicator	1923
Xerography	1938
Phototypesetting	1949
Inkjet printing	1951
Dye-sublimation	1957
Dot matrix printer	1968
Laser printing	1969
Thermal printing	c. 1972
3D printing	1984
Digital press	1993

V • T • E

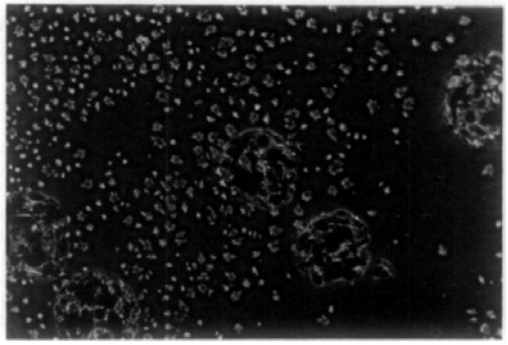
Cytoscribing: Using standard office equipment for the precise positioning of cells in 1988 (R. Klebe, experimental cell research)

Hewlett Packard 7470A graphics plotter



Filling of inc jet cardige with fibronectin

A piece of plastic was cytoscribed with fibronectin with the word “fibronectin”. SV-T2 cells (4x 106) were applied and unattached cells were decanted after 1.5 h, revealing the word “fibronectin.”



Bright field at higher magnification, dots represent letters

Additive manufacture (AM): solids are produced through the sequential deposition of solid layers or slices.

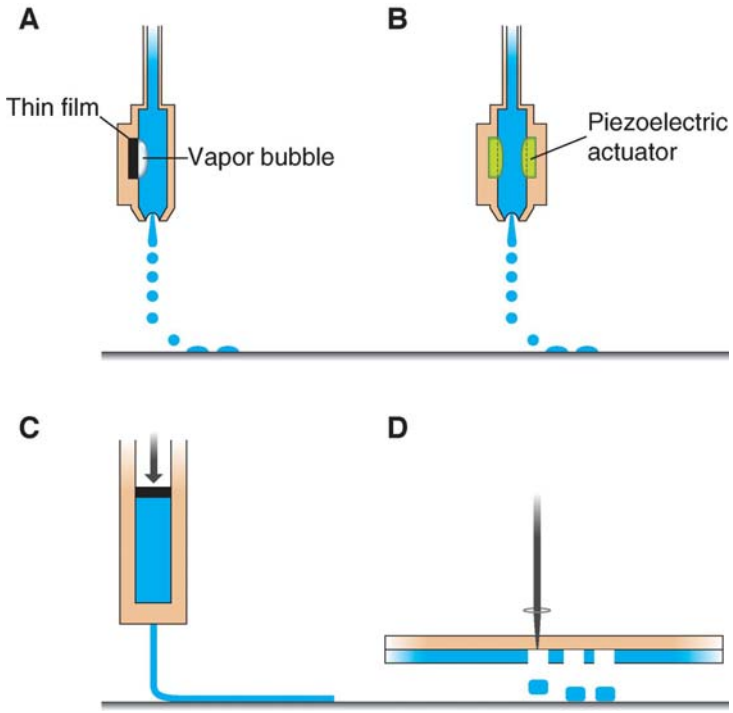
Scaffold: Important concept in **tissue engineering**, a three-dimensional highly **porous** substrate. Cells donated by the patient are expanded in culture and are then transferred to the scaffold.

Conventional machining: Using moulds and removing material in a **subtractive** manner.

Additive manufacture:

- Production of a complex 3D object
- 3D design file decomposed in parallel slices
- Production by reproducing this slices a layer at a time
- Adding of material **layer-by-layer**

Inkjet printing: A and B
Microextrusion of Filament plotting : C
Laser forward transfer: D



B Derby Science 2012;338:921-926

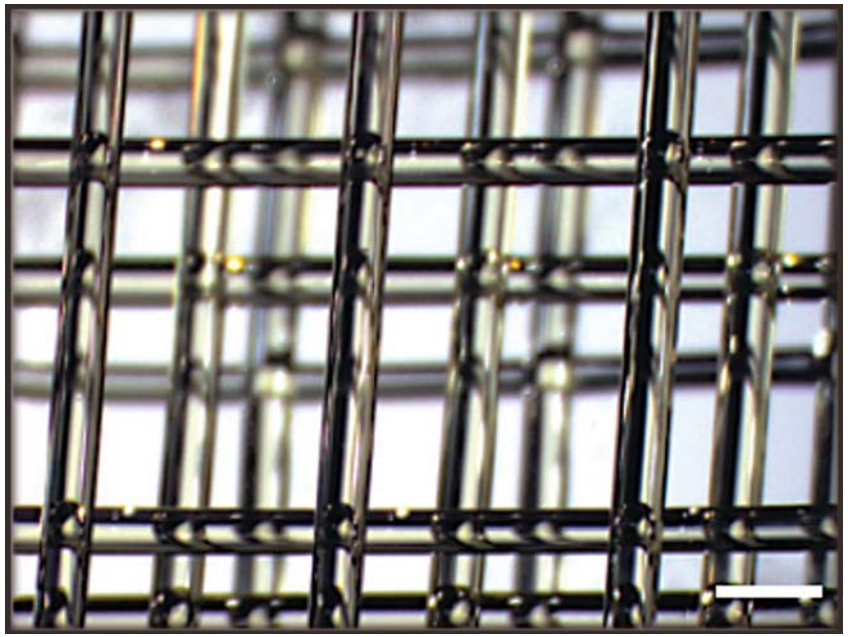
Key problem in the manufactures of scaffold structures by AM methods: Need to **fabricate internal cavities**

Cavities and pores are required:

- to assist nutrient transport in a bioreactor
- To provide a framework to encourage neovascularization

Difficult to deposit material above an empty cavity:

- Temporary support structures
- **Leaching**: Sacrificial material that is leached away after manufacture (cytocompatible solvents!)



Leaching:
Three-dimensional lattice fabricated from a carbohydrate glass structure using a filament extrusion AM technique, that can be leached away with water

B Derby Science 2012;338:921-926

Bioprinting

Description:

- Distinct from AM technology to fabricate scaffolds
- Fabrication of structural material and deposition of cells
- Positioning of biochemical, biological material and living cells

Goal: To reproduce tissue structures

Concept: Combination of

- Different types of cells in defined loaction
- Supporting matrix or scaffold (if needed)
- **Biochemical cues** to control behaviour
- Combination with **bioelectronics** possible

Target Applications:

- **Modelling of tissue and tumours**
- **Toxicity and drug screening**
- Manufacturing of bio-sensors
- Reconstruction medicine

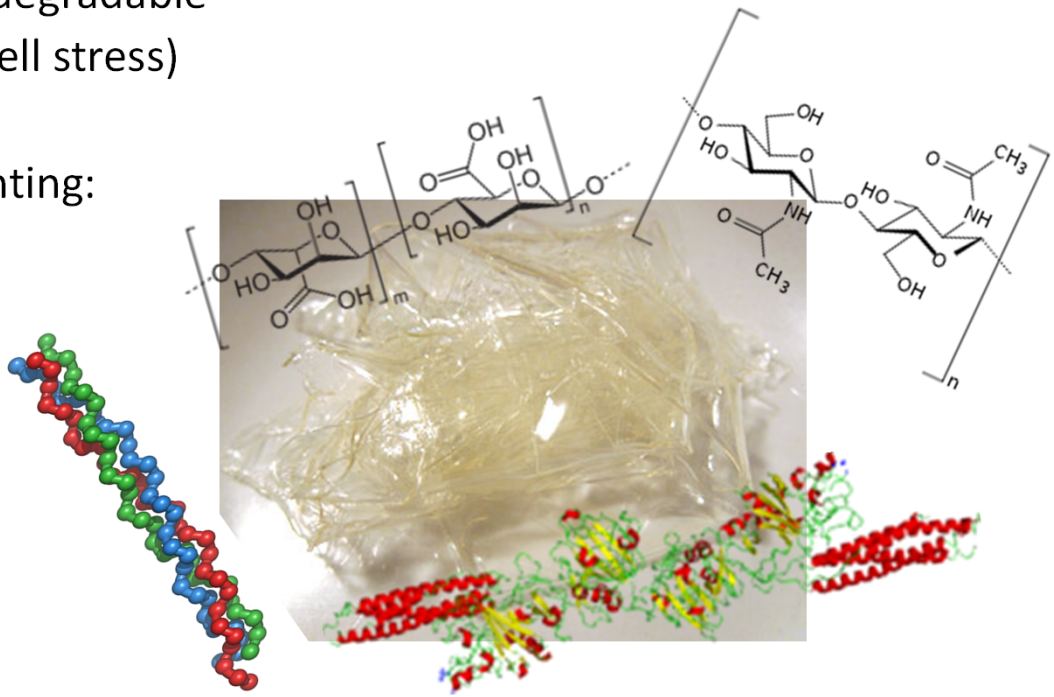
The concept of Bioink

Cells encapsulated in gelatinous substances / biological materials which:

- Behaves like a **liquid** during printing (enough fluid to print it to the relatively thin nozzle)
- **Soldify in response** to a specific stimuli in a controlled enviroment (thermo-, photo-, pH or molecule-sensitive)
- Nontoxic, biocompatible and biodegradable
- Low **shear-rate** during printing (cell stress)

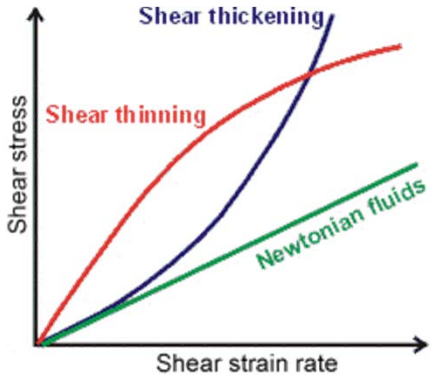
Material used in extrusion based printing:

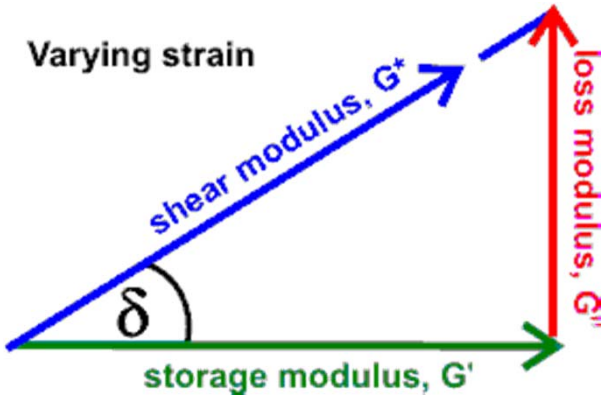
- Gelatin (collagen)
- Gelatin / Chitosan (chitin)
- Gelatin / Alginate
- Gelatin / Fibrinogen
- Lutrol F127 / Alginate
- Alginate

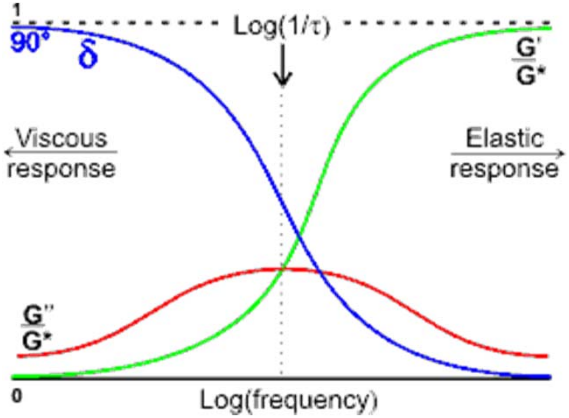


Rheology – Lessons from ketchup

Rheology: Study of the flow of matter
Viscosity: Resistance to flow of a fluid
Shear stress: force devised by the area parallel to the force
Shear strain rate: Velocity over separation height
Shear strain: Effect, Displacement over separation height
Shear thinning: The fluids viscosity decreases with an increasing shear stress
Shear thickening: The fluids viscosity increases with an increasing shear stress
Storage modulus G': elastic response
Loss modulus G'': Loss of energy / viscous response
Shear modulus G*: G' + i x G''







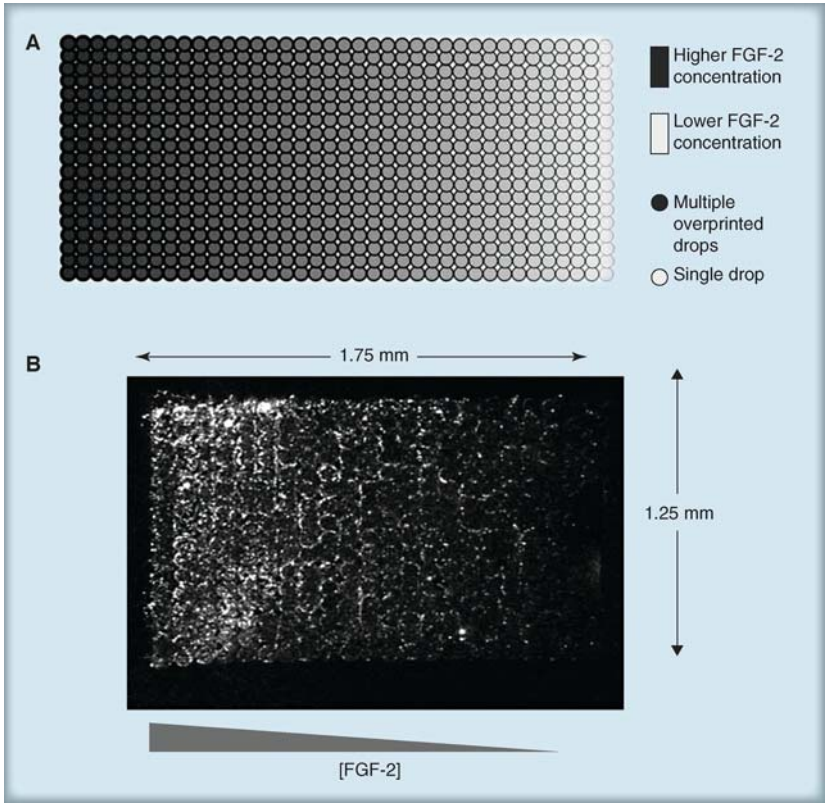
http://www1.lsbu.ac.uk/water/hyrhe.html

Illustration of how digital printing methods can be used to print spatially defined concentration gradients of biochemical

