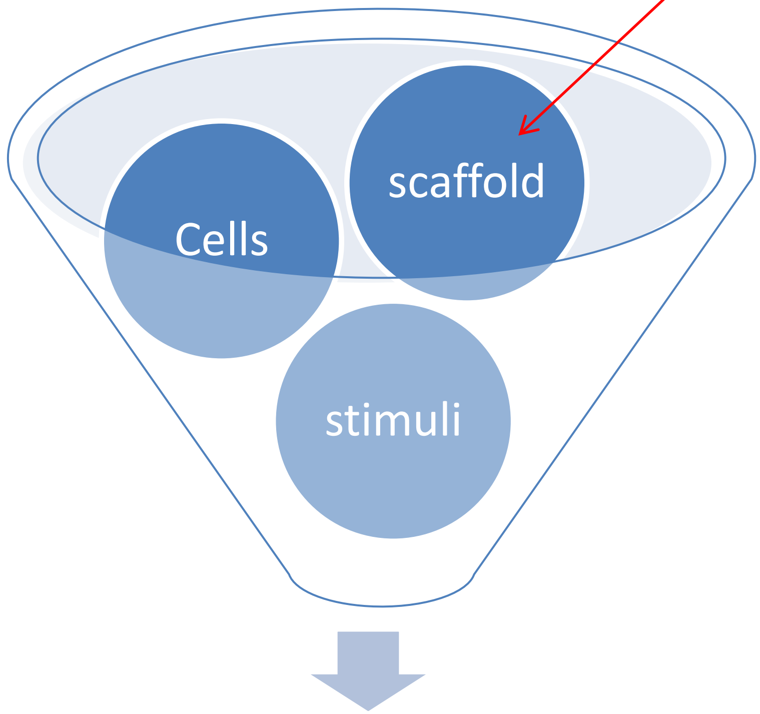
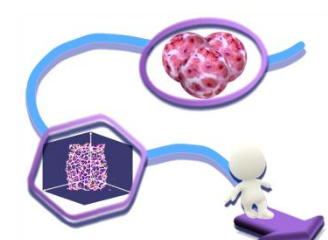
Not required in all ATMP

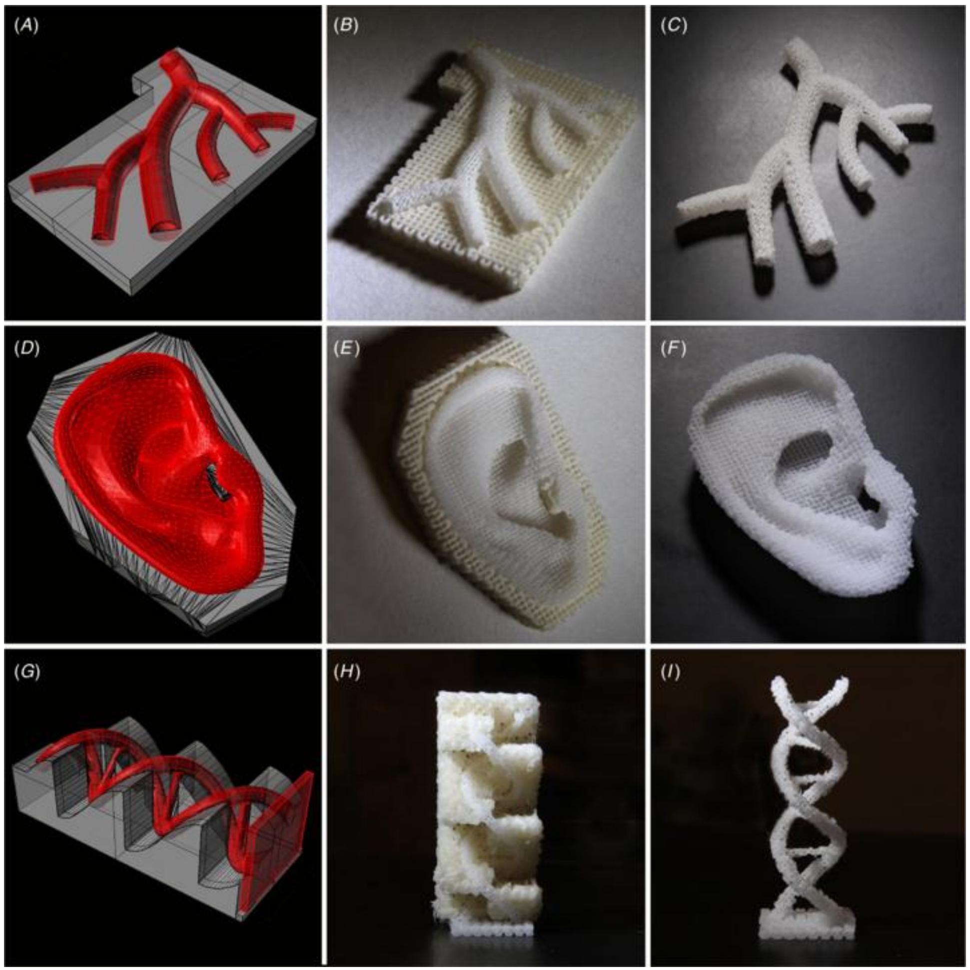


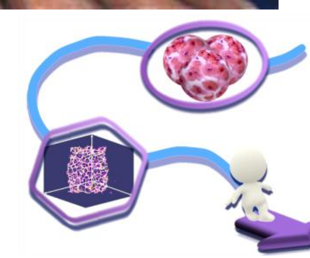
Tissue engineered product



What is a scaffold?

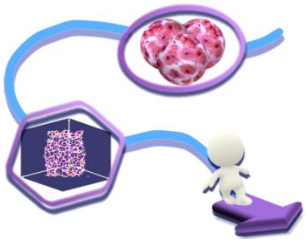
A 3D structure which supports 3D tissue growth





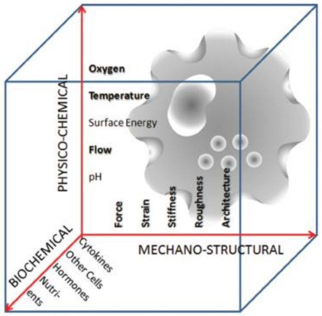
What are the features of an ideal scaffold?

- 3D. Biocompatible, cell adhesive, bioerodable and *bioactive*
- Mechanical properties *similar* to those of natural tissue
- Optimal meso, micro- pores
- Well-defined, or *quantifiable* topology at meso- micro- and nanoscales
- 3D-matrix adhesions differ in content, structure, location, and function from classically described in vitro adhesion, e.g., focal and fibrillar adhesions
- - cell adhesion in 3D-matrix more efficient (6-fold increase)
- - cell morphology is that of more in vivo-like (spindle shape)
- - cell migration speed increased by ~ 50%



Extracellular matrix features

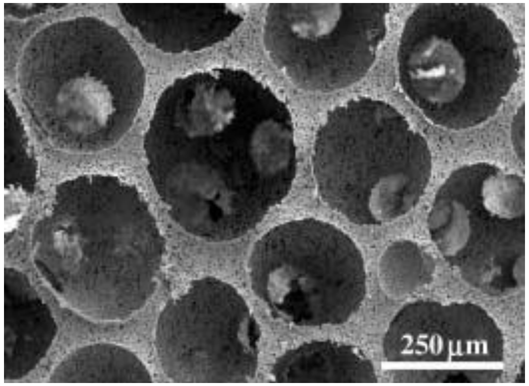
- High degree of porosity
- Appropriate pore size
- High surface to volume ratio
- High degree of pore interconnectivity
- Biochemical factors & ECM features able to guide cell function

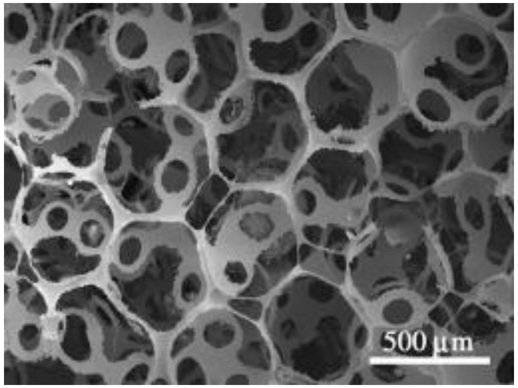


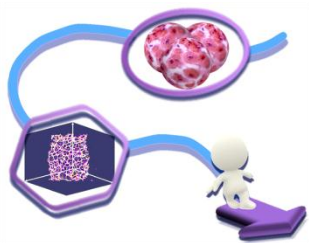
Porosity and architecture

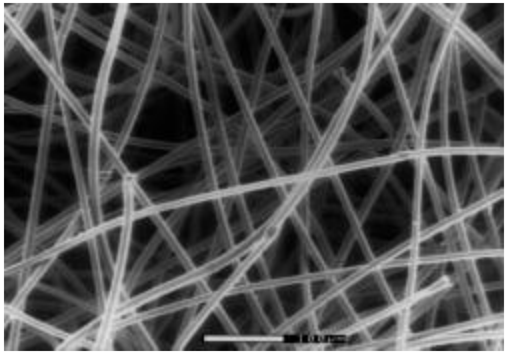
Pore size, pore connectivity, porosity, pore distribution are all critical

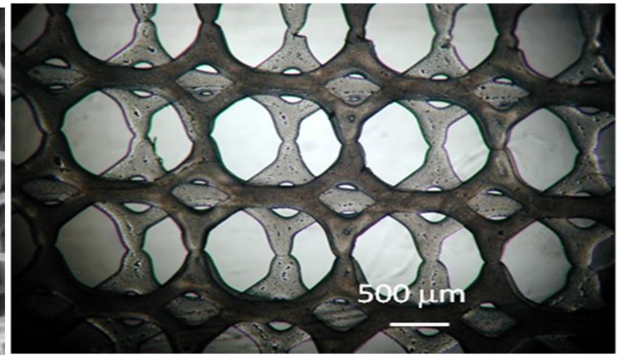
- To fit cells
- Fit at least a functional unit
- Allow nutrient perfusion

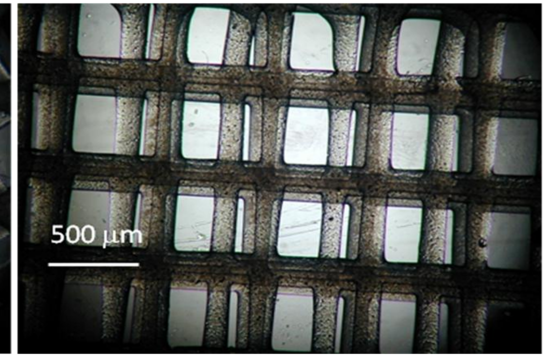


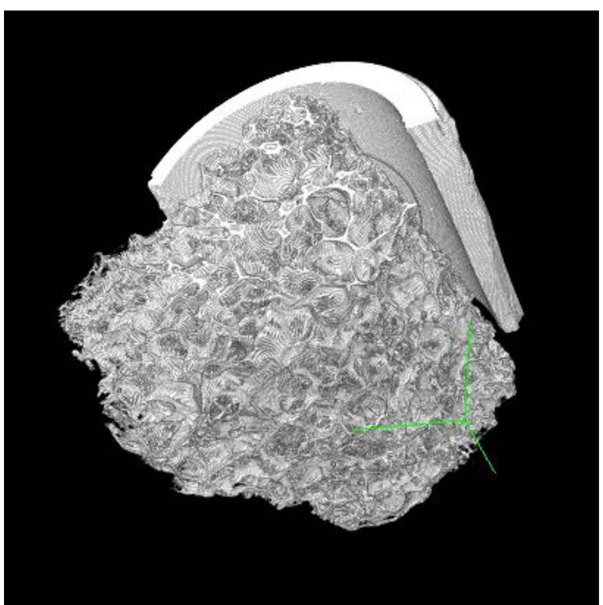












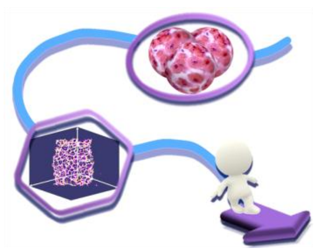
Liver ECM

All polymers (materials) have to be porous in order to support 3 D tissue ingrowth.

