Bioprinting Overview

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Tissue Engineering & 3D Printing

- Tissue Engineering Components:
  - The type or types of living cells being implanted (e.g. somatic, embryonic stem cells, adult stem cells, or induced-pluripotent stem cells).
  - Type of scaffolds supporting the cells (i.e. the mechanical cues provided to the cells).
  - Type of drugs, extra-cellular matrix (ECM), and growth factors conditioning the cells, (the additives that provide chemical cues to the cells).
- 3D Printing:
  - Computer assisted process for depositing biomaterials and living cells in a determinate configuration in order to produce a defined 3D biological structure.
  - Bioinks consist of various polymer materials, cells and additives.

Mosadegh, B., G. L. Xiong, S. Dunham, and J. K. Min. "Current Progress in 3d Printing for Cardiovascular Tissue Engineering." *Biomedical Materials* 10, no. 3 (Jun 2015).

Bioprinting Technology

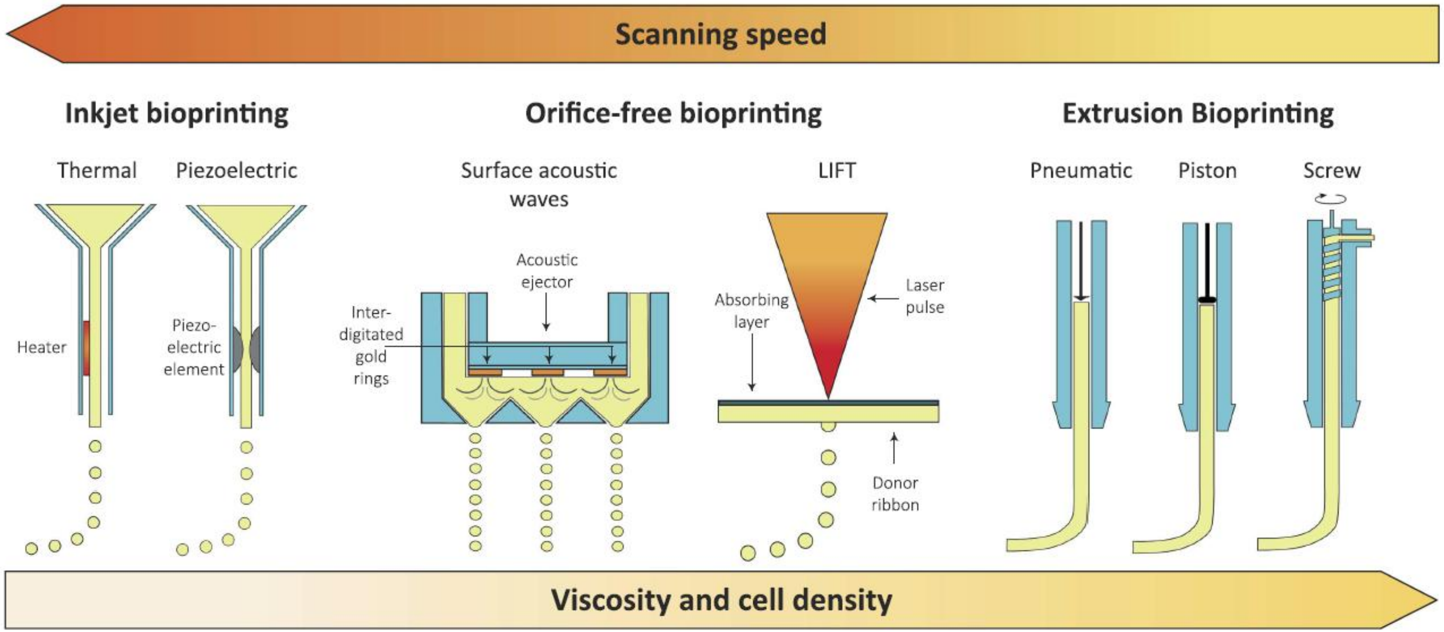
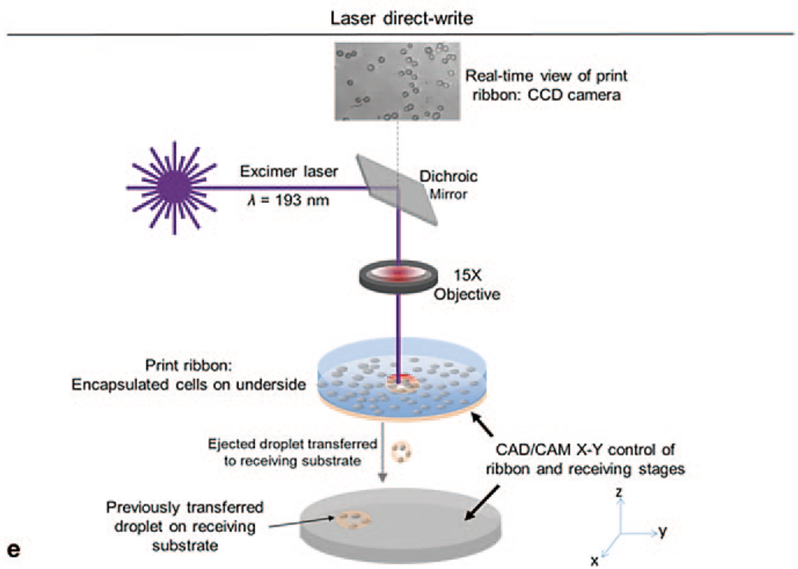


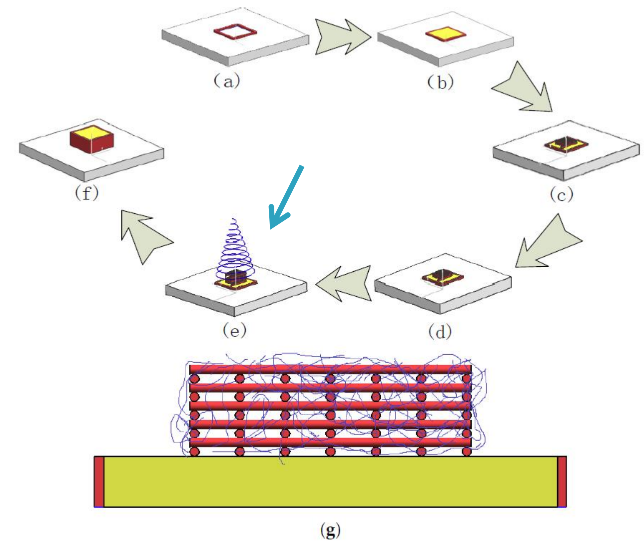
Figure 1. Overview of the most widespread bioprinting approaches and according parameters crucial for printability of the material.