Possibility to engineer variations in surface concentration through overprinting at different drop densities (incjet printing):

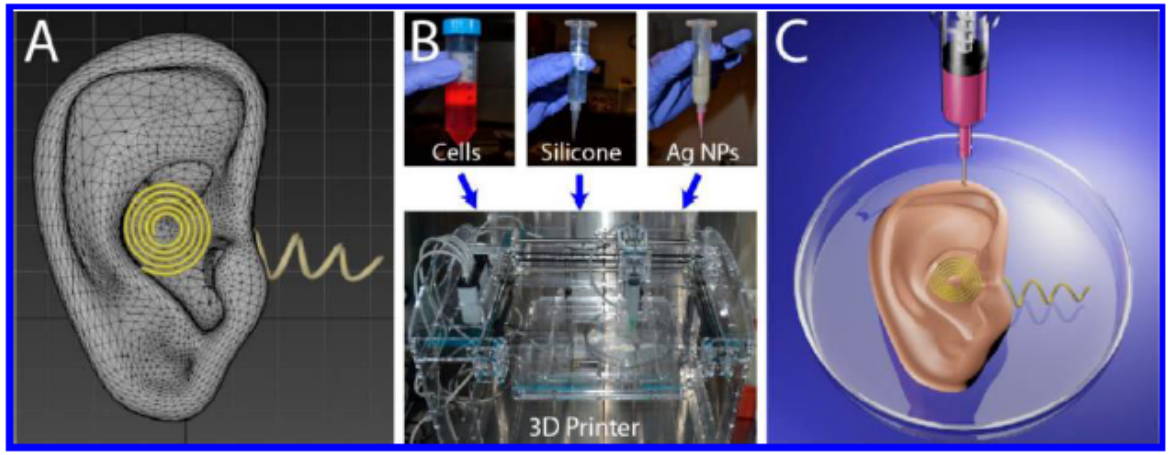
- Campell et al: Demonstration that the response of cells can directly be correlated with the surface concentration of printed hormones

- Surface concentration of growth factors are able to control stem cell fate / different pattern can be used to differentiate cell fate in the same culture dish



B Derby Science 2012;338:921-926

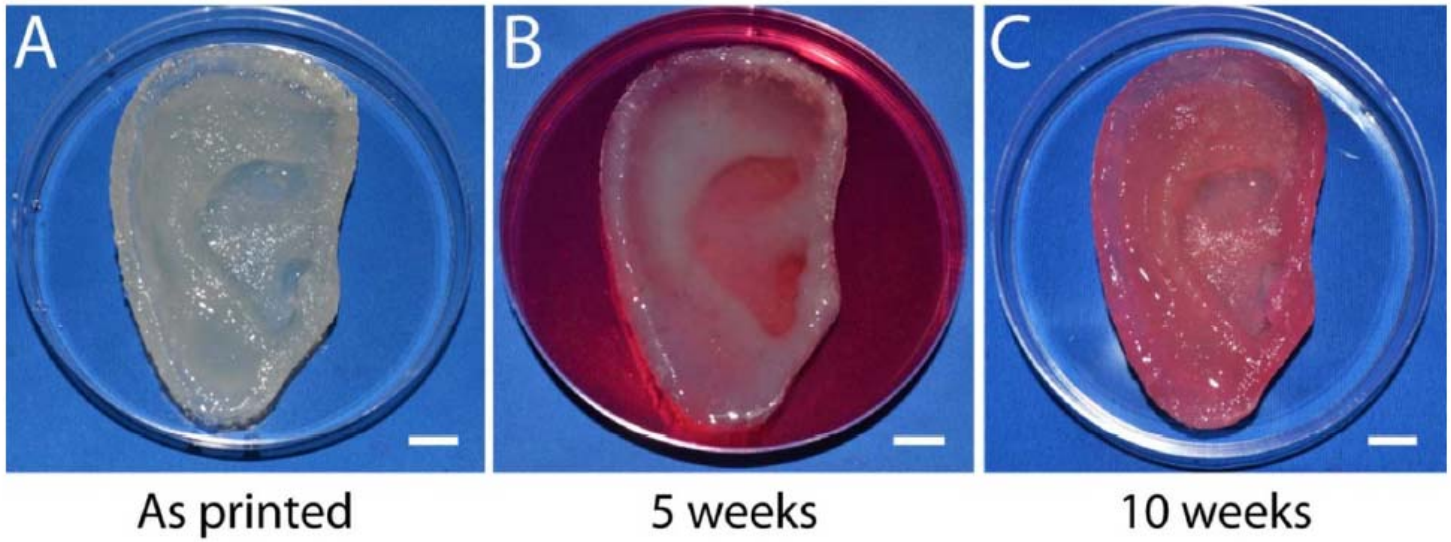
Interweaving functional electronic components with biological tissue: 3D Printed Bionic Ear, Mannoor et al. Nano Letter 2013, highlighted in Science and Nature in june and july 2013



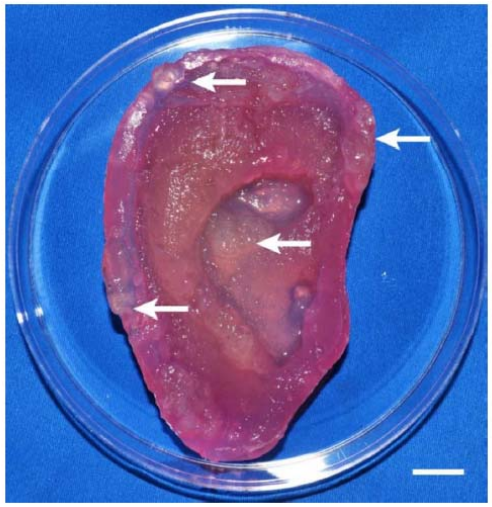
- Computer aided design (CAD)
- Bioink: Chondrocytes (≈ 60 million cells / ml from articular cartilage of a one month old calve) in **alginate hydrogel matrix**
- Electronic: conductive **silver nanoparticle (Ag NP)** for a cochlea shaped electrode connected to a coil antenna **supported on silicone**
- Commercial syringe **extrusion** based Fab@Home 3D printer (The NextFab Store, Albuquerque NM)

Aim: **Fully interweaving** functional electronic components with biological tissue via 3D printing of nanoelectronic material and viable cell-seeded hydrogels in the **precise geometries** of human organs

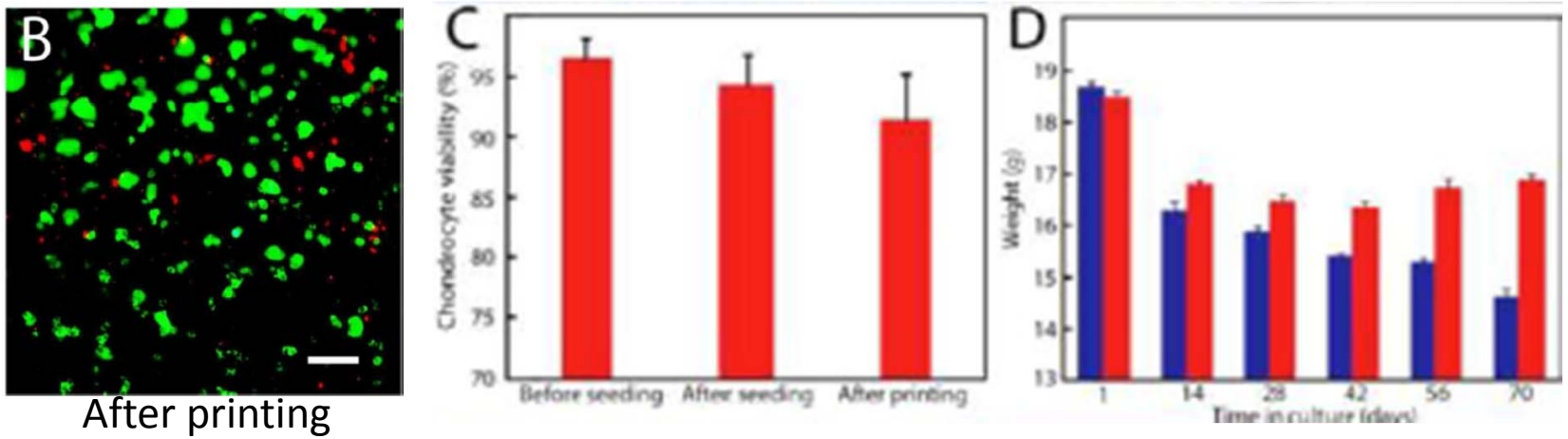
Growth and tissue Viability of the bionic right ear - I



- Culturing on chondrocyte culture media (10-20% FBS, refreshed every 1-2 days)
- Total **10 weeks** in culture
- Neocartilage outgrowth: bulbous outgrowth on the surface (arrows)



Growth and tissue Viability of the bionic ear - II



After printing

B/C: Chondrocyte viability assessed with calcein AM (green) and ethidium homodimer-1 (red) LIVE/Death® Viability Assay before, during and after the printing process

- After printing by talking specimens from various location
- Ratio of live cells over total cells

Limitation: just time points during printing, no controls, n=1

D: Weight of the printed ear over time: chondrocyte-seeded alignate (red), only allignate (blue), n=1 as an illustration of the absorbance of the polymer scaffold and the development of the cartilage tissue.

Biomechanical characterization of the 3D printed neocartilage tissue

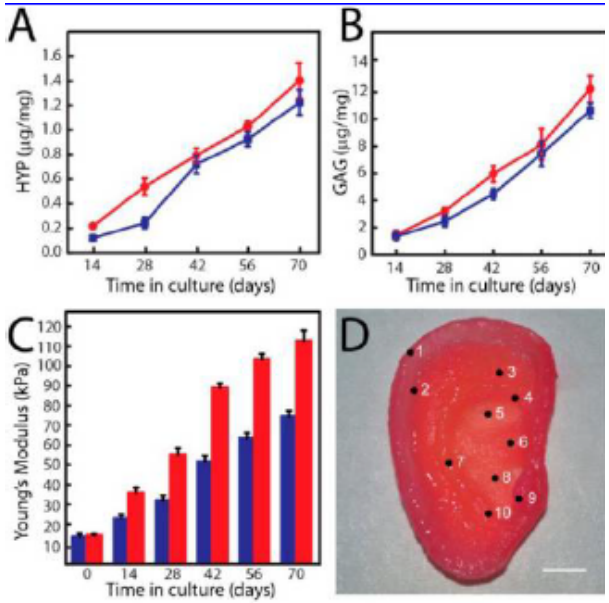


Table 1

Part	Mean Hardness (kPa)
1. Helix	44.85 ± 2.68
2. Scapha	38.93 ± 3.00
3. Fossa	42.40 ± 2.87
4. Crura Antihelix	45.47 ± 3.95
5. Cymba Conchae	41.53 ± 4.36
6. Crus of Helix	46.80 ± 4.72
7. Antihelix	40.67 ± 3.13
8. Cavum Conchae	38.50 ± 1.73
9. Tragus	40.10 ± 2.42
10. Antitragus	39.27 ± 3.26

Extra Cellular Matrix properties (ECM) accumulation evaluated by:

A) Hydroxyproline measurement as an estimation for **collagen content** (absorbance measurement / standard curve)

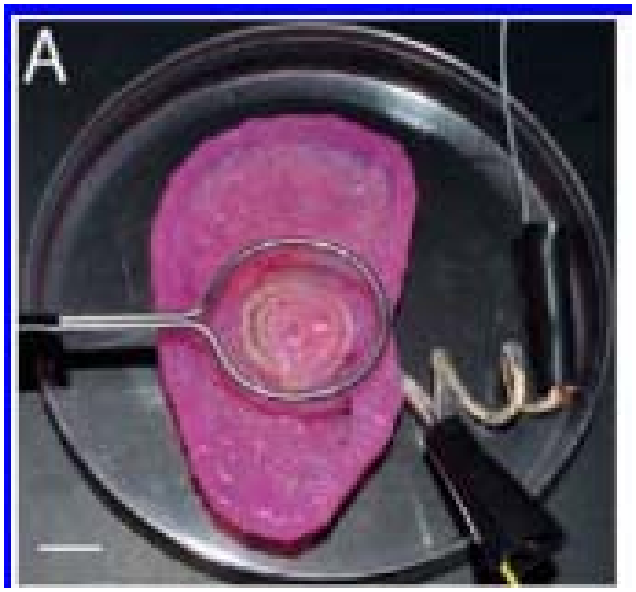
B) Measurement of sulfate glycosaminoglycans (GAG) as a marker of proteoglycans (1,9-dimethylmethyline blue dye / spectrometric measurement)

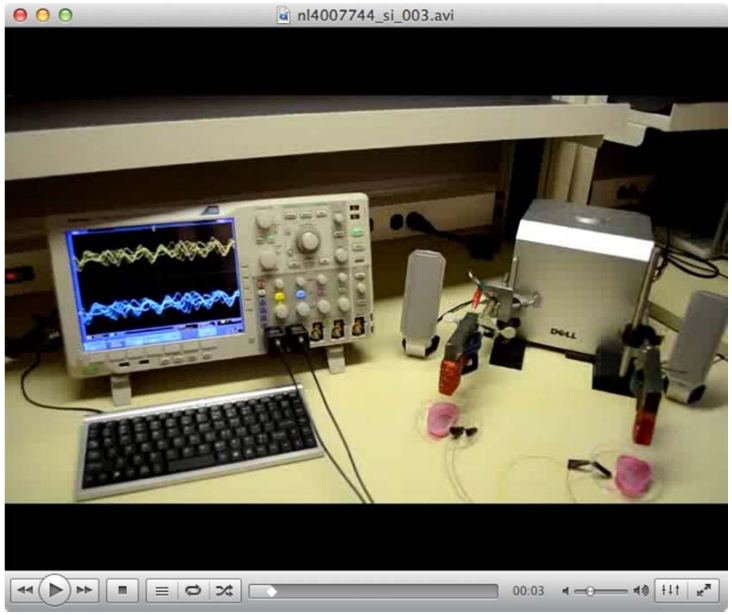
Tensile properties and hardness

C) Young‘s module (tensile)