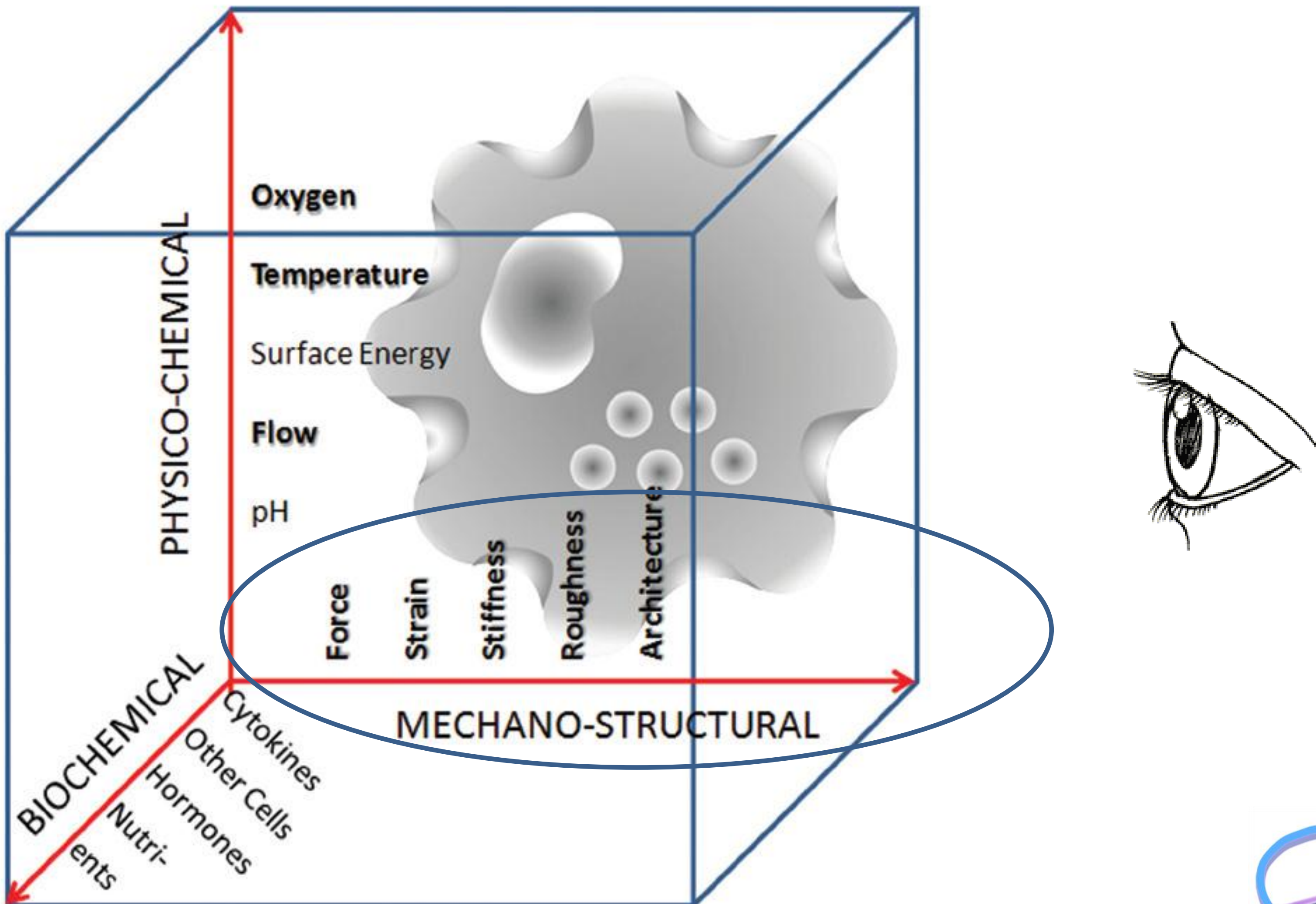
TABLE 2. STUDIES DEFINING OPTIMAL PORE SIZE FOR BONE REGENERATION[24]

Reference	*Scaffold pore size (μm)*	*Porosity*	*Mineralize tissue ingrowth/comments*
Klawitter et al.[40]	Type I: 2–6 μm	33.5%	No tissue ingrowth
	Type II: 15–40 μm	46.2%	No bone ingrowth, fibrous tissue ingrowth
	Type III: 30–100 μm 80% pores < 100 μm	46.9%	50 μm of bone ingrowth, osteoid and fibrous tissue ingrowth
	Type IV: 50–100 μm 63% pores < 100 μm	46.9%	20 μm of bone ingrowth by 11 weeks and 500 μm of ingrowth by 22 weeks, osteoid and fibrous tissue ingrowth
	Type V: 60–100 μm 37% < 100 μm	48.0%	600 μm of bone ingrowth by 11 weeks and 1,500 μm of ingrowth by 22 weeks, osteoid and fibrous tissue ingrowth
Whang et al.[24]	≤100 μm	35.3%	Not statistically different from untreated controls
	≤200 μm	51.0%	Not statistically different from untreated controls
	≤350 μm	73.9%	Statistically significant more bone than all other groups

Pores have to be interconnected (why, what is the difference between porosity and permeability?). Both porosity and permeability change when a material is degraded



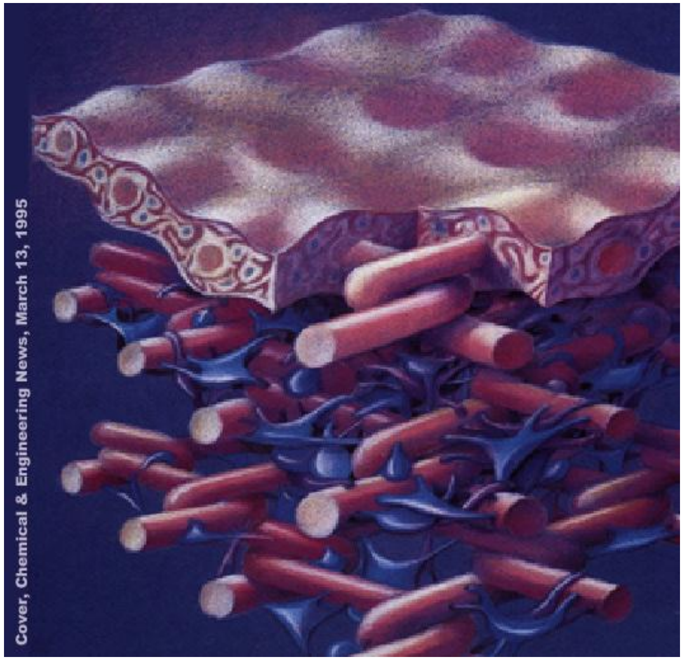
Stimuli- the tripartite axis

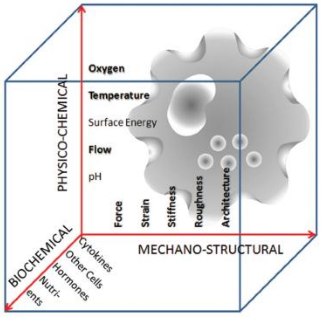


Engineering Quasi-Vivo In Vitro Organ Models. Sbrana & Ahluwalia. Methods Adv Exp Med Biol. 2012;745:138-53.

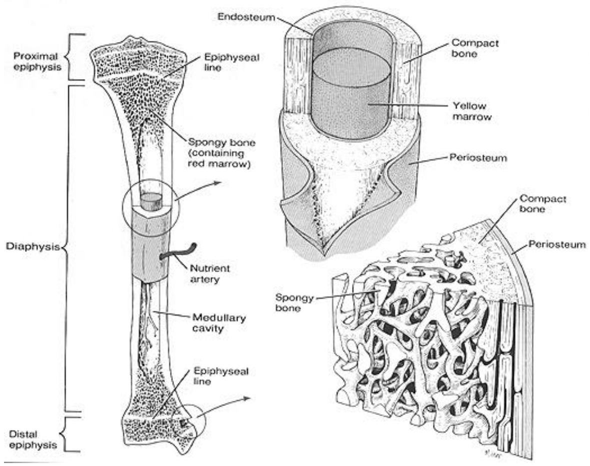
Biochemical stimuli in scaffolds

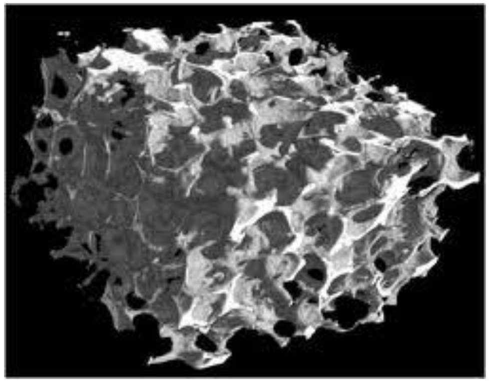
- Synthetic biomaterials with ligands /proteins
- Natural biomaterials
- Decellularized Tissue

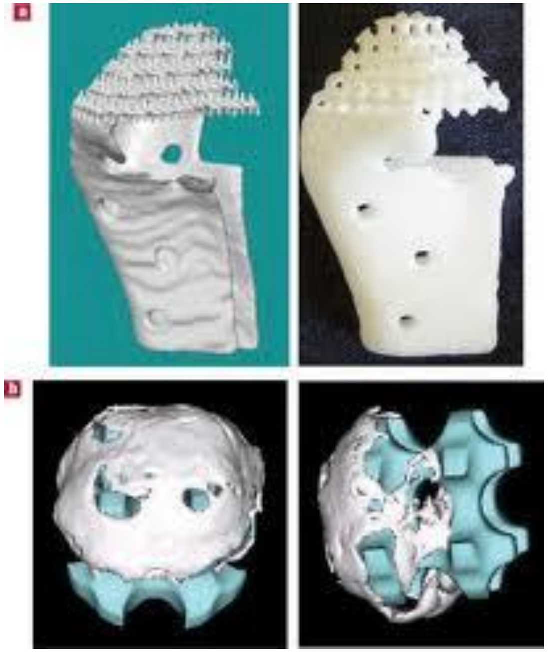


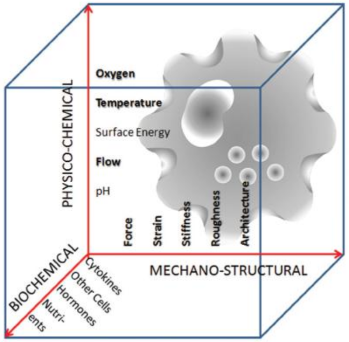


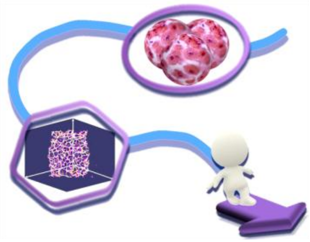
Mechano-structural stimulii









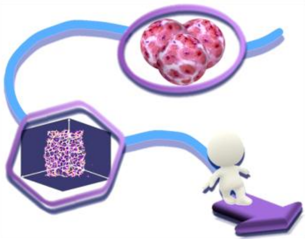


Forces are important

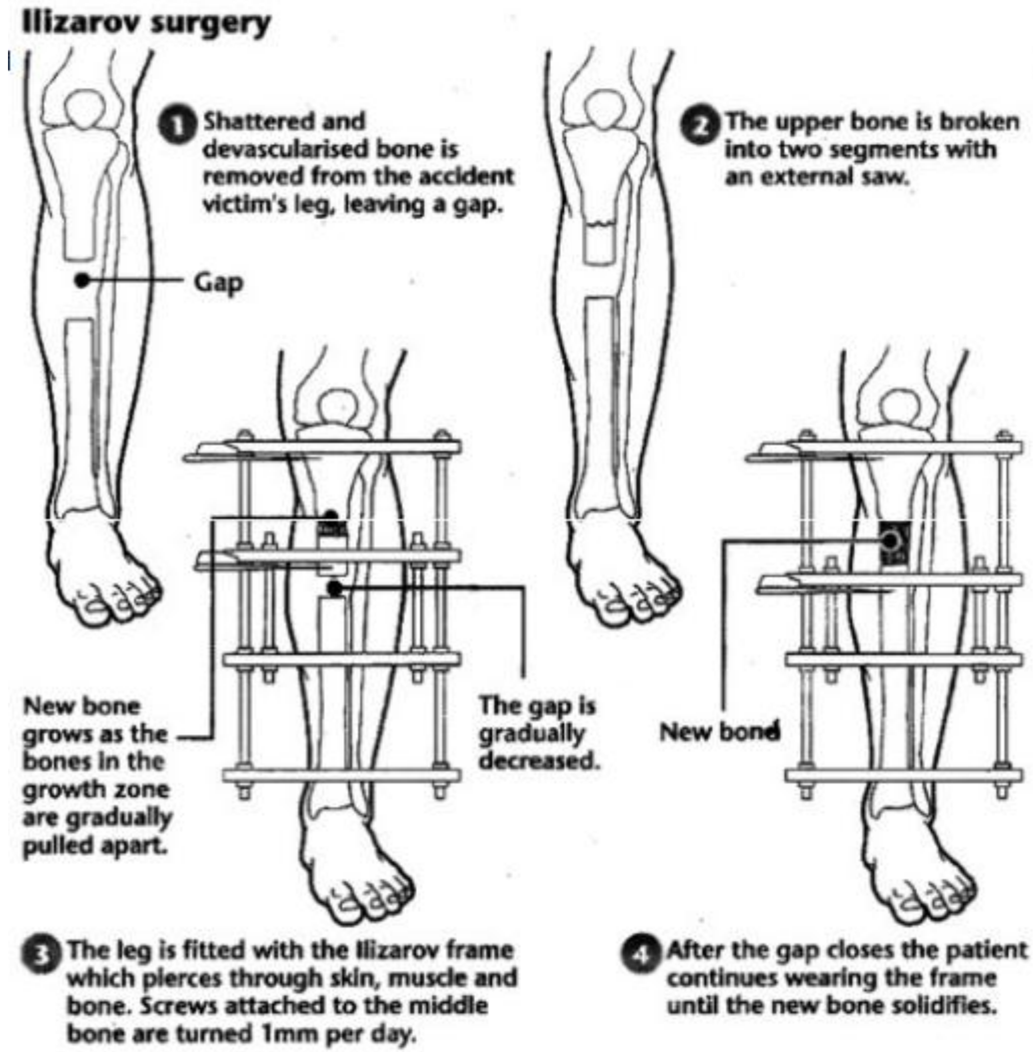
- Gravity
- Shear
- Pressure
- Tension
- Compression