Holzl, K., S. M. Lin, L. Tytgat, S. Van Vlierberghe, L. X. Gu, and A. Ovsianikov. "Bioink Properties before, During and after 3d Bioprinting." *Biofabrication* 8, no. 3 (Sep 2016).

Technologies…



Laser-Induced Forward Transfer

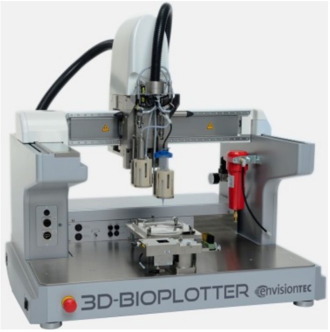


Electrospinning (e.g. Polycaprolactone - PCL)
Filament Extrusion

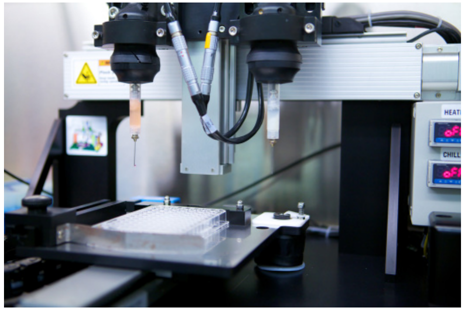
Lee, V. K. et al. *3D Bioprinting and 3D Imaging for Stem Cell Engineering*. Bioprinting in Regenerative Medicine. Edited by K. Turksen 2015. doi:10.1007/978-3-319-21386-6_1.

Sun, Y. S., Y. Y. Liu, S. Li, C. E. Liu, and Q. X. Hu. "Novel Compound-Forming Technology Using Bioprinting and Electrospinning for Patterning a 3d Scaffold Construct with Multiscale Channels." *Micromachines* 7, no. 12 (Dec 2016).

