Bioprinting. From technical set-up to biological applications

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NanoInnovation 2020 Rome

Additive manufacturing?
3D printing?
Bioprinting?
Different words for the same technology?

Additive manufacturing process

Virtual Model → Slicing → Printed Model

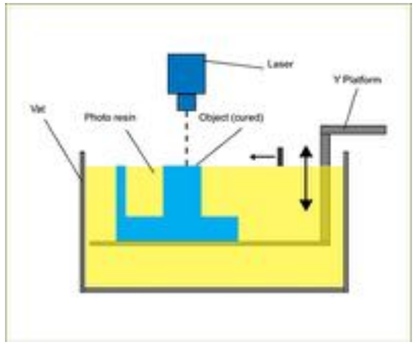
FFF: Fused Filament Fabrication
SLA: Stereolithography
DLP: Direct Light Processing
SLS: Selective Laser Sintering
DMLS: Direct Metal Laser Sintering
SLM: Selective Laser Melting

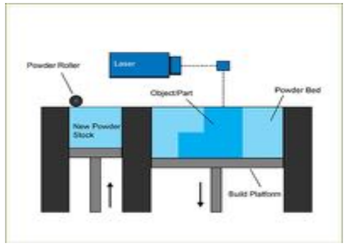
ISO/ASTM 52900:2015 (ASTM F2792)
Additive manufacturing -- General principles -- Terminology

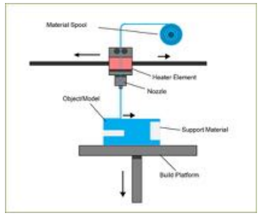
https://www.iso.org/standard/69669.html

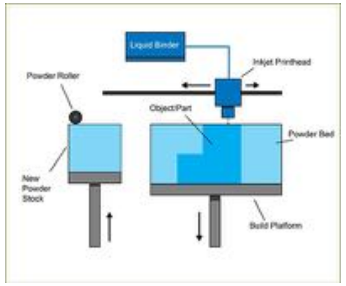
AM/3D printing technologies

- Material Extrusion
  - FFF (or FDM)
- Vat Polymerization
  - SLA
  - DLP
- Power bed fusion
  - Polymers
    - SLS
  - Metals
    - DMLS
    - SLM
    - EBM
- Material Jetting
  - Material Jetting
  - DOD
- Binder Jetting









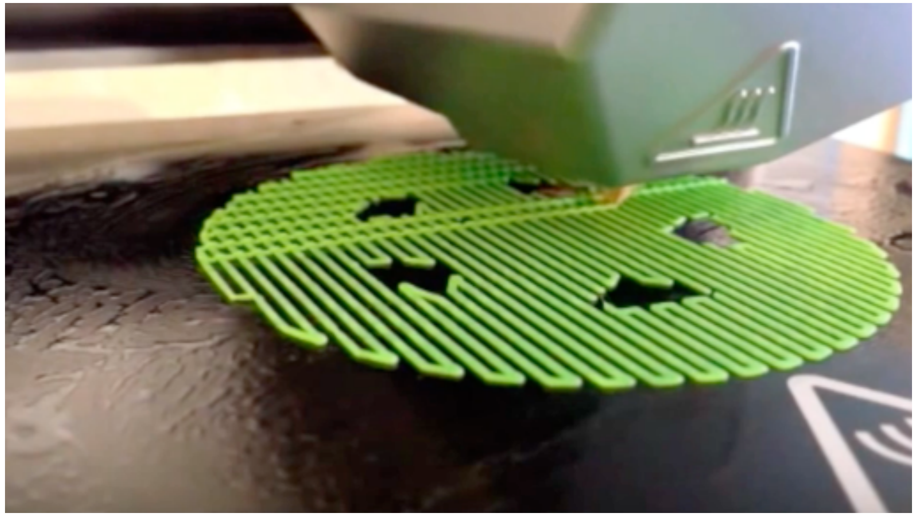
EBM: Electron Beam Melting
DOD drop on demand

3DP technologies

3DP technologies

- **Fused Deposition Modeling (FDM)**
- Stereolithography (SLA)
- Material Jetting
- Binder Jetting
- Selective Laser Melting (SLA)
- Electron Beam Melting (EBM)

A **thermo-plastic filament**, pushed through a **heating chamber** and extruded through a **small nozzle**



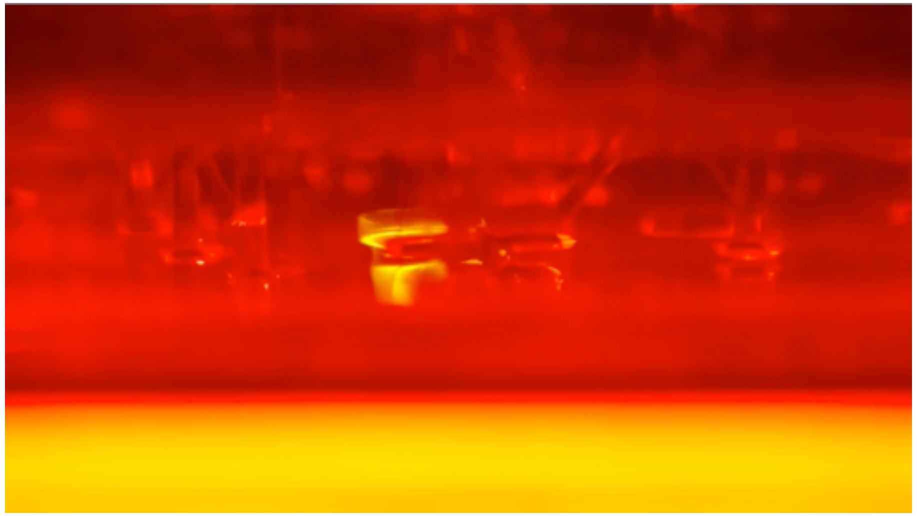
- Material: **thermoplastic filaments** (PLA, ABS, HIPS,TPU, TPE, PETG, Nylon, reinforced/charged materials)
- Curing: **temperature gradient**
- **Inexpensive** process
- Accuracy and speed are low when compared to other process

3DP technologies

3DP technologies

- Fused Deposition Modeling (FDM)
- **Stereolithography (SLA)**
- Material Jetting
- Binder Jetting
- Selective Laser Melting (SLA)
- Electron Beam Melting (EBM)

Uses a container of liquid **photopolymer resin**, from which the model is obtained using an **UV light** employed to harden the resin



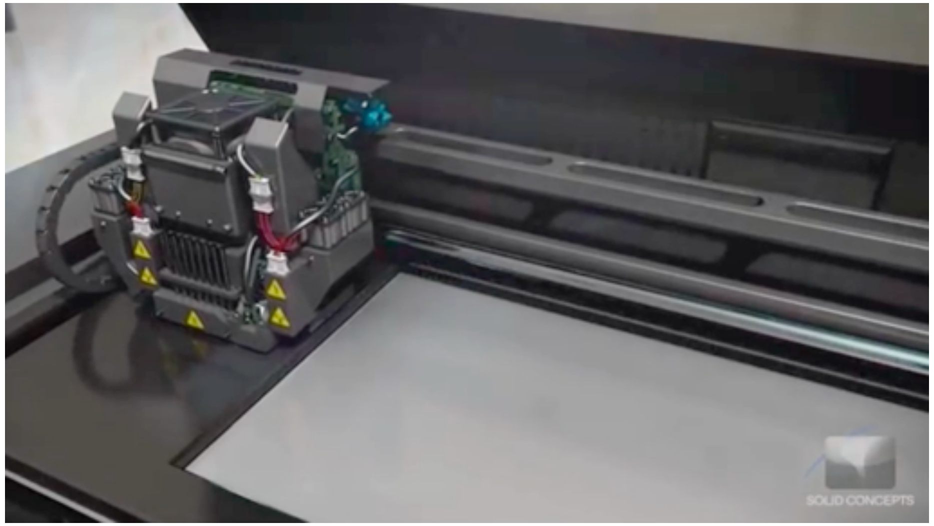
- Material: **photo-polymeric resins** hardened by UV light
- Curing: **UV laser**
- **Expensive** process, **high accuracy**

3DP technologies

3DP technologies

- Fused Deposition Modeling (FDM)
- Stereolithography (SLA)
- **Material Jetting**
- Binder Jetting
- Selective Laser Melting (SLA)
- Electron Beam Melting (EBM)

Printing material is dropped through **small diameter nozzles** (similar to inkjet paper print)



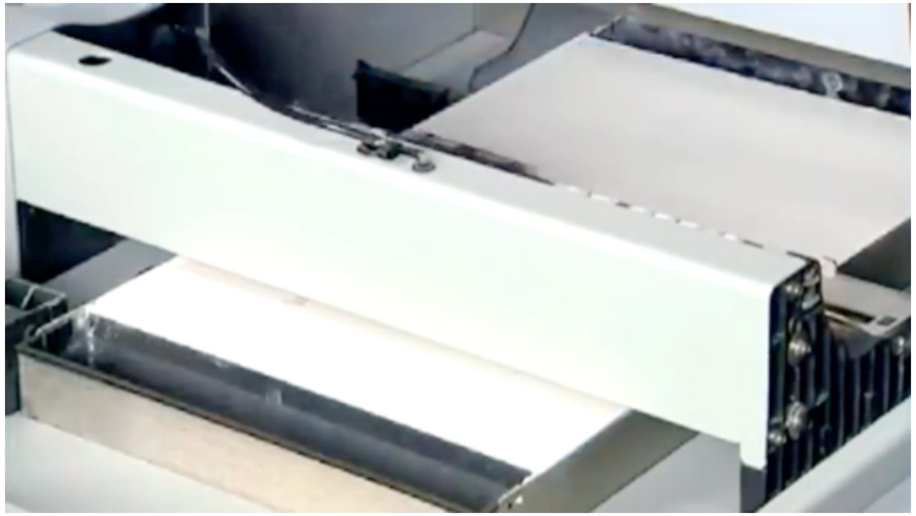
- Material: **photo-polymeric resins** hardened by UV light
- Curing: **UV light**
- **Multiple material,** parts & colors and **high accuracy**

3DP technologies

3DP technologies

- Fused Deposition Modeling (FDM)
- Stereolithography (SLA)
- Material Jetting
- **Binder Jetting**
- Selective Laser Melting (SLA)
- Electron Beam Melting (EBM)

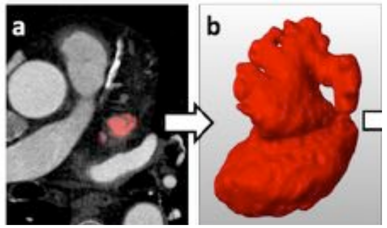
Powder is spread in equal layers & **binder** applied **through jet nozzles** that **"glue"** the powder particles



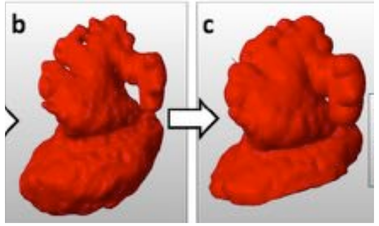
- Material: **binder powder**
- Curing: **binder (glue)**
- Parts can be made with a wide range of **different colours**

Additive manufacturing?
3D printing?
Bioprinting?
Different words for the same technology?

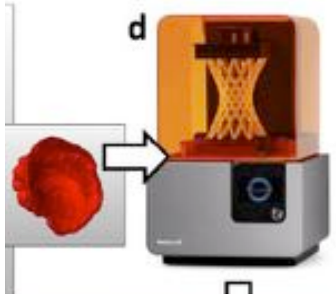
We have to deal with a class of technologies with their peculiar features, aims, and issues
With a common key idea ...



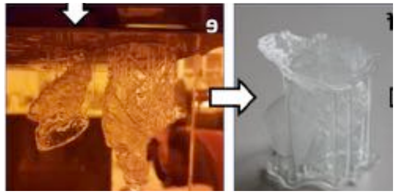
1. Producing a 3D file



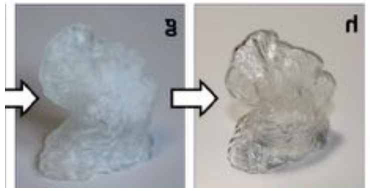
2. STL creation and file manipulation



3. Printing

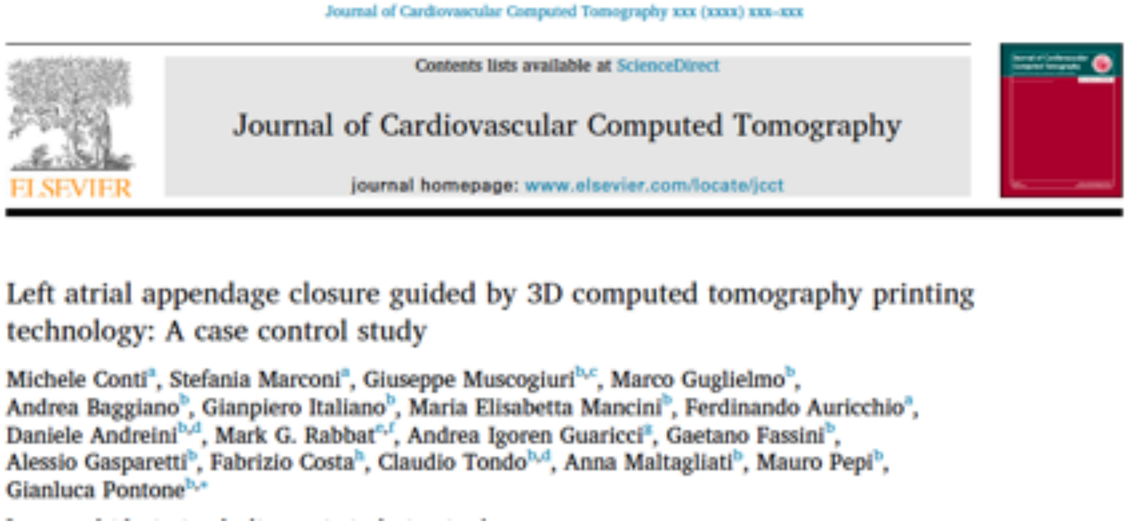


4. Removal of prints



5. Post processing

Workflow



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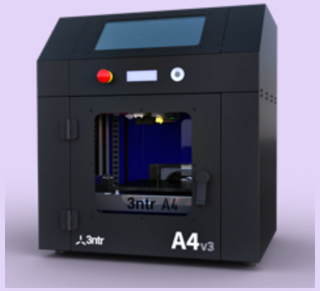
Left atrial appendage closure guided by 3D computed tomography printing technology: A case control study

Michele Conti[a], Stefania Marconi[a], Giuseppe Muscogiuri[b,c], Marco Guglielmo[b], Andrea Baggiano[b], Gianpiero Italiano[b], Maria Elisabetta Mancini[b], Ferdinando Auricchio[a], Daniele Andreini[b,d], Mark G. Rabbat[e,f], Andrea Igoren Guaricci[g], Gaetano Fassini[b], Alessio Gasparetti[b], Fabrizio Costa[h], Claudio Tondo[b,d], Anna Maltagliati[b], Mauro Pepi[b], Gianluca Pontone[b,*]

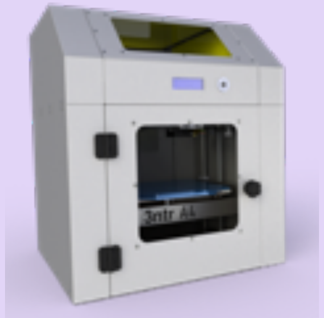
3D@UniPV

FDM

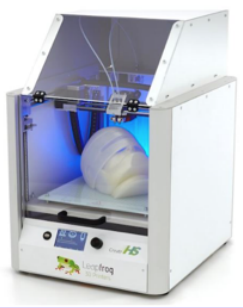
3NTR A4v3



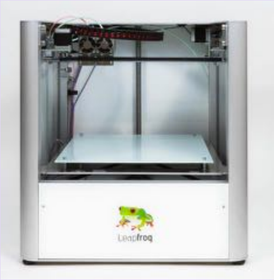
LeapFrog Creatr HS



3NTR A4v2

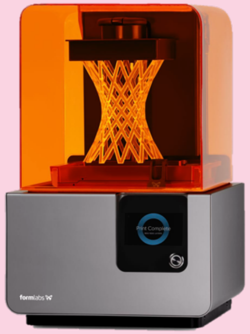


LeapFrog Creatr Dual Extruder



SLA

FORM 2 Desktop SLA



Material jet

ObJet 260 Connex 3

Binder jetting

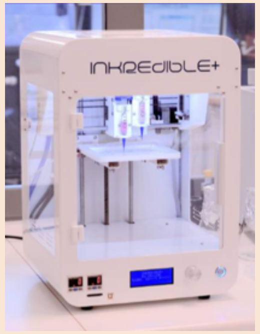
3DSystems ProJet 460 Plus

SLA

Renishaw AM 400

Bioprinting

Cellink Inkredible+



http://www-4.unipv.it/3d/

3D4MED - https://www.3d4med.eu

UniPV and IRCCS San Matteo
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From 3D printing to 3D BIOprinting

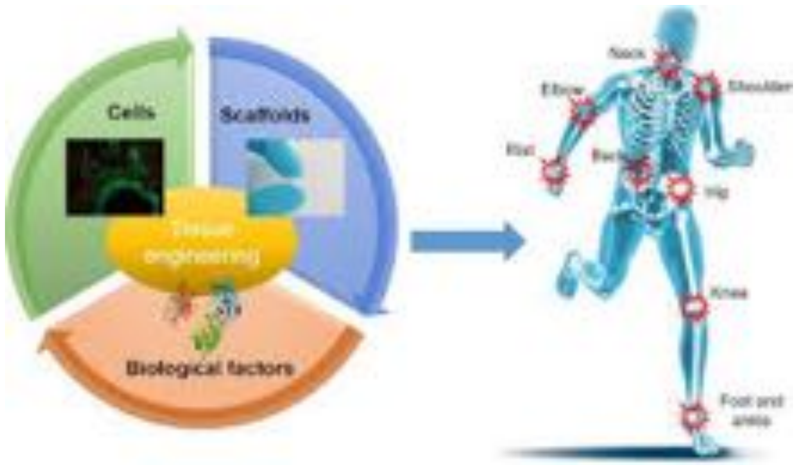
Functionalize 3D printed objects

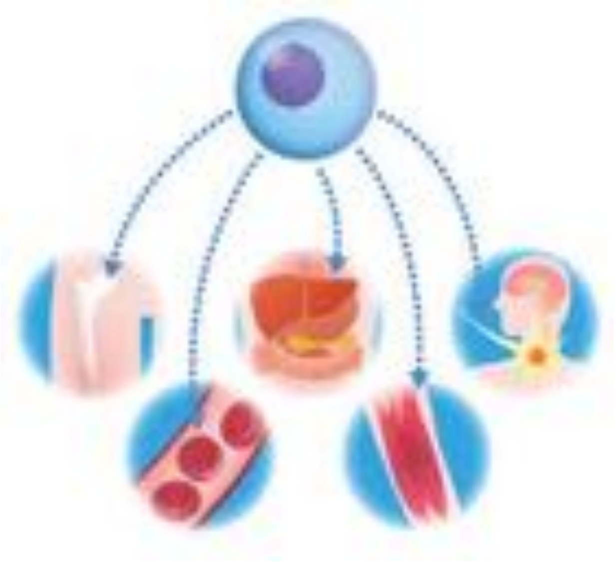
BIOprinting

Tissue engineering

An interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve biological tissue function or a whole organ.