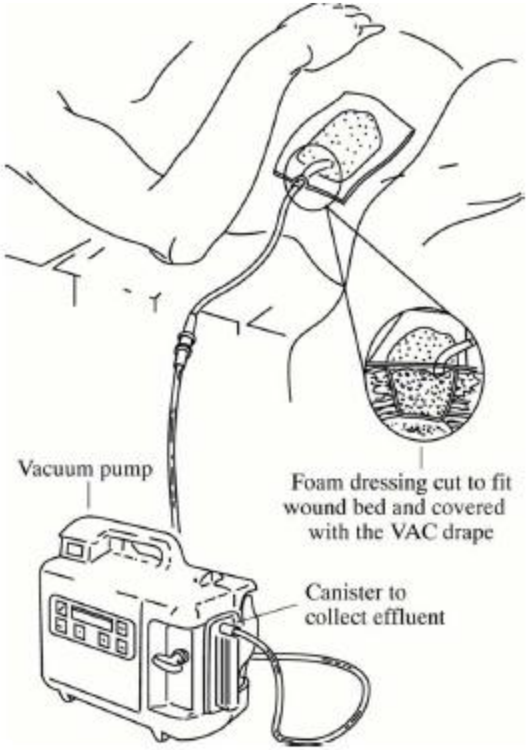
❑Cyclic forces

❑Static forces (materials in constant tension)



Distraction osteogenesis: bone is pulled apart to encourage growth





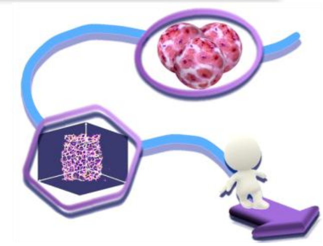
http://pmj.bmj.com

medicalconnectivity.com

Vacuum-assisted closure therapy (VAC)

Methods for generating (static) MS stimuli in scaffolds

- **Designer Scaffold**
  - Subtractive
  - Additive
- **Random Scaffold**
  - Organ processing
  - Biomaterial processing

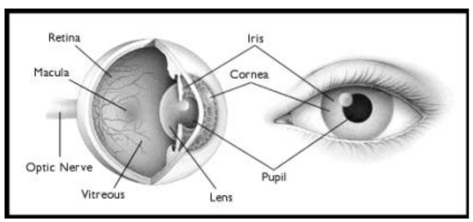


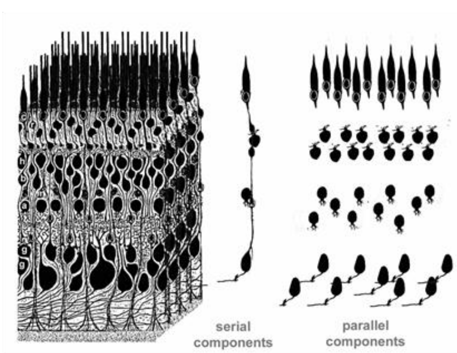
Designer or Random?

Structure

Function

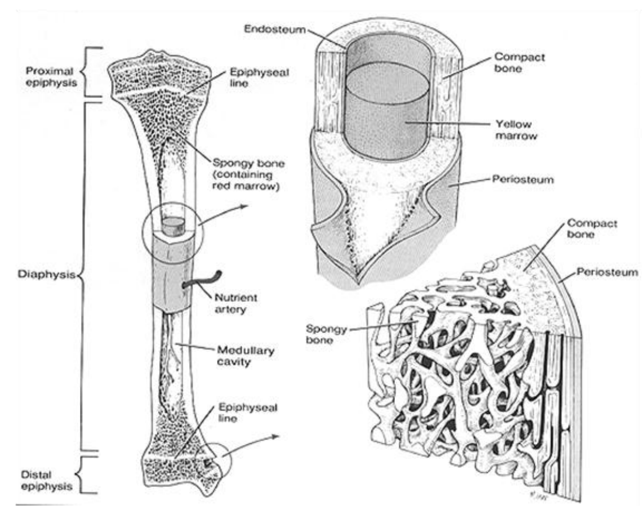
Retina



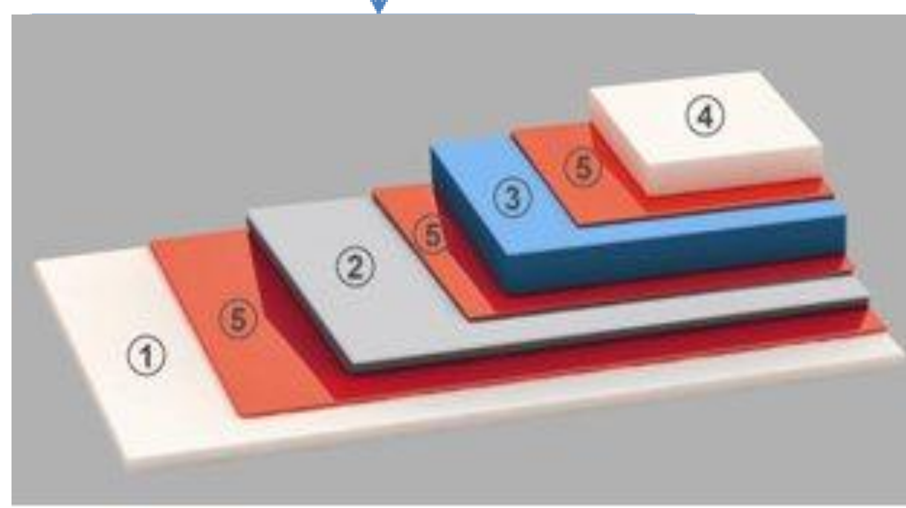


Liver

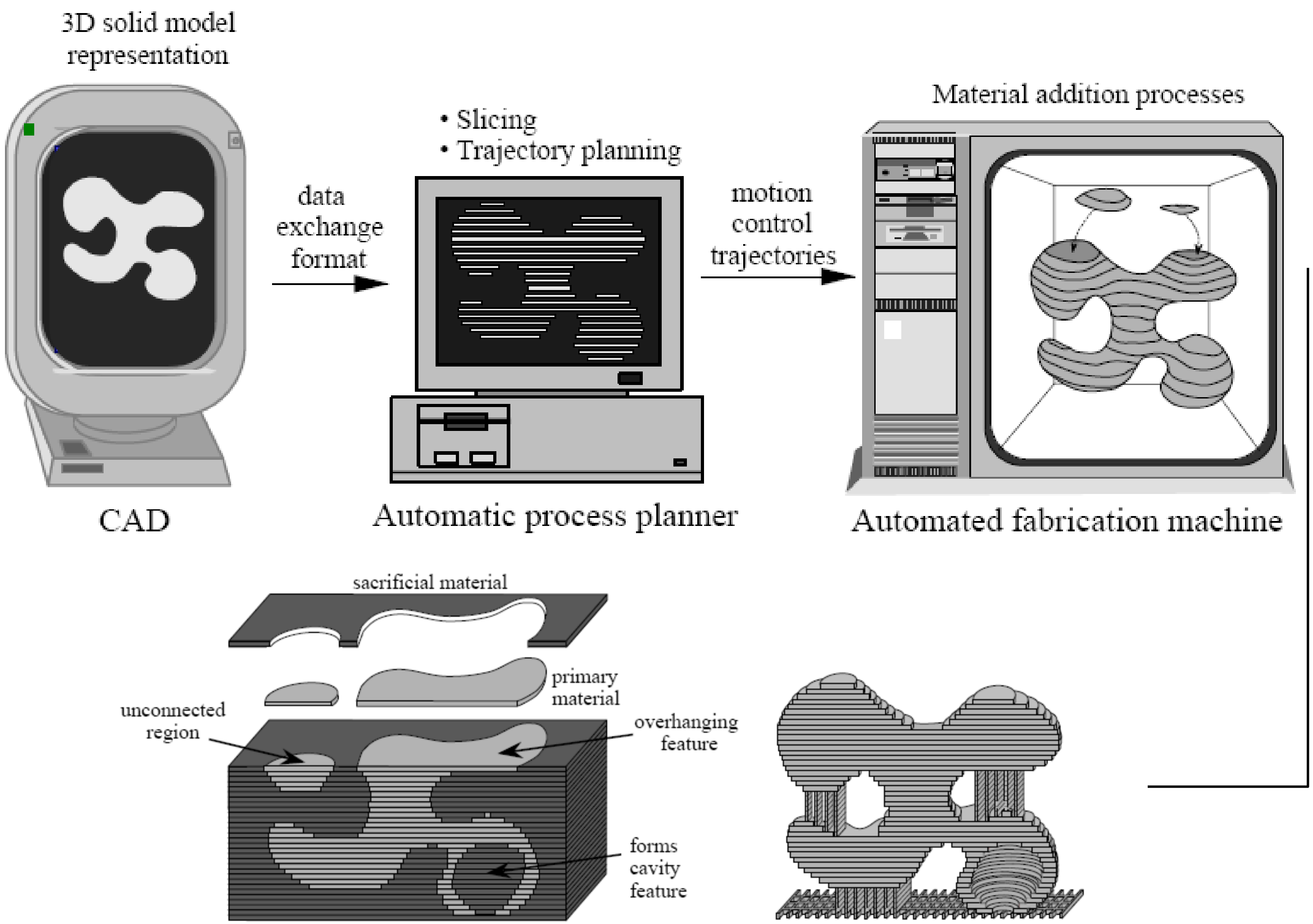
Bone



Designer Scaffold



Rapid prototyping: designer scaffolds



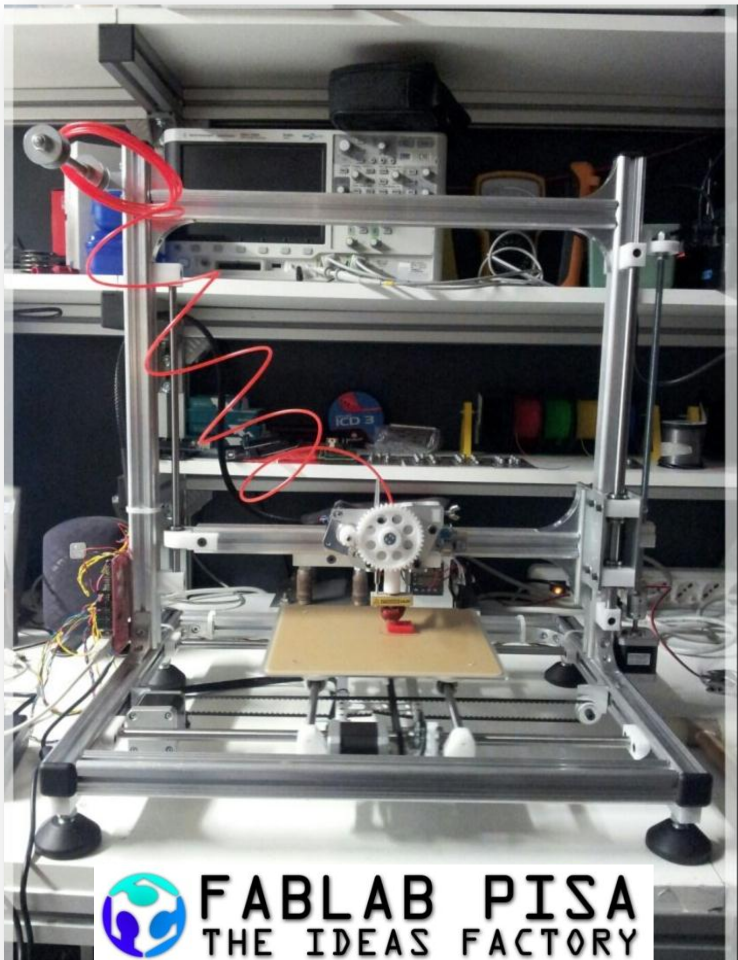
a. Complementary support.

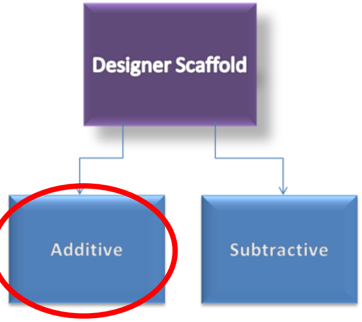
b. Explicit support.

