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3D Printing of Tissue Engineered Constructs for *in vitro* Modeling of Disease Progression and Drug Screening

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Abstract

2D cell culture and preclinical animal models have traditionally been implemented for investigating the underlying cellular mechanisms of human disease progression. However, the increasing significance of 3D versus 2D cell culture has initiated a new era in cell culture research in which 3D *in vitro* models are emerging as a bridge between traditional 2D cell culture and *in vivo* animal models. Additive manufacturing (AM, also known as 3D printing), defined as the layer-by-layer fabrication of parts directed by digital information from a 3D computer-aided design (CAD) file, offers the advantages of simultaneous rapid prototyping and biofunctionalization as well as the precise placement of cells and extracellular matrix with high resolution. In this review, we highlight recent advances in 3D printing of tissue engineered constructs (TECs) that recapitulate the physical and cellular properties of the tissue microenvironment for investigating mechanisms of disease progression and for screening drugs.

Keywords

Additive Manufacturing; 3D printing; bioprinting; drug screening; tissue engineered constructs

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Introduction

For decades biologists have relied on a combination of cell culture and mouse models for elucidating the underlying cellular mechanisms that lead to human diseases. While these models have their limitations, they have enabled major discoveries and greatly improved understanding of diseases. However, traditional cell culture and mouse models are limited in their ability to recapitulate the complex tissue microenvironment (μ EN). Thus, the increasing recognition of the physical and cellular properties of the μ EN as a significant factor contributing to disease progression has motivated the investigation of new techniques for fabrication of more complex 3D *in vitro* models.

Preclinical animal models are an established approach for studying the mechanisms of disease progression. However, the clinical translatability of animal models to human disease has been questioned, since the μ EN in animals differs from that in humans. The efficacy and toxicity of drugs evaluated in animal studies do not always predict the response in human patients. For example, liver toxicity of experimental drugs that was not discovered in preclinical studies is a leading cause of clinical trial failures.⁶⁴ Animal models also present uncontrollable variables that limit quantitative analysis of the mechanisms of interest in understanding disease progression at the molecular level. Furthermore, the cost and ethical concerns associated with animal testing have motivated research on less expensive, higher throughput, and more humane alternatives.⁵

Cells more closely mimic *in vivo* behavior when grown in 3D conditions, and a number of 3D culture systems have been reported that more accurately predict the cellular response compared to 2D culture.^{18,34,37,80,87,88,91} Tissue Engineered Constructs (TECs) have emerged as more physiologically relevant 3D *in vitro* models of organogenesis, disease progression, and drug screening due to their ability to recapitulate the physical and cellular properties of the tissue μ EN. Despite the advances in TECs utilizing conventional scaffold techniques, the inability of traditional methods to reproducibly fabricate structures with precise architectural features and spatial location of cells has motivated the search for new alternatives. Additive manufacturing (AM, also known as 3D printing), defined as the layer-by-layer fabrication of parts directed by digital information from a 3D computer-aided design (CAD) file⁷, offers the advantages of simultaneous rapid prototyping and biofunctionalization as well as the precise placement of cells and biomaterials with high resolution. In this review, we highlight recent advances in AM technology for 3D printing of TECs to investigate mechanisms of disease progression and screen drugs.

3D Tissue-Engineered Constructs (TECs) for Modeling Disease Progression and Drug Efficacy

3D *in vitro* models have been proposed as a bridge between cell culture and *in vivo* modeling⁴⁶ and even between animal modeling and human trials.²⁶ Early studies with 3D culture largely involved either explanted host tissue or natural biopolymers such as collagen, cell-extracted native extracellular matrix, or polyacrylamide gels. The explanted tissue models assumed very simple architectures, often layers less than 1 mm thick, that allowed for cells to migrate in 3D.⁸⁷ However, the use of native tissues *in vitro* often necessitates

arduous extraction and preparation techniques that limits their ease of application. The need for more precise control over mechanical, chemical, and surface properties, as well as simple preparation techniques has engendered a new approach for developing TECs. While initial studies targeted *in vivo* implantation of TECs in regenerative medicine applications, TECs have recently been investigated as *in vitro* tools for understanding molecular mechanisms and testing drug candidates prior to human trials. These *in vitro* models have potentially far-reaching effects in the fields of drug development and molecular biology that could reduce the need for organ and tissue implants.²⁵

Conventional scaffold synthesis methods employing synthetic and natural polymers include gas foaming, freeze-drying, phase separation, particulate leaching, liquid molding, fiber bonding, and electrospinning.⁴¹ These techniques have been used to create scaffolds with rigidity, strength, surface properties, porosities, and degradation kinetics targeted to host tissue.^{12,29} Spheroid cell culture has also been utilized to simulate *in vivo* cell morphology and behavior by facilitating aggregation of cells in a non-anchored environment. Spheroid culture is achieved either by using attachment-resistant cell culture surfaces with constant agitation, or by culturing cells in droplets that are hanging from a surface, also known as the hanging drop technique.⁴⁷ Spheroid cultures of tumor cells form hollow cores in which the central cells experience a hypoxic environment and become quiescent, similar to how tumor cells in the necrotic core behave *in vivo*.³ Spheroid cultures have exhibited proper differentiation abilities and chemical gradients, as well as the ability to resemble avascular tumor nodules and micrometastases.³⁵ A significant limitation of spheroid culture is its inability to control spheroid size and architecture.⁴⁷

Considerable progress has been made in designing scaffolds specifically for *in vitro* models using conventional synthetic scaffolds and spheroid cell culture to mimic a variety of tissue and organ types. A skin-replicating model has been developed to model melanoma for studying disease mechanisms and drug screening utilizing collagen scaffolds and tumor cell spheroids.⁸⁵ A liver-like model utilizing an immortalized hepatoma cell line and ECM-derived hydrogel has been reported to not only recover hepatocyte function in 3D but also provide a means of high-throughput screening of drugs to assess liver toxicity *in vitro*.⁶⁷ Other *in vitro* models have been developed for organs including bone⁴, myocardium¹⁶, trachea⁵⁶, vessels⁸⁹, nerves⁵¹, cartilage¹⁷, and cornea.¹

Despite these successes, traditional synthesis methods still lack control of some essential factors contributing to *in vivo* cellular function, in particular the tissue micro-architecture and precise placement of cells within the construct. The architecture in the tissue milieu of interest has proven to play a large role in cell differentiation, proliferation, metabolic activity, and motility.^{31,57,72} Thus, creating the appropriate architecture in addition to the chemical and mechanical properties of the specific tissue is necessary if *in vivo* conditions are to be accurately simulated.