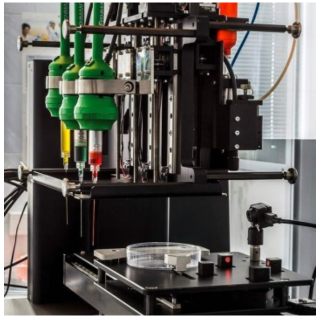
Commercial Bioprinters



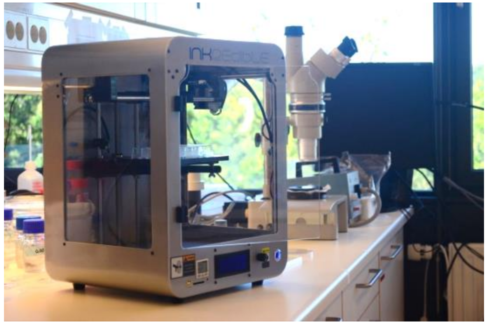
EnvisionTEC 3D-Bioplotter


Organovo's NovoGen MMX

RegenHU's 3DDiscovery + Biofactory

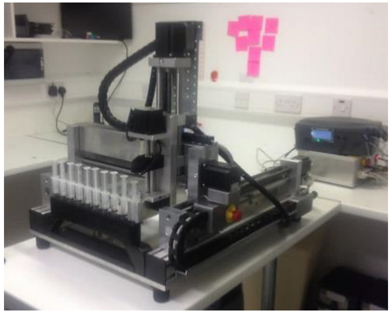

3D Bioprinting Solutions' FABION

BioBots BioBot


CELLINK Inkredible

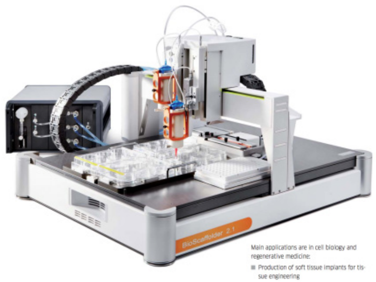
3dprintingindustry.com/news

Commercial Bioprinters…

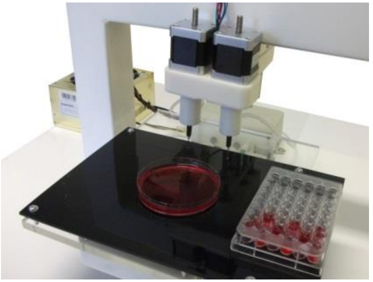


Ourobotics Revolution

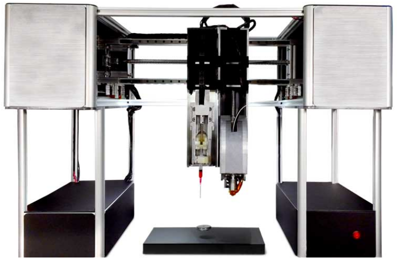
Advanced Solutions' BioAssemblyBot



GeSim's Bioscaffolder 2.1



3Dynamic Systems' Alpha & Omega



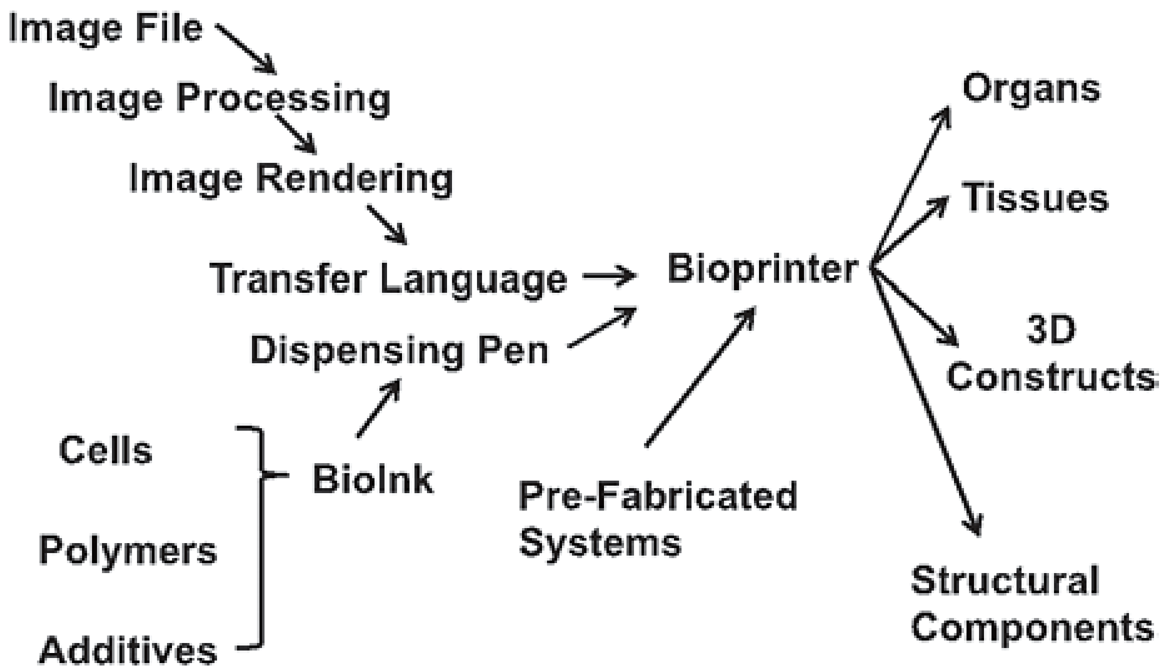
Bio3D's SYN^ and Explorer

Commercial Bioprinters…

Bioprinter and manufacturer	Fabrication technique	Specified resolution	Recommended materials
3Dn300TE, NScrypt	Extrusion-based	Line widths 20–100 μm	Not specified (viscosity range: 0.001–1000 Pa s)
3D-Bioplotter®, Envisiontec[a]	Extrusion-based	Minimum strand diameter 100 μm	Hydrogels, ceramic, metal pastes, thermoplasts
Bioscaffolder®, Gesim[a]	Extrusion-based	Not specified	Hydrogels, biopolymers (collagen, alginate) bone, cement paste, biocompatible silicones and metling polymers (CPL, PLA)
Biobot 1, Biobots[a]	Extrusion-based	Layer resolution 100 μm	Hydrogels, biopolymers (viscosity range: 100–10^4 Pa s, see table 3 for more details)
Inkredible+, Cellink[a]	Extrusion-based	Layer resolution 50–100 μm	Hydrogels (see table 3)
Biofactory®, RegenHU[a]	Extrusion-based Inkjet	Not specified	Bioink, Osteoink (see table 3 for more details)
Revolution, Ourobotics	Extrusion-based	Not specified	Collagen, gelatin, alginates, chitosan
Bio3D Explorers, Bio3D technologies[a]	Extrusion-based	Not specified	Not specified
CellJet Cell Printer, Digilab		Droplet size 20 nl–4 μl	Water-based, hydrogels, alginate, polyethylene glycol
BioAssemblyBot, advanced solutions	Extrusion-based	Not specified	Not specified
Regenova, Cyfuse	Spheroid assembly	Related to spheroid diameter	Cells only (scaffold/biomaterial-free approach)
NovoGen MMX, Organovo[b]	Inkjet	20 μm	Cellular hydrogels
Dimatix Materials Printer, Fujifilm	Inkjet	20 μm	Water-based, solvent, acidic or basic fluids
Poietis[b]	LIFT	20 μm	Not specified

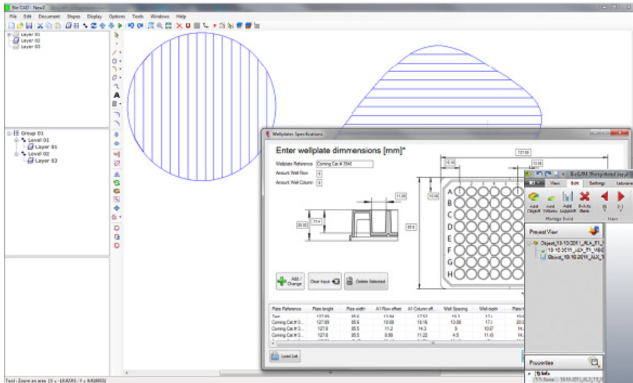
Holzl, K., S. M. Lin, L. Tytgat, S. Van Vlierberghe, L. X. Gu, and A. Ovsianikov. "Bioink Properties before, During and after 3d Bioprinting." *Biofabrication* 8, no. 3 (Sep 2016).

Components of Bioprinting

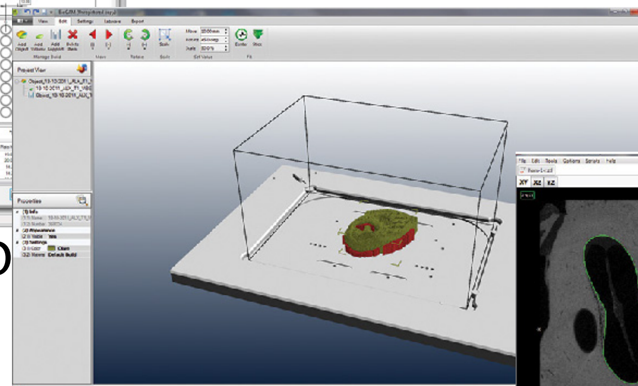


Williams, S. K., and J. B. Hoying. *Bioinks for Bioprinting*. Bioprinting in Regenerative Medicine. Edited by K. Turksen 2015. doi:10.1007/978-3-319-21386-6_1.

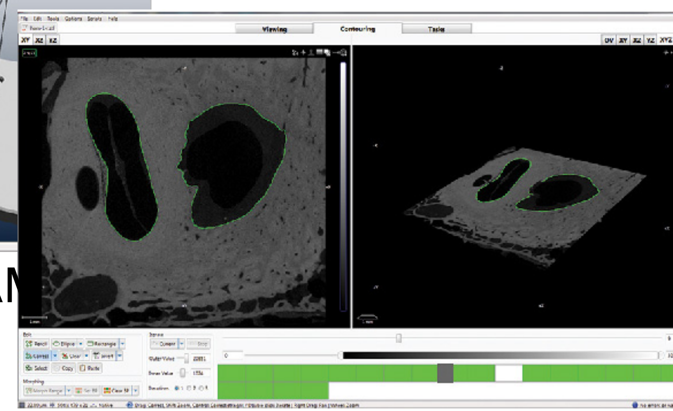
CAD Software



regenHU’s BioCAD



regenHU’s BioCAM



regenHU’s BioCUT

Images courtesy of regenHU

Embryonic Stem Cells (ESC)

- Embryonic stem cells (ESCs) are cells derived from the inner cell mass of the preimplantation blastocyst that retain the ability to differentiate into all three germ layers.
- Theoretically, ESCs are capable of being expanded in culture indefinitely, which is due to their active telomerase enzymes that prevent telomere shortening, senescence, and rapid apoptosis.
- No new ESC lines generated with federal funds as declared by the Dickey–Wicker amendment in 1996.
  - Currently, the excess unused eggs from in vitro fertilization (IVF) are the main source of new ESC lines in the USA.