D) Nanoindentation measurement (hardness)

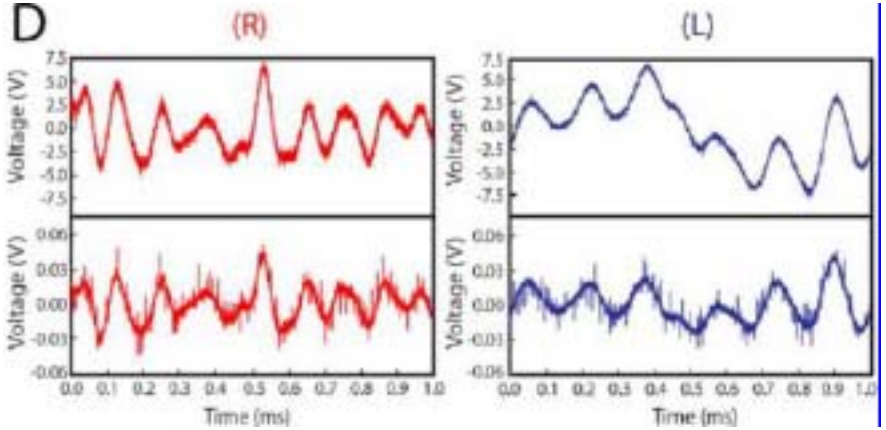
Electronic characterication of the bionic ear





- Exposure to a signal from a transmitted loop antenna
- Output is collected to two electrodes

Transmission of signals across an extended frequency spectrum beyond normal audible signal frequencies



Summary & Discussion

Aim: **Fully interweaving** functional electronic components with biological tissue via 3D printing of nanoelectronic material and viable cell-seeded hydrogels in the **precise geometries** of human organs

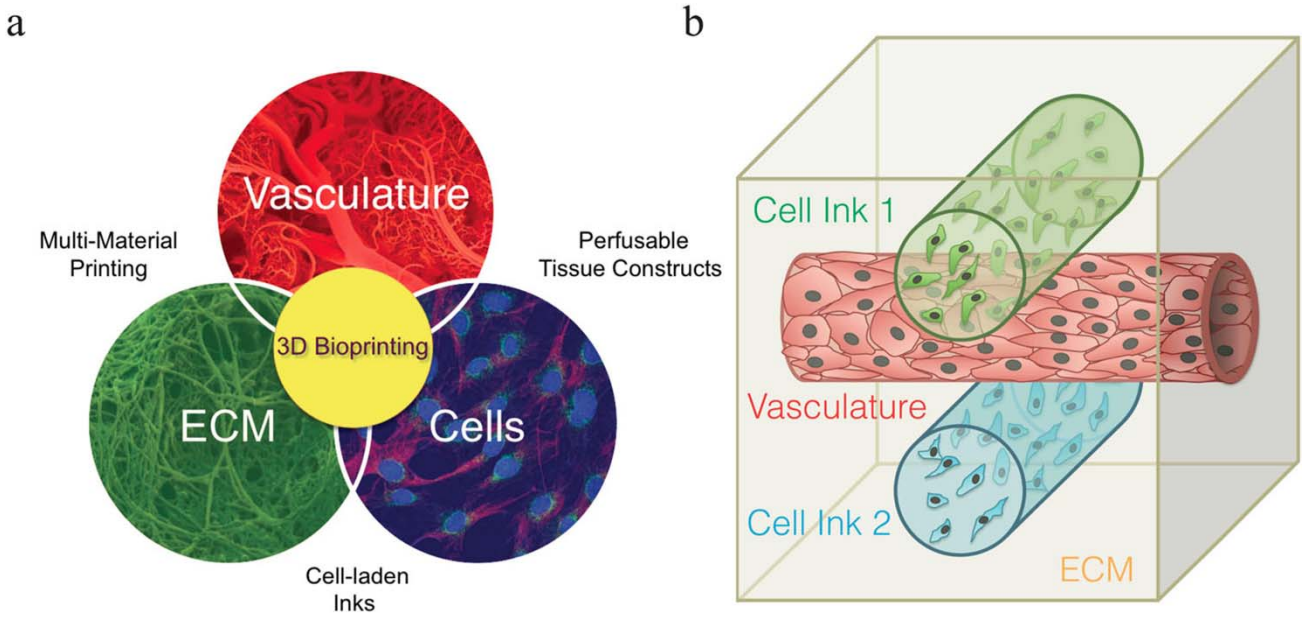
Achieved:

- Interweaving of functional electronic components with biological tissue
- Bioprinting of a precise geometry of a human organ using a computer aided design

Limitation:

- No repetition of experiments / no control experiment
- Limited viability assessment
- Problematic outgrowth of cartilage after 10 weeks
- Electronic caractericaton again without control

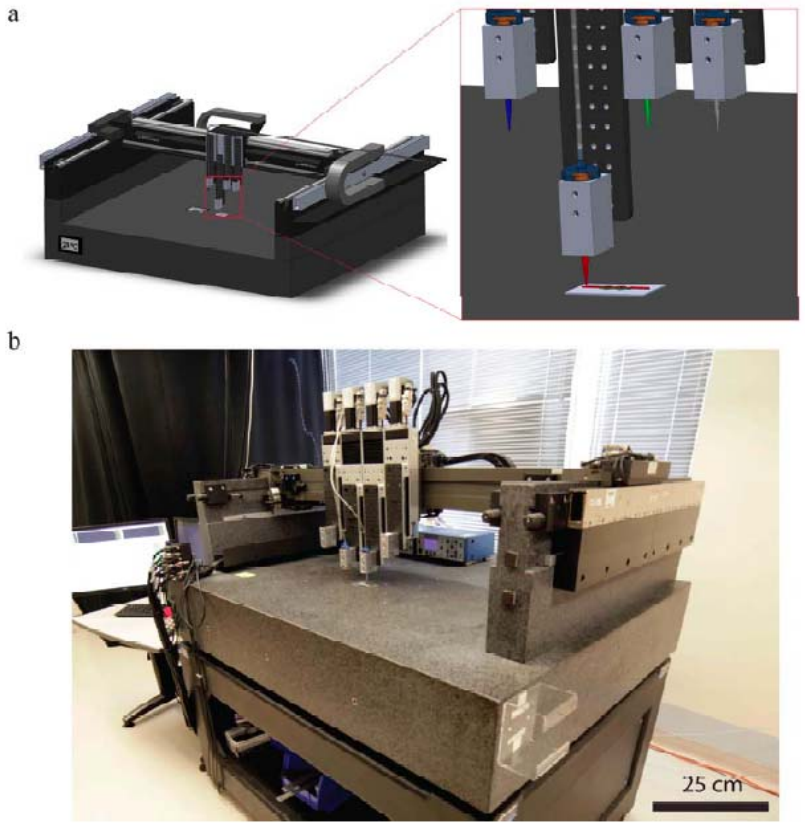
3D bioprinting of Vascularized, Heterogeneous Cell-Laden Tissue Constructs: Kolesky et al., Advances Materials, 2014



Limitation on the approach of using **carbohydrate glass structure**: With this printing-molding technique just simple tissue architecture composed of homogenous cell-laden matrices can be produced.

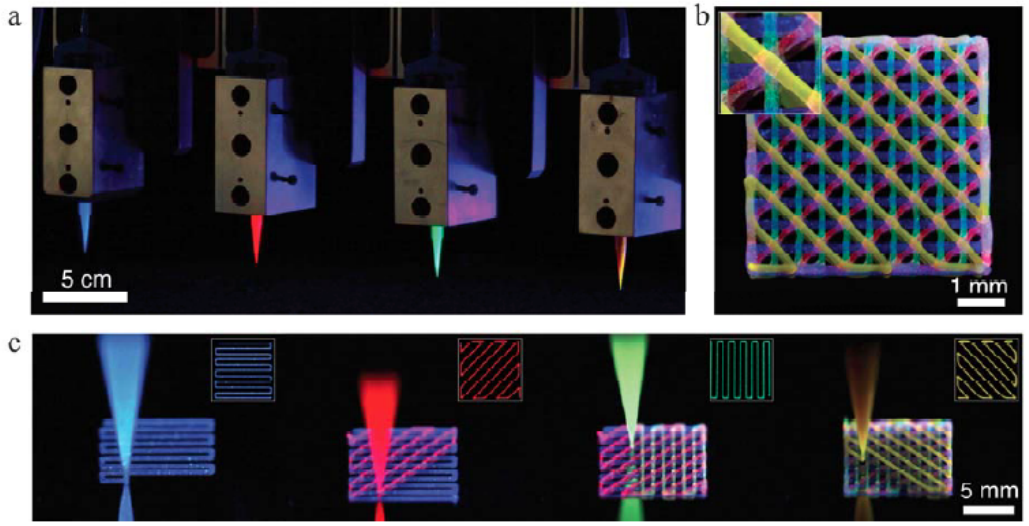
Aim: A 3D bioprining method for fabricating engineered tissue constructs replete with vasculature, multiple types of cells and ECM.

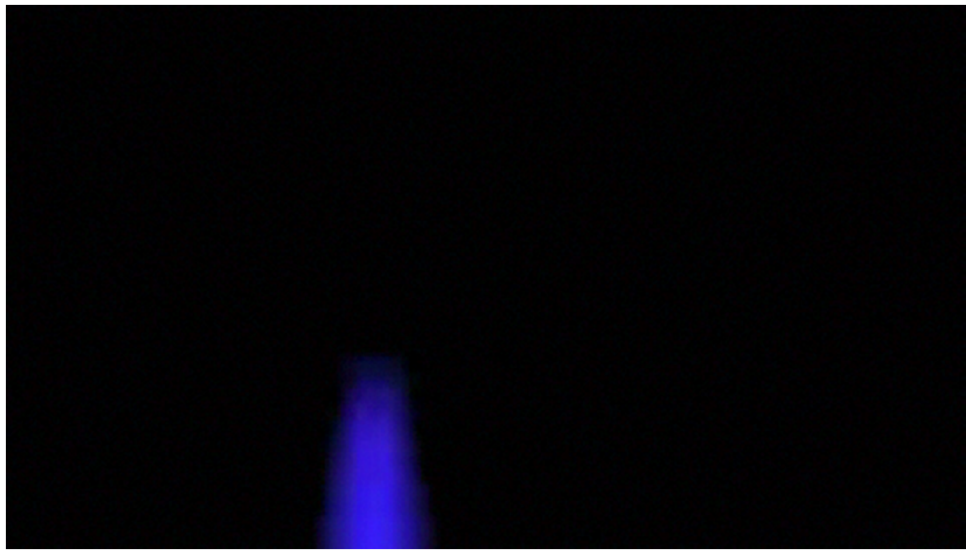
The printer – a custom built 3D printer



725 x 650 x 150 mm / Aerotech Inc. Pittsburgh, PA USA
Four independent, zaxis controlled ink

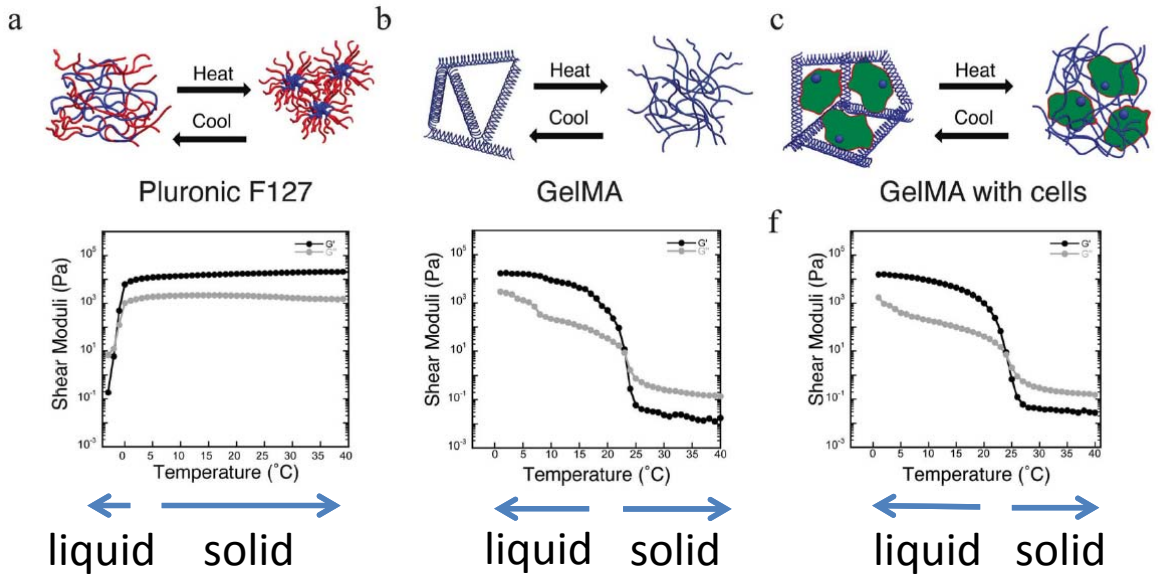
The printer at work





- Four independently adressable printheads
- For demonstration printing of poly(dimethyl siloxane) PMDS inks each dyed with a different fluophore

The inks – generating **three distinct processing windows**



G‘: shear elastic
G‘‘: loss moduli

Pluronic F127: Is a **surfactant** which undergoes thermally reversible gelation above a critical micelle concentration (CMC = 21 wt%) and temperature, which decrease from 10°C to 4°C depending on CMC ➔ **high CMC of 40% wt% used**

GelMA: Gelatin methacrylate (GelMa) for use as a bulk matrix and cell carrier. Denatured **collagen** that is modified with photopolymerizable methacrrylate (MA), **covalently cross-linked by UV light after printing (stable also at culturing conditions)**