Langer R, Vacanti JP. Tissue engineering. Science 1993, 260(5110), 920–926



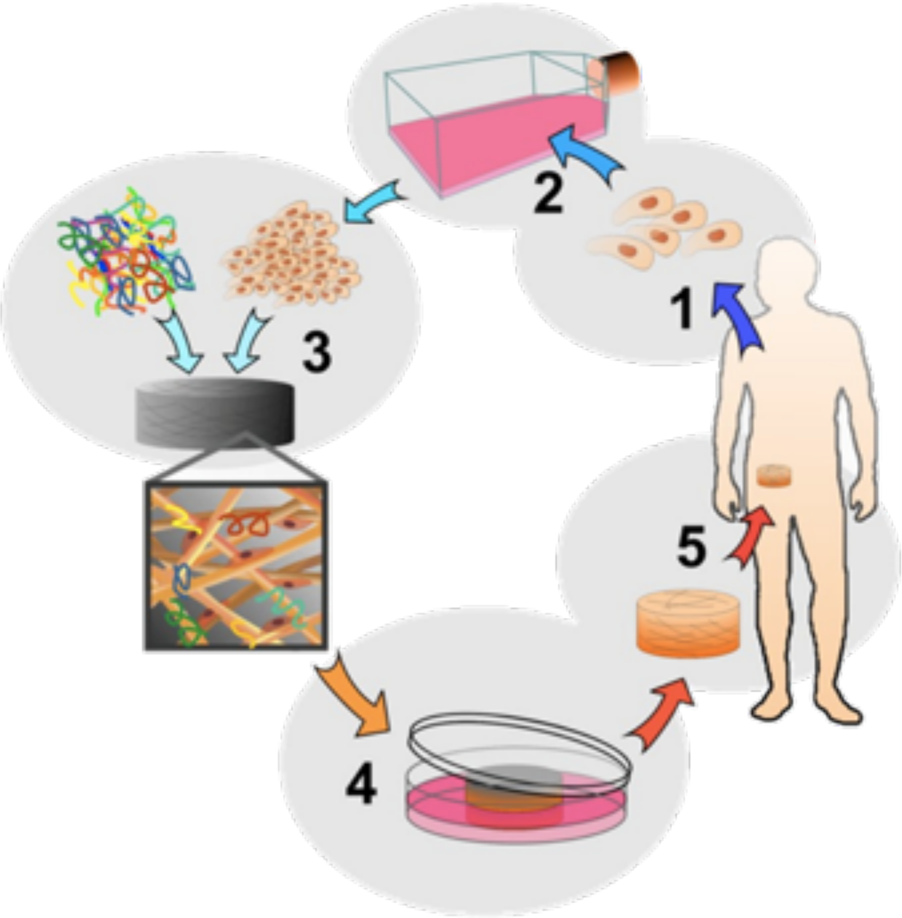


Regenerative medicine

Application of tissue science, tissue engineering, and related biological and engineering principles that restore the structure and function of damaged tissues and organs

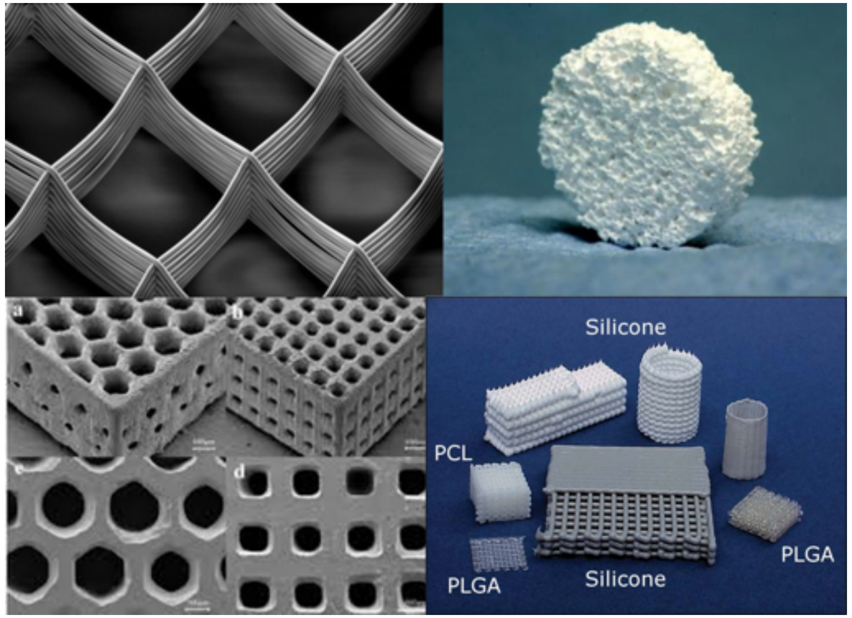
U.S. department of health and human services, 2006: A New Vision - A Future for Regenerative Medicine,

Classic paradigm in TE



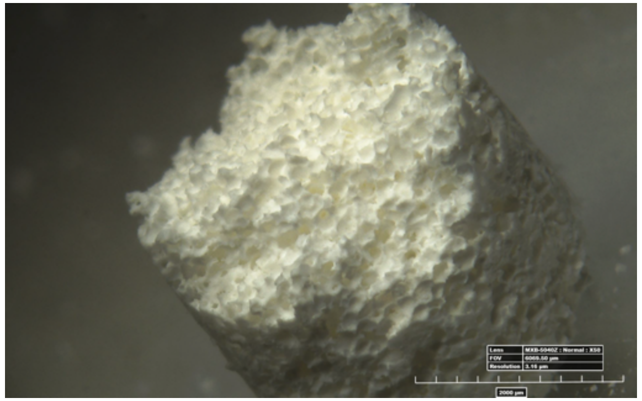
- Scaffolds
- Cells
- Growth Factors

Scaffold



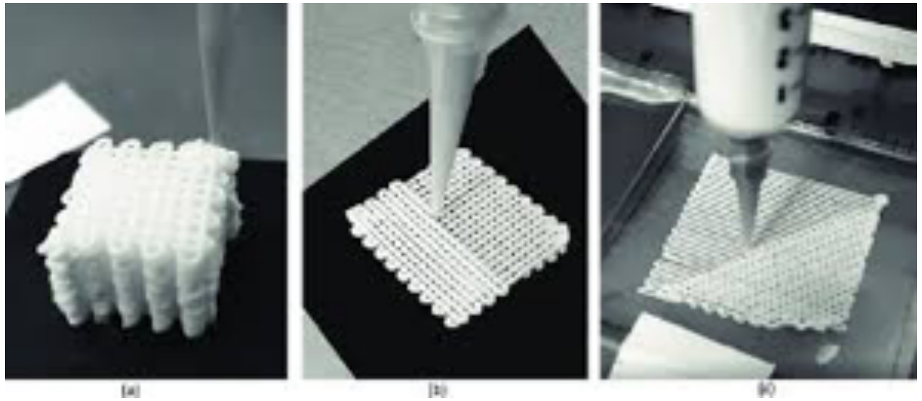
- 3D and porous
- to mimic host tissue with its mechanical
- properties
- to promote cell activities
- to give cells suitable and ECM-like cues
- …

Conventional fabrication technique



- Solvent casting and particulate leaching
- Gas foaming
- Phase separation
- Freeze drying
- …..

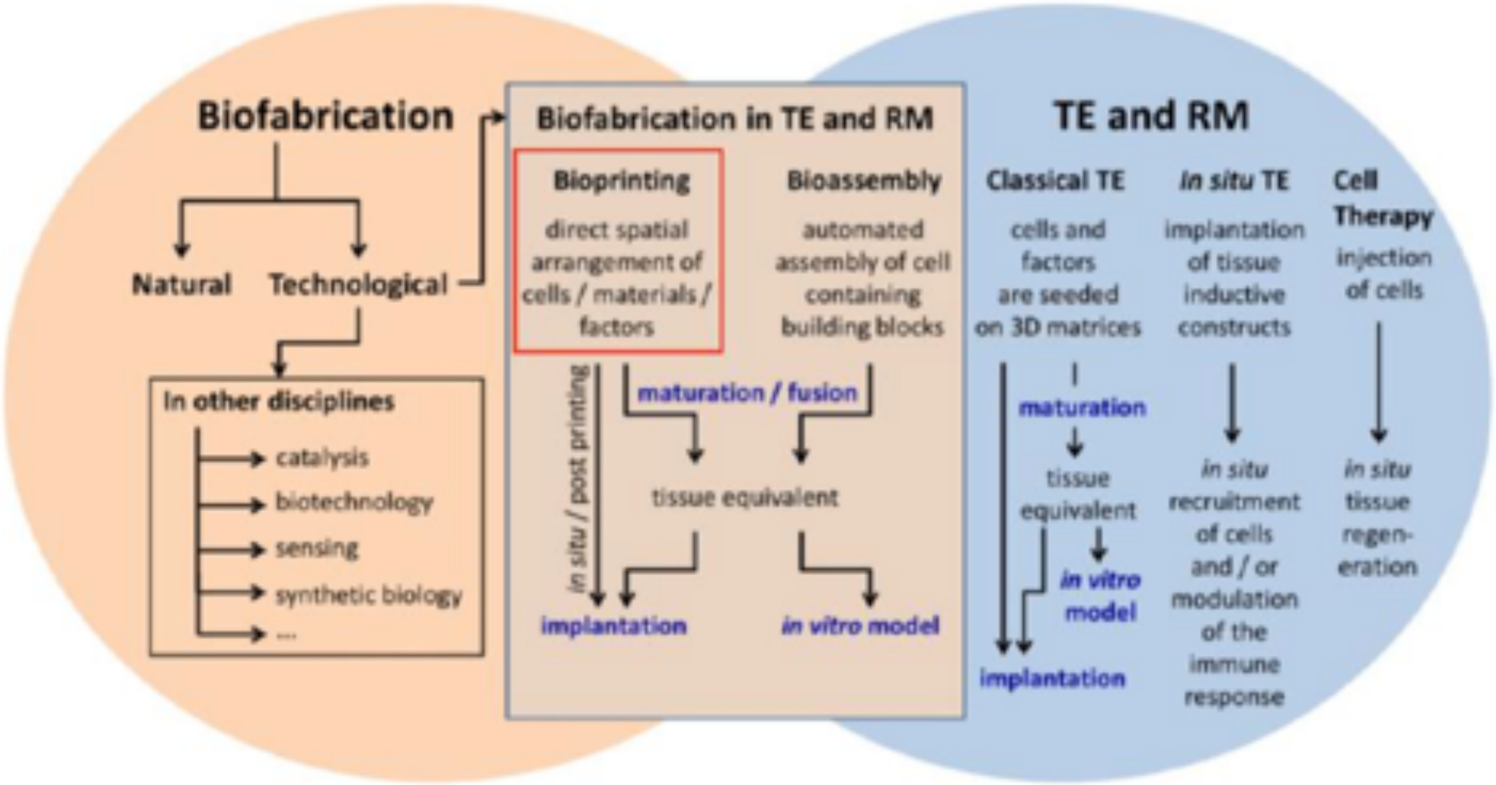
ADDITIVE MANUFACTURING



- 3D printing
- Light based techniques
- Extrusion based technique
- Electrospinning
- ….

BIOFABRICATION

Generation of biologically functional products with structural organization from living cells, micro-tissues or hybrid tissue constructs, bioactive molecules or biomaterials either through top-down (**Bioprinting**) or bottom-up (**Bioassembly**) strategies and subsequent tissue maturation processes



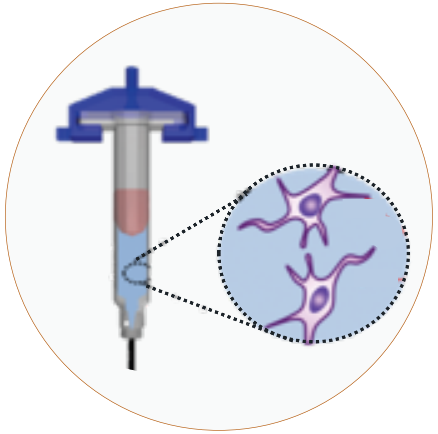
Groll J., et al. Biofabrication, 2016

Moroni L et al., Biofabrication: A Guide to Technology and Terminology. Trends Biotechnol. 2017 Nov 11. pii: S0167-7799(17)30279-2