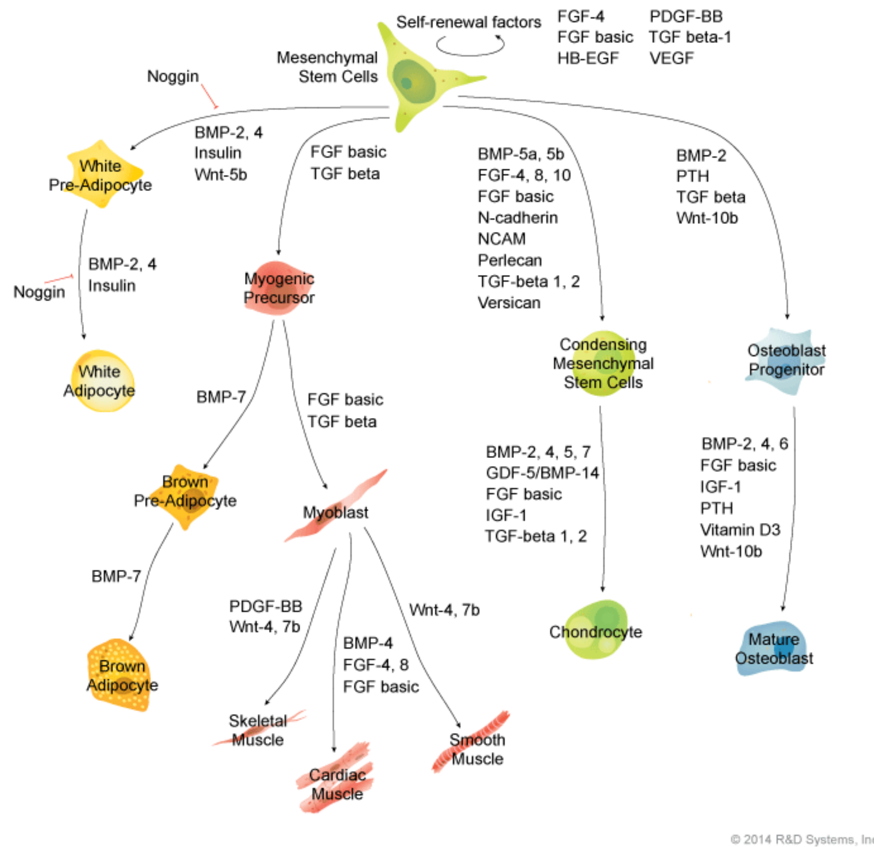
Anderson, C. W., et al. "Stem Cells in Cardiovascular Medicine: The Road to Regenerative Therapies." *Current Cardiology Reports* 19, no. 4 (Apr 2017).

Mesenchymal Stem Cells (MSC)

- MSCs are adult stem cells traditionally found in the bone marrow, but also present in cord blood, peripheral blood, fallopian tube, and fetal liver and lung.
- They are multipotent stromal cells that can differentiate into a variety of cell types, including: osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells, cardiomyocytes) and adipocytes (fat cells).
- Morphologically, MSCs have long thin cell bodies with a large nucleus.
- MSCs have a high capacity for self renewal while maintaining multipotency.
- Bone marrow derived (BMSCs) and adipose derived (ADMSC) cells may be useful in cardiovascular regenerative medicine.

R&D Systems, https://www.rndsystems.com/research-area/mesenchymal-stem-cells

Mesenchymal Stem Cells (MSC)…



White Adipocytes
Brown Adipocytes
Myocytes
Chondrocytes
Osteocytes

R&D Systems, https://www.rndsystems.com/research-area/mesenchymal-stem-cells

Induced Pluripotential Stem Cells (iPSCs)

- In 2006, Shinya Yamanaka described successful reprogramming of human somatic cells into a pluripotent state that was similar to ESCs in both its phenotype and transcriptome.
- This was accomplished by using retroviral transduction of what have become known as the Yamanaka factors (Oct3/4, c-MYC, Klf4, Sox-2).

Anderson, C. W., et al. "Stem Cells in Cardiovascular Medicine: The Road to Regenerative Therapies." *Current Cardiology Reports* 19, no. 4 (Apr 2017).

Commercially Available Bioinks

Company	Bioink	Material	Features
Bioink Solutions, Inc.	Gel4Cell®	Gelatin-based	UV-crosslinkable
			Cell viability >90%
	Gel4Cell®-BMP	Conjugated with different growth factors	Osteoinductive
	Gel4Cell®-VEGF		Angiogenic
	Gel4Cell®-TGF		Chondrogenic
CELLINK	CELLINK	Nano-cellulose/alginate mixture	Shear thinning
			Fast crosslinking
			For soft tissue engineering
RegenHU	BioInk®	PEG/gelatin/hyaluronic acid-based	Good cell adhesion properties
			Biodegradable
			Mimics the natural ECM
			Possible combination with Osteoink™
	Osteoink™	Calcium phosphate paste	Osteoconductive
			Chemical composition similar to human bone
			For hard tissue engineering
Biobot	Bio127	Pluronic F127-based	Gels at room temperature
			Dissolves when cooled
	BioGel	Gelatin Methacrylate based	When combined with GelKey it
			Covalently crosslinks when exposed to light

Holzl, K., S. M. Lin, L. Tytgat, S. Van Vlierberghe, L. X. Gu, and A. Ovsianikov. "Bioink Properties before, During and after 3d Bioprinting." *Biofabrication* 8, no. 3 (Sep 2016).

Bioinks

Table 1 Common Materials Used in Bioinks and Mechanism of Gel Formation

Compound	Mechanism gel formation	Chemical structure
Agar	Thermal	Polysaccharide
Collagen	Spontaneous gelation/ photoinitiation	Protein
Alginate	Ionic	Polysaccharide
PLGA-g-PEG	Thermal	Poly(lactic-*co*-glycolic acid)
PEGDMA	Thermal/chemical	Poly(ethylene glycol) dimethacrylate
Pluronic	Thermal	Poly(ethylene glycol)-poly(propylene glycol)-poly(ethylene glycol)
Agarose	Thermal	Polysaccharide
Carageenen	Thermal	Polysaccharide
Fibrin	Spontaneous gelation	Protein
Elastin	Photoinitiation	Protein
Silk	Photoinitiation	Protein
Chitosan	Chemical	Polysaccharide
Hyaluronic acid	Chemical	Glycosoaminoglycan
NIPAAM	Thermal	N-isopropyl acrylamide/N-t-butyl acrylamide copolymer

Williams, S. K., and J. B. Hoying. *Bioinks for Bioprinting*. Bioprinting in Regenerative Medicine. Edited by K. Turksen 2015. doi:10.1007/978-3-319-21386-6_1.