< 4°C : F127 is liquid and flows readily / GelMa and GelMa with cells is stiff

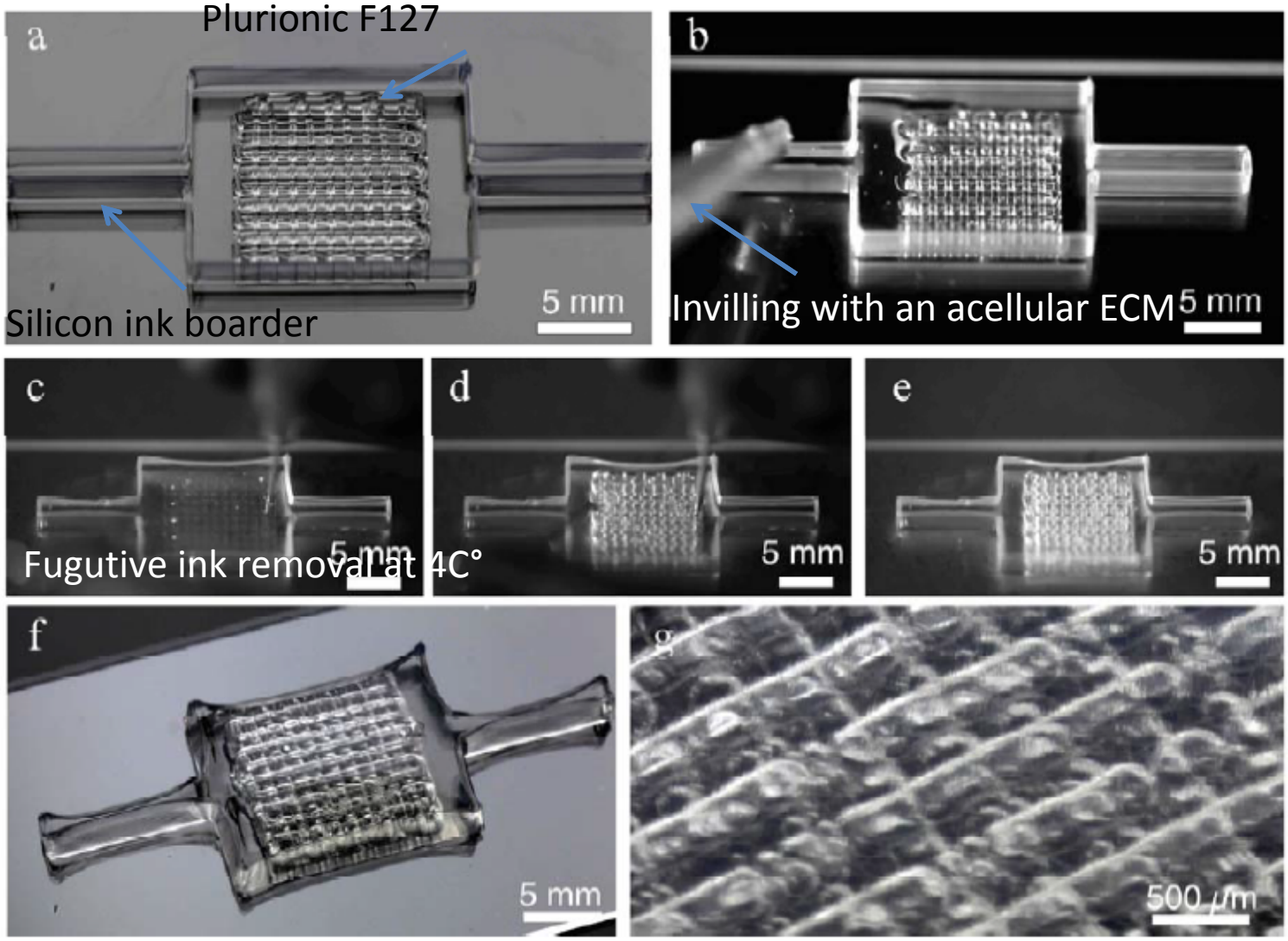
4°C – 22° C: Each ink is stiff and exhibits a solid like response

> 22° C: Plurionic F127 is stiff and solid-like (G’ > G’’) while the pure and cell-laden GelMa inks are liquid (G’ > G’’)

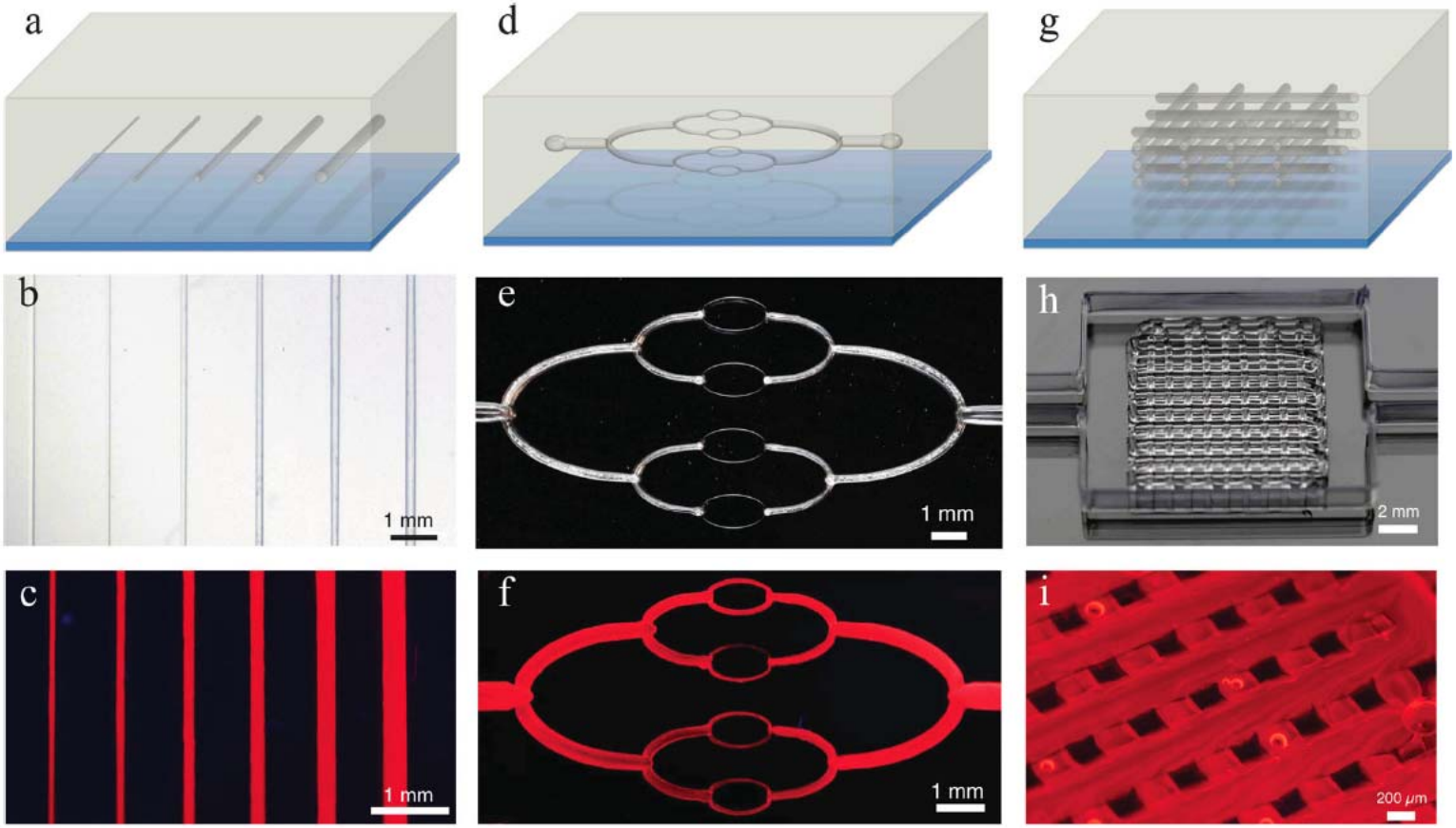
Principle: printing, infilling and fugutive ink removal

Printed 3D vascular structure

Plurionic F127

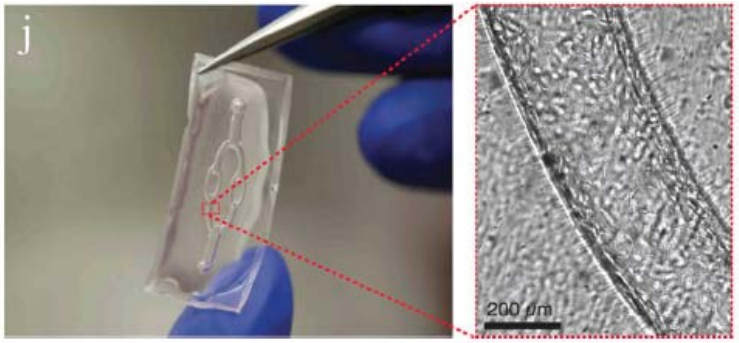


1D, 2D and 3D vascular network

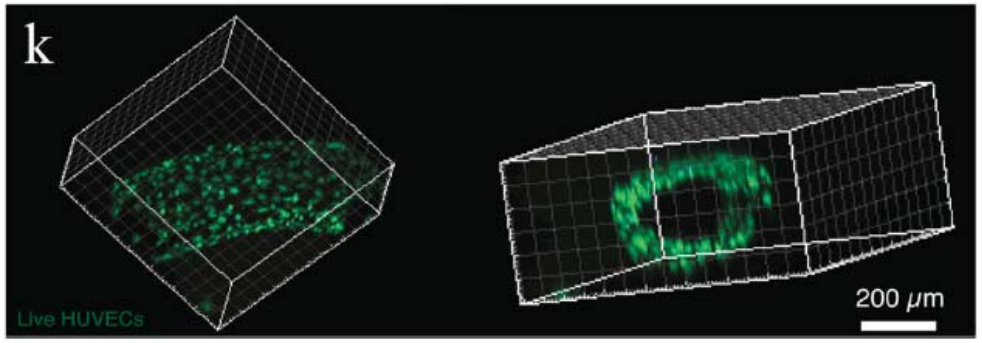


Perfusion with a water soluble fluorescent dye

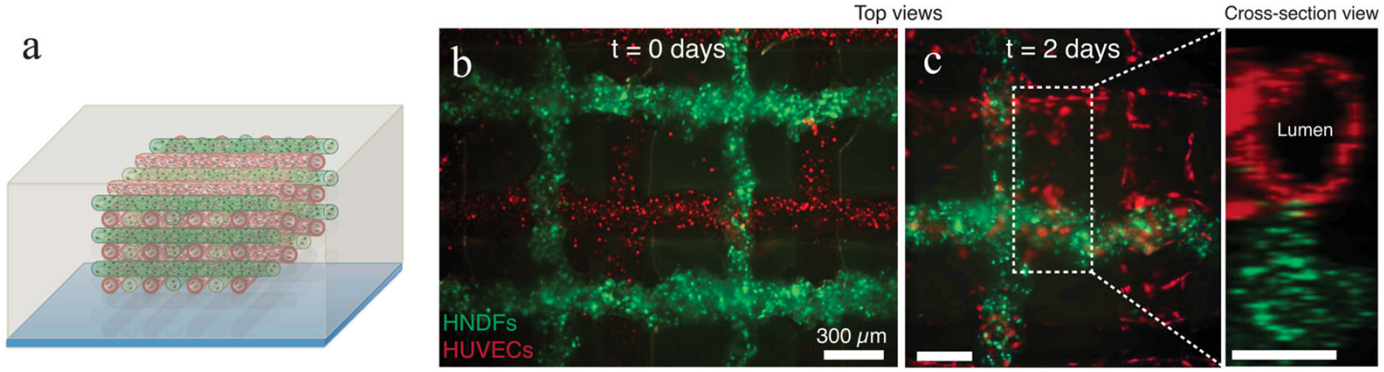
Promotion of endothelialisation by injection of a human umbilical vein endothelial cell suspension (HUVEC)