BioP: key elements

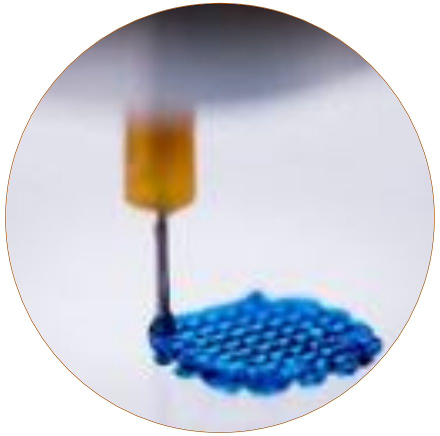
Bioprinting

=



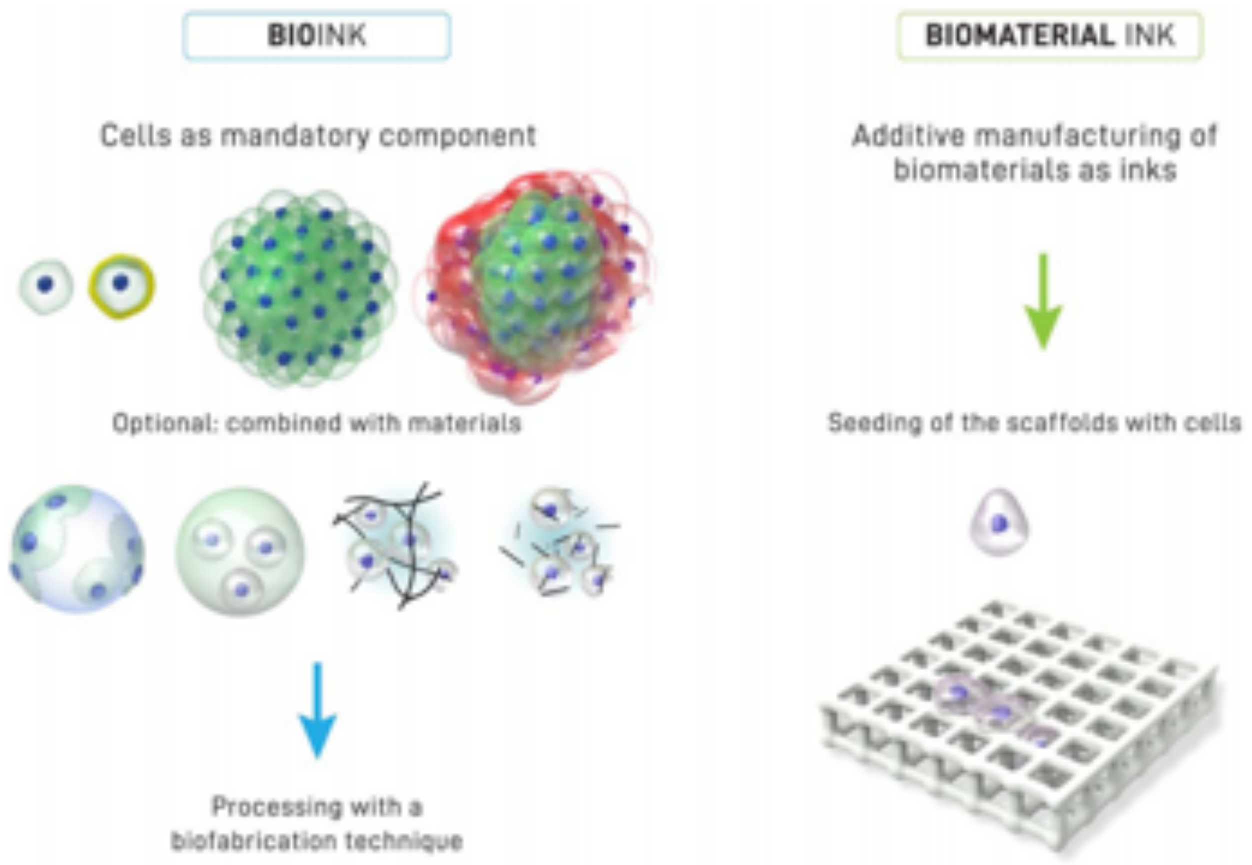
Bio-ink

+



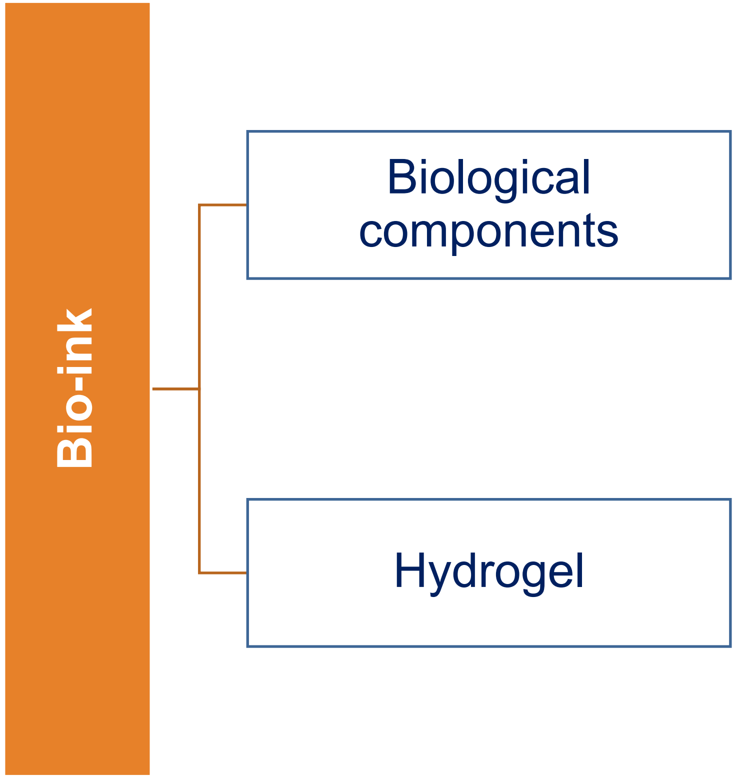
Bio-plotter

Bioink vs Biomaterial ink

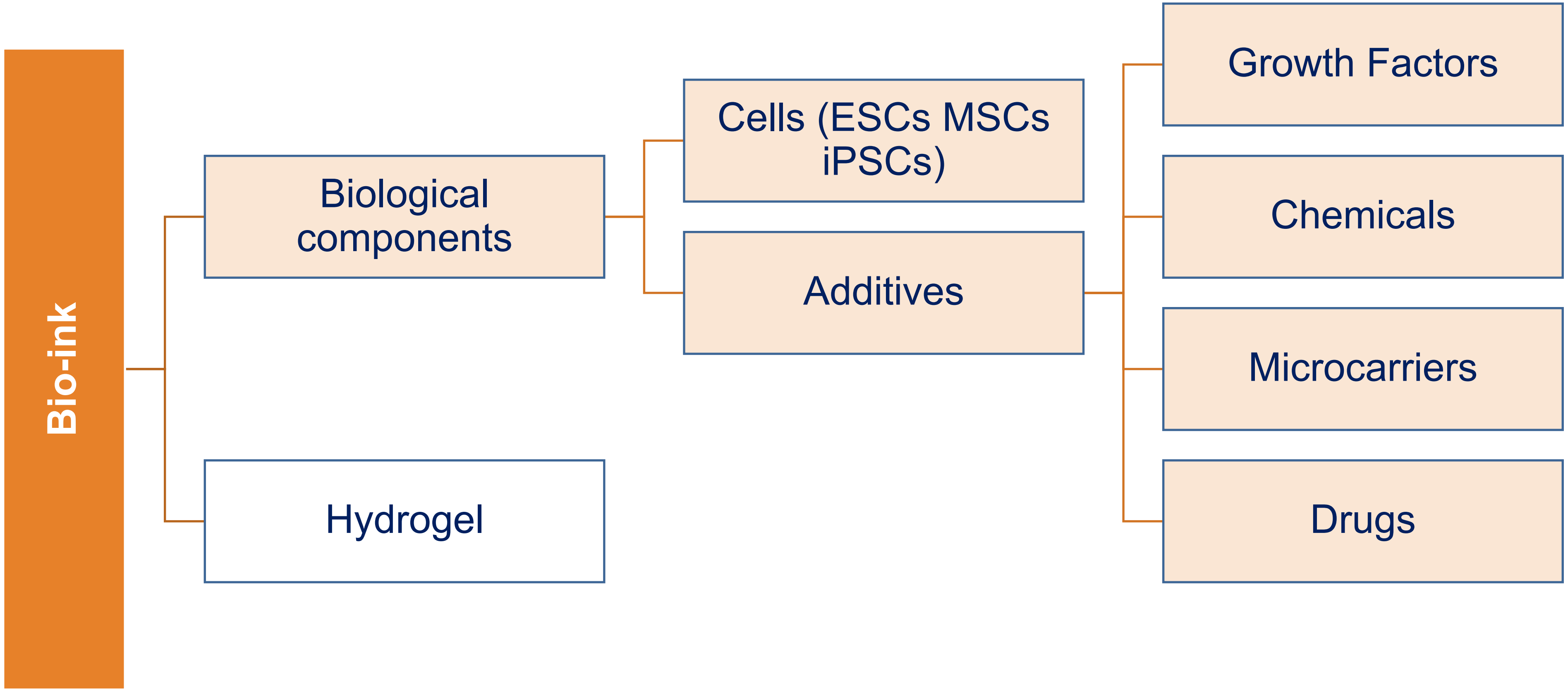


Groll, J., et al. "A definition of bioinks and their distinction from biomaterial inks." Biofabrication 11.1 (2018): 013001.

BioP: bio-ink



BioP: bio-ink BioP: bio-ink

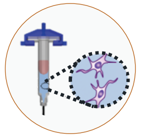


BioP: bio-ink

- Bio-ink
  - Biological components
  - Hydrogel
    - Natural
    - Synthetic

BioP: bio-ink

- **Bio-ink**
  - Biological components
  - Hydrogel
    - Natural
      - Alginate
      - Agarose
      - Collagen
      - Hyaluronic acid
      - Fibrin
      - Cellulose
      - Extracellular matrix
    - Synthetic
      - Pluronic
      - Poly(ethyleneglycol)
      - Polycaprolactone
  - **Crosslinking** → Hydrogel



Hydrogel crosslinking

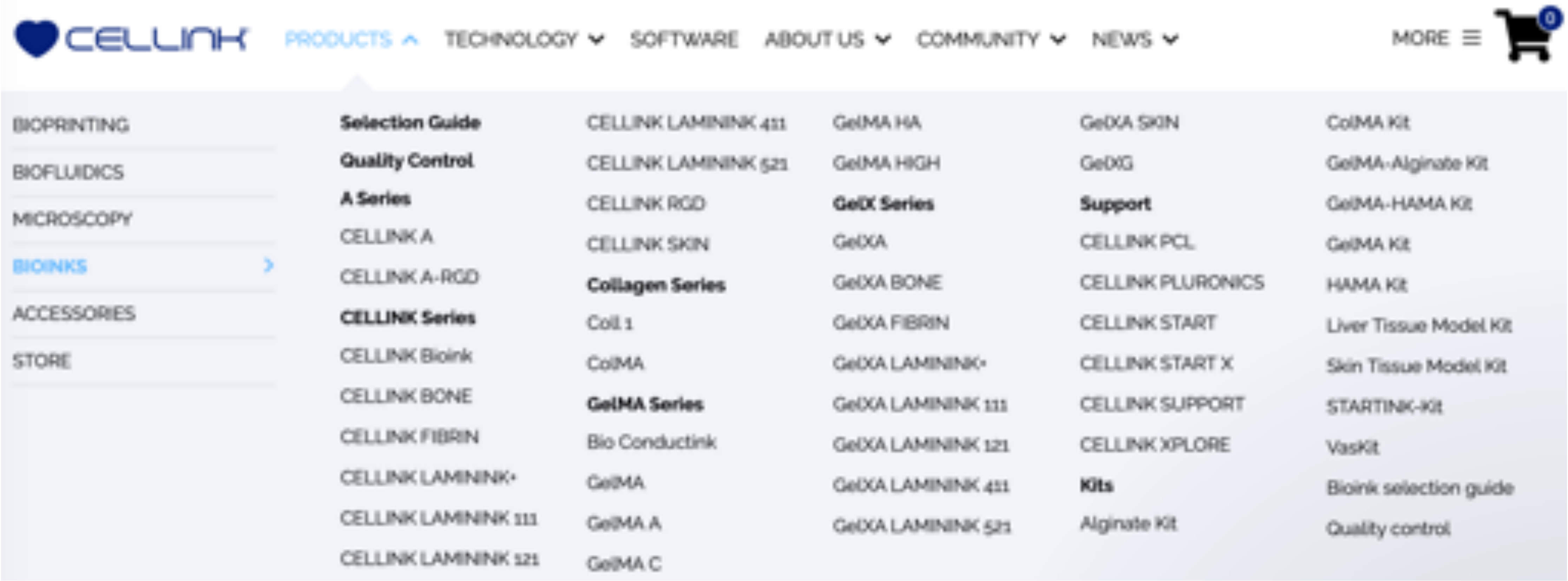
Polymer

+ Crosslinker

Ionic or covalent crosslinking
Photo crosslinking
Thermic

Hydrogel

BioP: bio-ink



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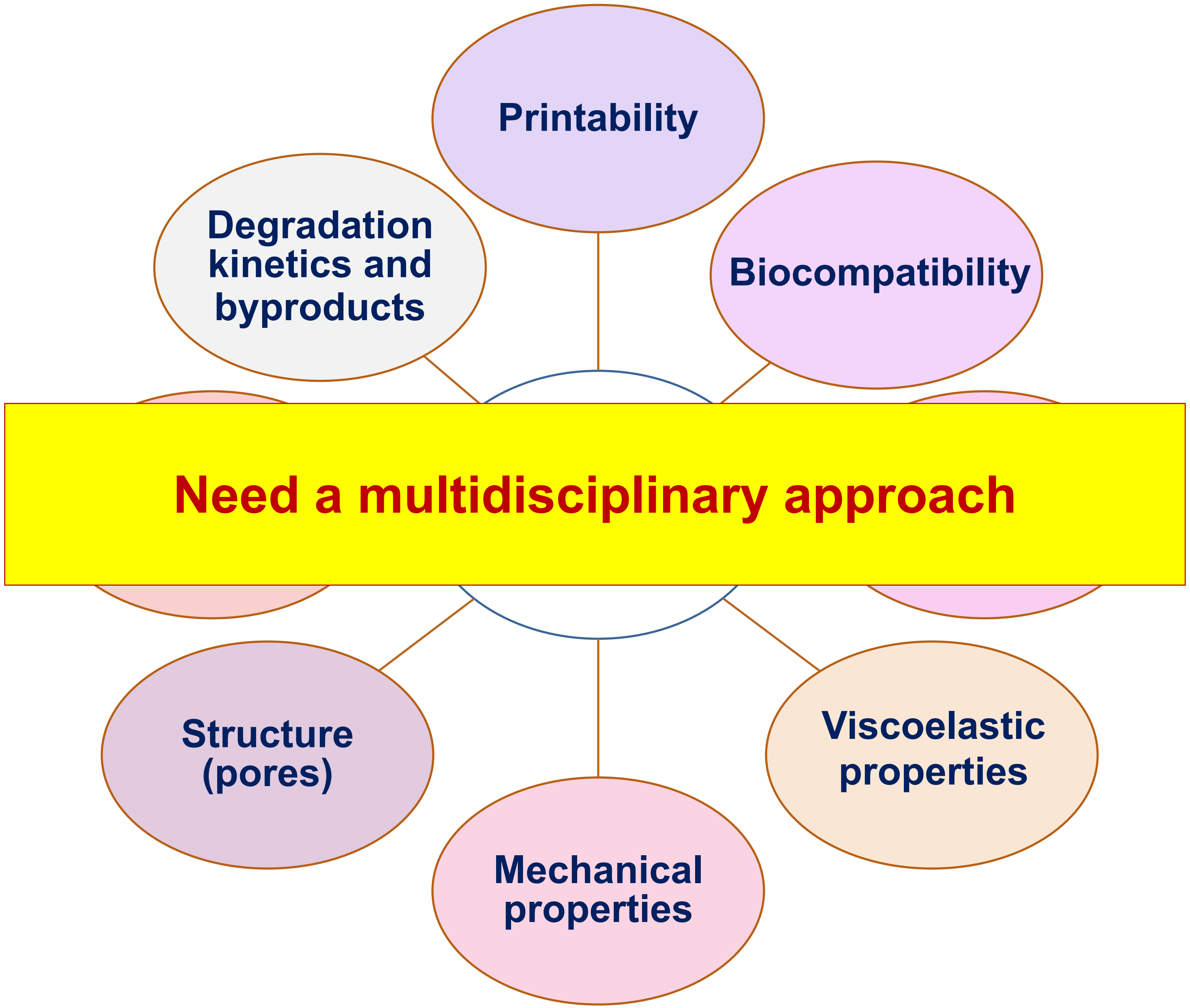
CELLINK LAMININK 111
CELLINK LAMININK 121
CELLINK LAMININK 411
CELLINK LAMININK 521
CELLINK RGD
CELLINK SKIN
Collagen Series
Coll 1
ColMA
GelMA Series
Bio Conductink
GelMA
GelMA A
GelMA C
GelMA HA
GelMA HIGH
GelX Series
GelXA
GelXA BONE
GelXA FIBRIN
GelXA LAMININK+
GelXA LAMININK 111
GelXA LAMININK 121
GelXA LAMININK 411
GelXA LAMININK 521
GelXA SKIN
GelXG
Support
CELLINK PCL
CELLINK PLURONICS
CELLINK START
CELLINK START X
CELLINK SUPPORT
CELLINK XPLORE
Kits
Alginate Kit
ColMA Kit
GelMA-Alginate Kit
GelMA-HAMA Kit
GelMA Kit
HAMA Kit
Liver Tissue Model Kit
Skin Tissue Model Kit
STARTINK-Kit
VasKit
Bioink selection guide
Quality control

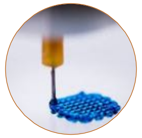
Bio-ink features for 3D BioP



BioP: bio-plotter

Bio-plotter

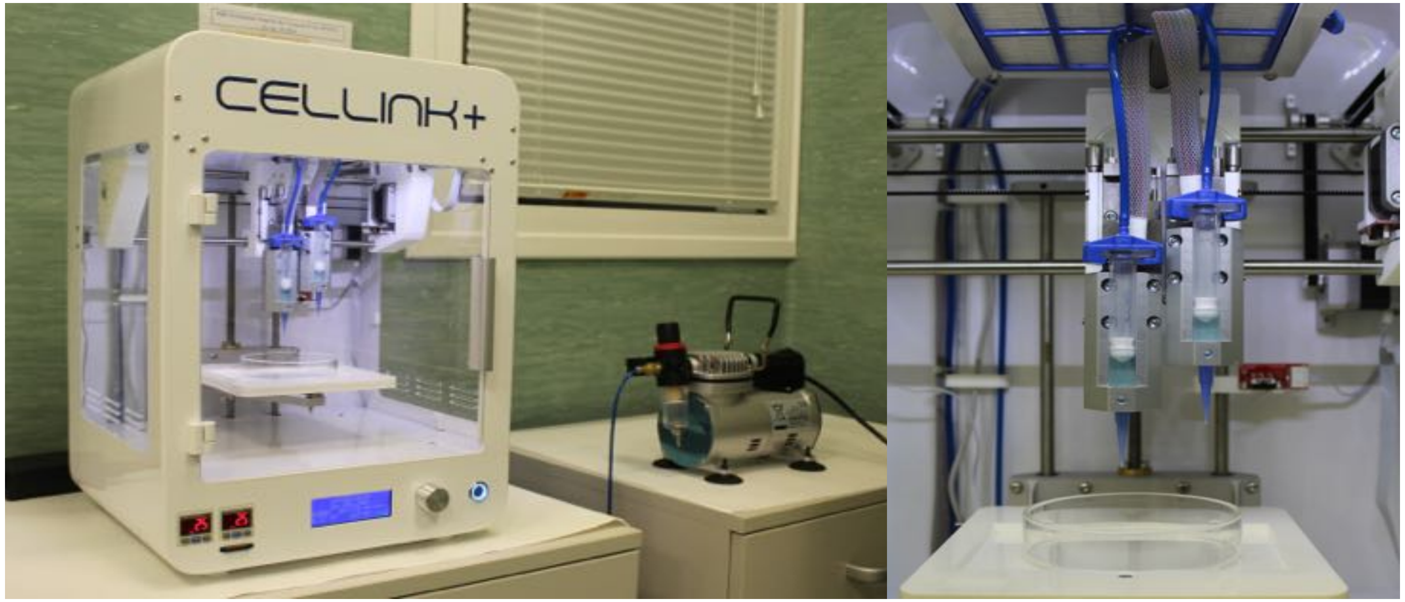
- Ink-jet
- Extrusion
- Laser Assisted
- Stereolithography