Fabrication of 3D Scaffolds by Additive Manufacturing (AM)

Since the advent of AM in the late 1980s, many techniques have been developed to create micro-scale precision 3D structures from a variety of source materials. In addition to the

plethora of techniques, the industry's Moore's law-type increase in speed, precision, and cost reduction has made it an appealing tool to create TECs with defined mechanical, topological, and cellular properties. Anatomically accurate models can be reliably and economically created by using medical imaging in conjunction with the computer-aided design (CAD) technology employed by most AM machines. The ability to create structures that can be tailored to an individual patient's anatomy through medical imaging has made AM an emerging regenerative medicine approach for organ replacement. However, AM is also being increasingly applied to create *in vitro* models of disease progression and drug screening, which has potential to advance drug discovery.

Current Methods of Scaffold Synthesis via AM

AM, also known as rapid prototyping (RP), layer manufacturing, 3D printing, and solid freeform fabrication, refers to the fabrication of parts by addition of layers that is directed by a CAD file, most frequently in the stereolithography (STL) file format created by 3D Systems.⁷ It can be inferred from this broad definition that there are many ways to achieve layer-by-layer manufacturing, and indeed there are a growing number of commercially available techniques. An American Society of the International Association for Testing and Materials (ASTM International) committee categorized AM techniques into seven groups: binder jetting, directed energy deposition, material extrusion, material jetting, powder bed fusion, sheet lamination, and vat polymerization.⁶ While these terms describe the general techniques used, not all are applicable to biomaterials applications. Currently, four AM technologies have been predominantly used for TECs: stereolithography, fused deposition modeling, material jetting (inkjetting), and bioprinting.

Stereolithography

Stereolithography (SLA) is widely considered the first AM technique developed.⁴⁰ SLA is a form of vat polymerization that utilizes photopolymerization to solidify a liquid monomer resin layer by layer. The liquid monomer is contained in a vat with a moveable base such that a UV laser, directed by a CAD file, selectively "draws" a layer of the desired part onto the liquid in the container. The UV light crosslinks the monomer to solidify the polymer in the location of UV exposure. After a layer is finished, the base drops the height of the previously polymerized layer and the process repeats. The base-lowering step is often accompanied by the sweep of a recoater blade to ensure there is a fresh, uniform layer of liquid monomer. Thus the part is built bottom-up, usually accompanied by support material to hold the part in place and prevent lateral deformation during the blade sweep or vertical deformation due to gravity. After completion, the part is washed in a chemical bath to remove any uncured monomer resin, the support material is removed, and the resulting part is post-cured in a UV oven.

While the bottom-up approach is the most abundantly used technology, an increasing number of SLA machines utilize a top-down approach in which the UV light is directed upward from under the vat and the base moves up as each layer is finished. Top-down manufacturing has multiple advantages, including a smooth polymerization surface, elimination of the need for a recoater blade, reduction in the volume of monomer, and elimination of atmospheric exposure, which mitigates oxygen inhibition.⁵⁹ Another

advancement in SLA technology, called microstereolithography (μ SLA), can achieve sub-micron features using focused light spot scanning. The major drawback to this process is that many photo masks must be used in parallel, which makes processing time and cost unfeasible. However, the use of a single dynamic mask (normally in the form of a digital micromirror display (DMD)) and a reducing lens has achieved sub-micron features at speeds comparable to normal SLA.⁷⁹

The lack of available photo-sensitive resins useable in SLA machines has been considered one of the limitations of the technology. In addition to being capable of photopolymerization, the resin must be a low-viscosity liquid. Despite these restrictions, SLA has found use in biomedical applications as a means to create patient-specific models of body parts for use as surgical guides, implantable devices, and tissue engineered grafts. Major advancements in SLA resins have made this shift toward biomedical devices possible. TECs fabricated from biodegradable resins such as poly(propylene fumarate), poly(ϵ -caprolactone), and poly(lactide) have been successfully evaluated in animals and shown to support cell adhesion and growth *in vitro*.⁵⁹ These resins have also been augmented with ceramic particles such as β -tricalcium phosphate (β -TCP) or hydroxyapatite to fabricate bone-like TECs.⁹ Despite the advances in polymer resins available to SLA technology, the major limitation to the progress of SLA in tissue engineering applications is its restriction to a single resin per structure.⁵⁹

Fused Deposition Modeling

Fused deposition modeling (FDM)¹⁴ is a material extrusion technique in which a filament is drawn through a computer-guided nozzle, heated above the glass transition temperature (T_g), extruded, and deposited in a layer-by-layer fashion to construct the part in a bottom-up fashion. Material is drawn by two rollers into a heating element, and the resulting semi-molten filament is then extruded through the nozzle tip and deposited. By remaining intact, the filament creates “roads” of deposited material rather than individual “dots”. Multiple parameters, including FDM head speed, nozzle tip width, roller speed, direction of deposition, and material temperature, can be adjusted to tailor the resulting product to desired specifications.⁹⁰ Furthermore, multiple materials may be used in a single print by the addition of separate nozzles and filaments to allow for more versatility in design. In most commercial cases, a second material is used as support material to build support for any structure on the part that exhibits an overhang angle less than 45° .² While the primary use of a second material is for support, this also allows for multi-composition structures with different chemical and mechanical properties for the construction of hierarchical designer scaffolds.³⁶