Representative microchannel
Within a 2D vascular network
Perfused with HUVEC

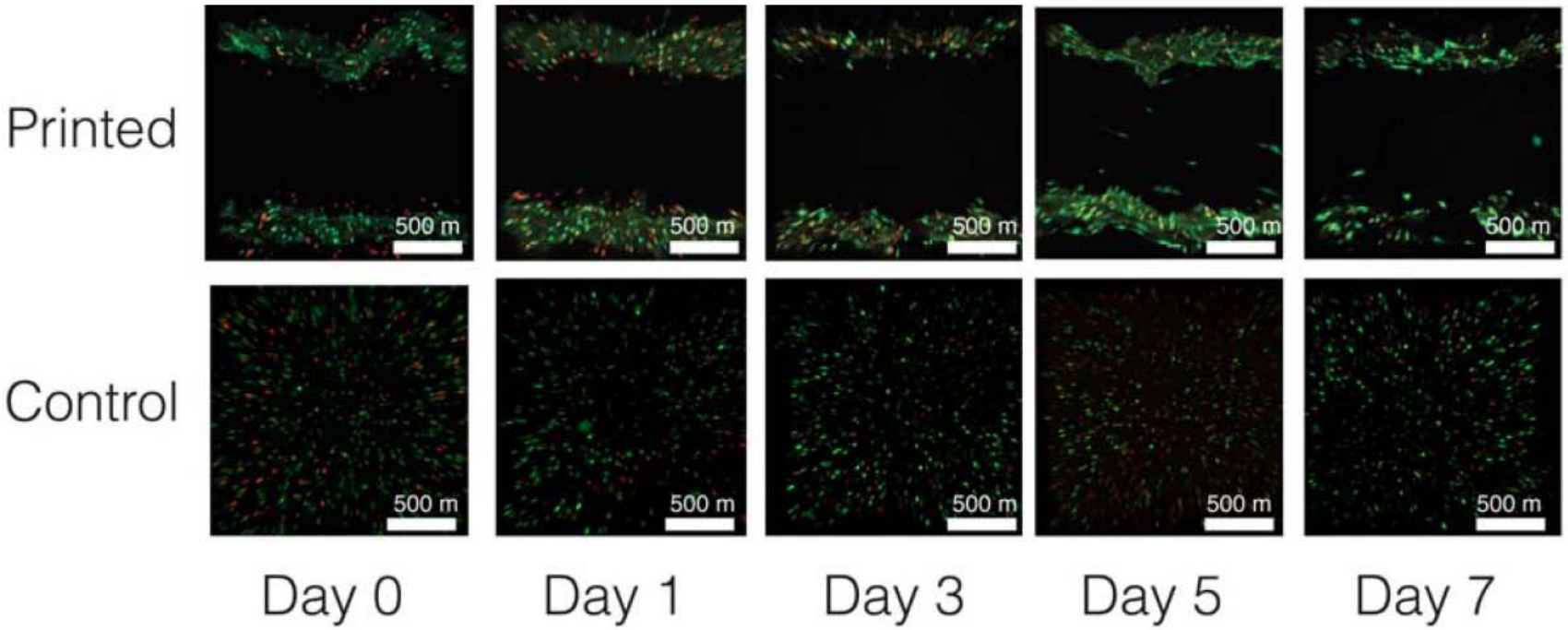


Confocal images of HUVEC cells
Lining the microvasculatur walls



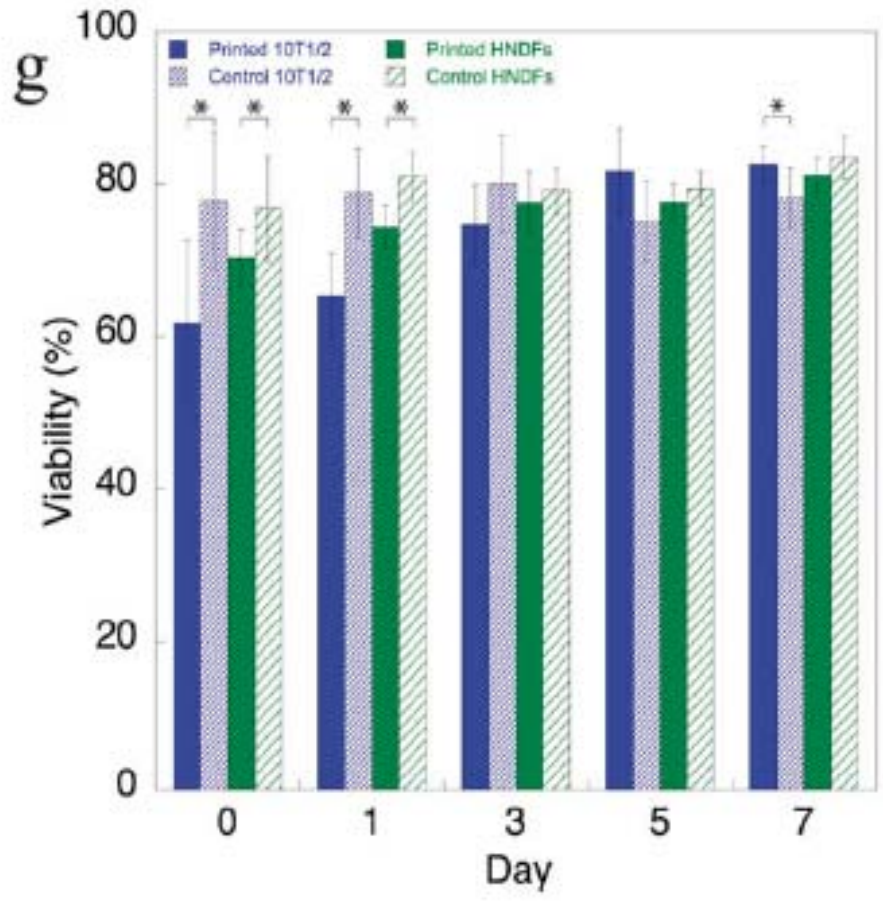
Engeneered 3D tissue at 0 and 3 day
Red: red fluorescent protein expressing HUVECs
Green: green fluorescent protein expresing human dermal fibroblasts (HNDFs / $2x10^6$ cells mL-1)

Cell viability over 1 week - 1



Confocal images (Z-projection) of live-dead staining, **calcein (green/live) and ethidium homodimer (red/dead)**, of printed filaments and control samples composed of 10T1/2 fibroblast cells in GelMA

Cell viability over 1 week – 2

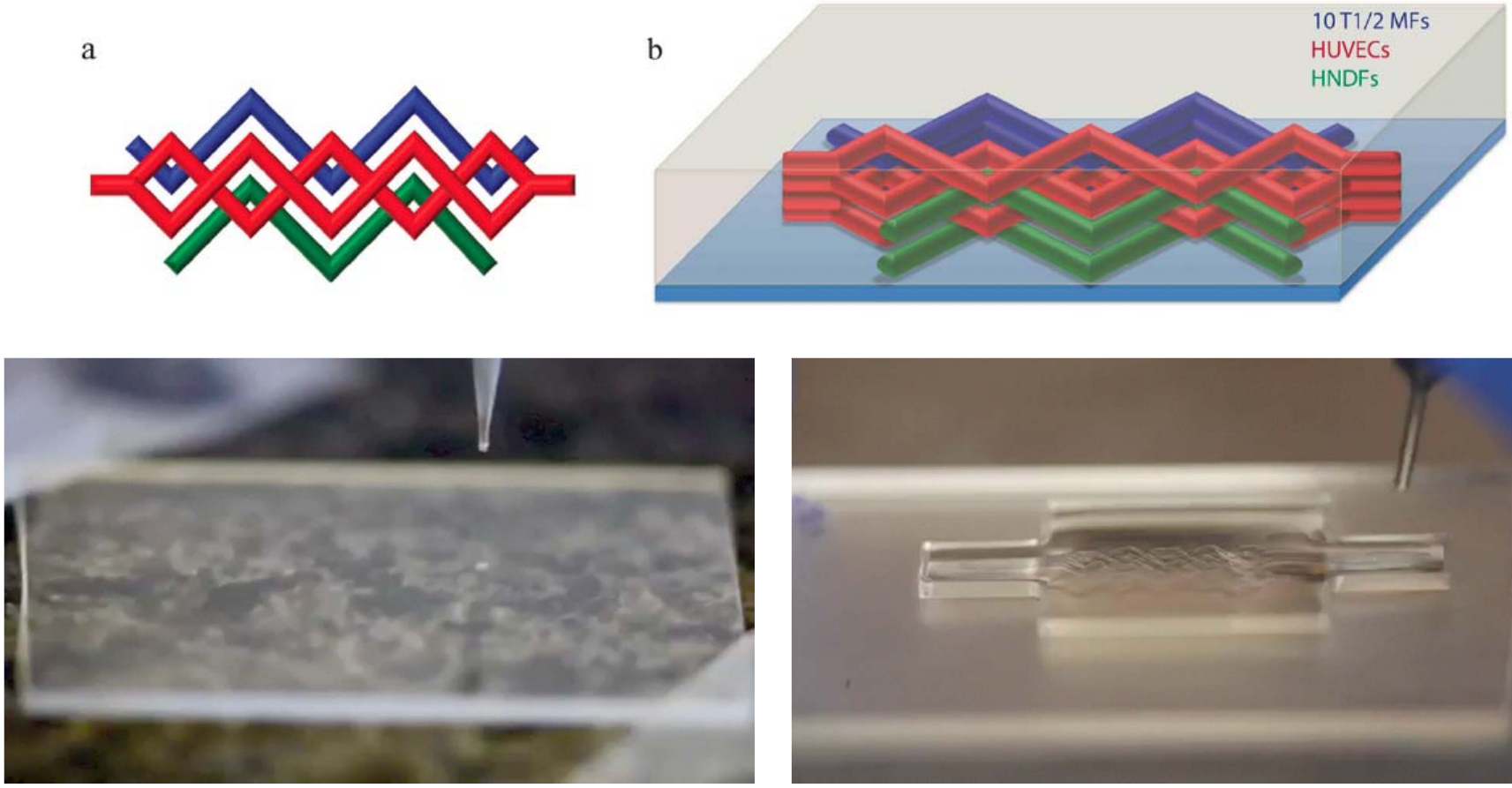


Control sample:
Casting cell-laden GelMa films composed of the same cell type, density and GelMa composite and exposed to the same curring process

N=10

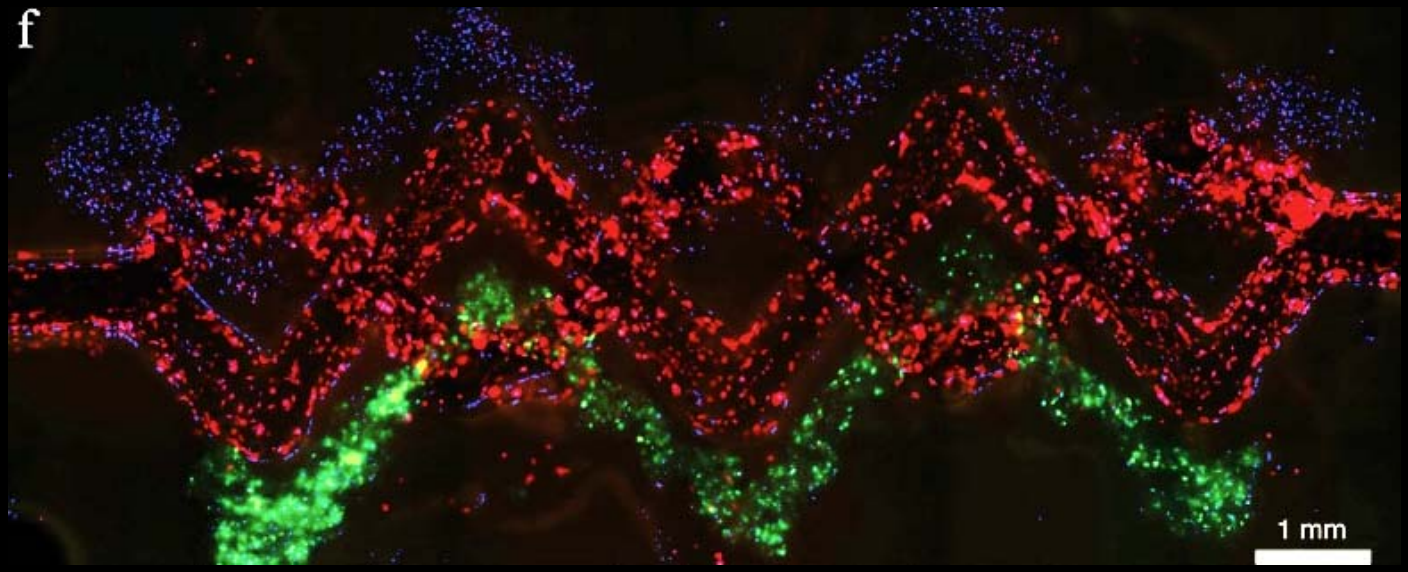
Student T-test

Tissue construct composed of semi-woven features printed in and out of plane



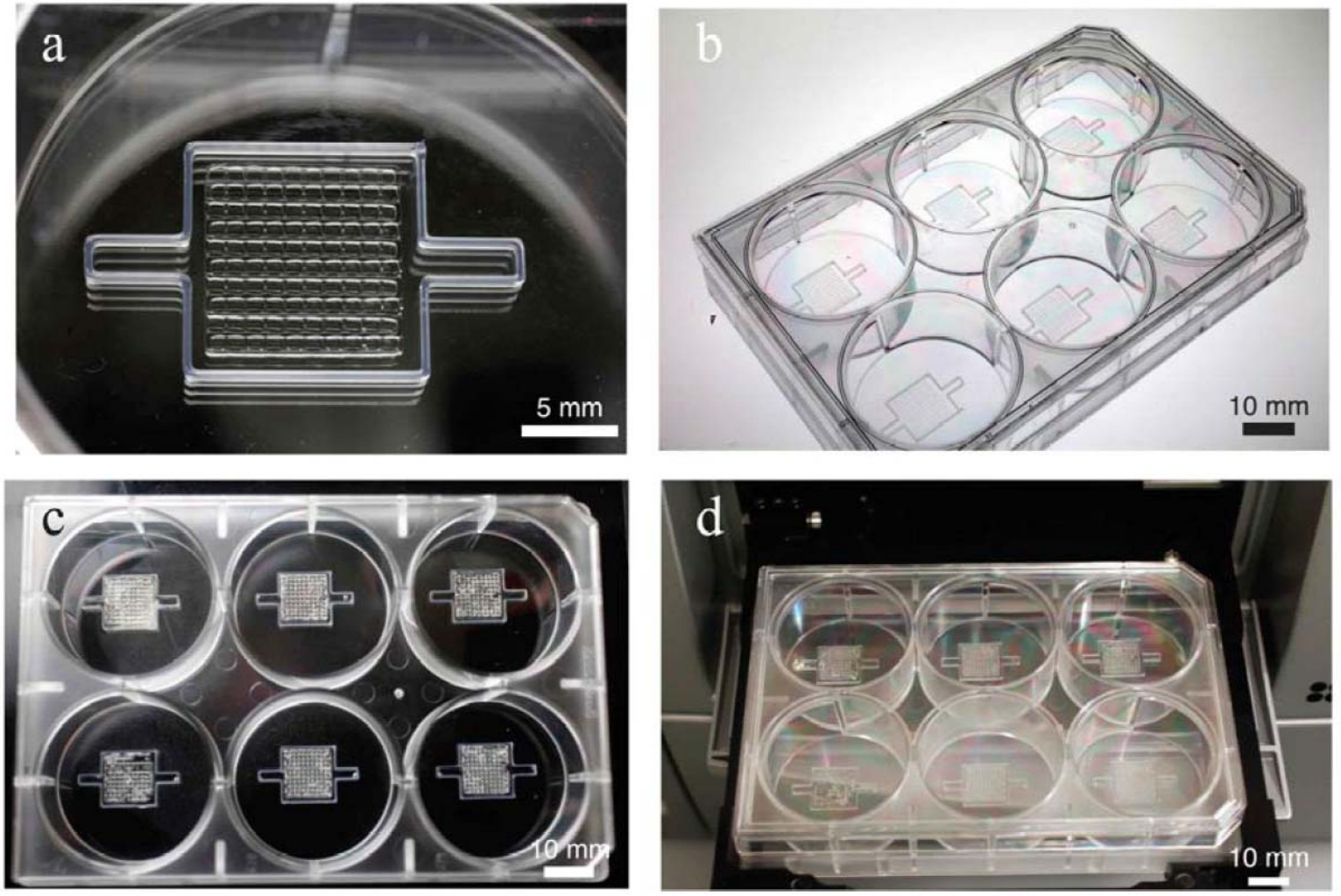
4 inks: PDMS, fugutive Pluronic F127, two different cell-laden GelMa

Composite image of 3D printed tissue construct



- 10T1/2 fibroblasts (blue)
- HNDFs (green)
- HUVECs (red)

Printed within 6-well plates



Summary & Discussion

Aim: A 3D bioprining method for fabricating engineered tissue constructs replete with **vasculature**, **multiple types of cells** and **ECM**.