BioP: our bio-plotter

Bio-plotter

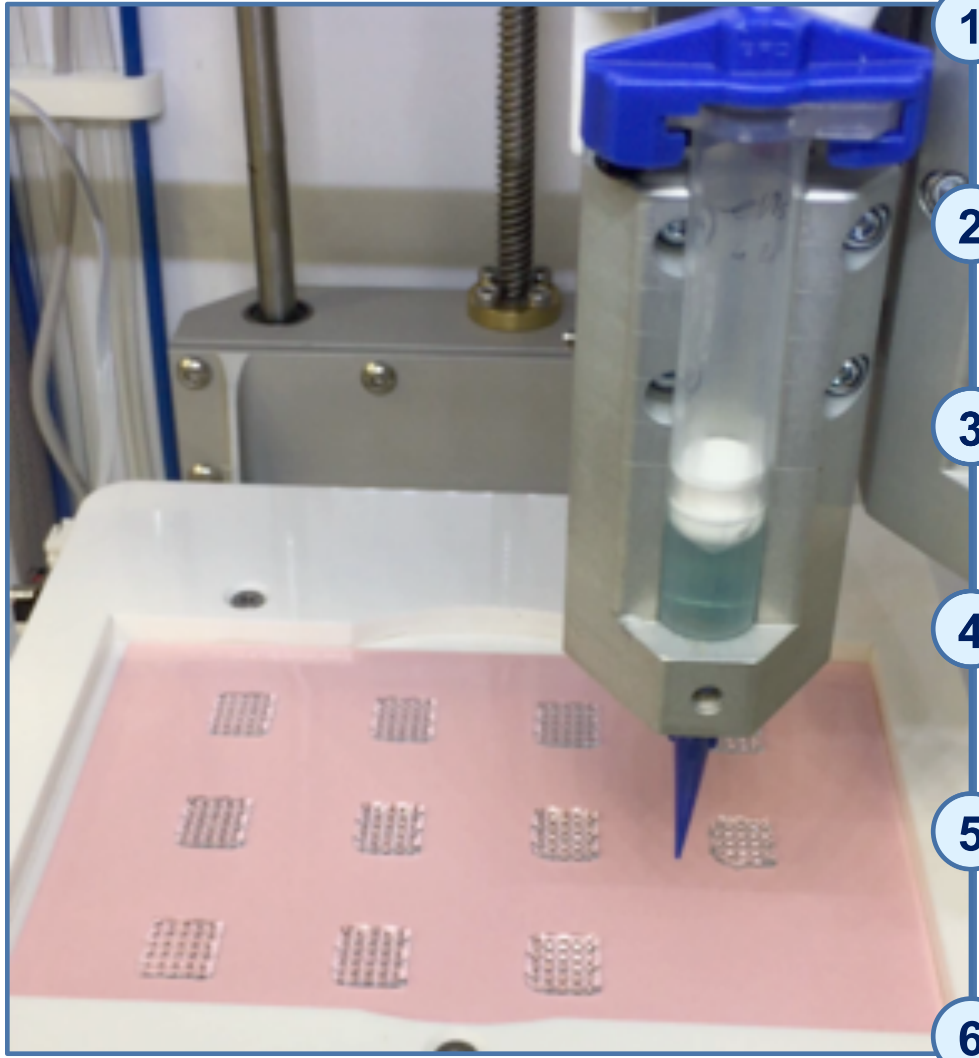
- Ink-jet
- Extrusion
- Laser Assisted
- Stereolithography



Cellink INKREDIBLE+ (Cellink AB)

Our pneumatic extrusion-based 3D bio-plotter

BioP process

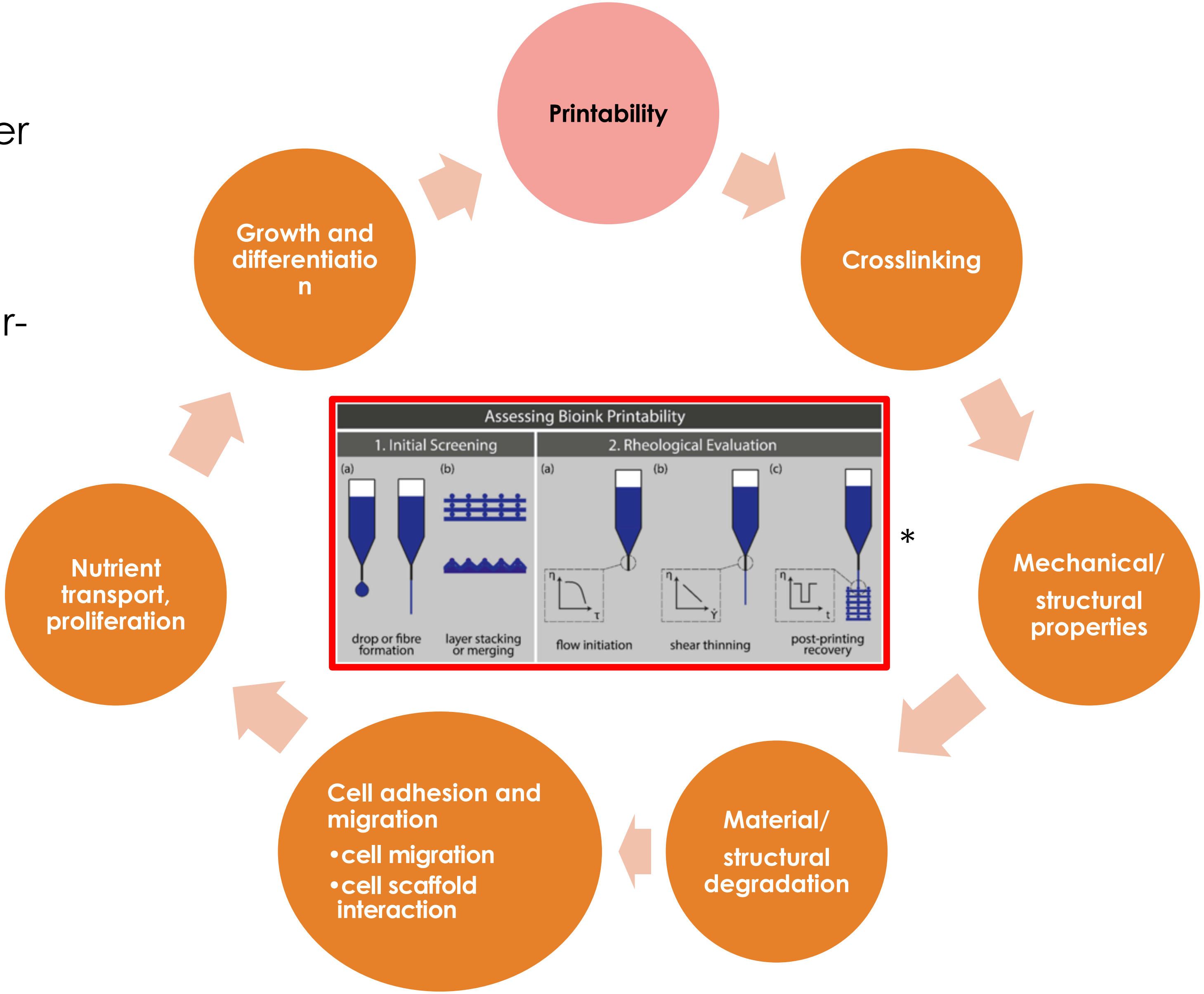


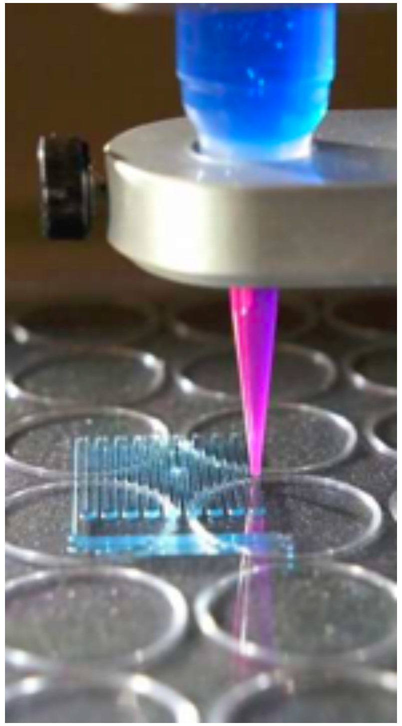
1. **Bio-ink**
   - **Hydrogel + Biological components, Drugs**
2. **Bio-plotter**
3. **Bioprinting**
4. **Crosslinking**
5. **Cell Colture at 37 °C**
6. **Bioprinted construct**
   - Transplantation
   - Drug Discovery
   - *In-vitro* biological study

Bioprinting: one word for a complex process

Bioprinting: one word for a complex process

- Printing resolution
  - nozzle diameter
  - speed head
  - Z-distance
  - pressure
  - Viscosity (shear-thinning)

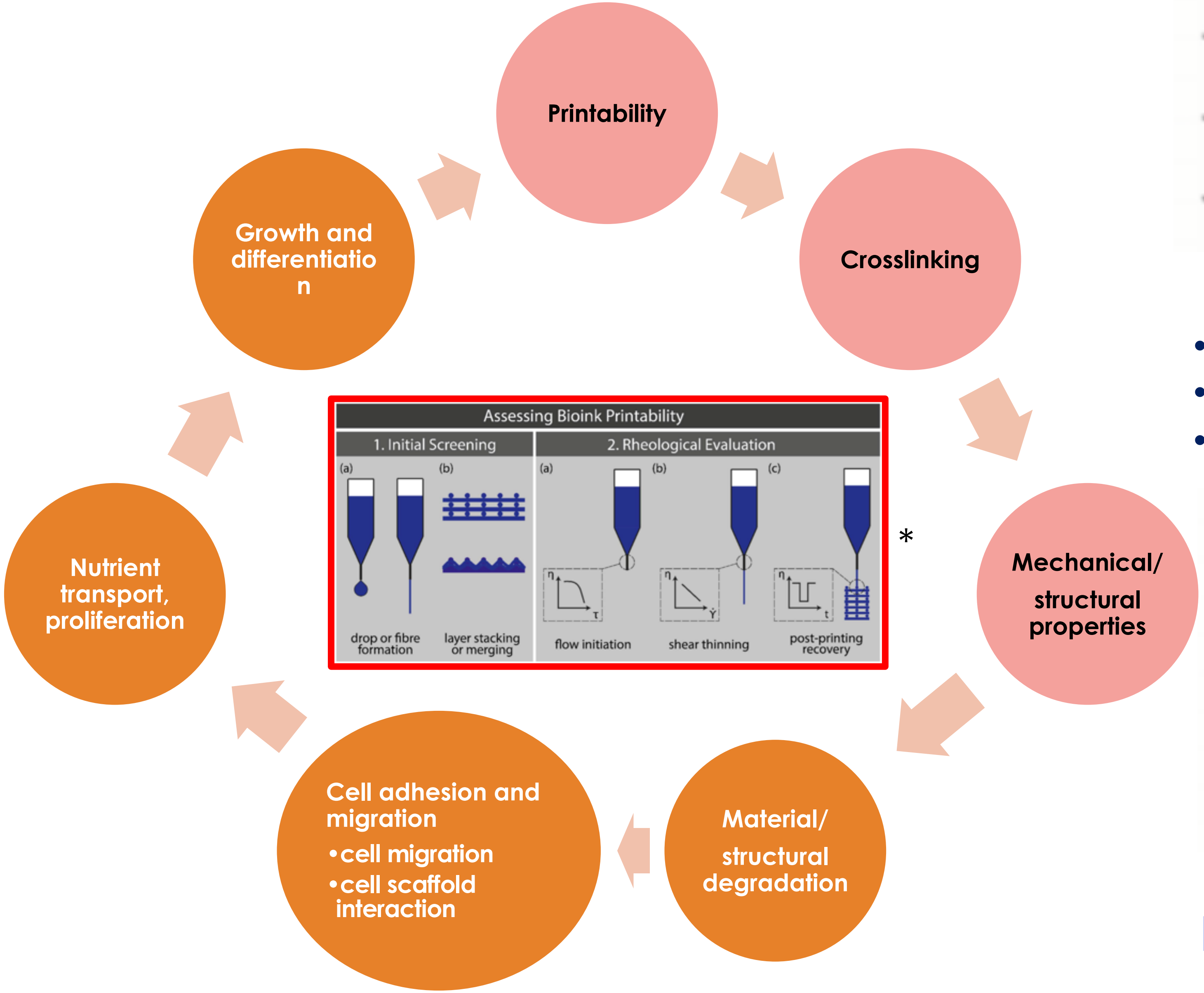




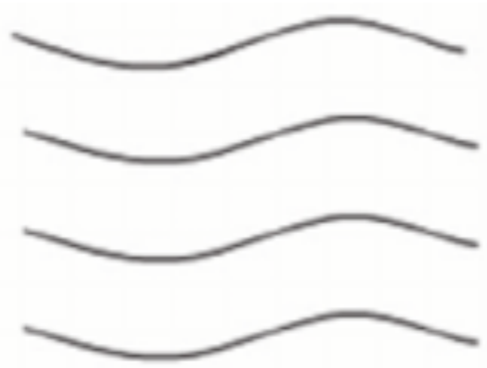
*Paxton, Naomi, et al. "Proposal to assess printability of bioinks for extrusion-based bioprinting and evaluation of rheological properties governing bioprintability." Biofabrication 9.4 (2017): 044107.

Bioprinting: one word for a complex process

- Crosslinker concentration
- Model size
- Exposure time
- Primary and secondary



Polymer



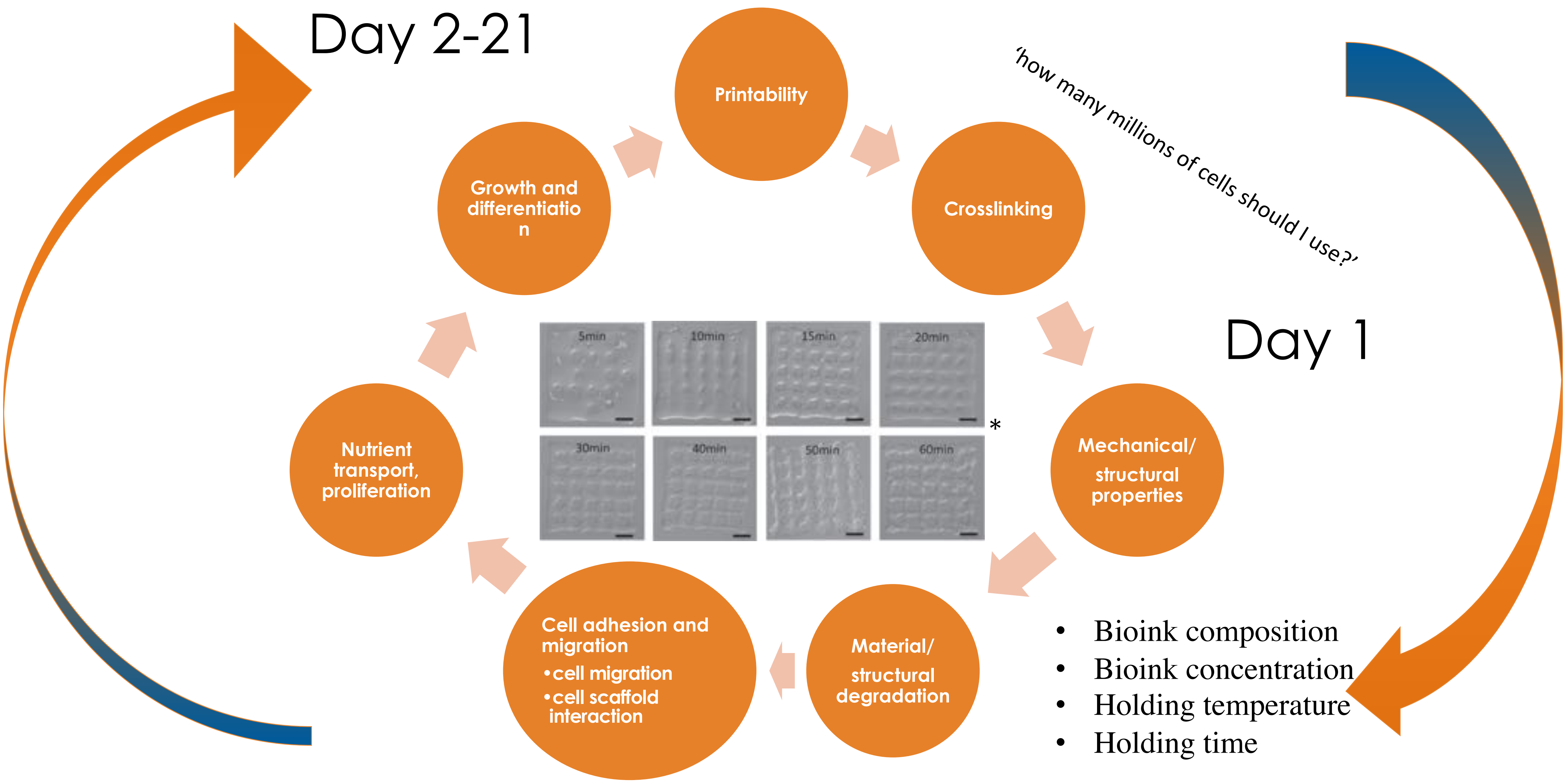
+ Crosslinker

- Ionic crosslinking
- Photocrosslinking
- Thermic

Hydrogel network

*Paxton, Naomi, et al. "Proposal to assess printability of bioinks for extrusion-based bioprinting and evaluation of rheological properties governing bioprintability." Biofabrication 9.4 (2017): 044107.