Designer Scaffold

Additive | Subtractive

3D Printing/Digital Fabrication & RP





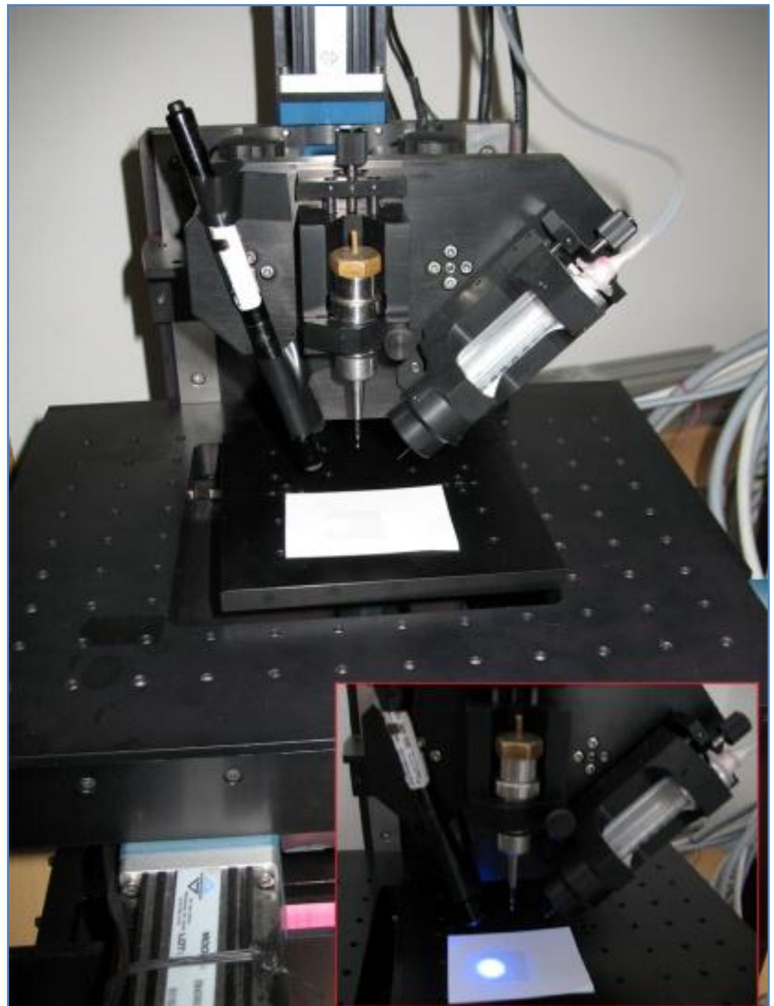
The PAM2 system

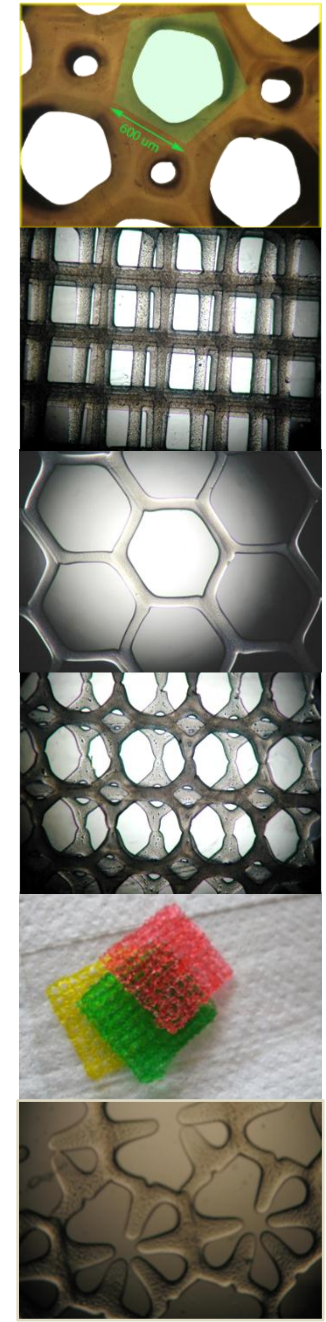
Robotic 3 axis micropositioner.

- ✓ PAM
- ✓ PAM2
- ✓ Diode laser
- ✓ Temperature control
- ✓ PAM^2 software

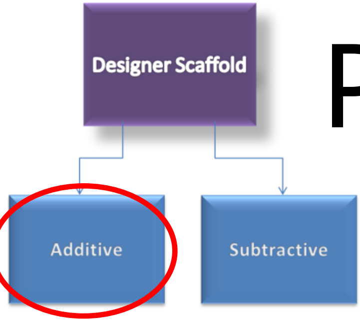
- 4 Position controlled brushless motors (resolution of 10 μm ± 1 μm)
- Working space 100×100×80 mm
- Working velocity 1-15 mm ·s^{-1}
- Design of *z*-stage to locate several modules

Materials?
Speed?
Price?
Fidelity?

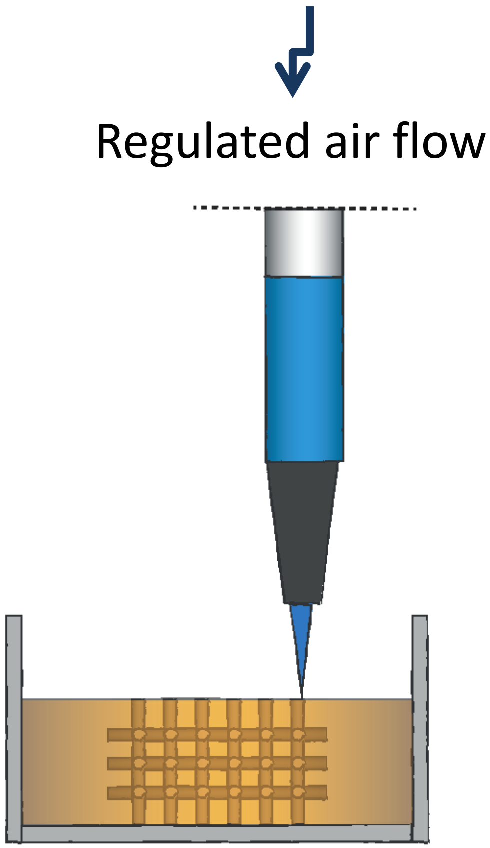


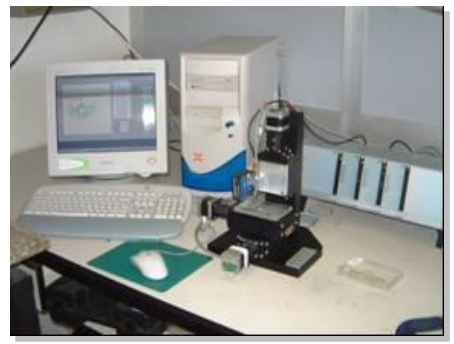


Tirella, De Maria, Vozzi, Ahluwalia Rapid Prot. J (2012);

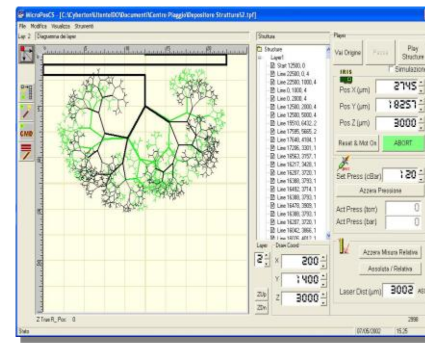


Pressure Assisted Microsyringe (PAM)

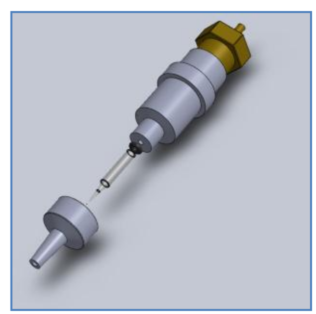




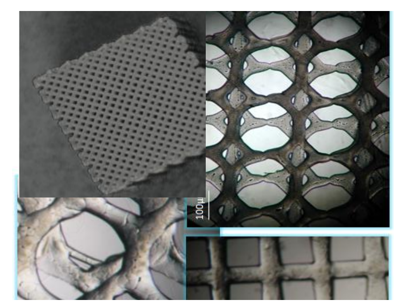
PAM system



Software



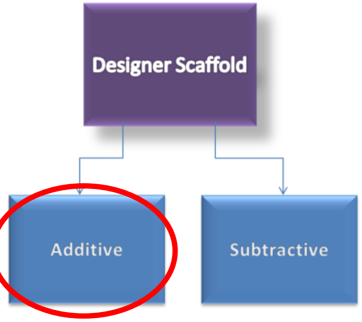
Syringe design



Software

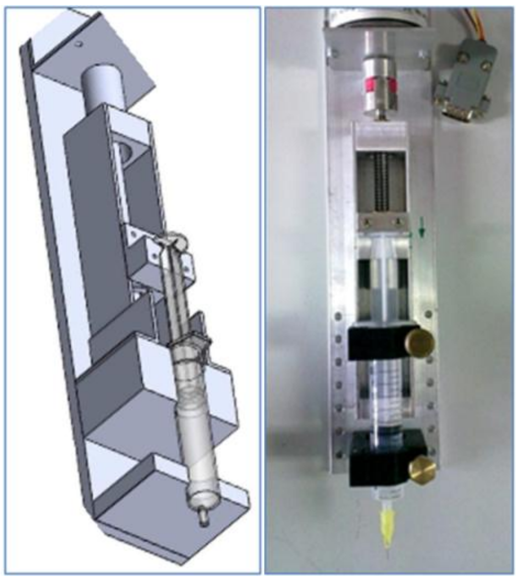
Materials?
Speed?
Price?
Fidelity?

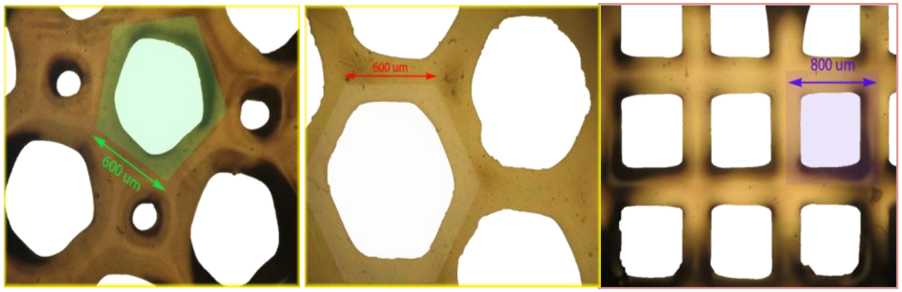
Vozzi et al,. Tissue Engineering, 8, 34, 2002. Vozzi et al , Biomaterials, 24, 2533, 2003, Vozzi et al, JBMRA, 71A, 326, 2004. Mariani et al., Tissue Eng. 12, 547, 2006. Bianchi et. Al. JBMR 81, 462, 2007.

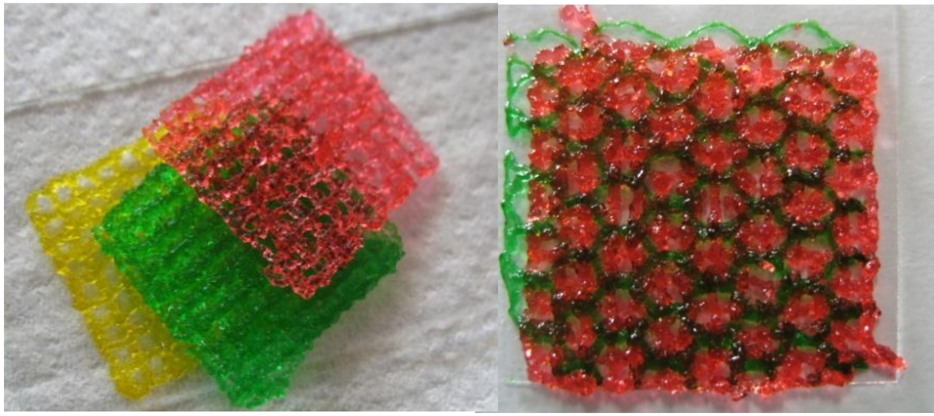


Piston Assisted Microsyringe (PAM2)