Time:

- It would require 3 days to print an engineered tissue construct of ca 1000 cm3 (one nozzel)
  ➔ With 64-multinozzel array the same volume could be print in 1h

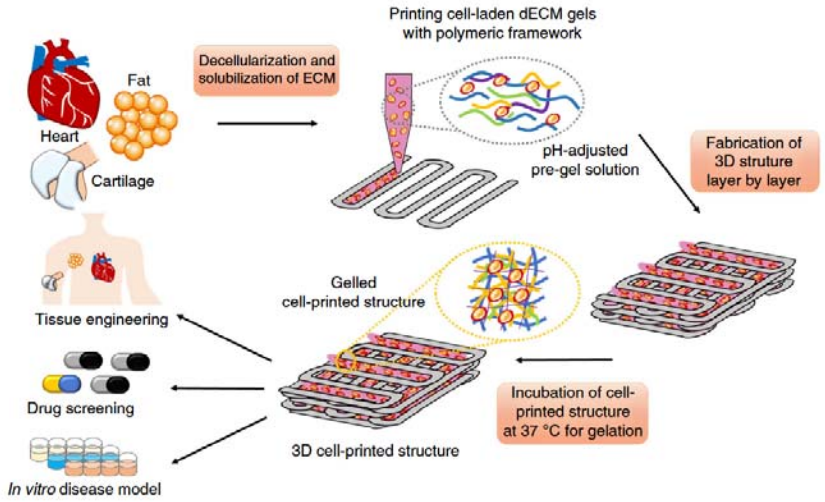
Achieved:

- New approach for the generation of vessel like structures
- Printing of multiple types of cells into one construct
- Good viability of cells over 7 days

Limitation:

- **Exposure to UV light for cross-linking**
- Maximal survival just tested up to 7 days
- Generation of „epithelialized tubulus“ - no real vessels with a complete multilayer architecture
- ECM just contains collagen

Printing three-dimensional tissue analogues with **decellularized extracellular matrix** bioink, Nature communication 2014.

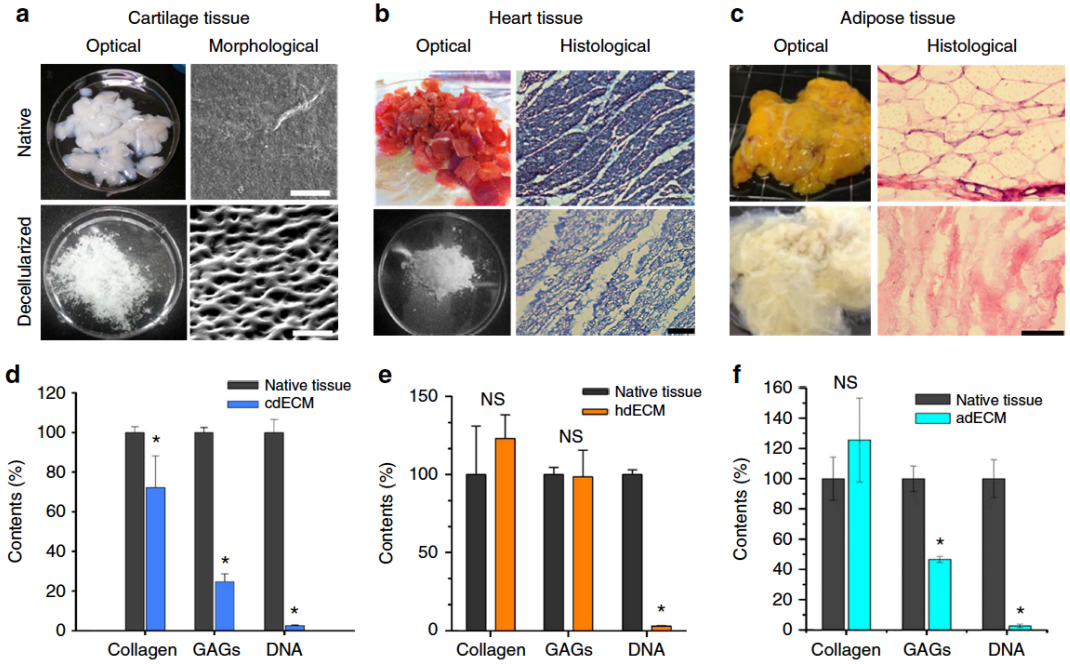


Actual used material in extrusion based printing:

- **Limited cell differentiation of stem cells**
- Cell are unable to adhere or degrade the surrounding alignate gel matrix
- Inadequate to recreate a microenvironment with cell-cell connections
- No three-dimensional cellular organization

Aim: Bioprinting method for printing of cell-laden structure with novel ECM bioink capable of providing an optimized microenvironment conducive to the growth of 3D structured tissue, where the intrinsic cellular and morphologies and function can be reconstituted

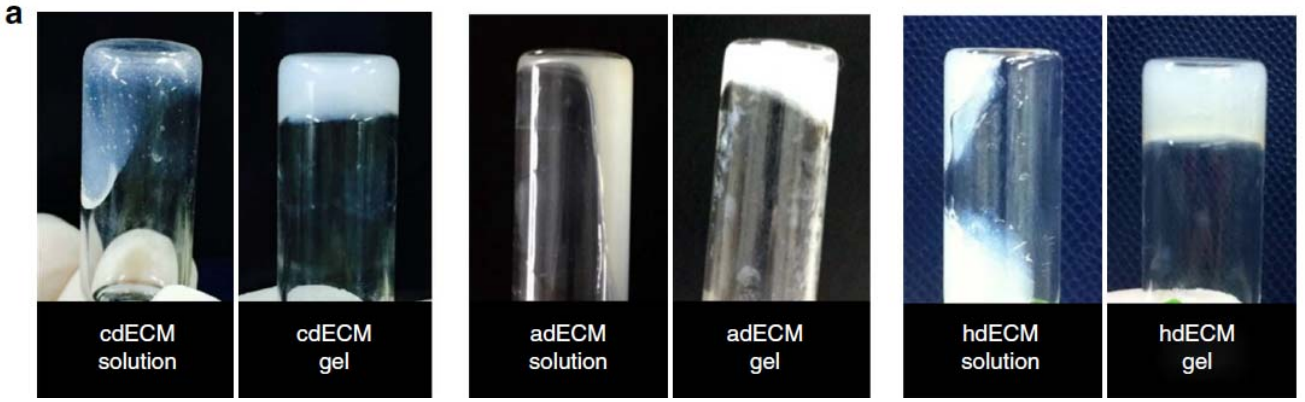
Decellularization of the native tissue and their biochemical analysis



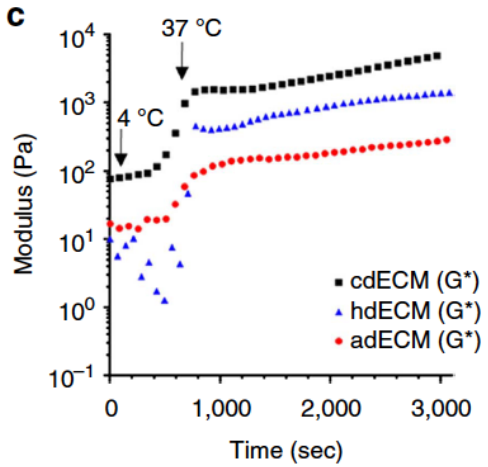
Decellularization process depending on tissue: **Physical** (chopping, freezing, thawing, centrifugation), **chemical** (1% SDS, Triton-X, Tris-HCL), **enzymatic process** (DNAse, RNAse).

- DNA quantification assay to evaluate the efficiency (Hoechst 33258 assay)
- Estimation of the ECM components: Collagen (COL, hydroxyprolin assay) and Glycosaminoglycans (GAGs, dimethylmethylene blue assay)

Rheological behaviour of the dECM pre-gel



Storage modulus G‘



Gelation kinetics:
Gelation beyond 15°C

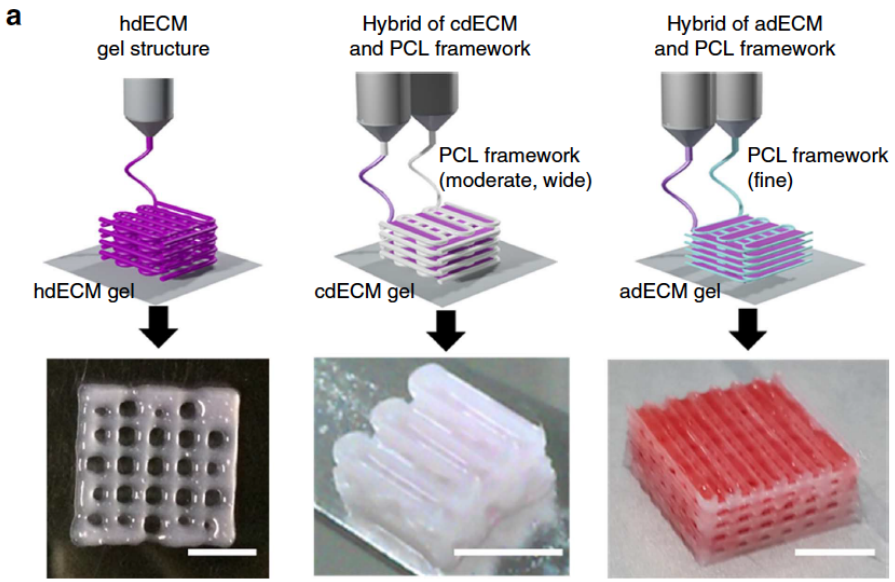
dECM pre-gel: dECM solubilized to a final concentration of 3% + pH adjusted

Tissue specific differences in **storage moduli: softer gel for adipocytes**

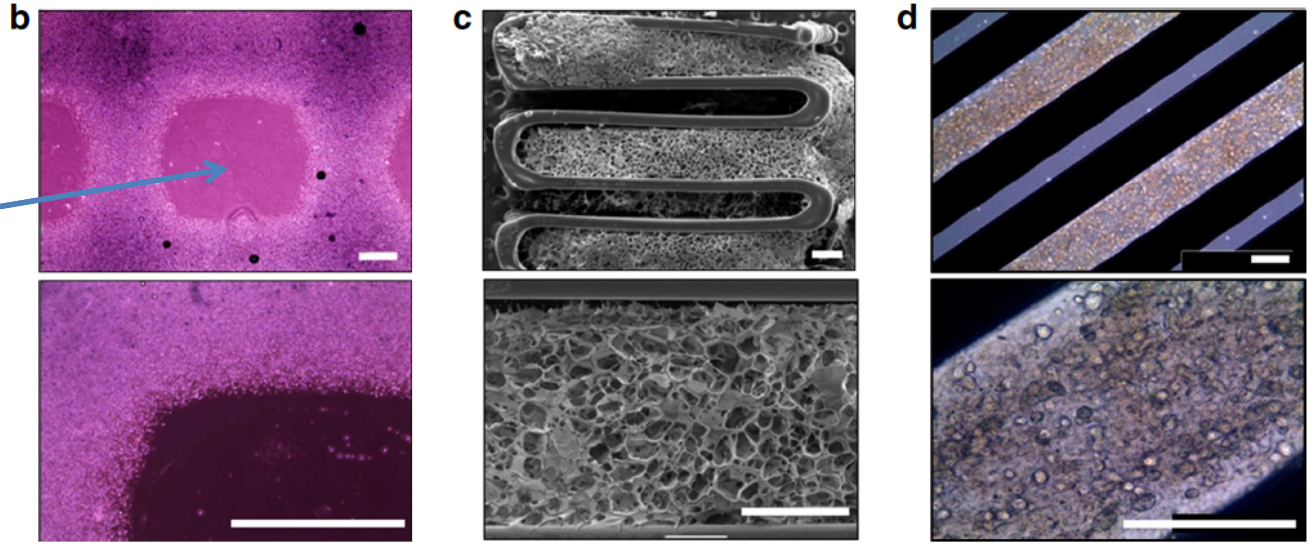
Printing process of particular tissue constructs with dECM bioink

Polycaprolactone (PCL): as framework
Machine: Multi-head tissue/organ building system
Gelling: In a humified incubator at 37C° for 30 minutes
Cells:

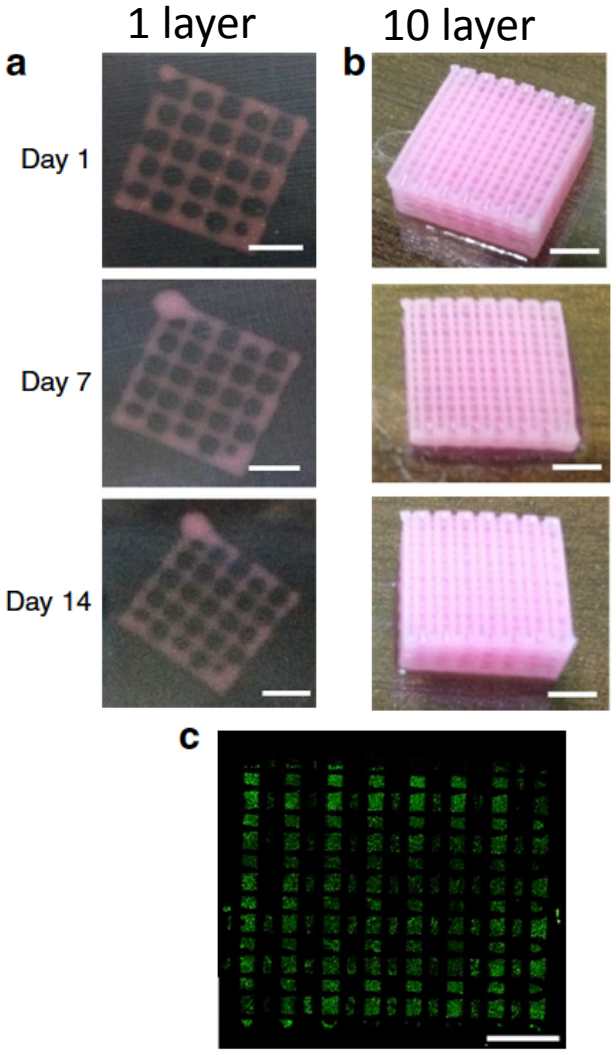
- Human adipose derived stem cells (hASCs)
- Human Turbinate-tissue derived mesenchymal stromal cells (hTMSCs)
- Rat myoblast cells (L6, ATCC CRL-1458)



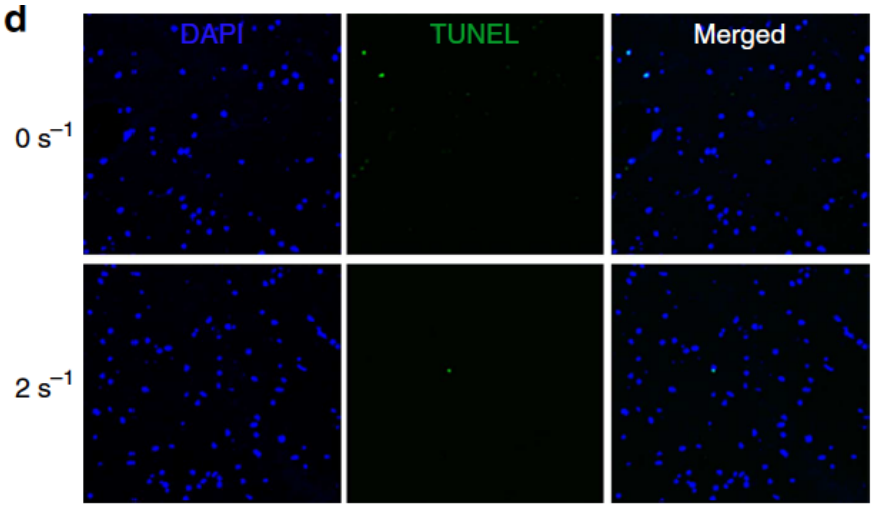
pore

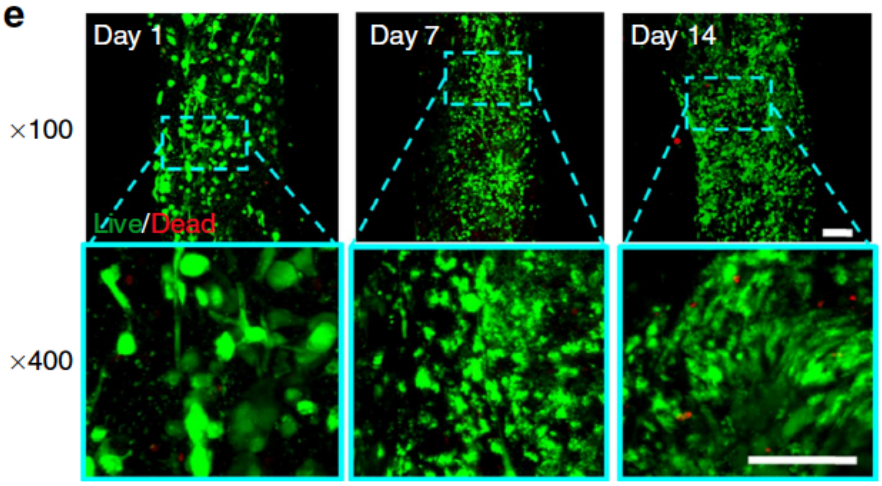


Stability and cell survival of the printed tissue constructs



Cell death through shear force: printed vs non-printed



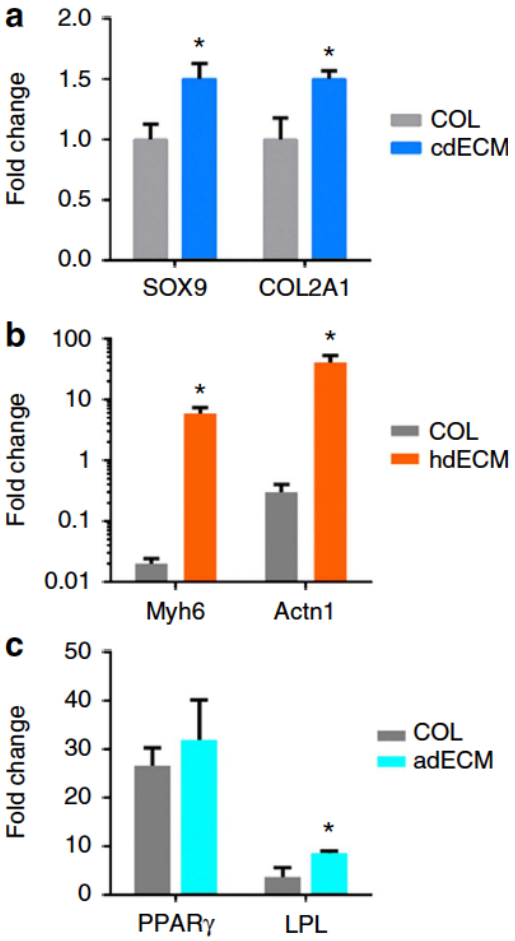


LIVE/Death® Viability Assay Cell viability over 90%

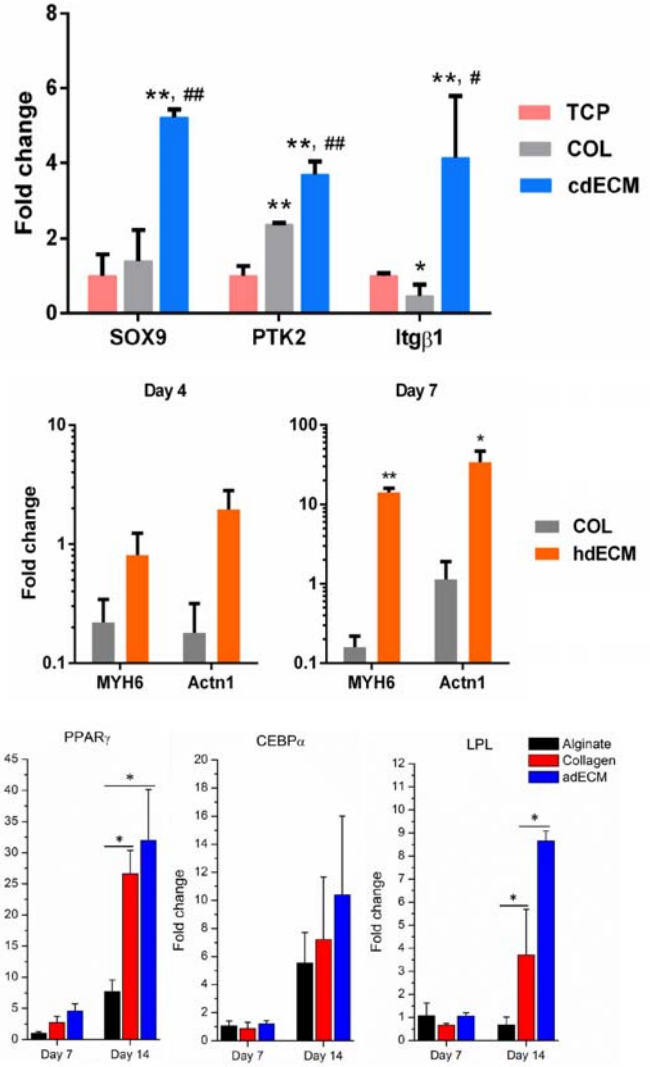
Cell to cell conections

Evaluation of differentiation into tissue-specific lineages
Gene expression through Reverse Transcript PCR

Day 14:



Day 3:



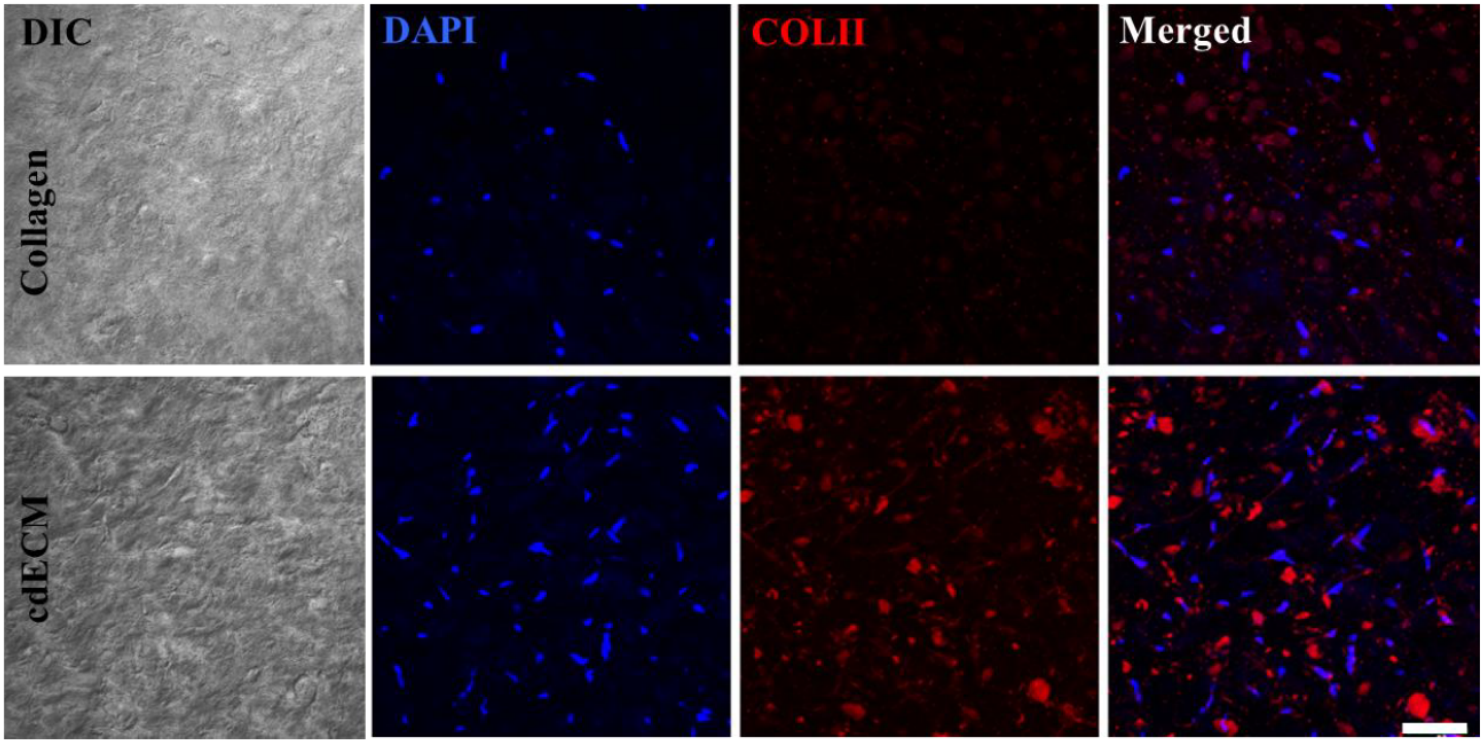
COL: Collagen type one / cells printed in collagen one as a control

SOX9: chondrogenic transcription factor
PTK2, Itgbeta1: adhesion genes

Myh6: Myosin heavy chain 6
Actn1: Alpha-sacromeric actinin

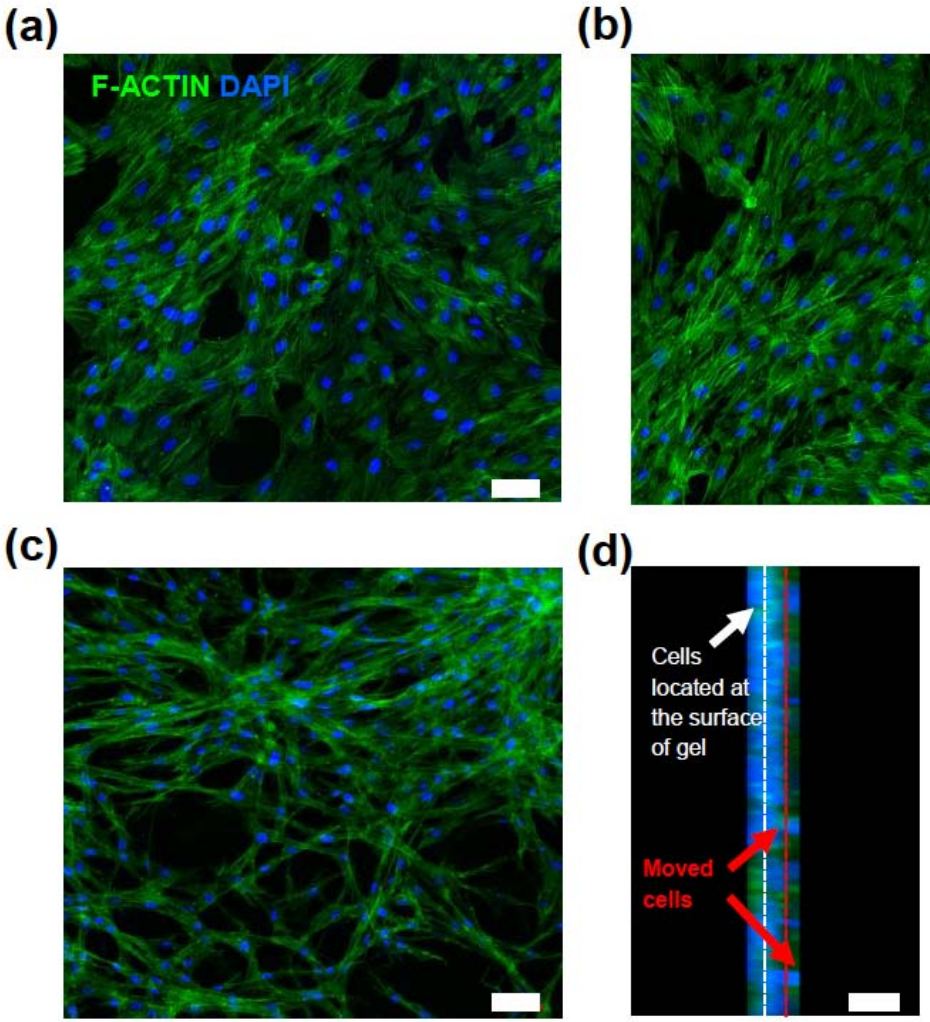
Peroxisome proliferator receptor gamma
LPL: Lipoprotein Lipase