Fused deposition modeling most commonly employs thermoplastics with relatively low T_g and high thermal stability. Polylactic acid (PLA), polyamide (PA), acrylonitrile butadiene styrene (ABS), and polycaprolactone (PCL) are commonly used FDM resins.⁹⁰ Metal wires, metal-polymer composites, and ceramic-polymer composites have also been utilized.⁷ This versatility in resins and capability for use of multiple materials renders FDM an appealing method of scaffold generation for tissue engineering. Multiple groups have created TECs to not only mimic tissue architecture, but also impart appropriate mechanical properties.⁴² The

major limitation of FDM is its low resolution compared to SLA and inkjet printing. Because FDM relies on semi-molten “roads” of material opposed to drops, the resolution is inherently lower and susceptible to surface imperfections in the resulting part. Therefore, FDM is limited to applications where a tolerance $>100\ \mu\text{m}$ is acceptable.²²

Material Jetting (Inkjet Printing)

Material jetting is analogous to 2D inkjet printing, in which a CAD-guided nozzle deposits an ink that is heated just past its melting temperature onto a solid base. Upon deposition, the material cools and solidifies in place. Once a layer is complete, the base moves down the height of a single layer and the process continues in a bottom-up fashion until completion. There are two well-established means of depositing the ink droplets: continuous and drop-on-demand (DOD) modes.²³ In the continuous mode, a constant pressure is applied to the ink chamber such that a steady stream of the fluid exits the nozzle. Upon exiting the nozzle, the stream forms droplets due to Rayleigh scattering. In DOD mode, an actuator creates pulses of pressure such that droplets are formed at the exit of the nozzle, and the frequency of pulses can be adjusted. The DOD mode is the most preferred due to the smaller drop size and placement accuracy.²³ Due to the temperature constraints required by material jetting, the type and number of useable materials is fairly limited compared to other AM techniques. Waxy, low molecular weight polymers and acrylate photopolymers are most often used in inkjet printing, which often limits the technique’s biomaterials applications to mold castings for use as an indirect means of scaffold generation.

Bioprinting

In contrast to the previously described AM methodologies, the term “bioprinting” is more conceptual and is not restricted to a specific technology. Bioprinting has been defined as “the use of computer-aided transfer processes for patterning and assembling living and non-living materials with a prescribed 2D or 3D organization in order to produce bio-engineered structures serving in regenerative medicine, pharmacokinetic and basic cell biology studies.”^{28,30} Multiple AM technologies have been applied to bioprinting, which is also referred to as bioplotting, cell writing, laser-assisted bioprinting, and microextrusion.^{15,48} Maintaining cell viability during and post-printing is the essential requirement for bioprinting that limits the AM techniques that can be feasibly used. Consequently, it is imperative that sterility of materials and equipment can be maintained during the printing process. Equipment and materials must be capable of being sterilized, most commonly by autoclave, UV light, or ethanol treatment, and it is common to conduct the bioprinting within a laminar flow hood to promote a clean environment. Further, sterility tests are often conducted by printing cell-containing material and culturing in antibiotic-free media.⁶⁹ SLA, material jetting, and material extrusion are the three ASTM-defined techniques that have been proven capable of bioprinting. New AM techniques not defined by ASTM International, known as laser-guided direct cell printing and laser-induced direct cell printing, utilize a laser to guide and deposit cells onto substrates.⁶² System temperature, pressure, surrounding media, matrix or scaffold composition, and associated chemical and solvents are all parameters that must be considered to optimize cell viability. New approaches have been developed to address these restrictions, such as nozzles that use piezoelectric actuators to create droplets to alleviate thermal stress, and the use of near-

infrared lasers to limit the risk of overheating cells in the laser-guided technique.^{48,81} Cell media must also be supplied before, during, and after printing to ensure sufficient cell nutrition, which has limited the matrix to hydrogels such as Matrigel and PEG gels that are cyto-compatible and nano-porous. Use of metals, ceramics, and composite materials with high mechanical properties are thus restricted to cell-seeding after scaffold preparation as opposed to direct bioprinting.⁸¹ While bioprinting is growing both in efficiency and complexity of constructs, the inability of this approach to fabricate sophisticated tissue architectures is a significant limitation.

3D Models of Disease Progression and Drug Screening

The design of TECs that mimic the μ EN, facilitate studies on the spatio-temporal dynamics of disease progression, and assess drug response has been recognized as a pressing need.⁷⁴ Compared to more conventional fabrication techniques, advances in AM have enabled more precise control over topological properties⁸⁴ such as porosity, pore size, pore shape, and curvature, as well as precise placement of cells.⁸⁸ Consequently, 3D printing is emerging as a powerful tool for recapitulating the mechanical, topological, and cellular properties of both hard and soft tissue. In this section, the design of 3D-printed TECs for modeling disease progression and drug response in a variety of tissue types are reviewed.

Hard Tissue Models

Bone—In a transformative study, viable human bone grafts that recapitulate the temporomandibular joint (TMJ) were fabricated from decellularized trabecular bone.²⁴ Clinical CT images of the joint were digitized and imported into a computer-aided design (CAD) tool, which was used to machine a TMJ-shaped graft. Human mesenchymal stem cells (MSCs) were cultured on the grafts in a perfusion bioreactor for 5 weeks, resulting in the formation of lamellar bone. While this study represented an important step toward *in vitro* culture of patient-specific bone grafts, recent advances in AM have aimed to fabricate clinically sized, anatomically shaped bone grafts from synthetic materials. In a follow-up study, anatomically shaped PCL scaffolds were printed by FDM from CT scans of the maxilla and the mandible in human patients (Fig. 1A).⁸³ AM has also been applied to manufacture TECs in which a scaffold and bioreactor chamber are fabricated simultaneously as a custom-designed device designed to match a patient's anatomy (Fig. 1B).¹³ A 3-cm section of an ovine tibia was imaged by μ CT, from which a computational 3D model was generated that captured the anatomical features of the tibia. Using a CAD tool, a shell wall was then created that enabled fluid flow. The resulting device was printed by FDM in a single step using two thermoplastic polymers: poly(lactic acid) (PLA) for the scaffold and acrylonitrile butadiene styrene (ABS) for the bioreactor (Fig. 1B). Primary human osteoblasts were dynamically seeded in the bioreactor and cultured under bi-directional perfusion. The cells maintained viability for up to 6 weeks.