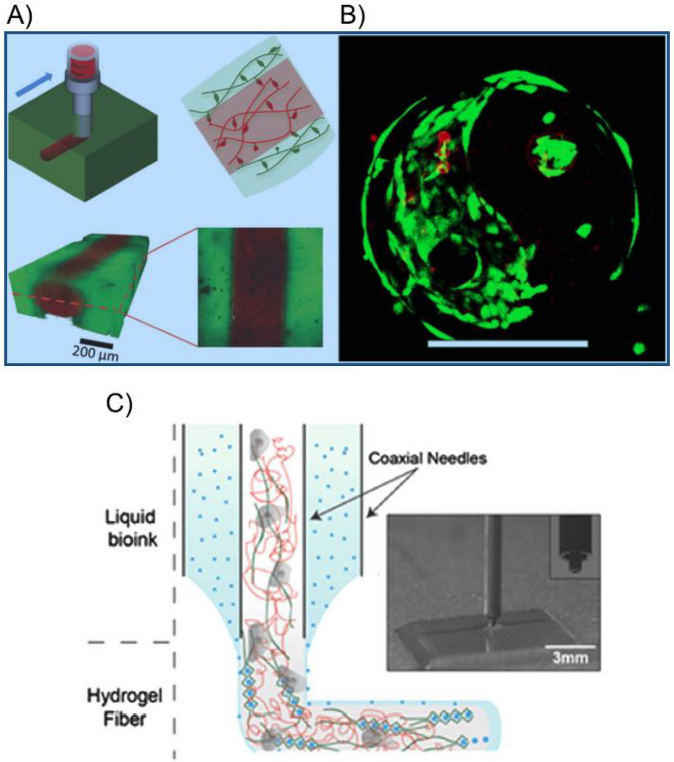
- Agar
  - Isolated from algae. Agar was the first gel used extensively in bioprinting (Wilson and Boland 2003)
  - Major components are agarose and agaropectin.
  - Ability to transition between a solution at high temperatures and forms a gel at lower temperatures. (Temperature will influence cell viability.)
- Agarose
  - Purified from agar.
  - Biocompatible
  - Various sol-gel temperatures.
  - Suitable for scaffolds that can be washed away by changing temperature.

Williams, S. K., and J. B. Hoying. *Bioinks for Bioprinting*. Bioprinting in Regenerative Medicine. Edited by K. Turksen 2015. doi:10.1007/978-3-319-21386-6_1.

- Alignate
  - Found in cell walls of brown algae and seaweed.
  - Binds 300 times its weight with water, forming a gum like material (e.g. dental impressions).
  - Used as a bandage, implantable material, encapsulation material, gel for 3D culture of cells and as a gel forming material for 3D bioprinting.
  - Ionic crosslinking (gel formation) can occur with various salts – e.g. $CaCl_2$ solutions.
  - Can be rapidly crosslinked through chelation of divalent cations by the carboxylic acid groups founds on adjacent strands of the component β– D –mannuronate or α– L –guluronate epimers.

Williams, S. K., and J. B. Hoying. *Bioinks for Bioprinting*. Bioprinting in Regenerative Medicine. Edited by K. Turksen 2015. doi:10.1007/978-3-319-21386-6_1.

Extruding Hydrogels & Alginate



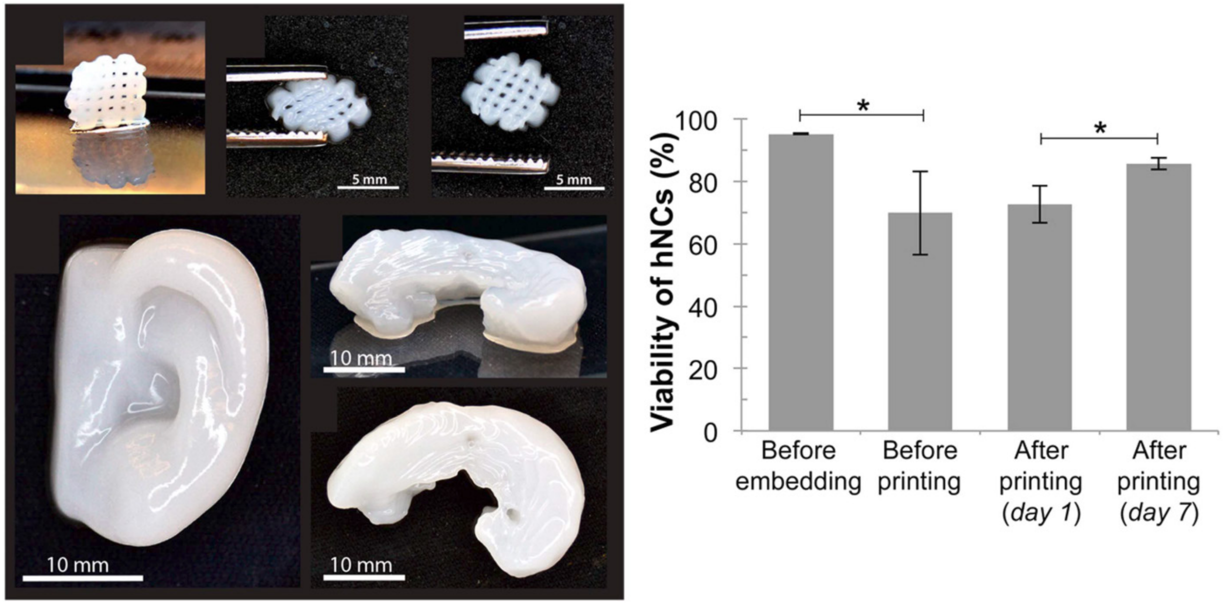
(A)Extrusion of a shear-thinning bioink into a self-healing support hydrogel allowing printing of high-resolution and multimaterial structures encapsulating cells.
(B)Proliferation of cells in a construct printed using two-photon polymerization, scale bar 200 µm(right).
(C)Bioprinting using coaxial needle system. The inner needle contains the bioink consisting of gelatin methacroyl (red dashed lines), alginate (green lines), photoinitiator and cells. The outer needle contains the CaCl2 (blue dots), which induces gelation after mixing.