Comparative evaluation of differentiation of hTMSCs into chodrgenic lineage in colagen and cartilage and dECM



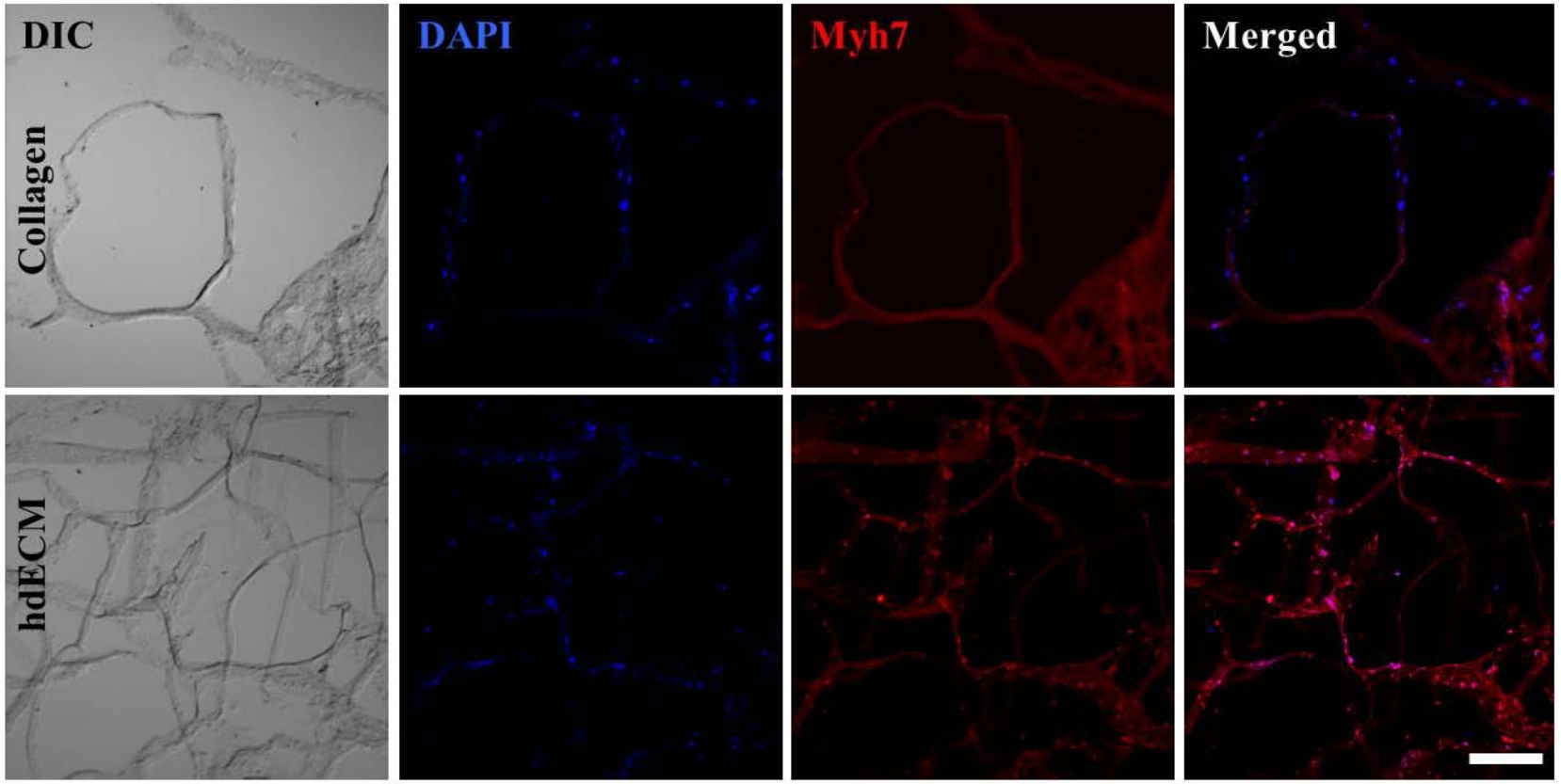
Synthesized typ II colagen by the cells

F-actin distribution of the htMSCs on tissue culture plate (TCP), collagen and cdECM

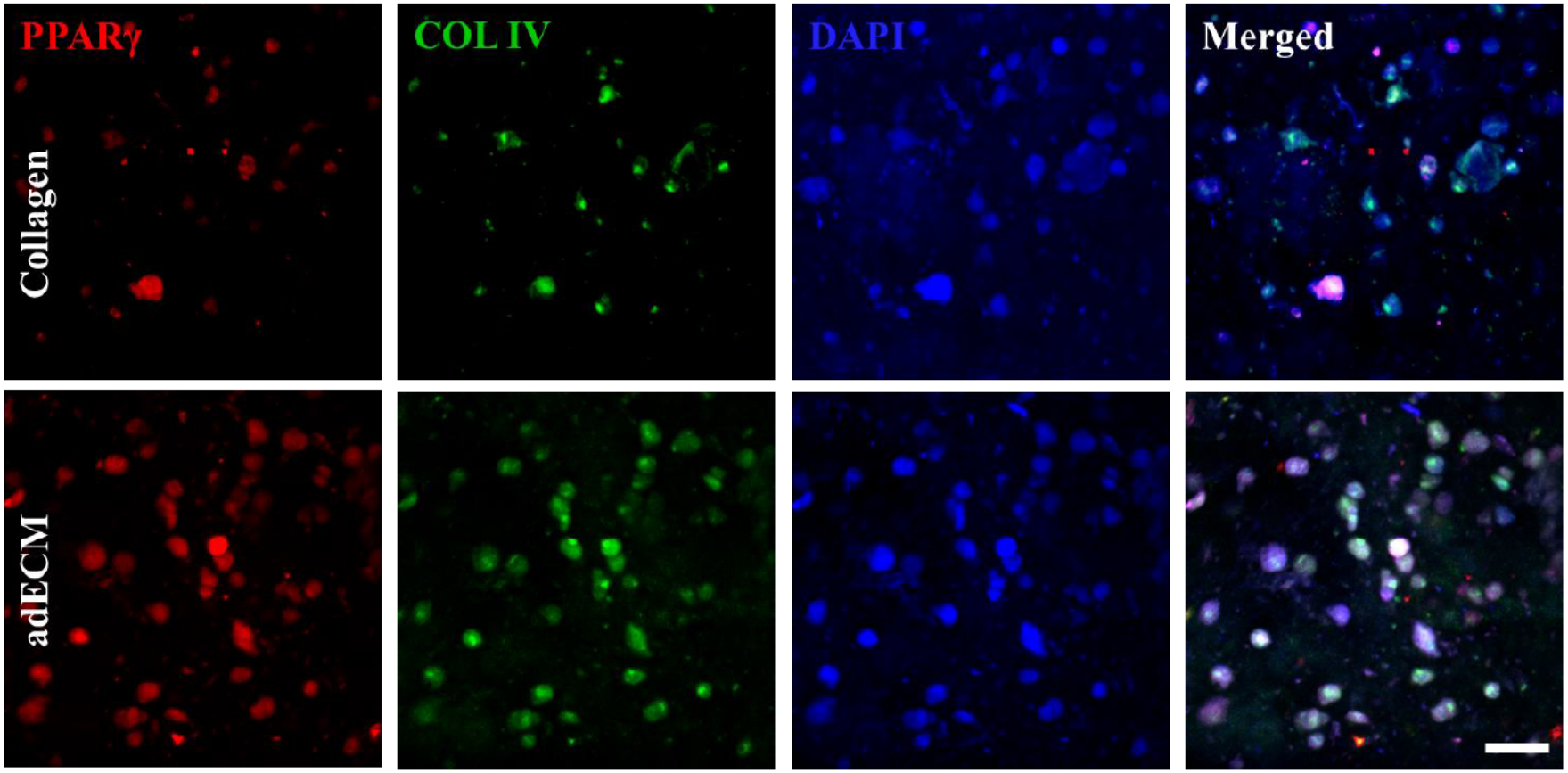


- Spread morphology on TCP and COL
- Migration into the depth of the cdECM

Comparative evaluation of differentiation of myoblasts into chodrgenic lineage in colaagen and cartilage and dECM



Comparative evaluation of differentiation of hASCs into in colaagen and cartilage and dECM



Summary & Discussion

Aim: Bioprinting method for printing of cell-laden structure with **novel ECM bionik** capable of providing an **optimized microenvironment** conducive to the growth of 3D structured tissue, where the intrinsic cellular and morphologies and function can be reconstituted

Achieved:

- Novel bionik using decellularized ECM
- Extended study of differentiation of cells (RT-PCR and morphology) is proofing advantages of the new ink over the use of standard ink (based on collagen)
- **No cross-linking procedures needed (no UV light or glutaraldehyde)**

Limitation:

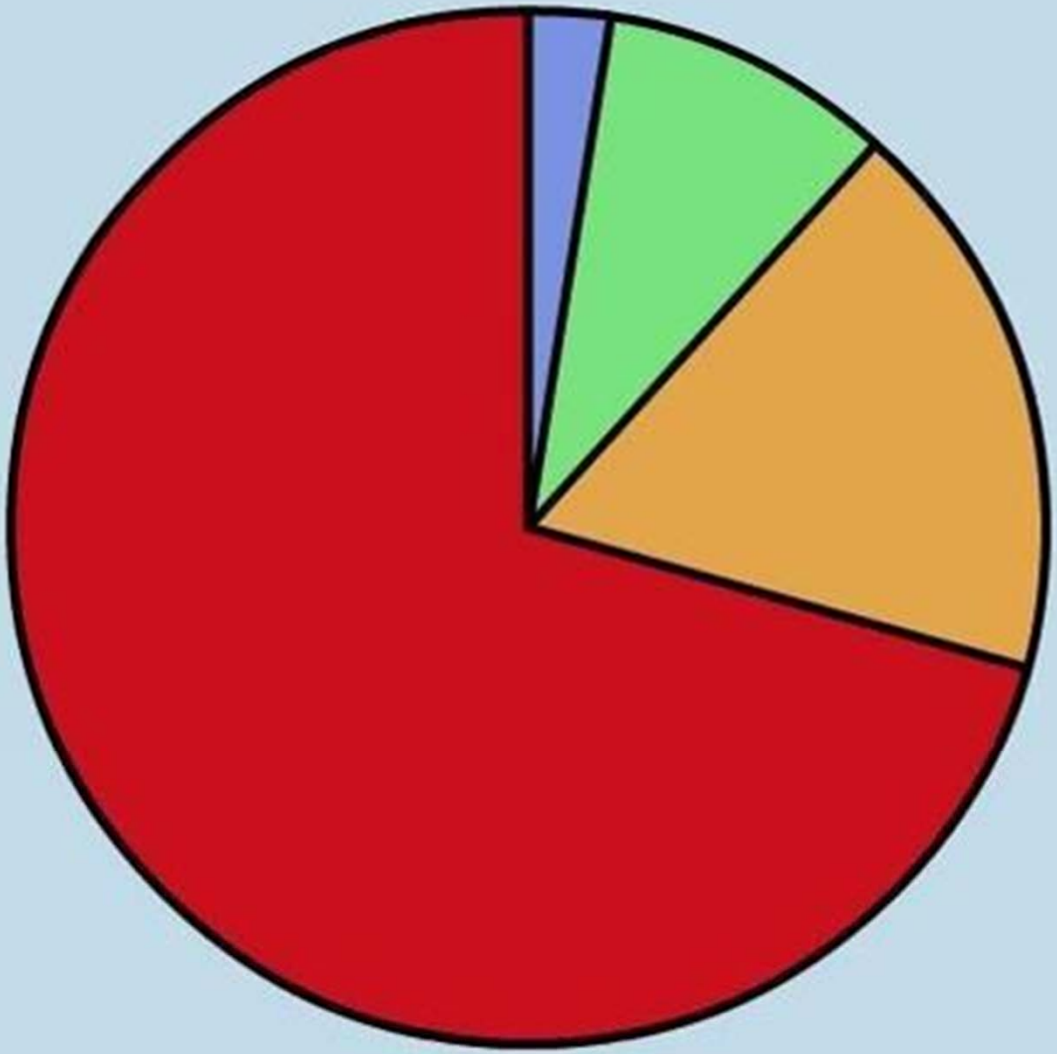
- Some quantifications (histology) are missing
- Survival shown just up to 14 days

Summary & Conclusion

Promissing:
- Tissue egeneering with different cell types
- Combination with electronic devices
- Combination with gradients of biochemical dyes
- Computer assistent design

Latest Achievements:
- Vasculatur Printing
- Improved ink with decellularized matrix
- Proof of principle for pinting cells in combination with electronic devices

WHY YOU CAN'T PRINT THAT



- Drivers aren't installed
- Forgot to load the paper. Whoops.
- You need cyan ink for a black and white document.
- No actual reason. Your printer just hates you.