Trabecular bone is differentiated from other tissues by its rod- and plate-like trabeculae spaced 600 – 800 μ m apart and its rigid mineralized ECM (93 - 365 MPa⁷⁵), which is several orders of magnitude higher than that of soft tissue.⁷¹ The progression of tumor-induced bone disease has been modeled *in vitro* using 3D scaffolds for metastatic breast

cancer⁵⁸, prostate cancer¹⁹, and Ewing sarcoma.²⁰ In these studies, cell culture on collagen or polymer scaffolds demonstrated that the 3D microenvironment substantially alters the tumor response to anti-cancer drugs compared to 2D monoculture. However, the substrate modulus and pore size of these scaffolds fabricated by conventional methods are generally not representative of trabecular bone. A templated-Fused Deposition Modeling (t-FDM) approach has recently been reported for fabrication of 3D scaffolds with tunable substrate moduli and pore sizes representative of trabecular bone (Fig. 1C).³¹ While pore sizes > 100 μm can be printed using FDM, the small number of thermoplastic polymers that can be printed limits the range of substrate moduli that can be achieved. In the t-FDM approach, a template is printed by FDM which is subsequently filled with a two-component reactive poly(ester urethane) with substrate moduli ranging from 20 MPa (collagen fibrils) to 266 MPa (trabecular bone) (Fig. 1C). Thus, the t-FDM method enables independent control of mechanical and topological properties. Rat bone marrow-derived mesenchymal stem cells (MSCs) were cultured on the 3D scaffolds for up to 21 days. Expression of markers of osteogenic differentiation, including the transcription factor *Runx2*, increased with increasing substrate rigidity and decreasing pore size. Furthermore, matrix mineralization increased with increasing substrate modulus and decreasing pore size, as assessed by Alizarin red staining and SEM imaging. Taken together, these findings highlight the utility of FDM methods for fabricating TECs for use in regenerative medicine and mechanistic studies investigating the effects of the μEN on cell fate.

Cartilage—Healing of damaged cartilage is often incomplete due to lack of vascularity and low cell density in cartilage tissue. Consequently, TEC approaches have aimed to promote cartilage regeneration through *ex vivo* engineering of cartilage tissue for implantation. However, limited clinical success has been achieved due to inferior mechanical and structural properties of the implanted or regenerated tissue.⁴⁹ Furthermore, zonal organization and integration of implanted articular cartilage tissue remains a challenge for clinical success.⁶¹ Researchers have shifted focus toward recapitulating the 3D structural properties of the *in vitro* μEN to not only improve the tissue quality of *ex vivo* cartilage for implantation, but also to investigate the cellular mechanisms involved in proper cartilage development.

In a recent study, a form of material extrusion bioprinting was used to fabricate hybrid constructs of PCL and chondrocyte-encapsulated alginate hydrogels.⁵² In this bioprinting system, called a multihead deposition system (MHDS), PCL was printed as the structural component and chondrocyte-containing hydrogels as the functional component for regeneration. Additionally, the growth factor TGF- β was incorporated in the chondrocyte hydrogels to promote cartilage tissue formation, which showed enhanced ECM formation compared to the hydrogels not supplemented with TGF- β *in vitro*. The scaffolds were implanted into the subcutaneous dorsal spaces of nude mice and tissue invasion and formation were observed. Histochemical staining showed that the engineered tissue supplemented with TGF- β exhibited improved cartilage formation with minimal adverse tissue response. This study highlights 3D bioprinting as a viable method for fabrication of TECs that direct cartilage regeneration *in vivo*.

An AM technique has also been used to create an *in vitro* biomimetic 3D μ EN to probe the signaling pathways involved in chondrogenesis as a tool to discover potential biomarkers for drug testing. In this study, a material jetting bioprinting system was used to deposit hydrogels containing human MSCs, TGF- β , and BMP-2 to create scaffolds with varying concentrations and gradients of the growth factors not feasible by conventional scaffold synthesis to assess their effect on fibrocartilage development.³³ An extensive quantitative real-time polymerase chain reaction (qRT-PCR) and pathway network analysis was conducted to assess the differentiation of the hMSCs based on the supplied conditions as well as the differentiation-related pathways involved in the model. The results showed that both osteogenesis and chondrogenesis-related genes were up-regulated in the bioprinted constructs that contained both growth factors as opposed to the constructs without growth factors. Furthermore, the pathway and network analysis showed that multiple bone- and cartilage-related differentiation pathways, including TGF- β , Wnt, and BMP pathways were all active in the model. This study highlights the capability of using AM techniques to recapitulate the structure and function of cells involved in bone and cartilage development, and further poses a tool for future research in drug discovery.

Soft Tissue Models

ECM analogues—The creation of vascularized 3D tissues *in vitro* would significantly advance the fields of tissue engineering and high-throughput drug screening. A number of challenges must be overcome for 3D bioprinting to achieve its full potential, including the development of new biomaterials that recapitulate the properties of the matrix and the fabrication of vascular trees with capillaries and microvessels that provide adequate blood supply.⁶² Recent studies have addressed these key technological limitations. Biomaterials used to prepare bioprinting inks typically lack the complexity of natural extracellular matrix, and therefore cannot recapitulate the cell-ECM interactions in the native tissue μ EN. To address this limitation, tissue-specific bioinks have been prepared from decellularized ECM (dECM) from adipose, cartilage, and heart tissue (Fig. 2A).⁶⁵ Porcine cartilage and heart tissue, as well as human adipose tissue, were decellularized and dissolved in pH-adjusted solutions that gelled at 37°C. Bioinks were used to bioprint structures encapsulating either human adipose-derived stem cells (hASCs) or human interior turbinate tissue-derived MSCs (hTMSCs) that supported the formation of structured 3D tissues. The printed scaffolds enhanced cell viability, commitment of the stem cells to a specific differential lineage, and deposition of new extracellular matrix compared to collagen controls. Due to the ability to print specific cells and ECMs, the dECM approach exhibits considerable potential for modeling disease progression and drug response for a broad variety of tissue types. Another recent study has applied 3D bioprinting to prepare constructs incorporating multiple cell types, ECM, and well-developed vasculature.⁵⁰ A custom 3D bioprinter with four independently controlled print heads was designed to concomitantly print cells, ECM, and vasculature. Gelatin methacrylate (GelMA) was used to print the cell-laden ECM, which was subsequently photo-crosslinked after printing. An aqueous solution of a poly(ethylene oxide) (PEO)-poly(propylene oxide) (PPO) triblock copolymer comprised the fugitive ink for bioprinting the microvascular network. After printing, the ink was removed by cooling to temperatures below 4°C to yield hierarchical, bifurcated vascular networks embedded in the ECM (Fig. 2B). In a proof-of-concept experiment, human neonatal dermal fibroblasts