Bioprinting: one word for a complex process



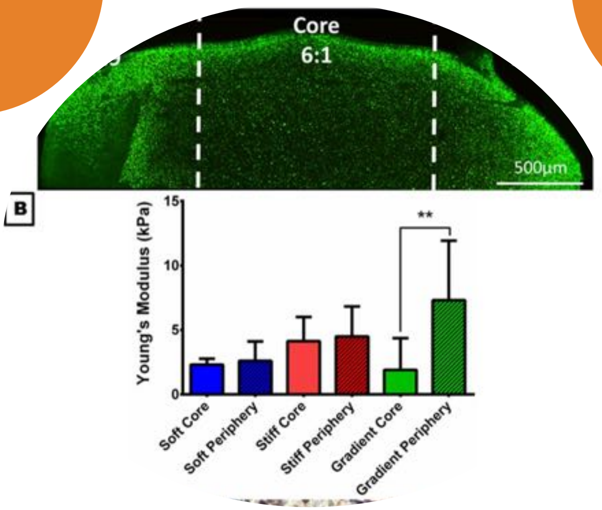
Zhao, Yu, et al. "The influence of printing parameters on cell survival rate and printability in microextrusion-based 3D cell printing technology." Biofabrication 7.4 (2015): 045002.

Bioprinting: one word for a complex process

Matrix stiffness is a key determinant *[of mesenchymal stem cell (MSC)]* differentiation

Modulation of *[alginate]* bioink mechanical properties

- Printability
- Crosslinking
- Mechanical/ structural properties
- Material/ structural degradation
- Cell adhesion and migration
  - cell migration
  - cell scaffold interaction
- Nutrient transport, proliferation
- Growth and differentiatio n

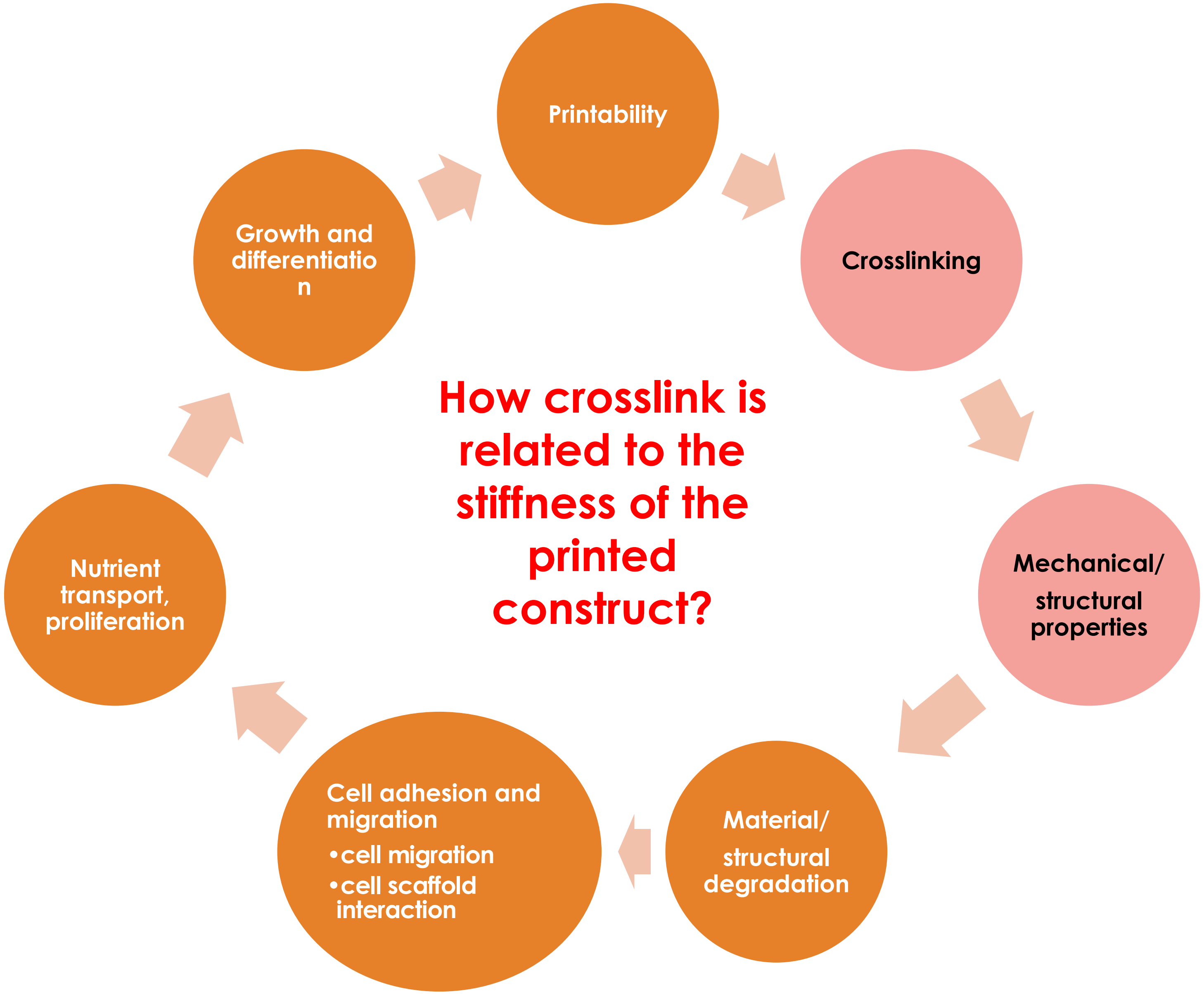


*

Spatial regulation of *[MSC]* fate within bioprinted tissues

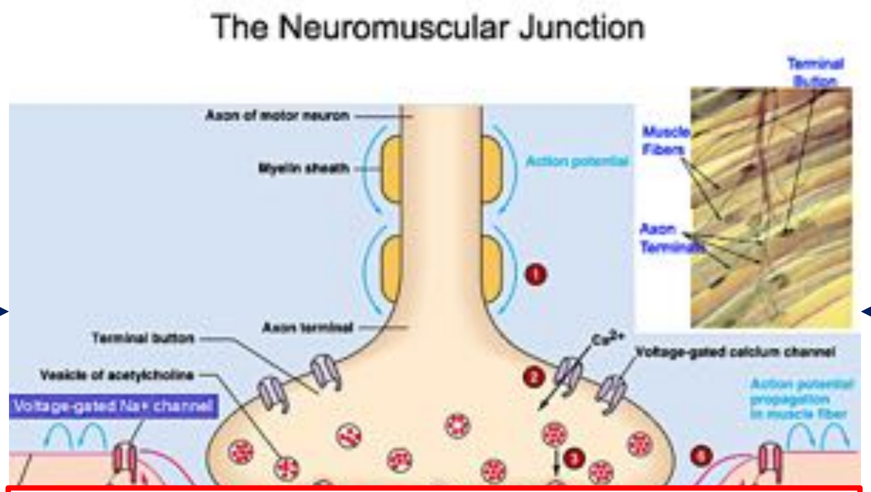
Freeman, Fiona E., and Daniel J. Kelly. "Tuning alginate bioink stiffness and composition for controlled growth factor delivery and to spatially direct MSC fate within bioprinted tissues." Scientific reports 7.1 (2017): 17042.

Bioprinting: one word for a complex process



Overall Goal

3D printing of NeuroMuscular Junction (NMJ)

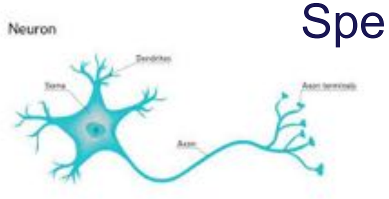


To create a model for the analysis of neurodegenerative disease

Fantini, V., Bordoni, M., Scocozza, F., Conti, M., Scarian, E., Carelli, S., ... & Cereda, C. (2019). Bioink composition and printing parameters for 3D modeling neural tissue. *Cells*, *8*(8), 830.

3D printing of muscle cells for tissue engineering applications

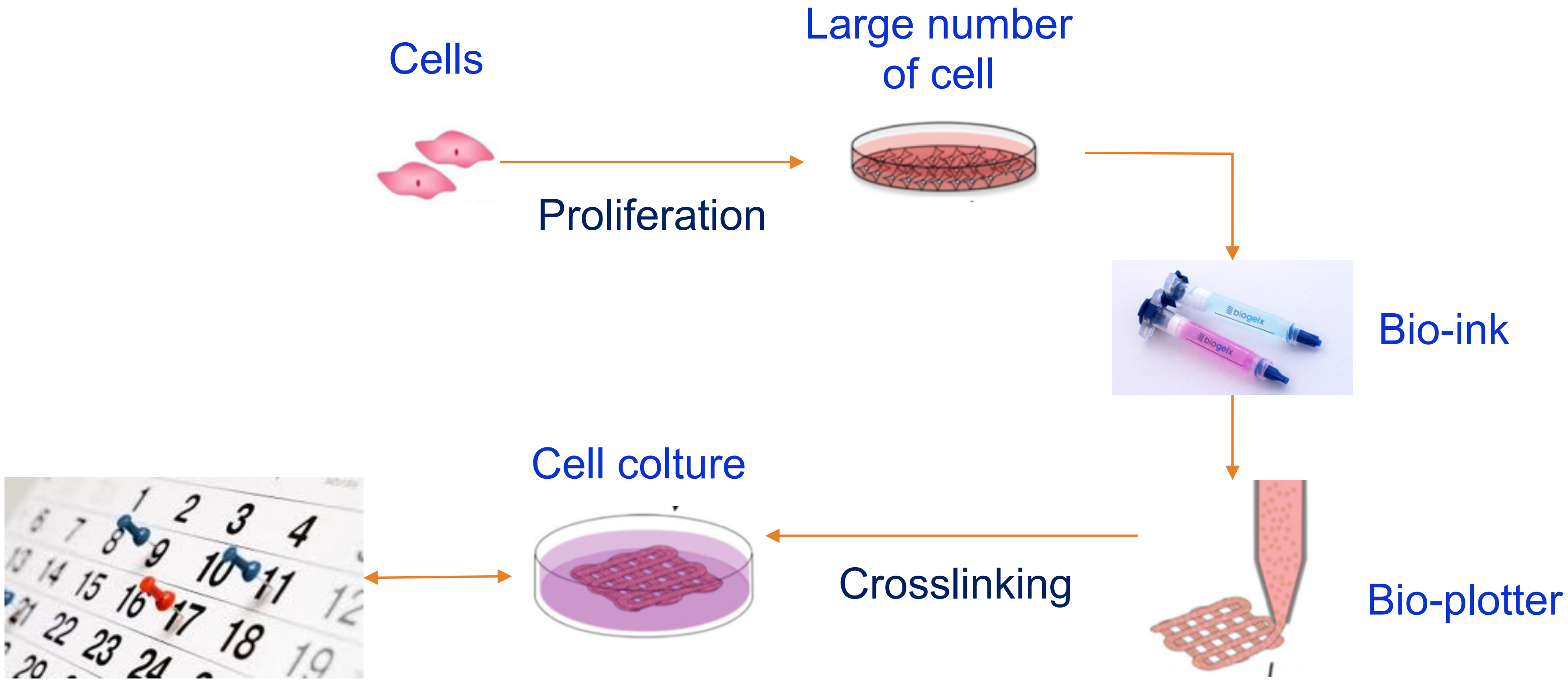
Collaboration with: G. Cusella, G. Ceccarelli, F. Ronzoni, M. Sampaolesi - Human Anatomy Unit, Dept. of PHEMF UniPV



3D printing of neurons

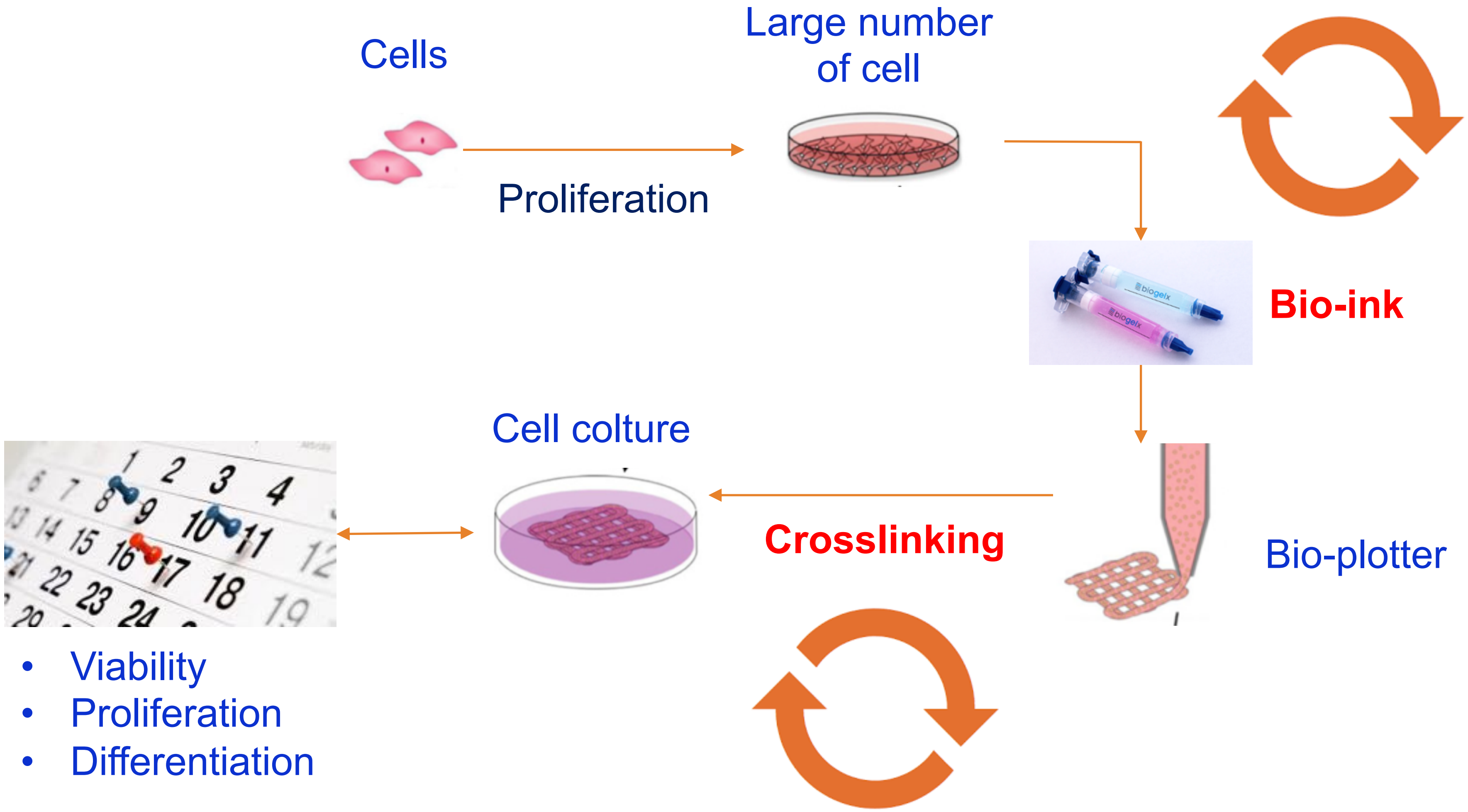
Collaboration with: C. Cereda, M. Bordoni, V. Fantini, Laboratorio di Neurobiologia Sperimentale, Inst. C. Mondino, PV