Plunger driven

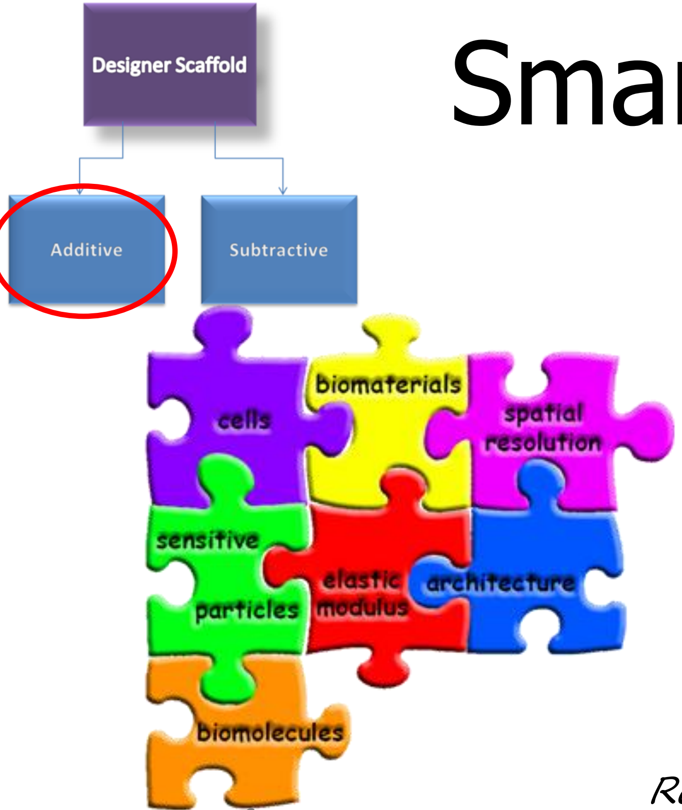


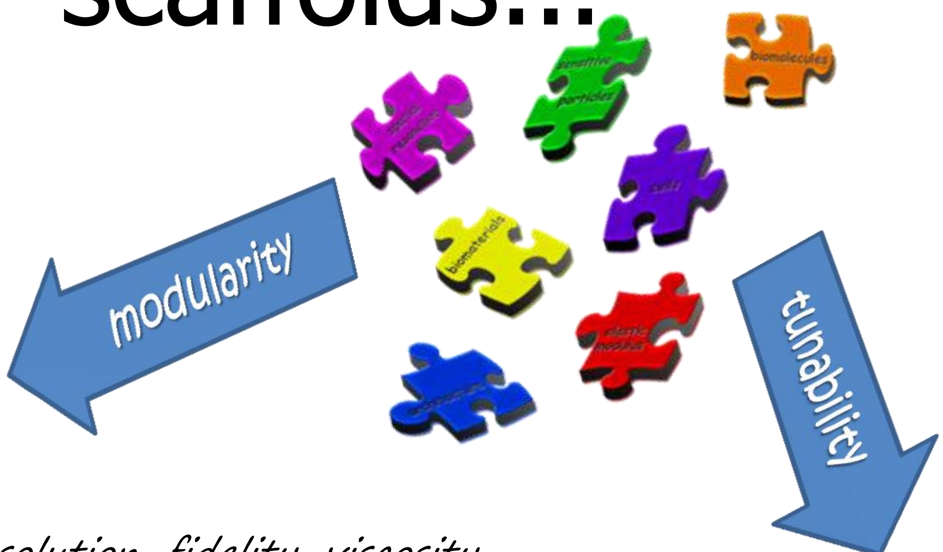


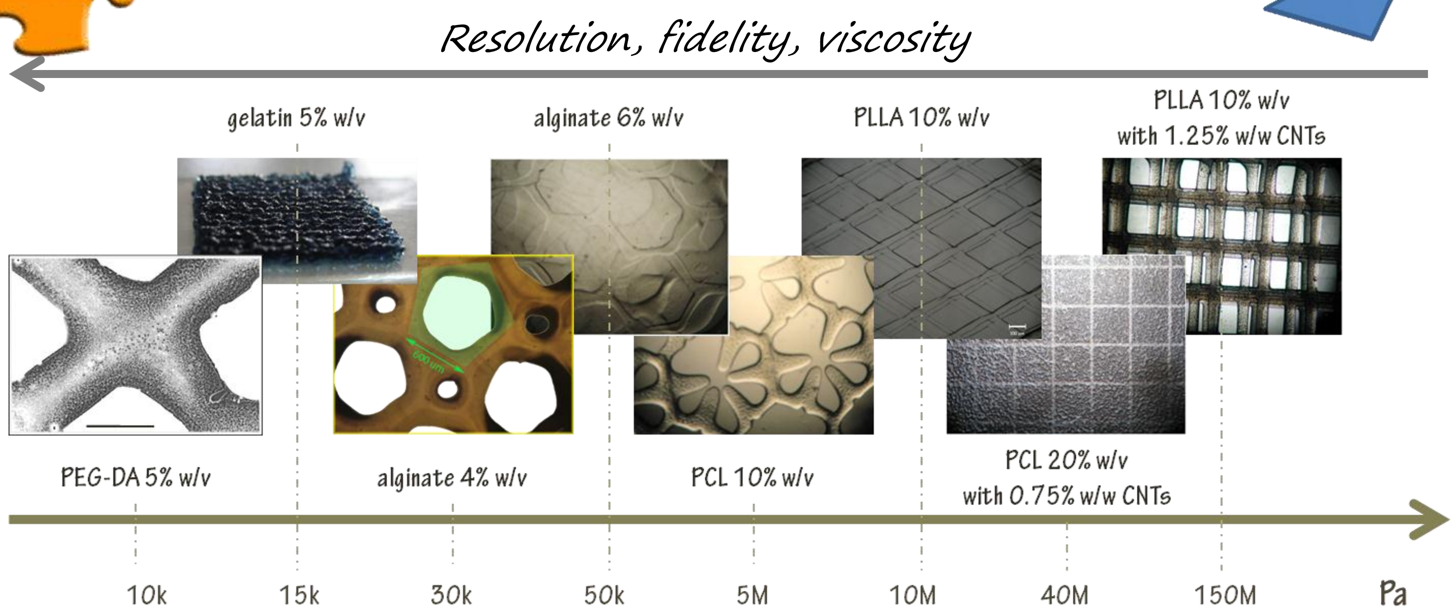


Materials?
Speed?
Price?
Fidelity?

Smart-tunable modular scaffolds…

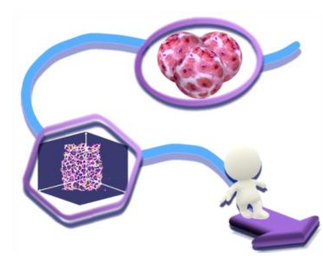






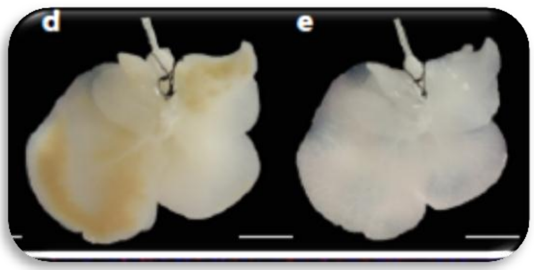
Development of a modular microfabrication system to engineer complex tissues

Technique	Material used	RTM ratio (cm^3/min)	Resolution (µm)	Cells used	Limits
Membrane Lamination	Bioerodable polymers (PLA, PLGA, etc), bio-ceramics	Low (<1)	1000	Osteoblasts	Structures not really porous, low resolution
Laser Sintering	Calcium Phosphates, polymers (PLA, PLGA, etc)	Medium to high	< 400	Osteoblasts	Presence of polymeric grains and of excess solvent
Photo-polymerisation	Photo-polymeric resins	0.5 (medium)	250	Osteoblasts	Use of photo sensitive polymers and initiators which may be toxic
Fused Deposition Modelling	Bioerodable polymers (PLA, PLGA,etc)	7 (very high)	200	Various types	Limited to non thermo labile materials. Layered structure very evident
3D™ Printing	Bioerodable polymers, (PLA, PLGA, etc) and hydroxyapatite	Medium (about 1)	300	Various types, mainly skeletal	Presence of polymeric grains and of excess solvent
iRP	Bioerodable polymers (PLA, PLGA, etc), collagen	0.1 (low)	300	Various types	Complex to realise, build materials limited, low fidelity.
PAM^2	Bioerodable polymers (PLA, PLGA, etc) and gels (alginate, gelatin)	1 (medium)	5-100	Neurons, endothelial cells, fibroblasts, hepatocytes, muscle	Highly water soluble materials cannot be used. Extrusion head very small.
InkJet	Water, solvents, nanoparticle suspensions	Very low (<0.01)	10	Various	Only low viscosity liquids.



Random Scaffold

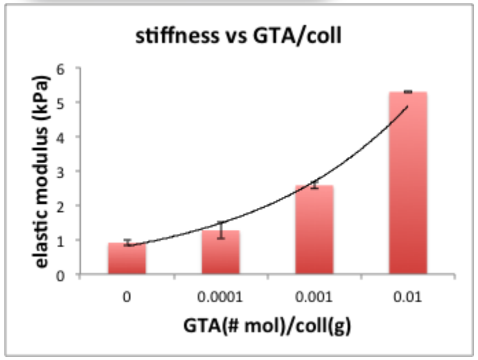
Organ processing

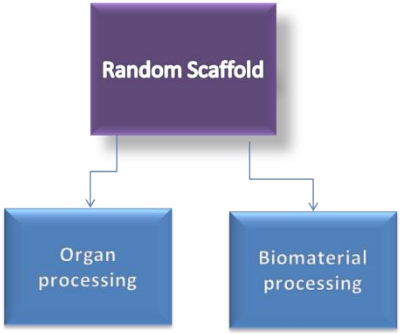


Uygun et al, Nature Med, 2010.

Mattei. et al, Biomat. Acta, 2013

Biomaterial processing

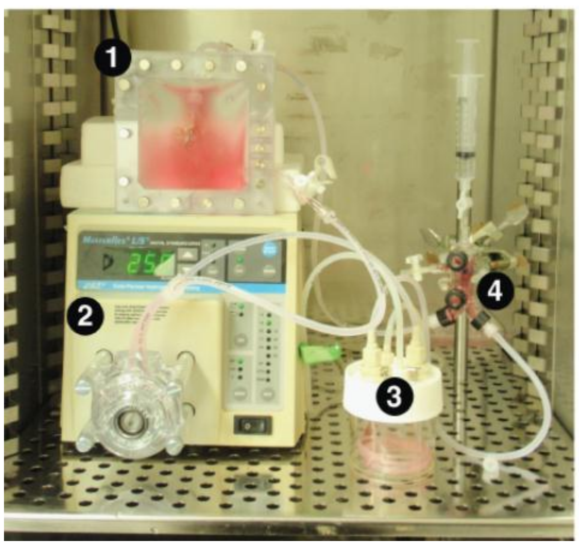




Organ Processing

Whole Organ Perfusion

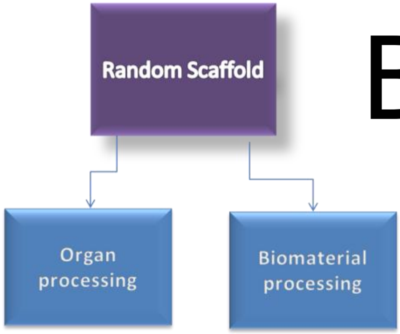
- Detergents
- Intact microvasculature
- Slow and costly



Price?
Materials?
Speed?
Repeatibility ?

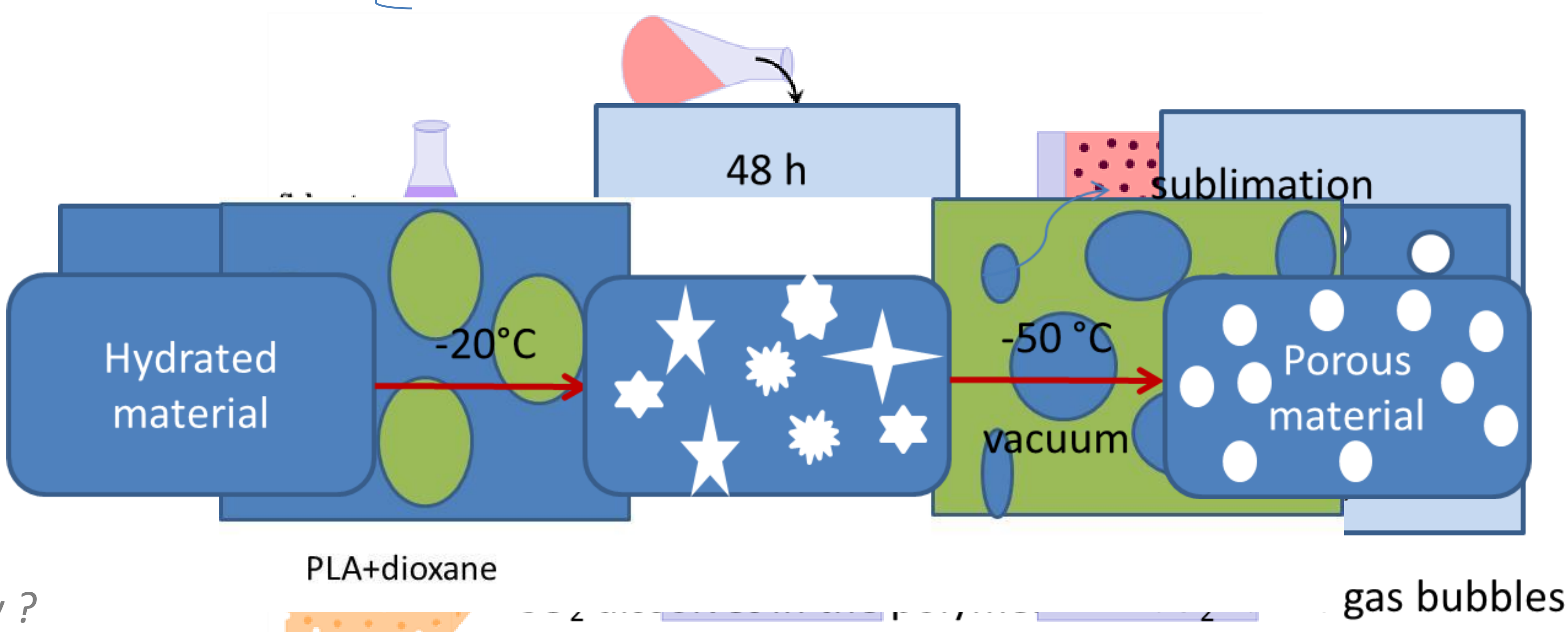
Tissue Decellularization

- Detergents
- Rapid, less wasteful

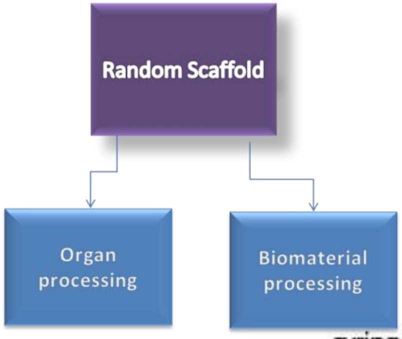


Biomaterial Processing

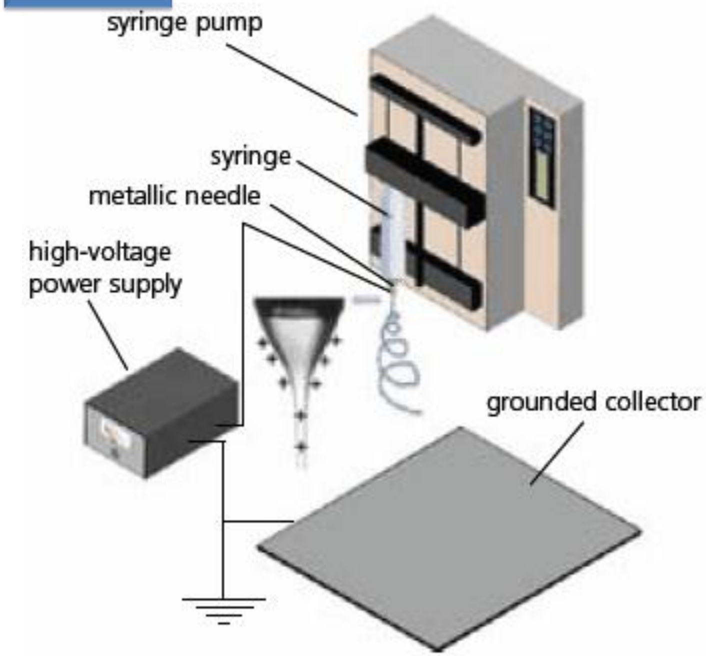
- ***Freeze drying***
- ***Phase separation***
- ***Gas foaming***
- ***Salt leaching***