(HNFs) and mouse 10T1/2s fibroblasts bioprinted in the vascularized constructs remained viable for up to 1 week in culture and lined the bioprinted channels (Fig. 2C). This new vascularization approach is scalable for mechanistic and high-throughput drug screening assays related to wound healing and angiogenesis, in which the 3D constructs could be printed in standard tissue culture plates.

Cutaneous tissue—Autologous split skin grafts (SSG) are the clinical “gold standard” for repair of cutaneous defects. However, autologous grafting is associated with scarring and is insufficient for extensive surgeries where a considerable amount of skin is needed.²⁷ Therefore, other directions in tissue engineering research have been taken to alleviate the need for autograft in repairing skin. *In vitro* platforms capable of producing tissue-engineered skin both for implants to replace autologous grafts and as models for drug and topical chemical compound screening have also been an area of extensive research. AM techniques have been utilized to recapitulate the hierarchical and layered nature of skin, which cannot be achieved using conventional approaches. A direct cell printing method of 3D bioprinting was utilized to create multi-layered TECs containing fibroblasts (FB), keratinocytes (KC), and a collagen-based hydrogel as the structural component to mimic skin layers.⁵⁴ A stratified skin layer was created by depositing a coat of sodium bicarbonate, a crosslinking agent, after the deposition of a layer of collagen and the desired cell type. The sodium bicarbonate crosslinks the recently deposited layer, fixing the cells within and allowing for the deposition of a new layer on top. Cell viability, proliferation and stratified structure of both the FBs and KCs were maintained after printing. Further, the authors proposed the use of this model to print additional cell lines such as melanomas and epithelial cells as a means of modeling skin disease for study of disease progression or a high-throughput drug-screening tool. In another study, the t-FDM scaffolds described in the Bone section above (Fig. 1C) were implanted subcutaneously in rats to investigate the effects of substrate modulus on cutaneous wound healing.³² Scaffolds with a modulus comparable to collagen fibrils minimized scar formation, Wnt signaling in fibroblasts, and polarization of macrophages toward the restorative phenotype compared to scaffolds that were more compliant or rigid.

Liver—The development of robust, reliable *in vitro* liver models is an area of continuous research due to the liver’s role in drug metabolism and associated toxicity.⁵³ In the past, most *in vitro* models relied on 2D monolayer hepatic culture, but 3D models utilizing AM techniques combined with perfusion culture are proving more capable of reliably modeling *in vivo* liver behavior. A direct cell printing approach in conjunction with perfusion culture has been developed to create a biomimetic liver micro-organ as a drug-screening tool.^{10,11} The direct cell writing (DCW) system utilized four nozzles capable of operating in extrusion or droplet mode to deposit alginate hydrogels encapsulating HepG2 liver cells. These alginate-encapsulated cells were printed into a three-layer TEC that mimicked the liver sinusoidal shape that was incorporated into a microchip device that allow for media circulation. Results showed that over 80% of HepG2 cells in the cross-linked, bioprinted construct remained viable after three days. Furthermore, viability was maintained after 24-hour perfusion flow, indicating that the perfusion system did not affect cell viability and could be used to perfuse a drug of interest through the system and assess its pharmacokinetic

behavior. A more recent study utilizing the DCW process incorporated both hepatic and epithelial cells in Matrigel to more closely mimic the liver sinusoid hierarchical structure.⁷⁶ In addition to successfully printing the dual-cellular construct, a radiation drug (amifostine) was perfused through the micro-organ system to test its efficacy in protecting cells from radiation damage. Results indicated that the drug caused a marked decrease in radiation damage compared to untreated cells.

Another study utilized an organ-on-a-chip device, LiverChip (CN Bio Innovations), and co-culture of hepatocytes and Kepffer cells to assess hydrocortisone pharmacokinetic behavior in an inflammation-induced liver environment.⁷³ The LiverChip is a microfluidic bioreactor model designed to recapitulate the liver capillary bed under perfusion and employs 12 bioreactors in series with 3D scaffolds capable of seeding cells. Endotoxin lipopolysaccharide (LPS) was introduced into the LiverChip co-culture to promote an inflammatory response by the cultured cells. Hydrocortisone, an anti-inflammatory drug, was then introduced into the perfusion medium and its disappearance and metabolism were determined using a form of liquid chromatography-mass spectrometry (LC-MS). The half-life, rate of elimination, clearance, and area-under-the-curve were assessed based on the LC-MS data, and an *in vitro/in vivo* correlation was established to extrapolate *in vivo* behavior from the *in vitro* culture. These results and correlations suggest that this *in vitro* system could be used as a tool to investigate drug metabolism and predict *in vivo* toxicology. Scaffolds that more closely mimic liver architecture and promote enhanced hepatocyte culture have also been investigated. Using SLA, PEG-based, photo-polymerizable hydrogels have been fabricated to improve hepatocyte cell seeding, proliferation, and duration of perfusion cultures in liver models (Fig. 3).⁶³