BioP process

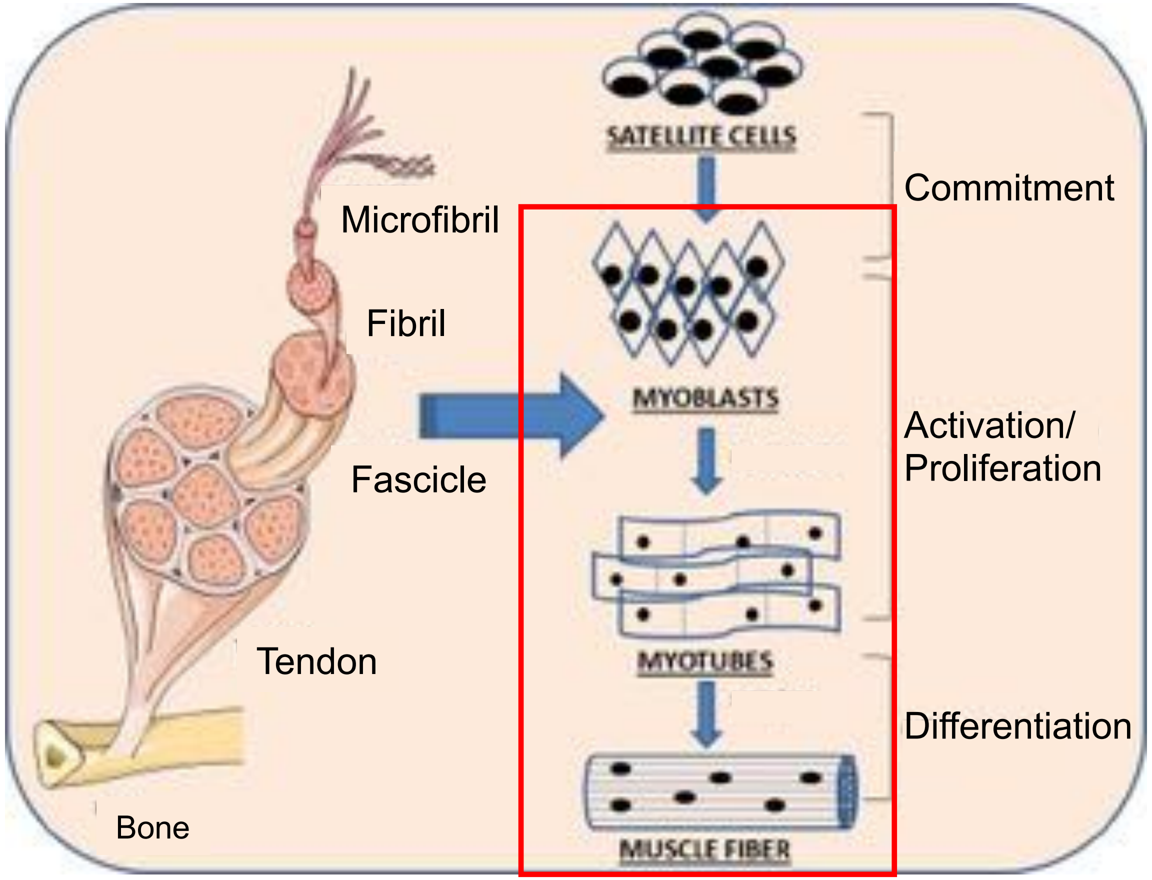


- Viability
- Proliferation
- Differentiation

BioP process



3DP of muscle cells for tissue engineering applications



Study goal
3D bioprinting mouse myoblast cell line (C2C12) to study cells differentiation into bio-ink and 3D modelling muscle fiber physiology

3DP of muscle cells for tissue engineering applications

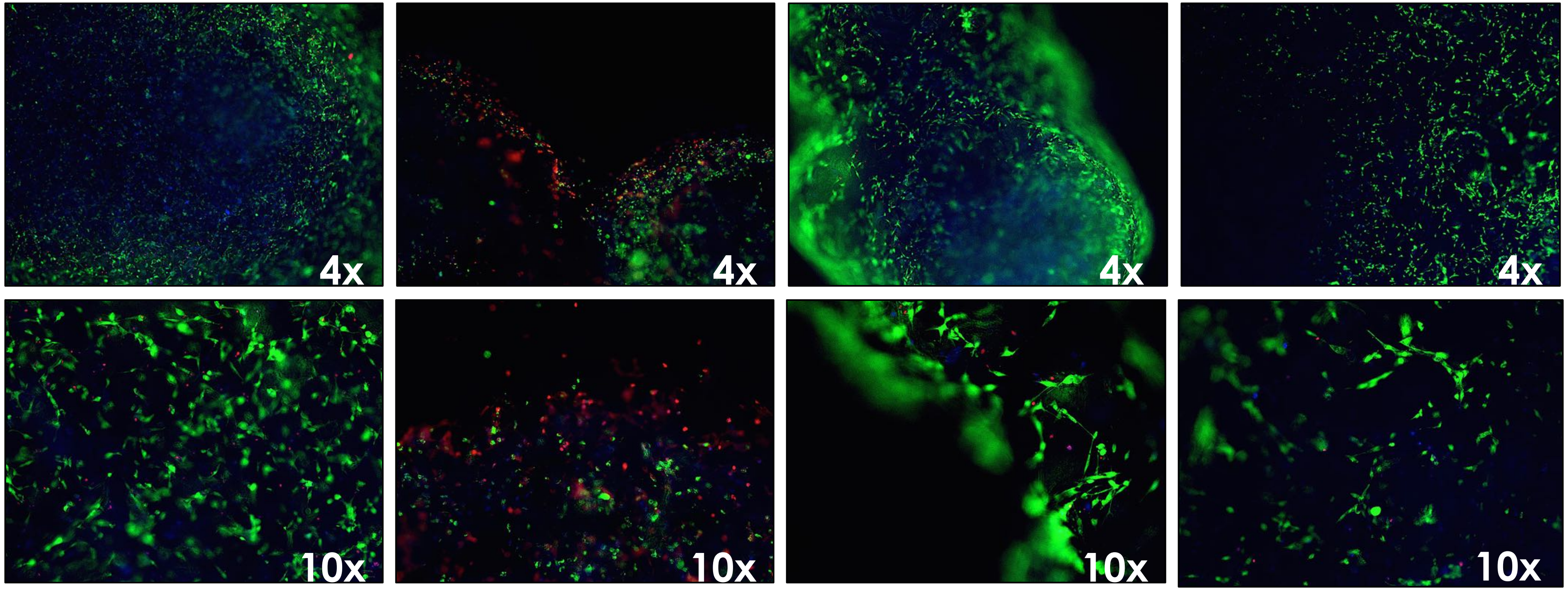
	Material	Crosslinking	3D BioP	# of Cell	Biological tests
1	Sodium Alginate and Gelatin	$CaCl_2$	Cube	$10x10^6$ cells/ml	Proliferation Differentiation Morphological analysis Gene expression analysis
2	FIBRIN Bio-ink (Cellink AB)	Thrombin/ $CaCl_2$	Line	$25x10^6$ cells/ml	
3	GelMa Bio-ink (Cellink AB)	UV (406 nm)	Line		
4	GelXA (Cellink AB)	$CaCl_2$+ UV (406 nm)	Line		

C2C12 cell line (immortal line of mouse skeletal myoblasts)

Proliferation test results

LIVE/DEAD

24 Hours | Day 4 | Day 7 | Day 14



- Good cell distribution and viability at 24 Hours
- At day 4 increases cell death, keeping round shape – not differentiated as in 2D
- At day 7 and day 14 cell differentiation is limited

3DP of muscle cells for tissue engineering applications

	Material	Crosslinking	3D BioP	# of Cell	Biological tests
✗	Sodium Alginate and Gelatin	$CaCl_2$	Cube	$10x10^6$ cells/ml	Proliferation Differentiation Morphological analysis Gene expression analysis
2	FIBRIN Bio-ink (Cellink AB)	Thrombin/ $CaCl_2$	Line	$25x10^6$ cells/ml	
3	GelMa Bio-ink (Cellink AB)	UV (406 nm)	Line		
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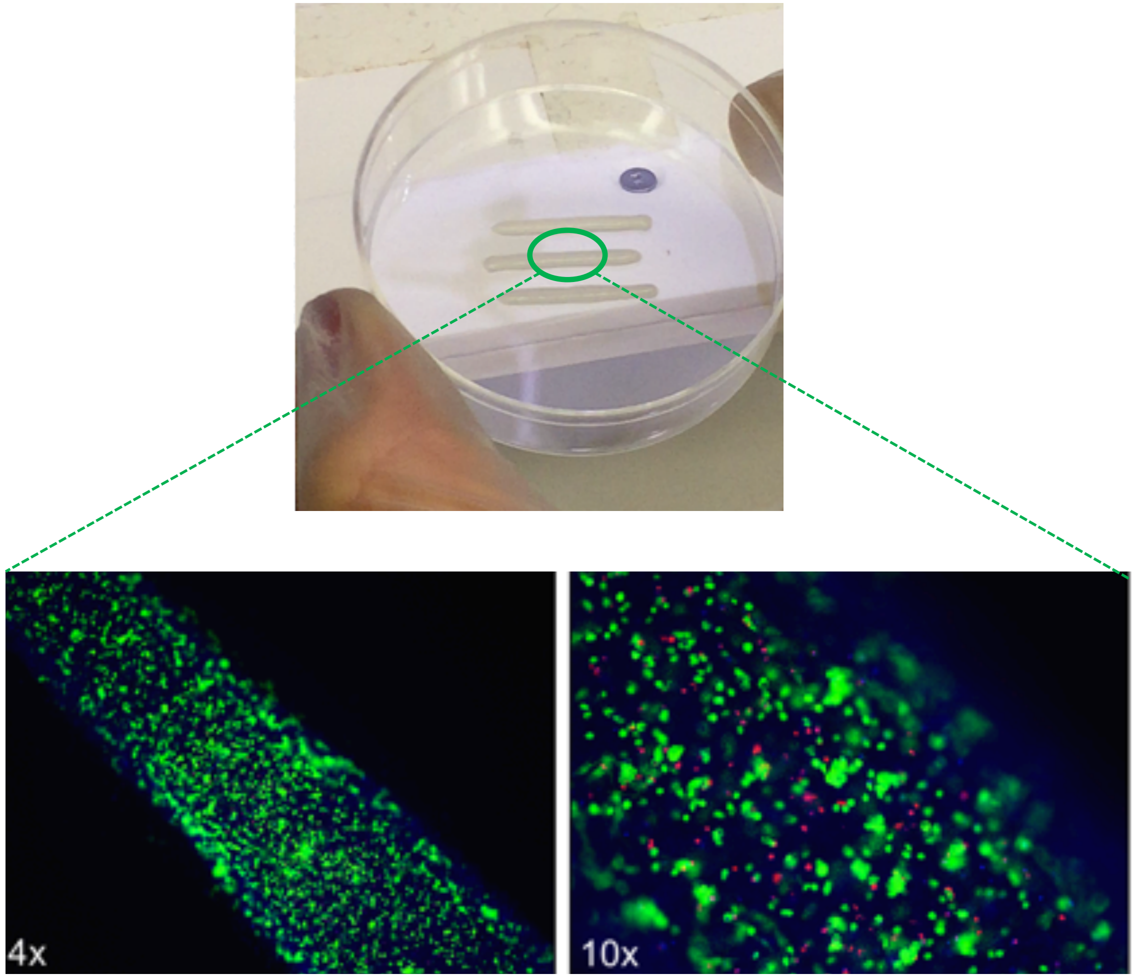
C2C12 cell line (immortal line of mouse skeletal myoblasts)

Proliferation test results

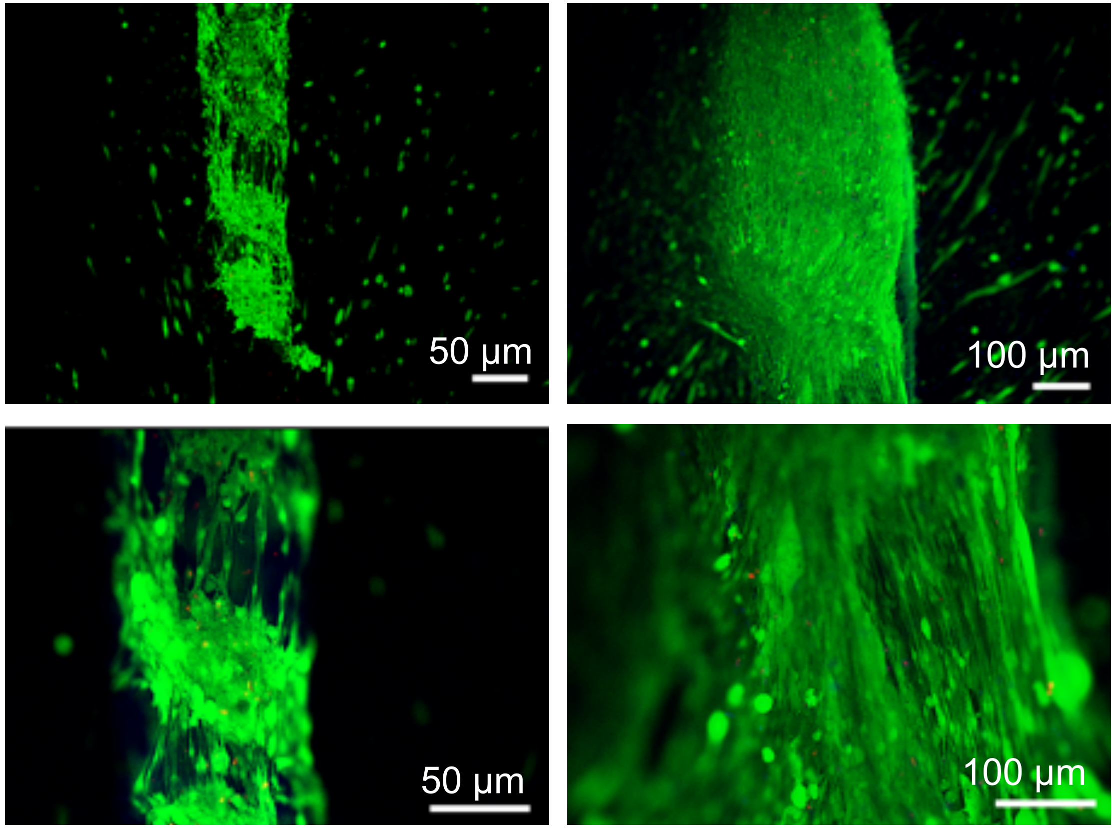


LIVE/DEAD - 24 Hours

Differentiation test results

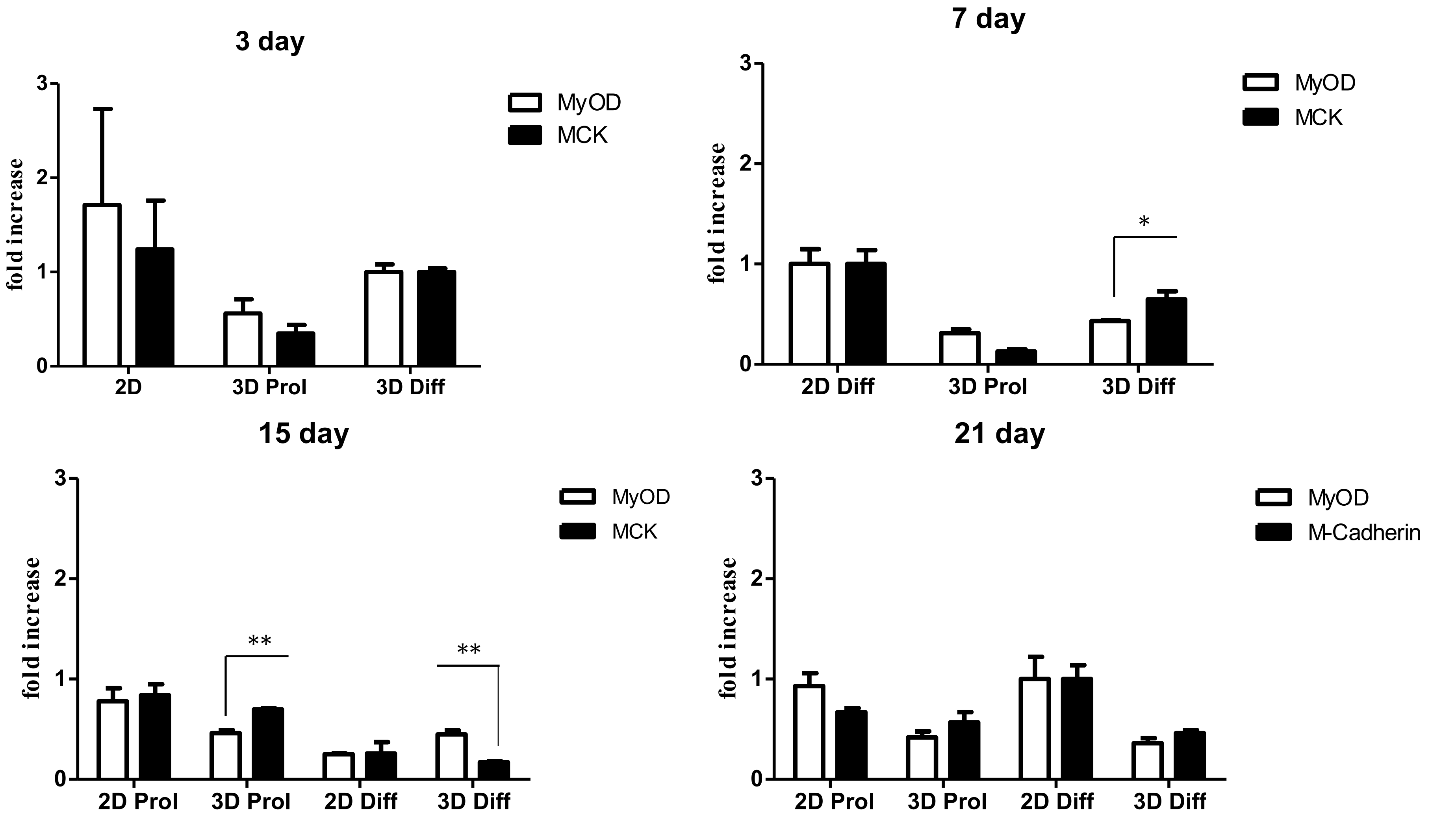
LIVE/DEAD

Day 14 | Day 28



- Uniform cell distribution,
- Optimal printability and good cell viability, even in long term
- Better cell differentiation on crosslinked edges
- Live/Dead Assay shows myotubes formation