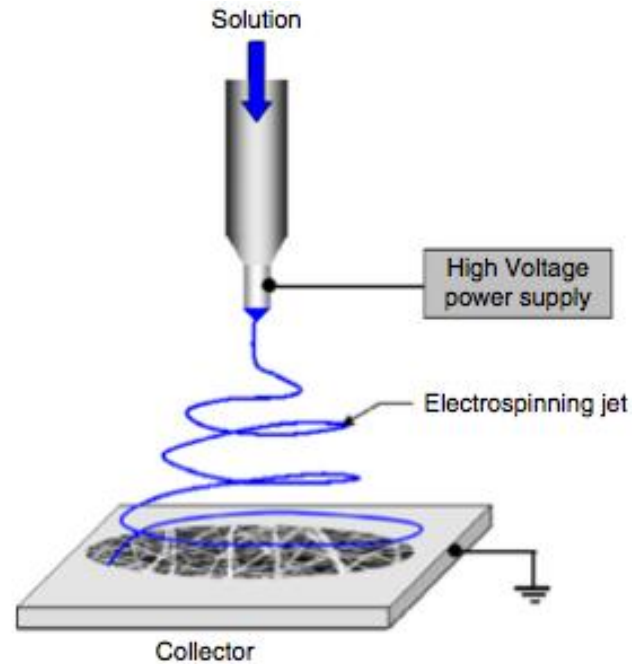


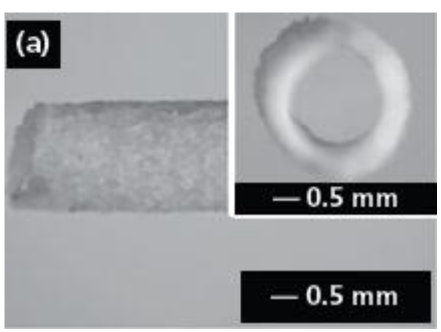
Price?
Materials?
Speed?
Repeatability ?

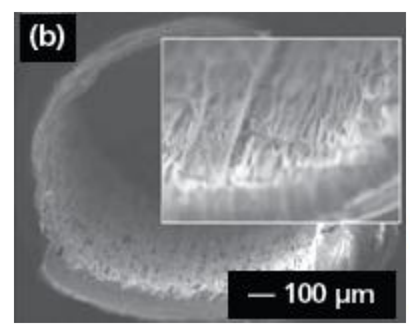


Electrospinning

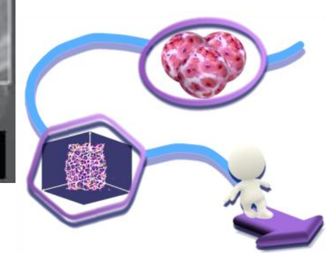






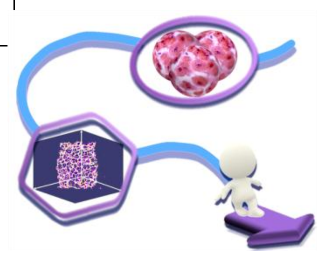


Price?
Materials?
Speed?
Repeatibility ?



Technique	Material used	RTM ratio (cm^2/min)	Cells used	Limits
Freeze drying	Proteins, carbohydrates, polyesters, hydroxyapatite	High	Variety	Wide distribution of pore size
Phase Inversion	Polyesters, PVA, polyurethanes, biogels (gelatin)	High	Variety	Low interconnectivity, difficult to control pore size
Salt leaching	Polyesters, polyurethanes, hydroxyapatite	High	Variety	Salt residues, limited connectivity
Gas foaming	Polyesters, PVA, polyurethanes, biogels (gelatin)	High	Variety	Quite expensive
Whole organ decell	Organs	High	Heart, liver, lung, etc	Whose organ? Detergents are aggressive
Tissue decell	Pieces of tissue	High	Many	
Electrospinning	Bioerodable polymers (PLA, PLGA, etc), proteins and gels (collagen, alginate, gelatin)	Very low (<1)	Variety	Gives rise to pseudo 3D "squashed" scaffolds

Stop here



Degradable Polymeric Biomaterials are materials which can be eliminated through hydrolytic degradation or enzyme attack. The synthetic ones are almost all polyesters (polycaprolactone, polyglycolide, polylactide)

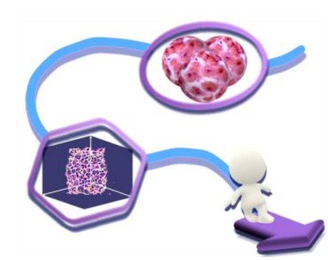
Polyesters

$$\begin{array}{c} O \\ \| \\ R\text{-}C\text{-}O\text{–}R' \end{array}$$

- They do not give rise to a permanent and chronic “foreign body” response
- Some materials are capable of inducing tissue regeneration.
- They are used as temporary supports and scaffolds in tissue engineering. They cannot be used as permanent suppports but only for remodelling and repair.

Requisites for bioerodabile materials

1) Provide an adequate mechanical support for a short period of time without any problems after degradation.
2) Degradation rate match rate of new tissue generation
3) Provide an approriate biochemical environment for cell/cell and cell/ECM interaction and supply nutrients and growth factors as necessary.
4) Guide tissue response as appropriate (enhance or suppress).
5) Not induce an inflammatory response. Low or negligible toxicity of degradation products both locally and systemically.
6) Easy to produce and fabricate in large quantities
7) Compatible with drug delivery methods
8) Porous



Biodegradable biological polymers

<u>Collagen</u>: from animal sources. It is non immunogenic because it is a highly conserved protein.

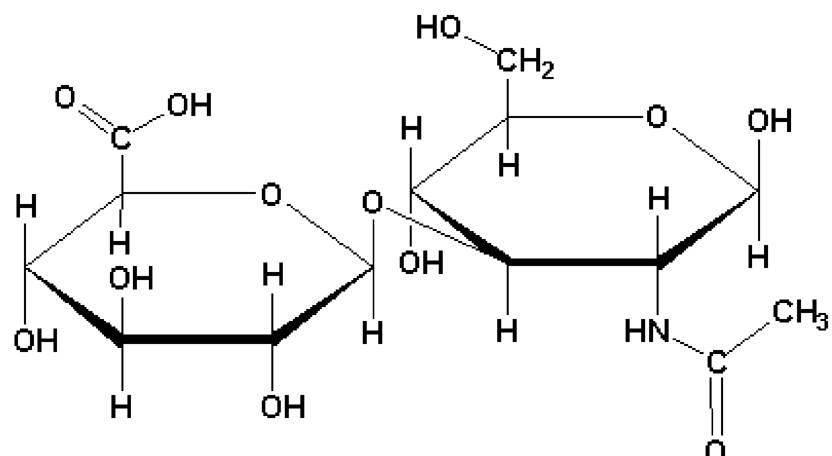
Can be crosslinked to render it more stable, more resistant, increase degradation time, less hydrophilic, les soluble and increase tensile strength.

Very common in tissue engineered products, eg Alpigraf (collagen gel, fibroblasts+keratinocytes)

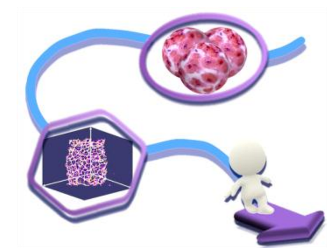
<u>GAG</u>: hyaluronic acid=gluconic acid+ glucoseamine .Main source is rooster combs or through transfected bacteria. This material is very viscous and hydrophilic, forming gels. The acid can be esterified with COOH to make it less viscous and more soluble.

Eg Hyaff

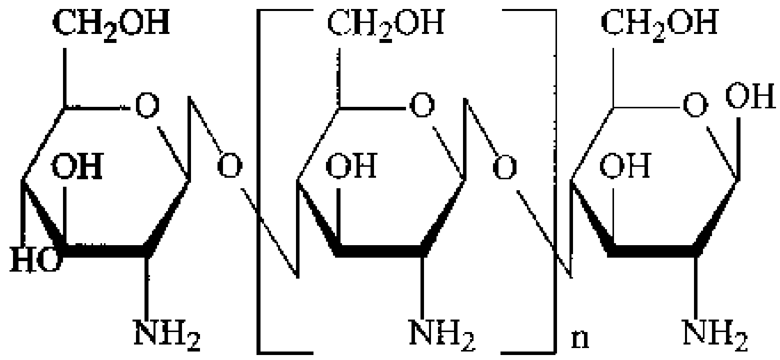
What is esterification?



Hyaluronic Acid



Chitosan:polysaccharide



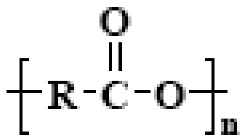
Chitosan

Chitosan has a high degree of biocompatibility but is not very resistant to loads or deformation. They are not reproducible (different sources are different) and may also carry infective agents.

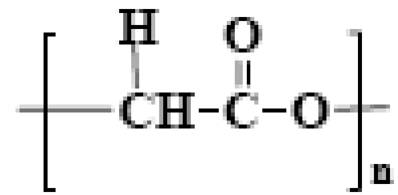
From crabs, for example. Approved for cosmetic use in Japan

<u>Synthetic biodegradables</u>

The most widespread are those approved by the FDA. Polycaprolactone, polyglycolic acid and polylactic acid. All 3 are polyesters.

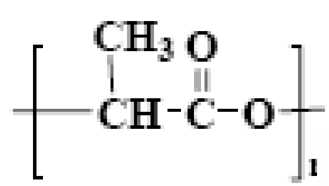


PGA : the simplest, crystalline (35-70%), insoluble (only in HFP), high mp (200C), used in sutures, Hydrophilic, degrades slowly



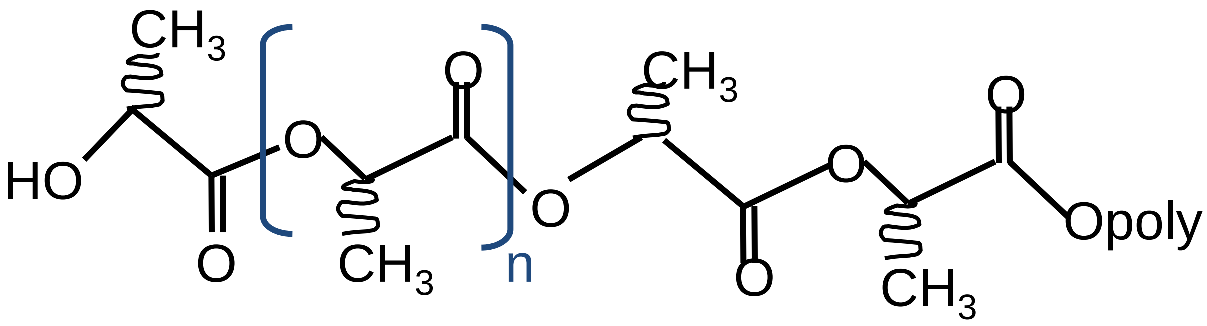
Poly(glycolic acid)

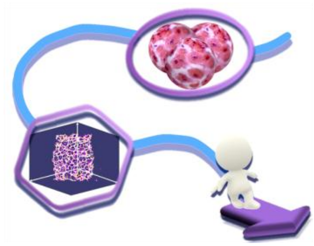
PLA: has an additional CH_3 , (35% crytstalline) hydrophobic, degrades more slowly than PGA, more soluble on organic solvents. Chiral, so found in 3 forms: l, d and ld



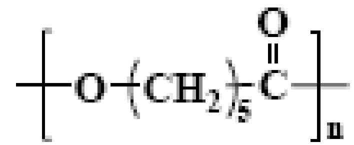
PLLA: semi crystalline, hard, mp=180C, less crytalline than PGA (35%)

PDLLA: random chiarality. Amorphous. Degrades faster than PLLA (2-12 months)





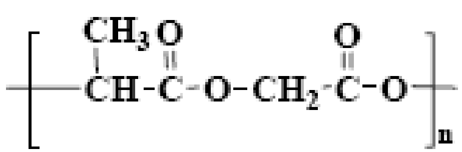
Polycaprolactone PCL:
semicrystalline,
degrades in 2 years.



Poly(ε-carprolactone)

All 3 polyesters degrade by alkaline hydrolysis releasing acid products. All are fairly rigid.