Brain and Nervous System—Capturing the complex structural organization of the brain and nervous system has been a major obstacle in creating *in vitro* models that could accelerate the development of new therapies for neurological disorders.⁸² Microfluidic mixing has been integrated with hydrogel functionalization to fabricate hydrogels with spatially controlled gradients of matrix and cells representative of glioblastomas⁶⁶, which could potentially support studies investigating how spatiotemporal gradients regulate tumor cell fate. Compared to these conventional approaches, 3D printing offers the advantage of simultaneous rapid prototyping and biofunctionalization as well as high-throughput production capacity, which has been applied to design TECs that recapitulate the function of glial cell-axon interfaces.⁴⁴ This TEC was prepared by 3D printing of three components: (1) microchannels to provide axonal guidance, (2) a sealant layer to prohibit exchange of fluids between chambers, and (3) a top tri-chamber to isolate different cell types. A 3D model of the central nervous system (CNS) was built by culturing hippocampal neurons in chamber 1, superior cervical ganglion (SCG) cells in chamber 2, and Schwann cells in chamber 3. When the peripheral (SCG) neurons were infected with pseudorabies virus (PRV), the viral particles were transported to the Schwann and hippocampal cells at a rate of $2 \mu\text{m s}^{-1}$. However, a bottleneck in the spread of the virus was observed. Thus, the 3D CNS model enabled two key findings related to transmission of viral infection in the central nervous system: (1) Schwann cells and hippocampal neurons are resistant to infection, and (2) Schwann cells transmit the infection response through axonal interaction. Furthermore, the

CNS TEC enabled quantitative measurements of the transport rate of viral particles. These findings underscore the significance of the model for interrogation of the nervous system's response to pathogens or therapies.

Until recently, the study of neuronal tissue was limited mainly to monolayer culture or to simple hydrogel scaffolds that lack the layered architecture exhibited by the human cortex.²¹ A syringe-based ink bioprinting technique has been employed to create layered, neuronal cell-encapsulated hydrogels to mimic layered human brain tissue.⁵⁵ A hand-held reactive bioextrusion technique was used to plot freeform 3D layers of gellan gum (GG) encapsulating cortical neurons and glial cells. To increase cell adhesion and proliferation, the GG gels were modified with an RGD peptide sequence and made into a bio-ink formulation to allow for bioprinting. Encapsulated neuronal and glial cells were shown to remain viable and proliferate within the cross-linked hydrogel, and importantly they exhibited appropriate morphologies and axonal development. This strategy for producing layers of different brain tissue cell subtypes provides a potential means of understanding neurodegenerative diseases to drug testing.

Lung—*In vitro* models of the biological barriers in the lung are critical for the development of new drugs and drug carriers for pulmonary delivery.⁷⁸ 3D *in vitro* models potentially enable the identification of physiological characteristics of the lung μ EN that must be considered in the design of novel carriers. Consequently, more accurate models of the air-blood barrier are needed to design carriers that minimize clearance and promote controlled release of the therapeutic. Co-culture models that capture cellular interactions, as well as microfluidic approaches that mimic the effects of fluid flow on the functionality of epithelial cells represent significant advances in the field. 3D bioprinting has been applied to fabricate air-blood tissue barrier analogues comprising endothelial cells, a basement membrane, and epithelial cells.³⁸ In a layer-by-layer bioprinting approach, A549 alveolar epithelial cells were separated from EA.hy926 endothelial cells by Matrigel, which was used to mimic the basement membrane. Compared to manually-seeded conventional constructs, the bioprinted analogues exhibited a thinner Matrigel layer and more homogeneously distributed monolayers of cells. Furthermore, the bioprinted endothelial cell layer was less permeable than the manually-seeded layer. Due to its ability to recapitulate the features of the alveolar μ EN, this automated and more reproducible 3D bioprinted air-blood tissue barrier model is anticipated to provide a more accurate approach to assessing inhalation hazards as well as screening of new therapeutics.

Vascular networks—Successful *in vitro* culture of organs requires vascularization of the construct, since cells must be less than 100 – 200 μ m from blood vessels and capillaries that supply oxygen and nutrients.⁴³ Due to its ability to precisely pattern cells and biomaterials, 3D printing of vascular networks is an emerging approach in which 3D printed sacrificial fibers are embedded in hydrogels to generate microchannels. In one study, template agarose fibers were printed by extrusion of the aqueous agarose solution from a glass capillary.⁸ After printing of the agarose fibers, a hydrogel was cast over the agarose fibers and photo-crosslinked, followed by removal of the agarose template. HUVECs cultured on GelMA hydrogels formed a confluent monolayer exhibiting high CD31 expression and formation of

cell-cell junctions. 3D printing of carbohydrate glass networks has also been utilized to prepare sacrificial templates.⁶⁰ Filament size was controlled by varying the velocity of the nozzle through which the carbohydrate glass was extruded. The resulting glass fibers were embedded in cell-laden hydrogels, including agarose, alginate, PEG, fibrin, or Matrigel, and the fibers removed by dissolution. The carbohydrate glass presents the advantages of sufficient strength to provide mechanical support during fabrication and the ability to be removed from the construct after gel encapsulation without harming the cells. Co-culture of 10T1/2 cells and HUVECs in fibrin gels resulted in the formation of three key components of vascularized tissue: the vascular lumen, endothelial cells lining the vascular wall, and the interstitial region. Furthermore, PEG hydrogels with vascular networks enhanced hepatocyte function compared to monolithic gels. In an alternative approach to generating vascular networks, a high-resolution μ SLA apparatus capable of patterning biomaterials and cells at resolutions $<5\ \mu\text{m}$ was designed to 3D print photo-crosslinked hydrogels with angiogenic patches.⁶⁸ Encapsulation of fibroblasts in PEG hydrogels with 3D-printed 100- μm channels enhanced the formation of neovasculature in a chick chorioallantoic membrane (CAM) model. The ability of these 3D printing techniques to control the architecture of the vascular network, as well as the types of cells and matrices used, may facilitate mechanistic studies of the relationship between vascular structure and mass transfer in tissue, as well as studies investigating disease progression and high-throughput drug screening.