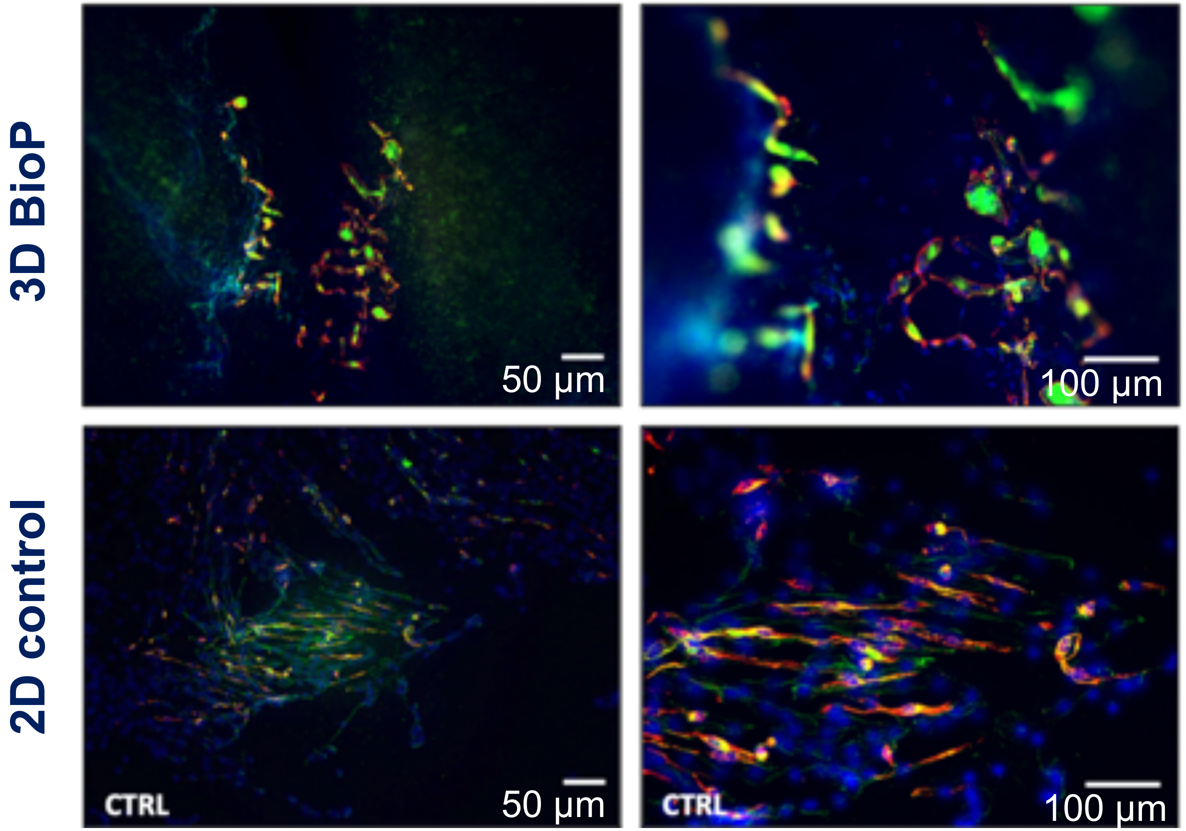
Gene expression



- Expression of two important master genes related to muscle differentiation (MyOD – late diff. and MCK – early diff.) highlight a positive effect of the 3D construct on the differentiation process

Immunofluorescence results

Phalloidin/MHC – Day 28 differentiation condition



➢ Analysis revealed myotubes laden into CELLINK FIBRIN bioink

https://www.cellink.com/technology/collaborator-data/university-of-pavia-collaboration/

* Phalloidin: actin filament. MHC: myosin filaments

3DP of muscle cells for tissue engineering applications

	Material	Crosslinking	3D BioP	# of Cell	Biological tests
✗	Sodium Alginate and Gelatin	$CaCl_2$	Cube	$10x10^6$ cells/ml	Proliferation Differentiation Morphological analysis Gene expression analysis
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3	GelMa Bio-ink (Cellink AB)	UV (406 nm)	Line		
4	GelXA (Cellink AB)	$CaCl_2$+ UV (406 nm)	Line		

Goal. Use UV to get an uniform crosslinking in the core in order to achieve also a differentiation in this region

3DP of muscle cells for tissue engineering applications

	Material	Crosslinking	3D BioP	# of Cell	Biological tests
✗	Sodium Alginate and Gelatin	$CaCl_2$	Cube	$10x10^6$ cells/ml	Proliferation Differentiation Morphological analysis Gene expression analysis
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✗	GelMa Bio-ink (Cellink AB)	UV (406 nm)	Line		
4	GelXA (Cellink AB)	$CaCl_2$+ UV (406 nm)	Line		

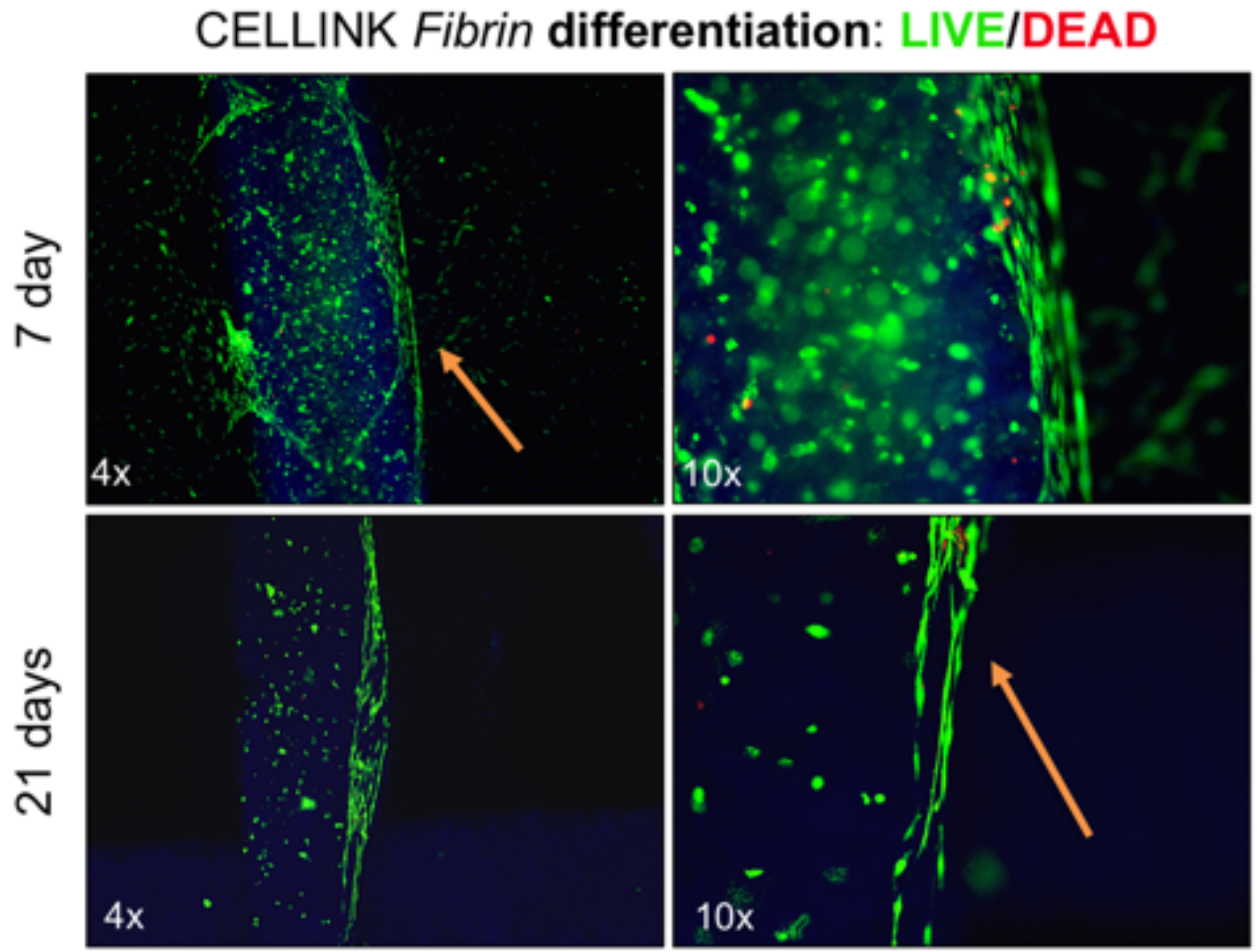
3D printing process extremely cumbersome – limited repeatability

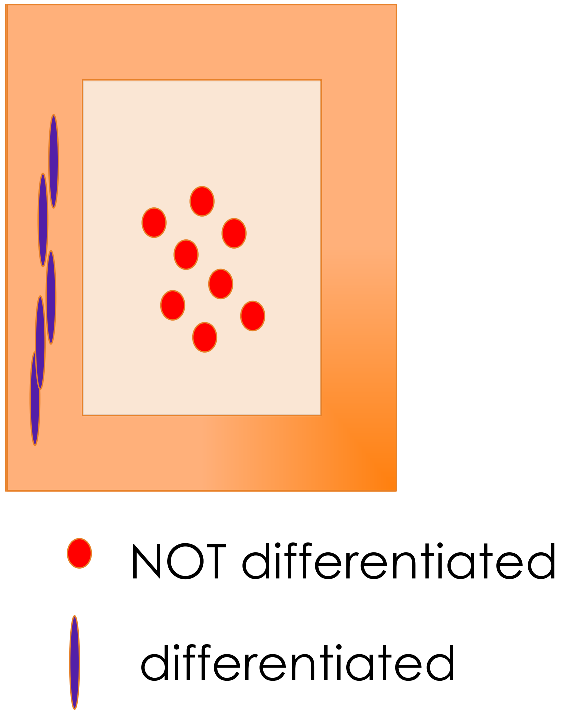
3DP of muscle cells for tissue engineering applications

	Material	Crosslinking	3D BioP	# of Cell	Biological tests
✗	Sodium Alginate and Gelatin	$CaCl_2$	Cube	$10x10^6$ cells/ml	Proliferation Differentiation Morphological analysis Gene expression analysis
✓	FIBRIN Bio-ink (Cellink AB)	Thrombin/ $CaCl_2$	Line	$25x10^6$ cells/ml	
✗	GelMa Bio-ink (Cellink AB)	UV (406 nm)	Line		
✗	GelXA (Cellink AB)	$CaCl_2$+ UV (406 nm)	Line		

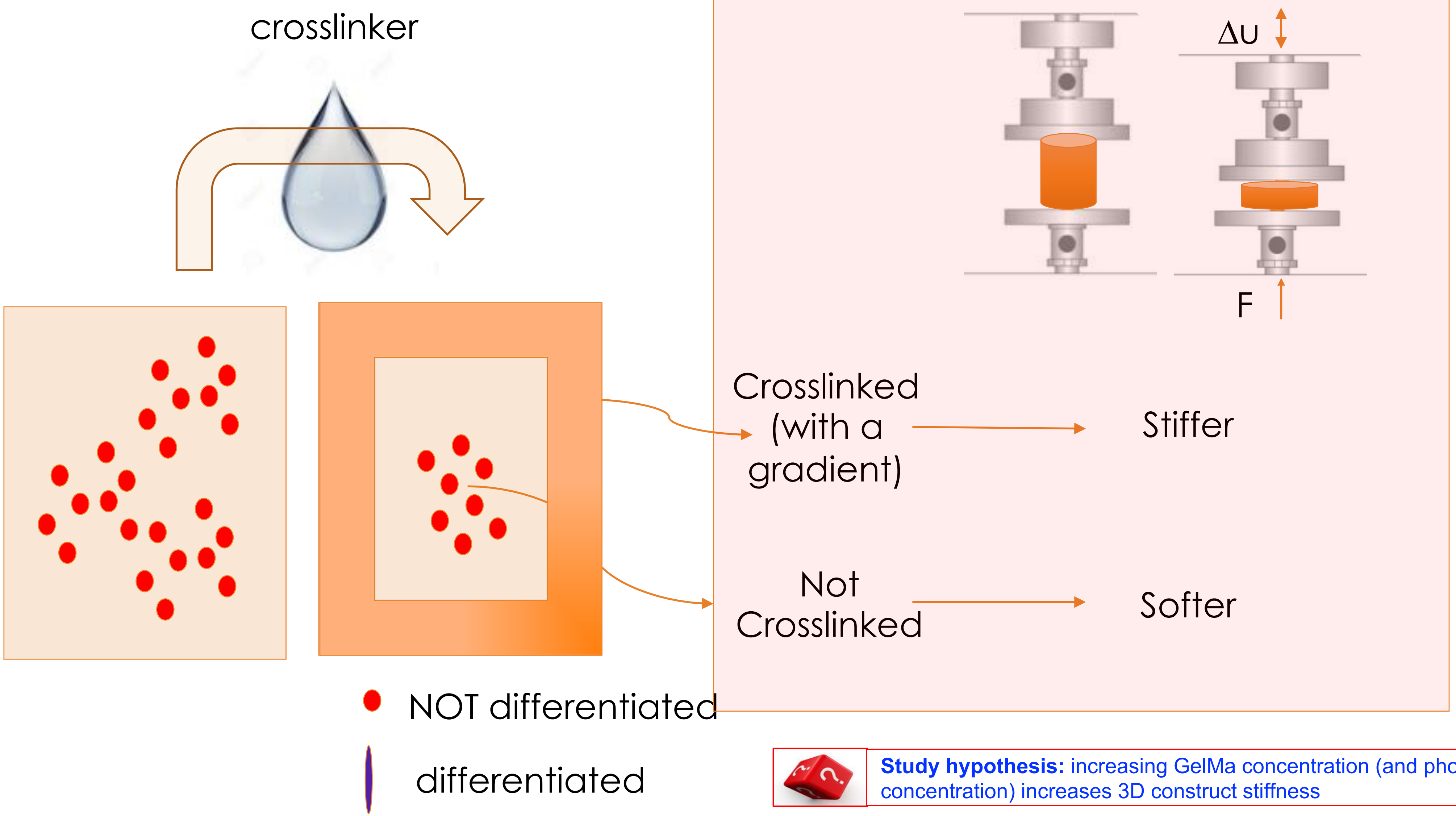
There is still not uniform differentiation inside the strand core

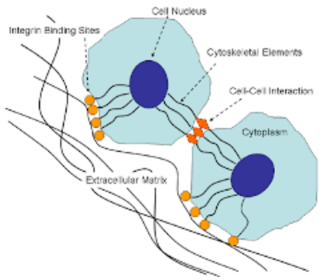
3DP of muscle cells for tissue engineering applications





Bioink. Crosslinking, stiffness, and biology

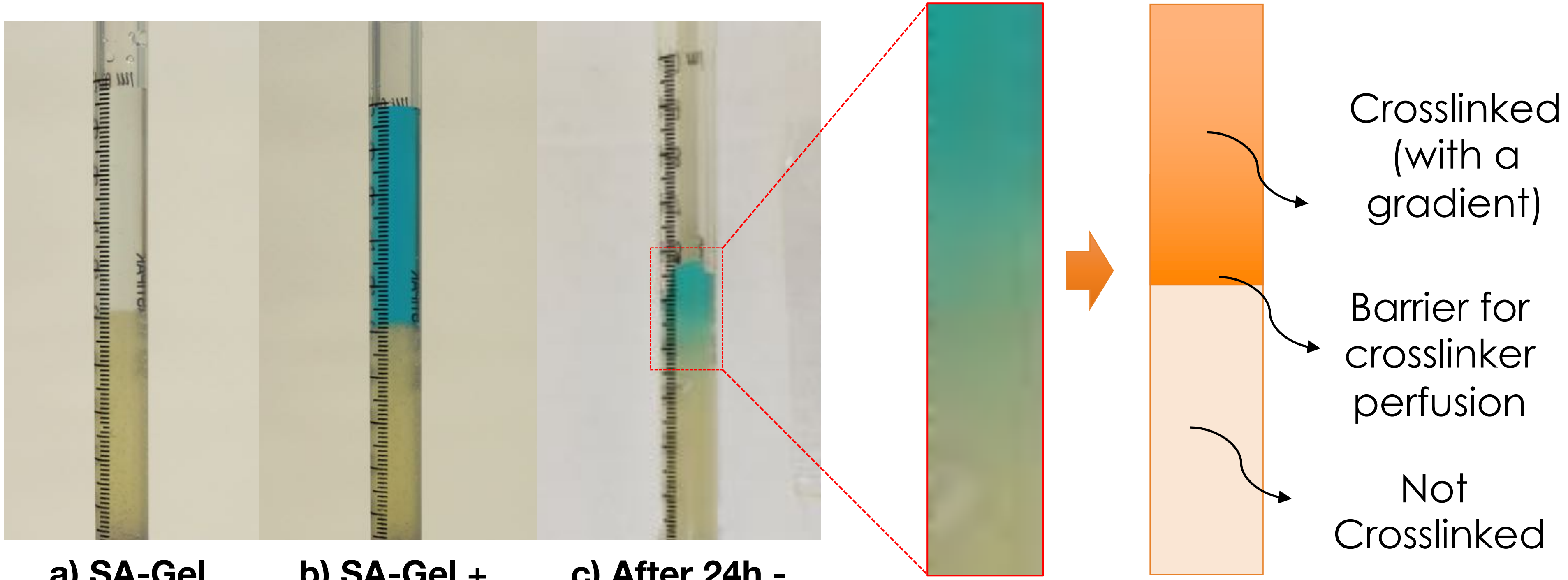




Study hypothesis: increasing GelMa concentration (and photocrosslinker concentration) increases 3D construct stiffness

Modeling bioprinting process

Study goal
Computational modeling of crosslinked sodium alginate and gelatin hydrogel used for bioprinting applications



a) SA-Gel **b) SA-Gel + $CaCl_2$** **c) After 24h - $CaCl_2$ removal**

Measure of $CaCl_2$ absorbed by SA-Gel was performed at 2, 5, 10, 15 , 20 minutes, 2, 24 and 48 hours. Test was repeated for 3 times.