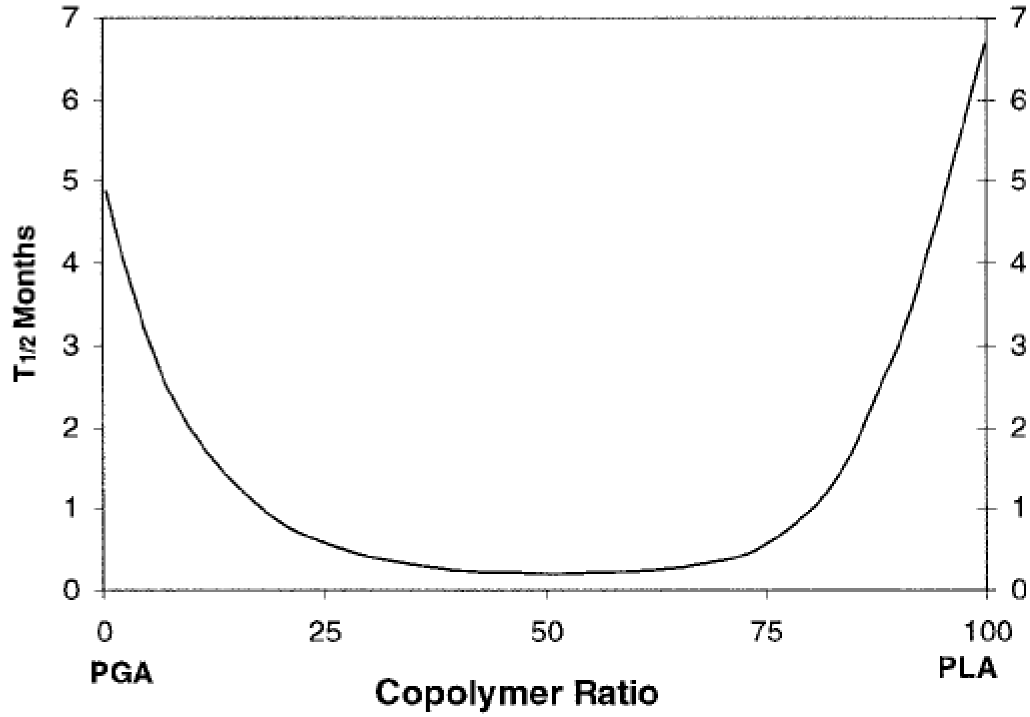
Copolymers: PLGA, Poly lactide co caprolactone etc. Their properties vary greatly. The most common is polylactide co glycolide. PLGA is available in different copolymer ratios. Eg Vicryl (fast degradation), polyglactin (slower). Dissolves in most organic solvents.



Poly(lactic-co-glycolic acid)

low MW copolymers can be obtained through condensation, whereas high MW copolymers require opening bonds

?



Copolymers are more amorphous

The degradation rate of PLGA depends on MW, hydrophilicity and the degree of crystallinity, pH and temperature

Problems with PGA e PLA e PCL:
They are rigid. Do not possess functional groups to modify and bind proteins. Can generate too much local acidity. (degrade by hydrolysis).
Question: write a reaction for hydrolysis of PGA (assume 3 monomers)

On the other hand,compared with biological polymers they are more reproducible and less likely to carry infective agents (BSE).
Moreover, biological polymers are not structurally strong.

Please note that there is a whole world of polymers out there- but only a handful actually approved for in-vivo use.

TABLE 1. PROPERTIES OF BIODEGRADABLE POLYMERS[27,29,31,32]

Polymer type	*Melting point (°C)*	*Glass trans. temp. (°C)*	*Degration time (months)*[a]	*Density (g/cm^3)*	*Tensile strength (MPa)*	*Elongation, %*	*Modulus (GPa)*
PLGA	Amorphous	45–55	Adjustable	1.27–1.34	41.4–55.2	3–10	1.4–2.8
DL-PLA	Amorphous	55–60	12–16	1.25	27.6–41.4	3–10	1.4–2.8
L-PLA	173–178	60–65	>24	1.24	55.2–82.7	5–10	2.8–4.2
PGA	225–230	35–40	6–12	1.53	>68.9	15–20	>6.9
PCL	58–63	−65	>24	1.11	20.7–34.5	300–500	0.21–0.34

[a]Time to complete mass loss. Time also depends on part geometry.

TABLE 3. MECHANICAL PROPERTIES OF HUMAN TISSUES

	Tensile strength (MPa)	*Compressive strength (MPa)*	*Youngs' modulus (GPa)*	*Fracture toughness (MPa.m1/2)*
Cancellous bone[56]	N/a	4–12	0.02–0.5	N/a
Cortrial bone[56]	60–160	130–180	3–30	2–12
Cartilage[57]	3.7–10.5	N/a	0.7–15.3 (MPa)	N/a
Ligament[58]	13–46	N/a	0.065–0.541	N/a
Tendon[58]	24–112	N/a	0.143–2.31	N/a

Degradation rate of a polymer

Depends on

1)Polymer intrinsic properties:MW, crystallinity etc

2) environment: shear, acidity etc

3) Surface area

Problem: consider a unit cell of biodegradeable mateial with a pore in the center.

How does

1)Porosity

2) Maximum load

3) Mass of material

change with time

Where are we today?

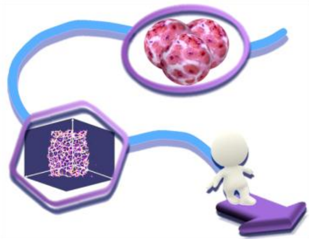
Humans

- Skin
- Cartilage
- Trachea
- Bladder
- Pancreas

- In-vitro meat

Animals

- nude mouse



Live scaffold fabrication

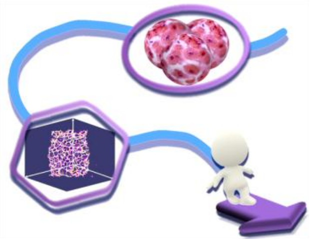
Direct Fabrication

Composite materials

Live Engineered Scaffold

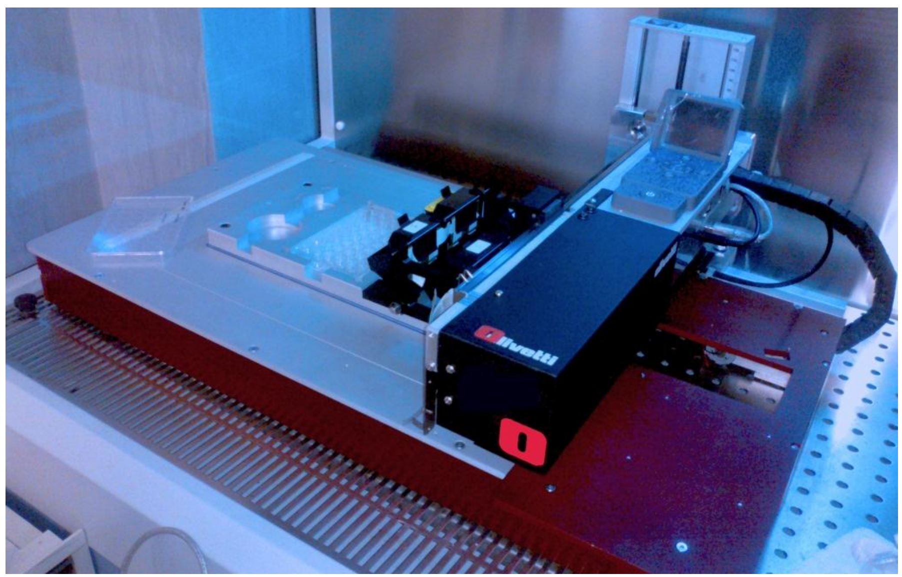
Cells

Biomaterial Processing



Cell Printing

- Cell Printing (inkjet)
- Organ Printing (nozzle based)
- Living Inks, bioinks, bioprinter, bioplotter



Olivetti NanoBioJet

Cell dispensers and Bioprinters

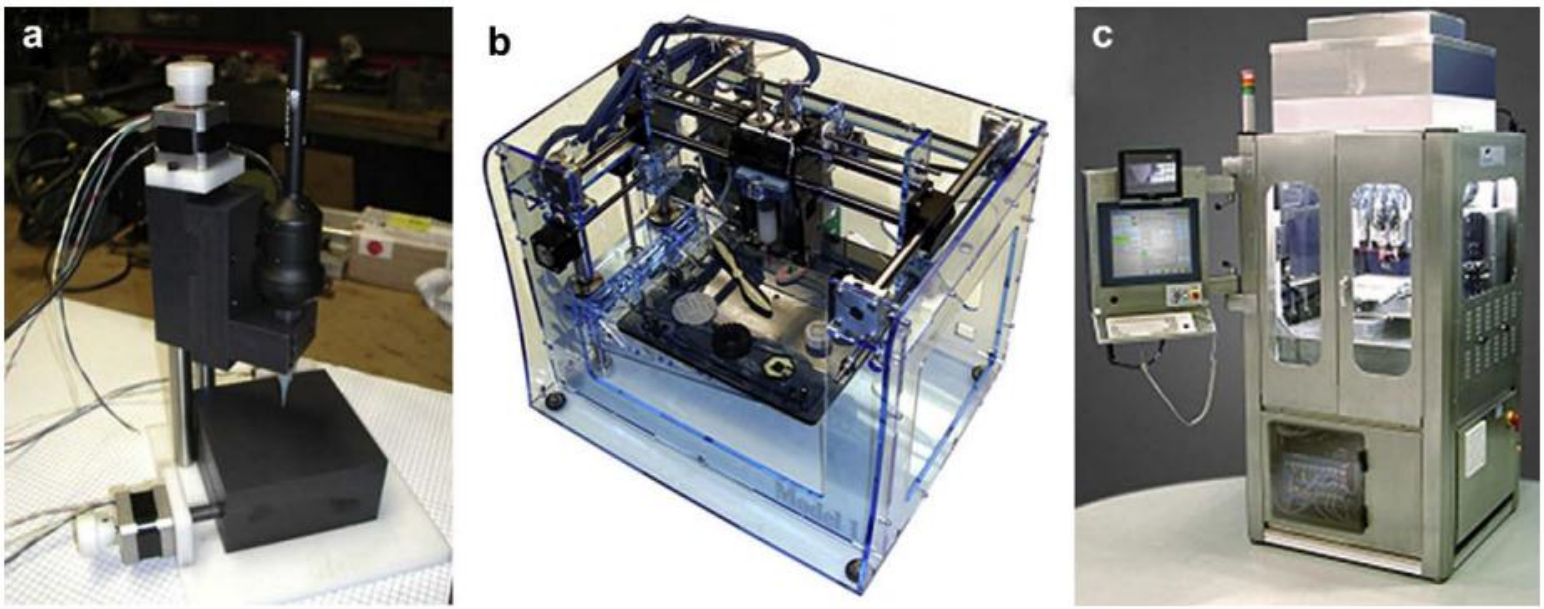
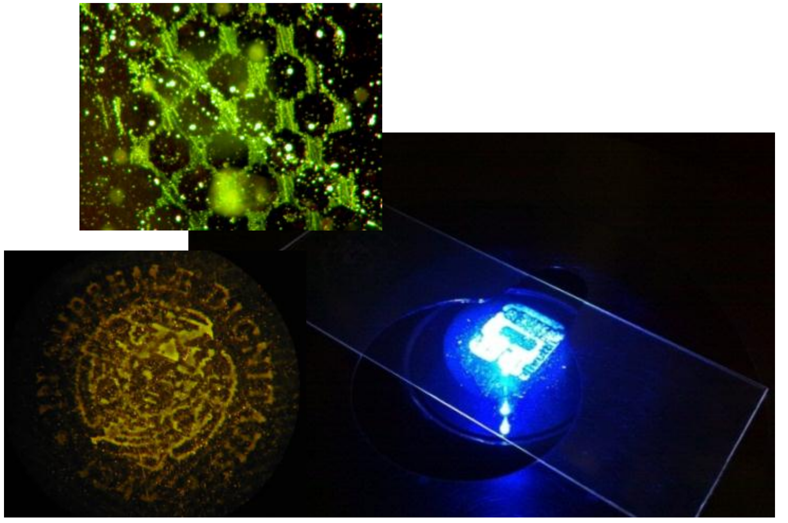
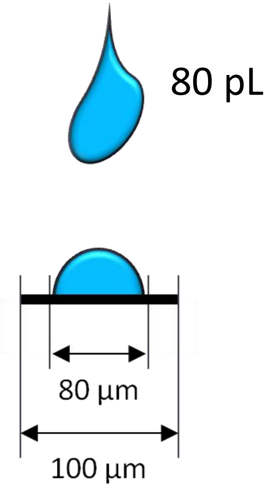


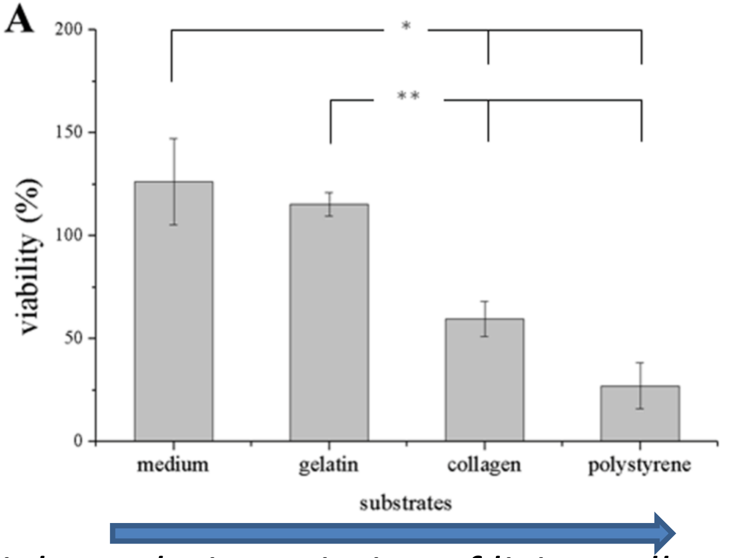
Fig. 3. Bioprinters: a) 3D dispensing Laboratory Bioprinter – 'LBP' (designed by Neatco, Toronto, Canada in cooperation with MUSC Bioprinting Research Center, Charleston, SC); b) 3D robotic printer – 'Fabber' (designed by Cornell University, USA); c) 3D robotic industrial bioprinter — 'BioAssembly Tool' (designed by Sciperio/nScript, Orlando, USA).