Cancer—Both the physical and cellular μ EN play a large role in cancer initiation, promotion, and metastasis; therefore, mimicking the 3D context of the *in vivo* milieu is essential to modeling the appropriate physical, chemical, and mechanical cues that contribute to tumorigenesis.³

3D bioprinting has been used to enhance a previously established 3D ovarian cell (OVCAR-5) model. In the original model, cells cultured on Matrigel spontaneously formed micronodules (acini), a commonly assumed physiological morphology *in vivo* representative of adherent micrometastatic disease.⁷⁰ To improve upon this model, a cell-patterning platform was developed to print two cell types, fibroblasts (MRC-5) and OVCAR-5, onto Matrigel to miniaturize the model, improve reproducibility, and make it amenable to high throughput screening. Furthermore, 3D cell-printing allowed for spatial control of the cancer and stromal cells to recapitulate their *in vivo* orientation.⁸⁶ Cells were printed using a dual-valve dispensing system and CAD program to independently and precisely deposit the MRC-5 and OVCAR-5 cell suspensions onto a bed of Matrigel in a predefined pattern. The patterning platform successfully produced a viable, cocultured system with reliable cell droplet size, cell density, spatial distribution, and appropriate morphological behavior.

A more recent study used bioprinting to create a cervical cancer model and tested chemoresistance of the cultured cervical cancer cells in 3D versus 2D.⁹² A 3D cell printer was used to deposit HeLa cervical cancer cells encapsulated in a gelatin, alginate, and fibrinogen hydrogel mixture into a gridded structure. The hydrogel was designed to mimic the ECM in which HeLa cells are known to proliferate. The printing method was capable of printing viable cells contained in the cross-linked matrix, and the cells showed greater proliferation over a 5-day period compared to 2D culture. Furthermore, this study also showed increased matrix metalloproteinase (MMP) expression on 3D culture versus 2D

culture, indicating a more metastatic phenotype in the HeLa cells cultured in 3D. Finally, this study compared treatment of a chemotherapeutic, Paclitaxel, between the 3D and 2D culture. While considerable HeLa cell death was exhibited in both groups, the 3D culture showed significant chemoresistance versus 2D.

Other studies have focused on cancer cell migration and motility utilizing AM techniques to pattern hydrogels. One such study utilized a form of SLA discussed previously, DMD-PP, to create honeycomb patterns in hydrogels that mimic vasculature to study migration of cancer cells versus non-cancerous cells.³⁹ Cancer cell migration speed increased with decreasing diameter of the bioprinted vessels. A similar study used the same SLA method to create PEG scaffolds to investigate how both cell morphology and substrate modulus affect cell migration.⁷⁷ Substrate modulus and cell morphology profoundly affected cell migration in 3D, whereas differences in 2D were not as significant. These models for cancer cell migration have the potential to provide insight into tumor behavior, progression, and invasion.

Perspective

AM has profoundly impacted the field of tissue engineering despite its only recent widespread use. While initial studies were motivated by the potential of organ printing for implantation, AM has recently found a niche in creating TECs for *in vitro* models of organogenesis, disease progression, and drug screening. 3D culture using conventional fabrication techniques, such as microfluidics, electrospinning, and porogen leaching, has underscored the need for 3D models that more accurately recapitulate the cellular and physical properties of the tissue μ EN. However, further research has indicated that parameters such as the matrix architecture, spatial arrangement of cells, hierarchical structure of tissues, and cellular cross-talk between adjacent tissues contribute substantially to cell behavior *in vivo*. AM techniques have the potential to bridge this gap between 3D models and the *in vivo* milieu due to their ability to precisely control scaffold architecture and spatial arrangement of cells. Progress in bioprinting has made possible the deposition of multiple cell types into different hierarchical structures, allowing for more sophisticated models capable of simulating the interaction between different tissue types. Furthermore, the continuously increasing speed of AM machines renders this approach useful for fabricating scaffolds for high throughput drug screening. Other advantages of AM compared to more conventional techniques include enhanced reproducibility, scalability from the tissue culture well plate to the anatomic scale, and the ability to combine rapid prototyping and biofunctionalization in a single step.

3D *in vitro* models based on AM techniques are still emerging, and thus a number of challenges need to be addressed to fully realize the potential of AM for the design of TECs. While *in vivo* validation of 3D *in vitro* TECs remains an ultimate long-term goal, more achievable and modest shorter term objectives would make substantial contributions to the field, such as the identification of physiological characteristics of the tissue μ EN that must be considered in the design of new therapies. Despite the fact that currently available 3D printed TECs cannot recapitulate all the properties of the μ EN, they still present a more realistic and stringent μ EN for drug screening compared to 2D culture. Consequently, 3D

printed TECs have the potential to reduce the need for preclinical testing by more stringently screening drug candidates *in vitro*.

Advances in AM have realized the ability to create TECs with precisely controlled structures beyond the capabilities of conventional approaches, but additional barriers must be overcome before these models can reliably predict drug efficacy or model disease progression in humans. The use of patient-derived cells rather than cell lines could potentially advance the development of personalized medicine, in which patient-specific therapies are identified on the basis of high-throughput *in vitro* drug screening. In addition, many current models rely on static culture, whereas the *in vivo* μ EN often experiences fluid flow that can affect cell behavior. Therefore, current models will need to support perfusion culture to more closely mimic the shear forces experienced by cells *in vivo*. Furthermore, maintaining a physiologic gradient of growth factors and other proteins within the tissues in perfusion culture would more accurately mimic the tissue μ EN. Bioprinting has made progress in creating TECs from different cell types; however, scaffold architectures are largely limited to simple constructs, such as grids. Therefore, bioprinting methods capable of constructing more sophisticated architectures could expand complexity and thus more accurately mimic the *in vivo* μ EN. More efficient simultaneous bioprinting of cells and biomaterials with tissue-specific mechanical properties could better recapitulate the mechanical, topological, and cellular properties of the μ EN, and significant progress is being made in this area. A new bioprinting system (ITOP) has been reported for fabrication of mechanically-stable, functional, human-scale constructs of the mandible, calvarial bone, cartilage, and skeletal muscle by simultaneously plotting cell-laden material with biodegradable structural polymers containing microchannels to allow for nutrient transport.⁴⁵ Finally, organs in the human body do not behave independently, but are interconnected and communicate in a complex manner. Consequently, *in vitro* 3D models should ideally capture the cross-talk not just between cells, but also between tissues and organs. For instance, promoting angiogenesis and innervation into TECs in perfusion culture would better replicate the biological forces experienced by the organ of interest. Incorporation of multiple tissues and organs will also require scale-up, which will in turn further necessitate vascular networks and other means of nutrient transport throughout the TEC, and this new complexity will need to be addressed. AM technology has the potential to address many of these limitations, due to the rapid growth in new technologies and approaches. Interdisciplinary research between tissue engineers, molecular biologists, and mechanical and electrical engineers in conjunction with AM technology has the potential to overcome the barriers that limit these models from becoming reliable tools for drug screening and understanding the underlying mechanisms contributing to disease.