A. Highley C et al. 2015 Direct 3d printing of shear-thinning hydrogels into self-healing hydrogels Adv. Mater. 27 5075-9

B. Ovsianikov A et al 2014 Laser photofabrication of cell containing hydrogel constructs Langmuir 30 3787-94

C. Wu W, et al. 2011 Omnidirectional printing of 3d microvascular networks Adv. Mater. 23

Nanofibrillated Cellulose/Alginate Bioink



(A) 3D printed constructs of the nanofibrillated cellulose/alginate bioink that show stability in size and shape.
(B) Viability of hNSCs before and after the printing process.

Markstedt K, et al. 2015 3D bioprinting human chondrocytes with nanocellulose-alginate bioink for cartilage tissue engineering applications Biomacromolecules 16 1489-96

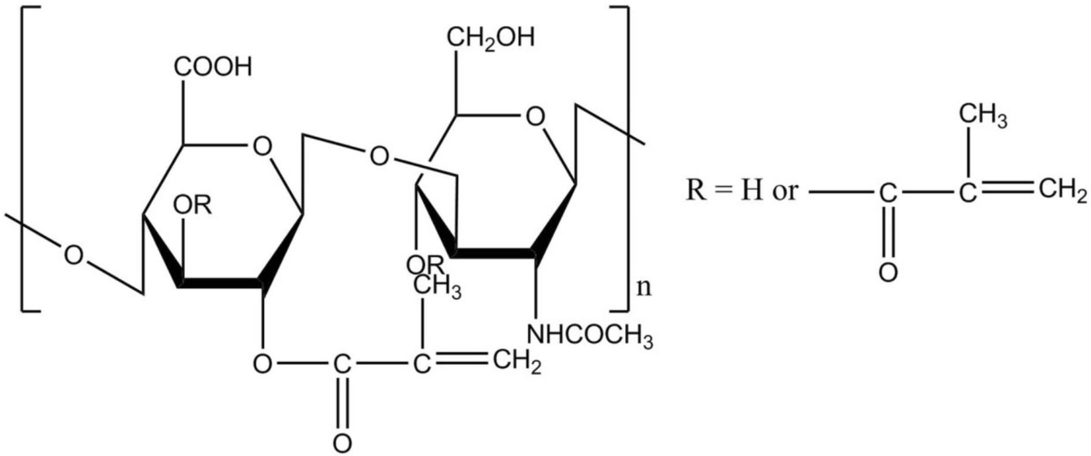
- Chitosan
  - Obtained from shrimp and other crustacean shells
  - Extrusion followed by chemical crosslinking with NaOH.
  - Scaffolds can be created by photopolymerization.
- Carrageenan
  - Seaweed derived – contains sulfur groups.
  - Porosity of scaffolds during gelation can be controlled to support cellular ingrowth.
- Collagen
  - Most abundant protein in the body (28 types, and types I to V are most common):
    - Type I or "Mature" collagen is found in skin, tendon, vascular ligature, organs, bone. This collagen is found in most scars after wounding.
      - Most common type used for gel formation.
      - Undergoes fibrillar collagen formation at 37°C and neutral pH.
      - Functionality is derived from various constituents including ions, peptides, proteins and the extracellular matrix proteins.

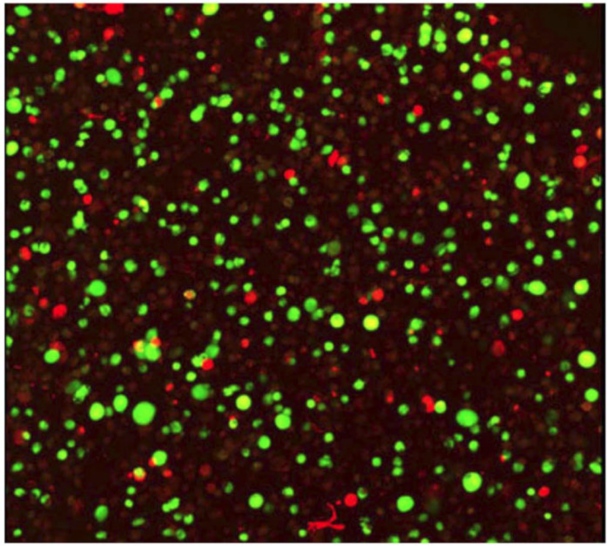
Williams, S. K., and J. B. Hoying. *Bioinks for Bioprinting*. Bioprinting in Regenerative Medicine. Edited by K. Turksen 2015. doi:10.1007/978-3-319-21386-6_1.

- Gelatin
  - Collagen that has been subjected to complete (usually thermal) hydrolysis.
- Hyaluronic Acid
  - Predominantly in connective, epithelial, and neural tissues.
  - High molecular weight >1 million.
  - Useful for scaffolds and subsequent treatment gels,
  - Used for heart valves.
  - Stable structures often use chemical or photo-crosslinking.
- Silk
  - Protein fiber composed mainly of fibroin and is produced by many insect larvae during the formation of cocoons.
  - Used for scaffold construction.

Williams, S. K., and J. B. Hoying. *Bioinks for Bioprinting*. Bioprinting in Regenerative Medicine. Edited by K. Turksen 2015. doi:10.1007/978-3-319-21386-6_1.

Methacrylated Hyaluronic Acid

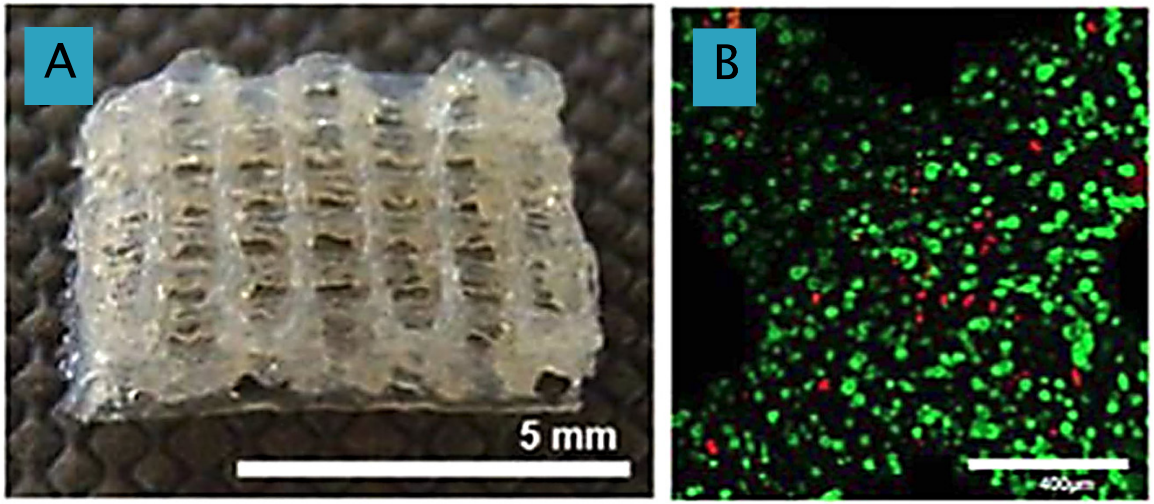




Live/dead staining results indicated that encapsulated cardiac valvular interstitial cells in methacrylated hyaluronic acid hydrogels remained viable after 1 week of cell culture