InkJet for Living Inks

- *2D…*
- *Small volumes* in high spatial resolution patterns
- *BioInk* (i.e. protein based solutions)
- Particle based inks
- *LivingInk* (i.e cell suspensions)





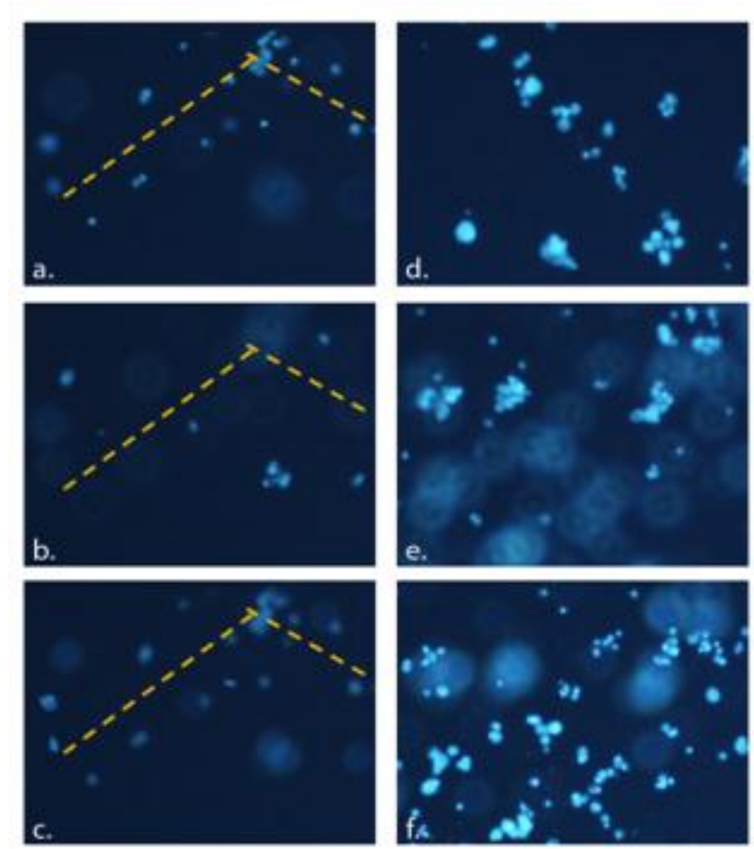


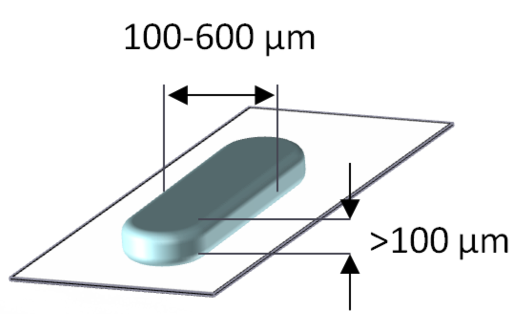
Tirella et. al, Substrate stiffness influences high resolution printing of living cells with an ink-jet system. J Biosci Bioeng. 2011

Nozzle systems for Living inks

- *...layer by layer*

- *Micro-resolution* of viscous biomaterials
- *Complex pattern s*and 3D architecture
- Liquid and viscous inks (including BioInk, particle based inks and LivingInks)





Organ Printing using *cell suspensions* as a *material*

V. Mironov et al. Biomaterials 30 (2009) 2164–2174

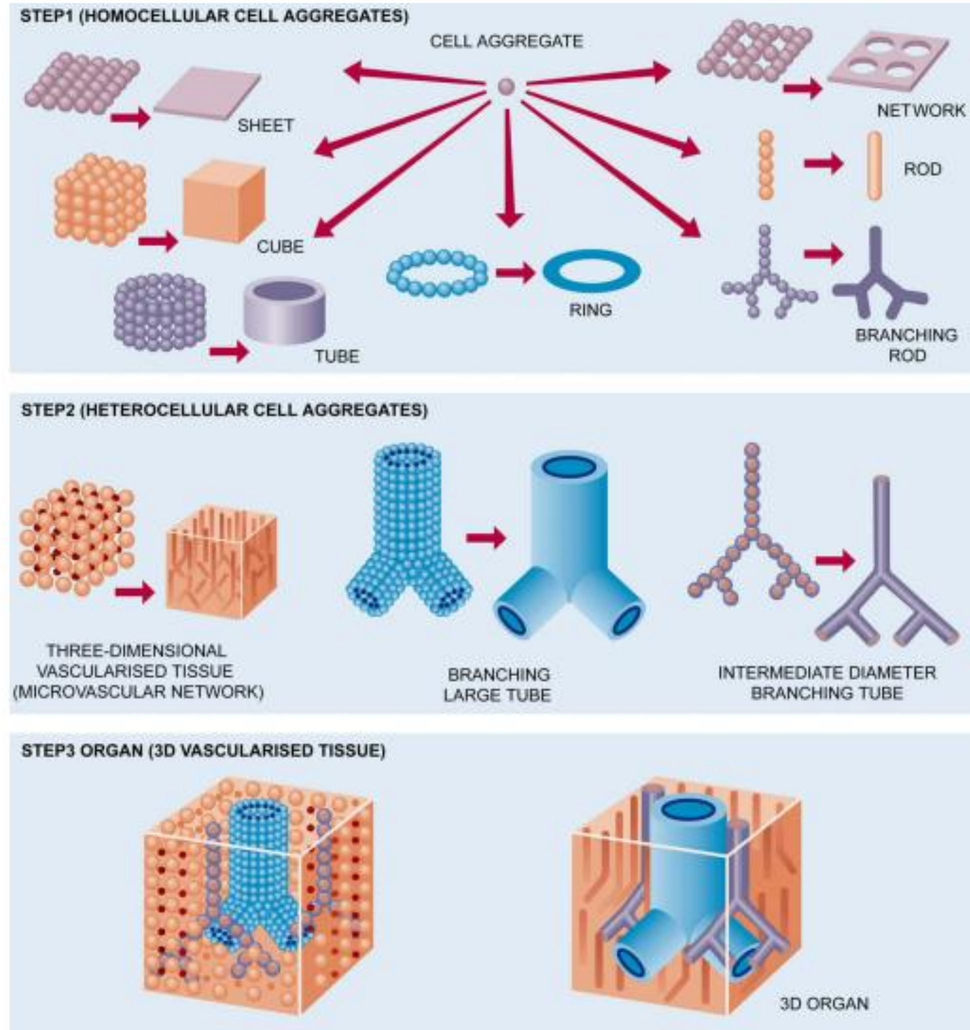
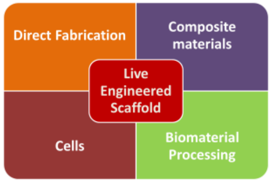


Fig. 4. Roadmap for organ printing.

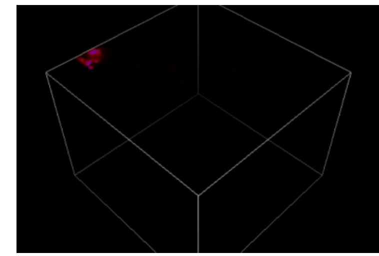
fusion is a ubiquitous process during embryonic development and can be recapitulated in vitro [45]. It has been shown that the kinetics of tissue fusion of two rounded embryonic heart cushion tissue explants placed in an hanging drop fits perfectly to fusion kinetics described for two droplets of fluids [46]. Moreover, based

physical laws and Malcolm Steinberg's "differential adhesion hypothesis" [28–30]. From another point, motile living cells, cytoskeleton and number, and redistribution and activation of cell adhesion receptors are also essential for the tissue fusion process [46,47]. The accumulation of ECM and associated restriction of cell



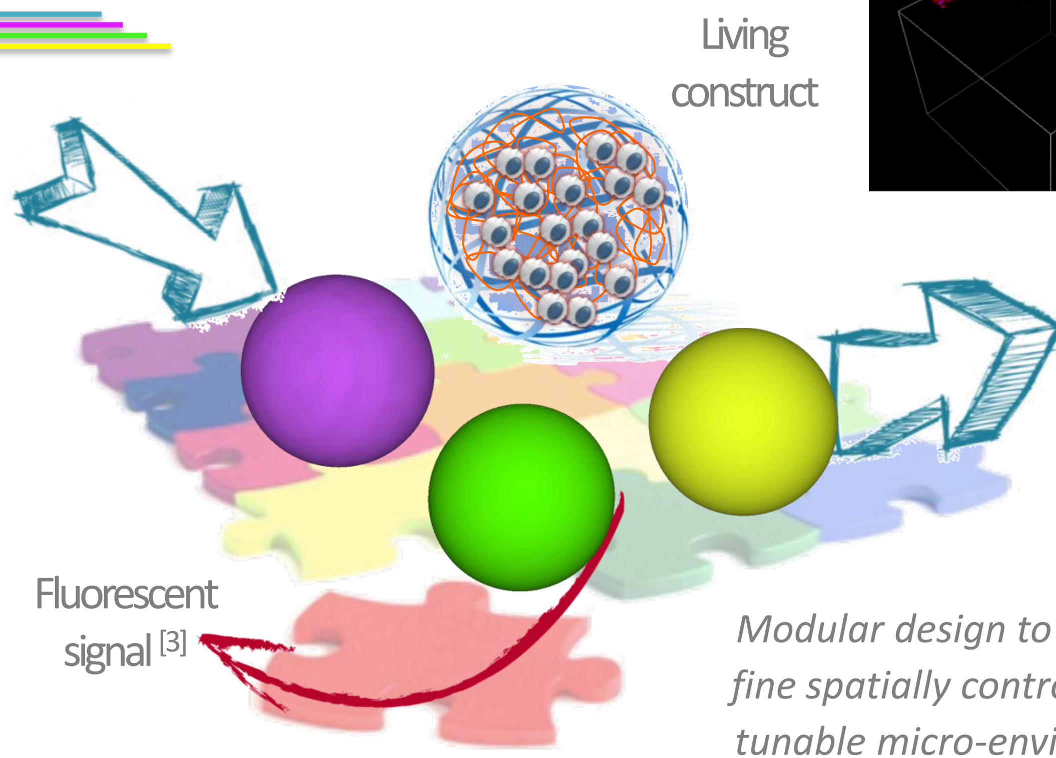
Nano-in-micro (NIM) Live Scaffold Fabrication

Recreate an *in vitro* microsystem able to interact and monitor living constructs in a non-invasive manner



Assembling:

- Living micro-spheres with controlled mechanical and properties and biomimetic composition;
- Having:
  - Cells
  - Tissue matrix
  - Release of known moieties (e.g. ROS, exogenous molecules)
  - Scavenger properties
  - Sensitive detectors[3]

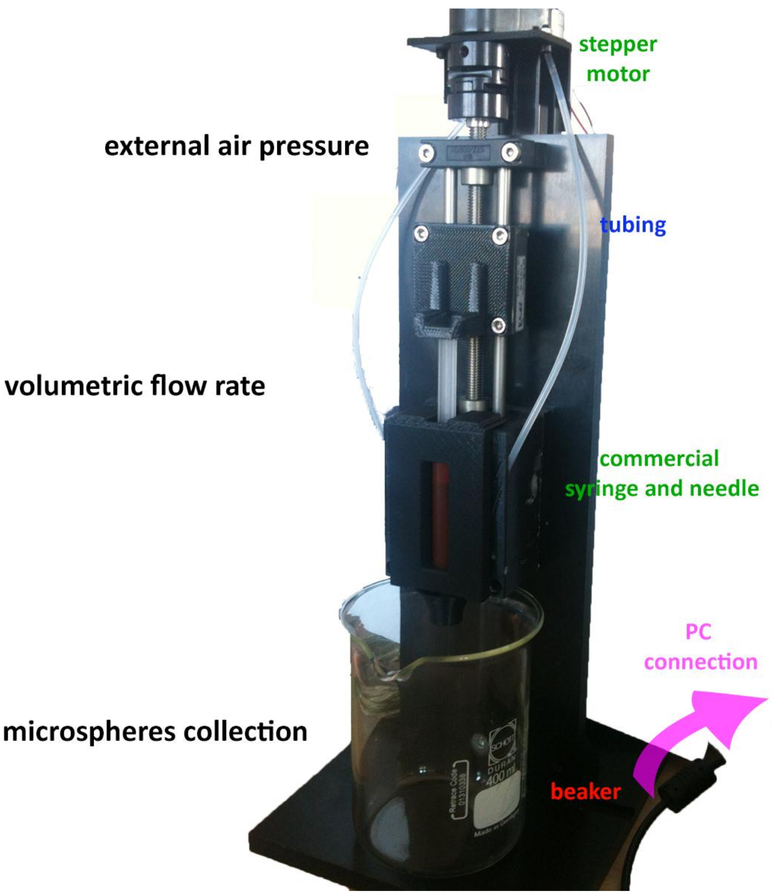


Modular design to obtain a fine spatially controlled and tunable micro-environment

[1] Tirella et al. JBMA 2013 ; [2] Mattei el all Biomat. Acta,; [3] Tirella , La Marca, Ahluwalia , Soft Matter submitted; [4] La Marca et al., ESB 2013.

Spherical Hydrogel Generator

Sensitive/Functional domains can be easily fabricated controlling sphere dimension, shape and composition



Size controlled hydrogel micro-spheres as function of system working parameters and solution properties:

- ✓ Solution viscosity (e.g. alginate w/v ratio, NPs concentration, cell concentration)
- ✓ Nozzle diameter
- ✓ Volumetric flow rate
- ✓ External air flow

Shape is fixed via rapid physical gelation, e.g. for alginate microspheres form a gel in a beaker containing a 0.1 M $CaCl_2$ solution in water.