* SA: Sodium Alginate; Gel: Gelatin; $CaCl_2$: Calcium Chloride

* SA: Sodium Alginate; Gel: Gelatin; $CaCl_2$: Calcium Chloride; CL: Crosslinked

Modeling bioprinting process

Experimental characterization and computational modeling of hydrogel cross-linking for bioprinting applications

Journal Title
XX(X):1–8

DOI: 10.1177/ToBeAssigned
www.sagepub.com/
SAGE

Aidin Hajikhani[1], Franca Scocozza[2], Michele Conti[2], Michele Marino[1], Ferdinando Auricchio[2], Peter Wriggers[1]

Modeling reactive-diffusive processes in sodium alginate hydrogels stabilized by $CaCl_2$ and accounting for the effects of cross-linking on the diffusion of gelation agents

$$\alpha = \frac{n_{cl}}{n_{tot}} \in [0,1]$$

1. gelation degree $\alpha = \alpha(x,t) \in [0,1]$;
2. calcium concentration $c = c(x,t)$.

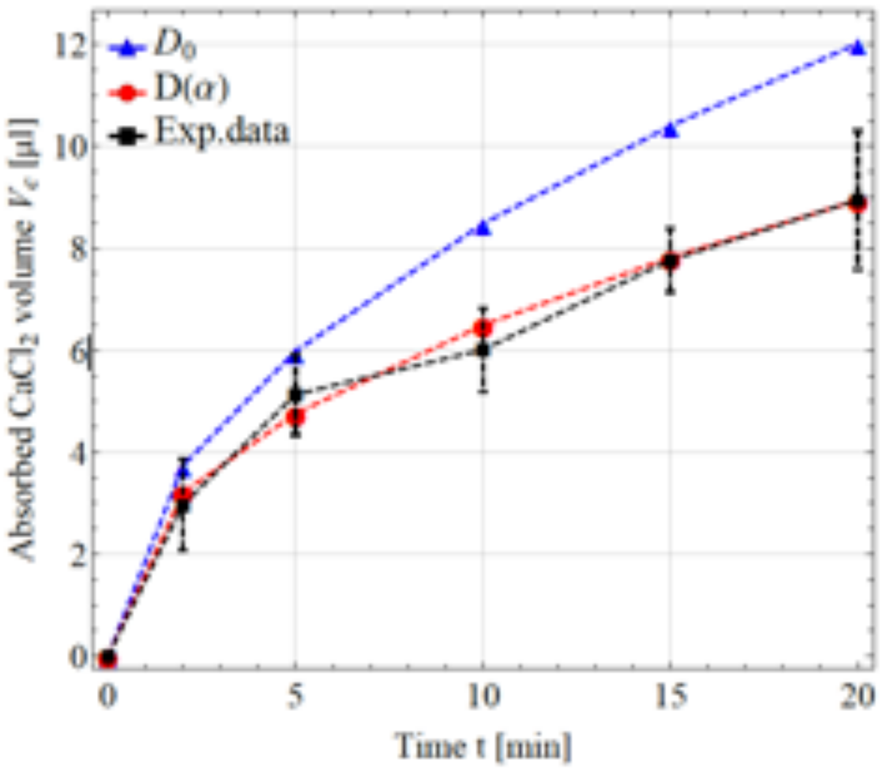
$$\frac{\partial c}{\partial t} - \frac{\partial}{\partial x}\left(D \frac{\partial c}{\partial x}\right) = r\,,$$

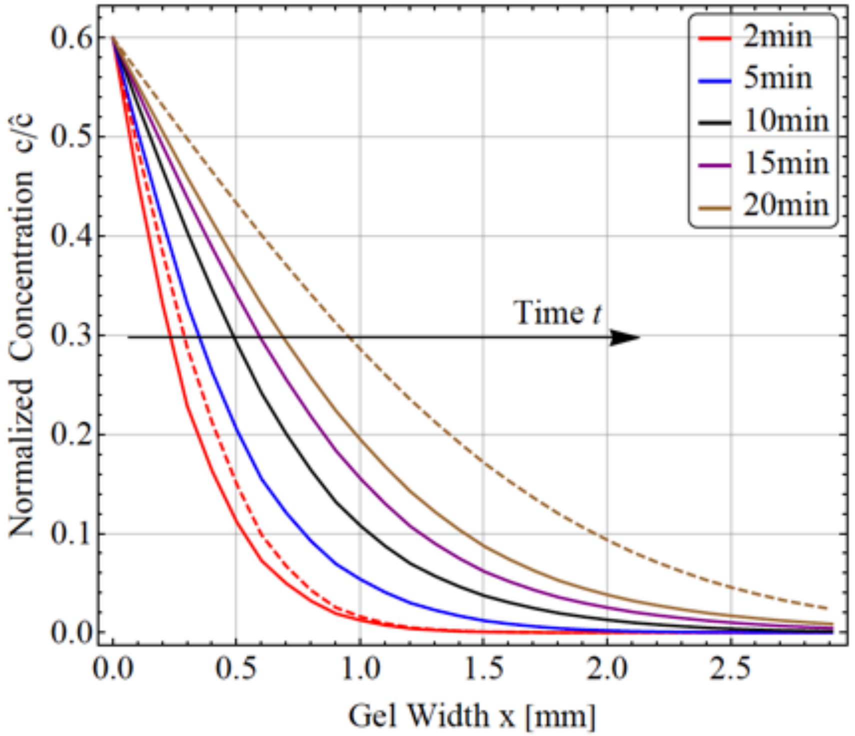
$$\frac{\partial \alpha}{\partial t} = K\,c\,(1-\alpha)$$

$$D(\alpha) = D_0 + (D_1 - D_0)\frac{\exp(-\alpha/\alpha_{\text{gel}}) - 1}{\exp(-1/\alpha_{\text{gel}}) - 1}\,.$$

Solved in a 3D domain by Finite element scheme (1D problem)

Modeling bioprinting process



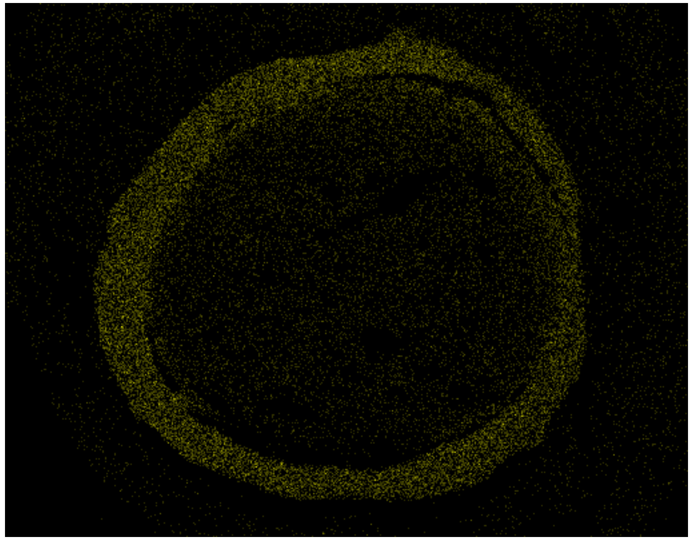


Inclusion of a diffusion coefficient as function of gelation allows to get experimental data

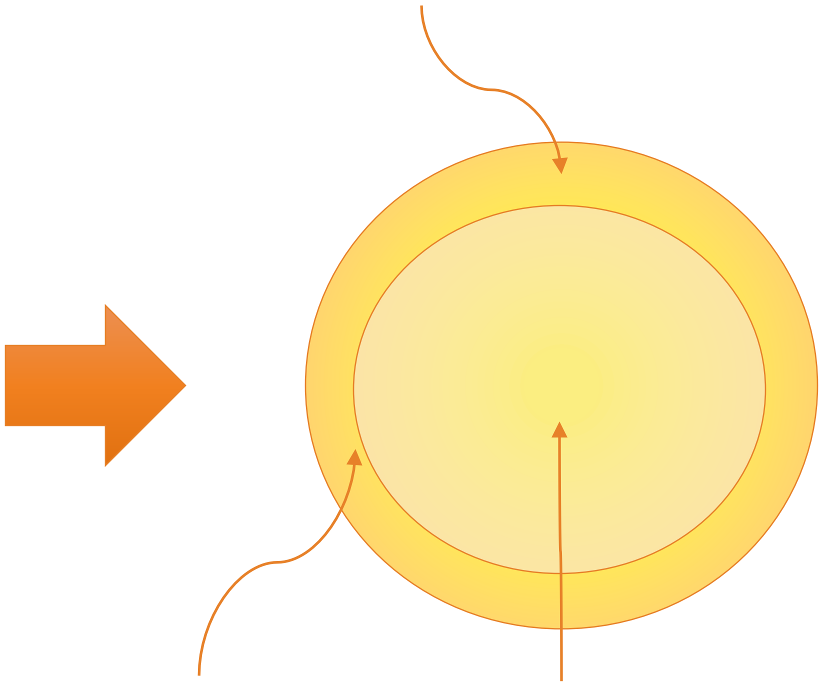
Crosslinker distribution is not uniform in space and does not diffuse linearly in time

Modeling bioprinting process

SEM of crosslinked hydrogel



Counts of Ca atoms



In collaboration with Dr. Michele Marino (UniTov) and Ilenia Tredici (UniPV)

Measuring percentage of hydrogel crosslinking - Experimental set-up

Materials

Hydrogel: 8% Sodium alginate (SA) and 4% Gelatin (Gel)

Crosslinking: 2% and 5% Calcium Chloride ($CaCl_2$)

Methods

- We 3D printed a cylinder (d: 8 mm, h: 2.1 mm) using Cellink INKREDIBLE+
- Crosslinking
  - We added 0,5 ml of $CaCl_2$ for **crosslinking 3D printed construct edges** (construct top face was not immersed)
  - We crosslinked construct for 2 and 5 minutes
- For each case we printed 5 samples (n_tot=20)
- Samples were dried for 24 h before microanalysis

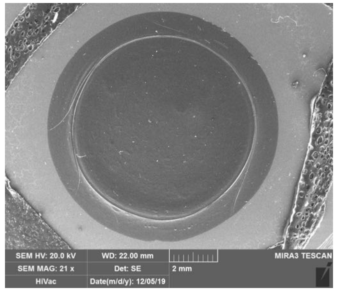
Measuring percentage of hydrogel crosslinking - Experimental set-up

- First, we analyzed constructs with stereomicroscope and then we choose the best 2 samples for SEM and microanalysis (n_tot=8)

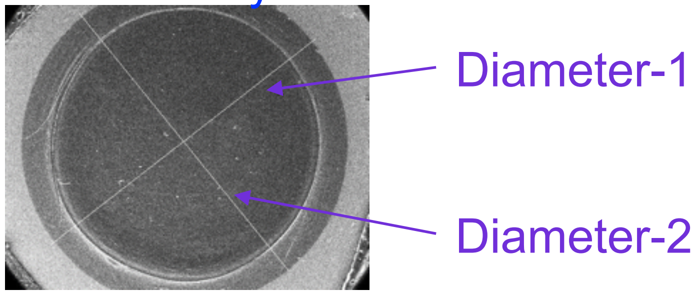
	2% CaCl2	5% CaCl2
5 min	A1, A2	C1,C2
10 min	B1, B2	D1,D2

- For each sample, we run microanalysis along two diameters (n_tot=16) of the same sample to extract chemical elements that make up the constructs (e.g. Ca, Cl, S, O, C, etc.)

SEM

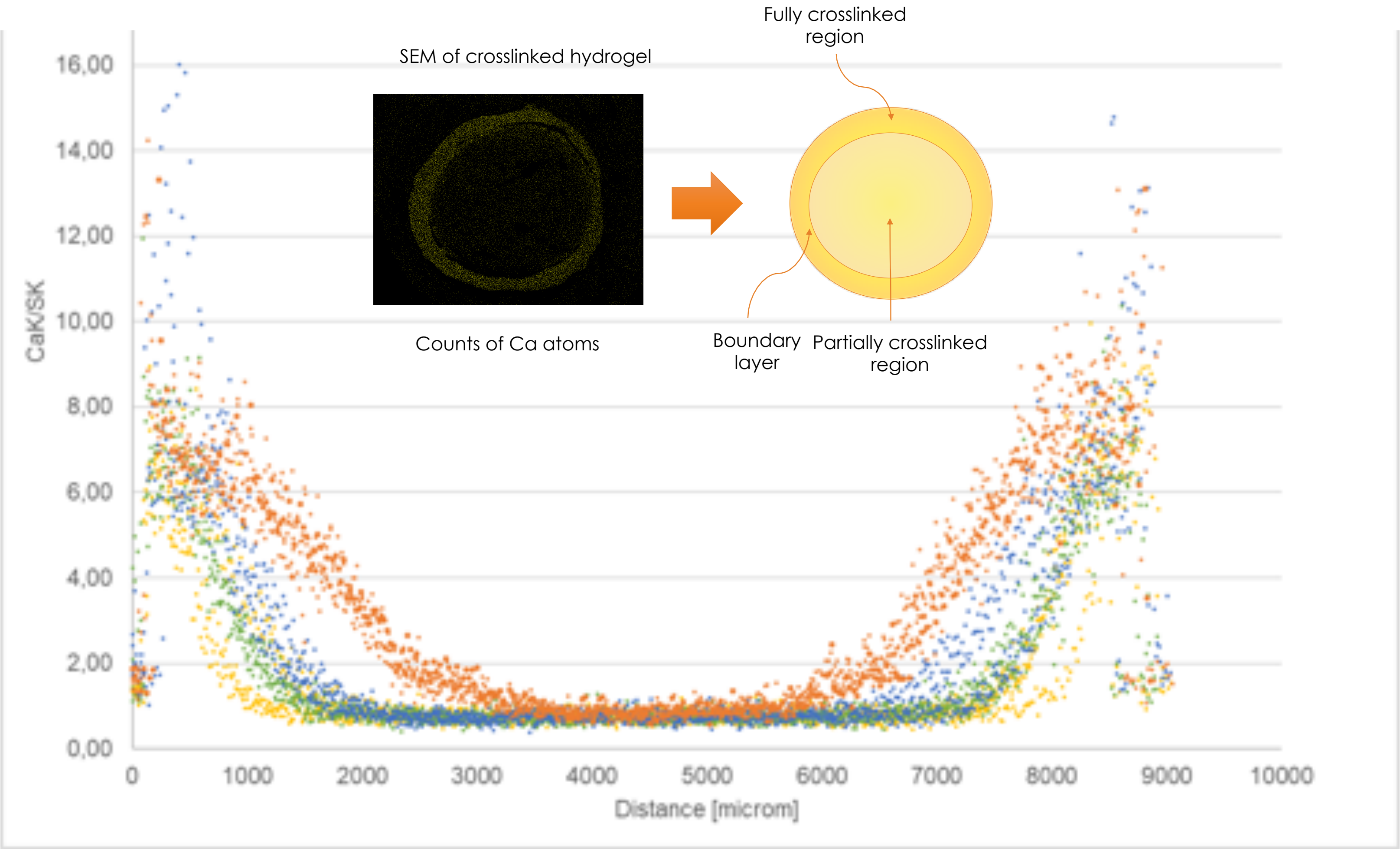


Microanalysis



- Calcium (Ca) were normalized with Sulfur (S) for avoiding shadow areas

Measuring percentage of hydrogel crosslinking - Experimental set-up



- 2% CaCl2 – 5 min
- 2% CaCl2 – 10 min
- 5% CaCl2 – 5 min
- 5% CaCl2 – 10 min

Take home messages

3D printing is **NOT** (yet) a **ONE-CLICK** technology

There is **NOT ONE** a 3D printing technology **FOR ALL** the applications
But there are a number of technologies and materials that can give you a solution

3DP is an **ENABLING** technology

CHANGE YOUR WAY OF DESIGNING

PERSONALIZATION

3D@UniPV

www.unipv.it/compmech/bioprinting_home.html

IV WORKSHOP BIOPRINTING:
DAL SET-UP DELLA STAMPA ALLE ANALISI IN LABORATORIO

29 Settembre 2020 – dalle 09:00 alle 13:00

Modalità TELEMATICA: iscrizioni tramite il seguente link

https://forms.gle/JMu2R1cQtYNe1qFJ9

Facoltà di Ingegneria Università degli Studi di Pavia,
Via Ferrata 3, Pavia

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Thanks for your attention

Bioprinting. From technical set-up to biological applications

MICHELE CONTI - UNIPV

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