Masters K.S at al. 2005 Crosslinked hyaluronan scaffolds as a biologically active carrier for valvular interstitial cells Biomaterials 26 2517–25

Silk-Fibroin Construct



(A) 3D bioprinted silk-fibroin construct
(B) Live/dead staining of hTMSCs encapsulated in the hydrogel

Das S, Pati F, et al. 2015 Bioprintable, cell-laden silk fibroin-gelatin hydrogel supporting multilineage differentiation of stem cells for fabrication of three dimensional tissue constructs Acta Biomater. 11 233-46

- Fibrin and Fibrinogen
  - Fibrin is formed when thrombin acts on fibrinogen in the body as part of the clotting mechanism.
  - Fibrous fibrin may have spontaneous gel formation.
  - Biocompatible and known cell and endothelial interactions.
  - Gels may undergo neovascularization.
- Elastin
  - Naturally occurring extracellular matrix protein.
  - May undergo transition between a coiled and elongated form.
  - Provides elasticity to tissues such as skin and large caliber blood vessels.
  - Scaffolds can be obtained from tissues samples using both enzymatic and chemical de-cellularization.
  - Often co-printed with collagen or other materials that provide spontaneous or chemically augment cross-linking and gelation.

Williams, S. K., and J. B. Hoying. *Bioinks for Bioprinting*. Bioprinting in Regenerative Medicine. Edited by K. Turksen 2015. doi:10.1007/978-3-319-21386-6_1.

- Hydrogels – Generally
  - Able to withstand extrusion, maintain structural fidelity, and permit adequate nutrient diffusion.
  - Biocompatible
  - Intrinsic porosity and capacity for high nutrient loading.
  - Gelation can be triggered by chemical bonding, photoactivated crosslinking, thermal setting, or shear-thinning.

Armstrong, J. P. K., et. al. "3d Bioprinting Using a Templated Porous Bioink." *Advanced Healthcare Materials* 5, no. 14 (Jul 2016): 1724-30.

- Thermosensitive Hydrogels
  - Temperature sensitive transition between a solution and gel form.
  - Ability to undergo transition from solution to gel at either a lower critical transition temperature (LCST) or upper critical transition temperature (UCST).
  - Ability to undergo repetitive gel to solution to gel transitions.
  - e.g. Poly(N-isopropylacrylamide) or PNIPAM (an LCST) can be bioprinted as a gel at temperatures below 32°C and then converted to a solution at temperatures above 32°C. Another LCST is poly(N-isopropylacrylamide) or PIPAAm.
  - Sheets of cells can be grown on the thermosensitive polymer gel at higher temperature and then the cell sheet can be released by simply lowering the temperature below 32°C.

Williams, S. K., and J. B. Hoying. *Bioinks for Bioprinting*. Bioprinting in Regenerative Medicine. Edited by K. Turksen 2015. doi:10.1007/978-3-319-21386-6_1.

- PEG Hydrogels
  - Synthetic crosslinked hydrogels of poly(ethylene) glycol (PEG).
  - PEG is also known as polyethylene oxide (PEO) or polyoxyethylene (POE), depending on the molecular weight.
  - Biocompatible
  - PEGylation is the process of attaching the strands of the polymer PEG to molecules, most typically proteins, drugs and antibodies, with the result of increased solubility and reduced immunogenicity.

Williams, S. K., and J. B. Hoying. *Bioinks for Bioprinting*. Bioprinting in Regenerative Medicine. Edited by K. Turksen 2015. doi:10.1007/978-3-319-21386-6_1.

- Poloxamers
  - Manufactured by BASF Corp. as *Synperonics*, *Pluronics* or *Koliphore.*
  - Triblock copolymers of poly(propylene oxide/PPO and poly(ethylene oxide/PEO.
  - In aqueous solutions they form micellar structures above critical micellar concentration.
  - Property of thermoreversible gelation. Pluronic127 is a liquid when refrigerated (4–5°C) and turns into gel form when brought to room temperature (> 16°C).
  - Useful for drug delivery systems and removable forms for making channels and voids.

Williams, S. K., and J. B. Hoying. *Bioinks for Bioprinting*. Bioprinting in Regenerative Medicine. Edited by K. Turksen 2015. doi:10.1007/978-3-319-21386-6_1.

Additives to Influence Cellular Behavior

- Incubation occurs either in vitro or in vivo, with resulting changes in cellular function:
  - Proliferation
  - Migration
  - Differentiation
  - Apoptosis
  - Self assemble
- Cellular activities are regulated by:
  - Soluble factors such as growth factors and cytokines.
  - The extracellular matrix proteins.
- The addition of Matrigel (a reconstructed basement membrane protein) to bioinks is an appropriate first step in identifying whether a complex mixture of components/additives can support desired cellular function in the printed structures.

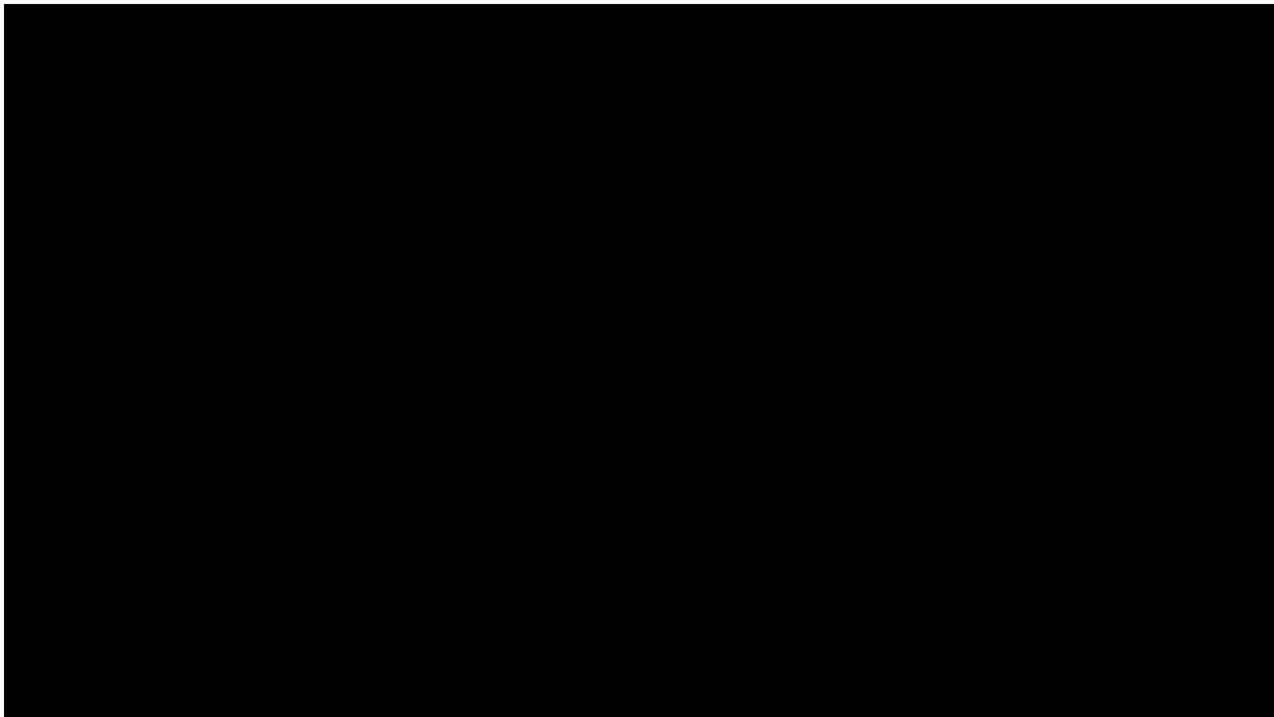
Williams, S. K., and J. B. Hoying. *Bioinks for Bioprinting*. Bioprinting in Regenerative Medicine. Edited by K. Turksen 2015. doi:10.1007/978-3-319-21386-6_1.

Chemical and Photo Cross Linkers

- Bioprinting of specific shapes, e.g. spheroid, rod and tube, require inherent viscosity or crosslinking (physical or chemical) for stabilization.
- Chemical cross linkers:
  - Glutaraldehyde (for collagen, possible toxicity)
  - Genipin (crosslink functional amine groups with little toxicity)
  - 1-Ethyl-3-(3 dimethylaminopropyl)
  - Carbodiimide/N-hydroxysuccinimide (EDC/NHS)
  - Tetrahedral polyethylene
  - Glycol
- Photoactivated cross linkers:
  - Irgacure (e.g. 2959, requires UV light at 276 nm) and Darocur are tradenames for a family of photoinitiator chemistries. These are Type 1 (cleavage) photoinitiators.
  - UV light can cause cell death and mutagenesis.
  - There are visible light (Type II) photoinitiators under development.

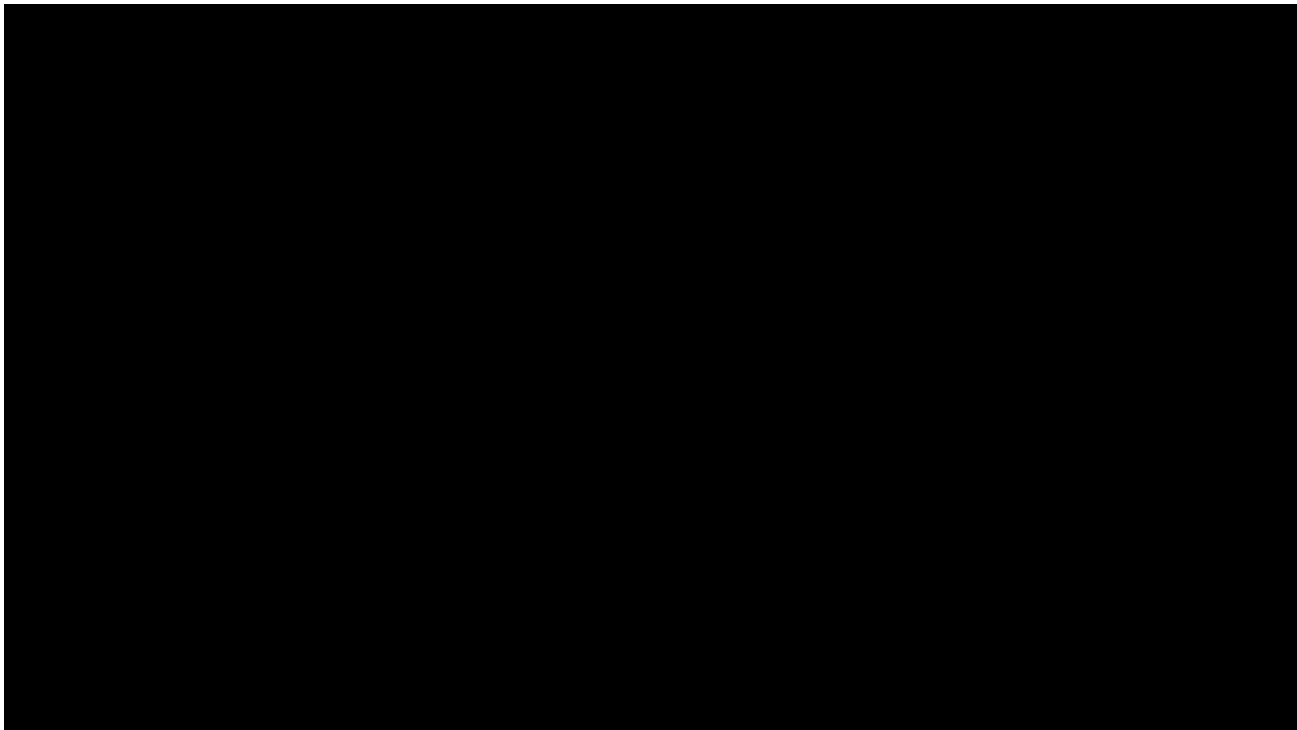
Williams, S. K., and J. B. Hoying. *Bioinks for Bioprinting*. Bioprinting in Regenerative Medicine. Edited by K. Turksen 2015. doi:10.1007/978-3-319-21386-6_1.

Bioprinters: BioBots 3D Printer



Run 5:45 of16:12

Organovo



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Summary

- Bioprinting Techniques
  - Inkjet
  - Extrusion
  - Laser Forward Transfer
  - Electrospinning
- Commercial Bioprinters
- Embryonic Stem Cells (ESC), Mesenchymal Stem Cells (MSC), Induced Pluripotential Stem Cells (iPSCs)
- Bioinks
- Additives to Influence Cellular Behavior
- Chemical and Photo Crosslinking