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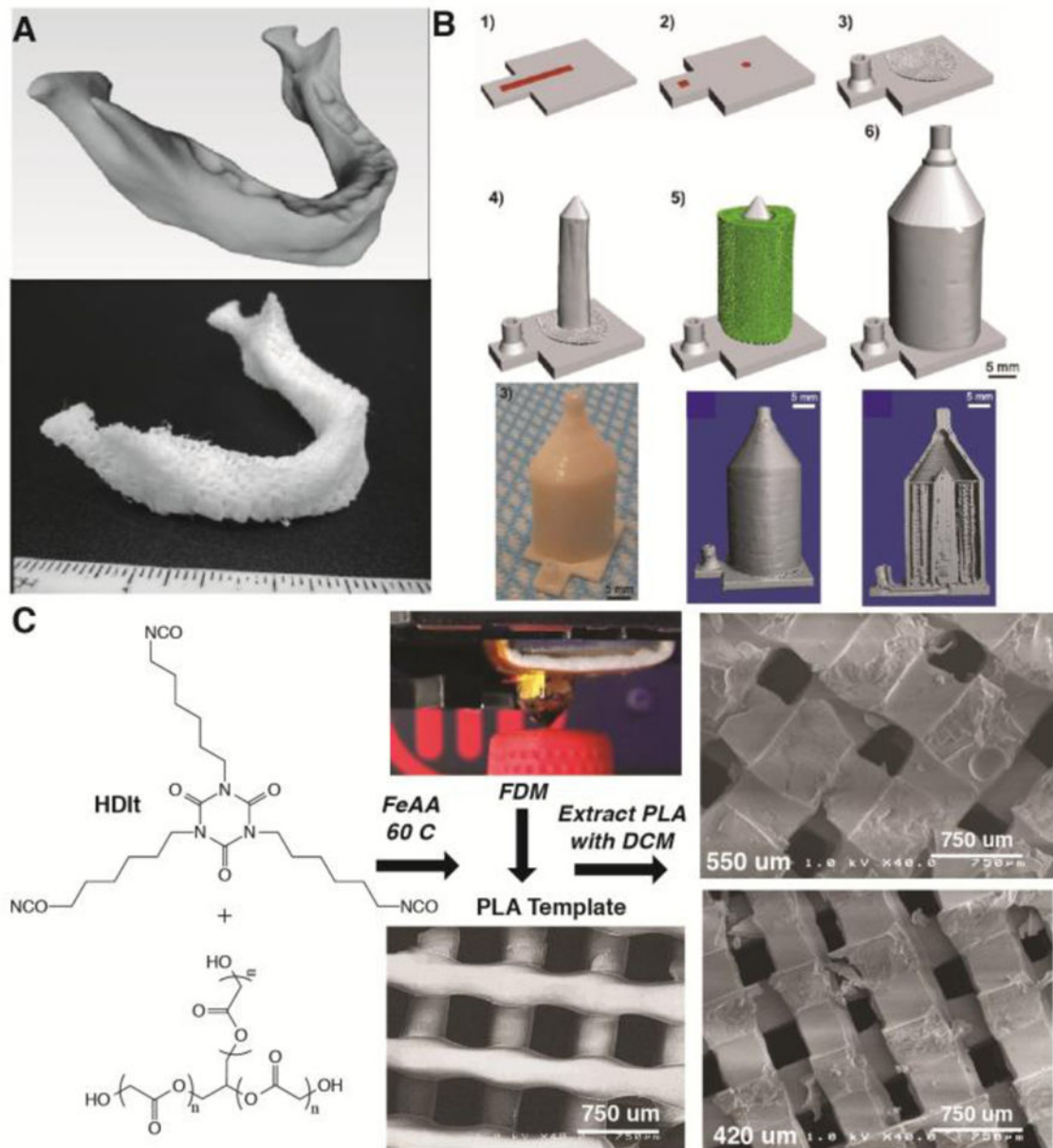


Figure 1.

3D-printed models of bone regeneration. (A) An anatomically shaped scaffold. Top: 3D image of the mandible. Bottom: A 3D-printed, porous PCL scaffold at 40% infill density. Adapted from Temple et al.⁸³ (B) 3D printing of Tissue-Engineered Constructs (TEC) comprising a scaffold and bioreactor. Top: Elements of the device to be prototyped. (1) The base of the device consisting in a lower plate designed with a mini-channel for supplying the culture medium (red) which is covered by another plate containing two holes. (2), (3) The medium inlet/outlet and porous structure positioned over the holes. (4) The filler column centrally positioned with regard to the scaffold. (5) The tailor-made porous scaffold (green) positioned around the filler column. (6) The bioreactor chamber surrounding the scaffold. Adapted from Costa et al.¹³ (C) Schematic of the t-FDM process for fabrication of 3D

scaffolds with defined mechanical and topological properties. Left: Hexamethylene diisocyanate trimer (HDI_t) was mixed with a polyester triol and iron acetylacetonate (FeAA) catalyst and poured into a poly(lactic acid) (PLA) template fabricated by a MakerBot® Replicator 2 3D printer. After curing overnight, the PLA template was dissolved in dichloromethane for 18 h. Right: SEM images of scaffolds with 550 and 420 μm pores. Adapted from Guo et al.³¹

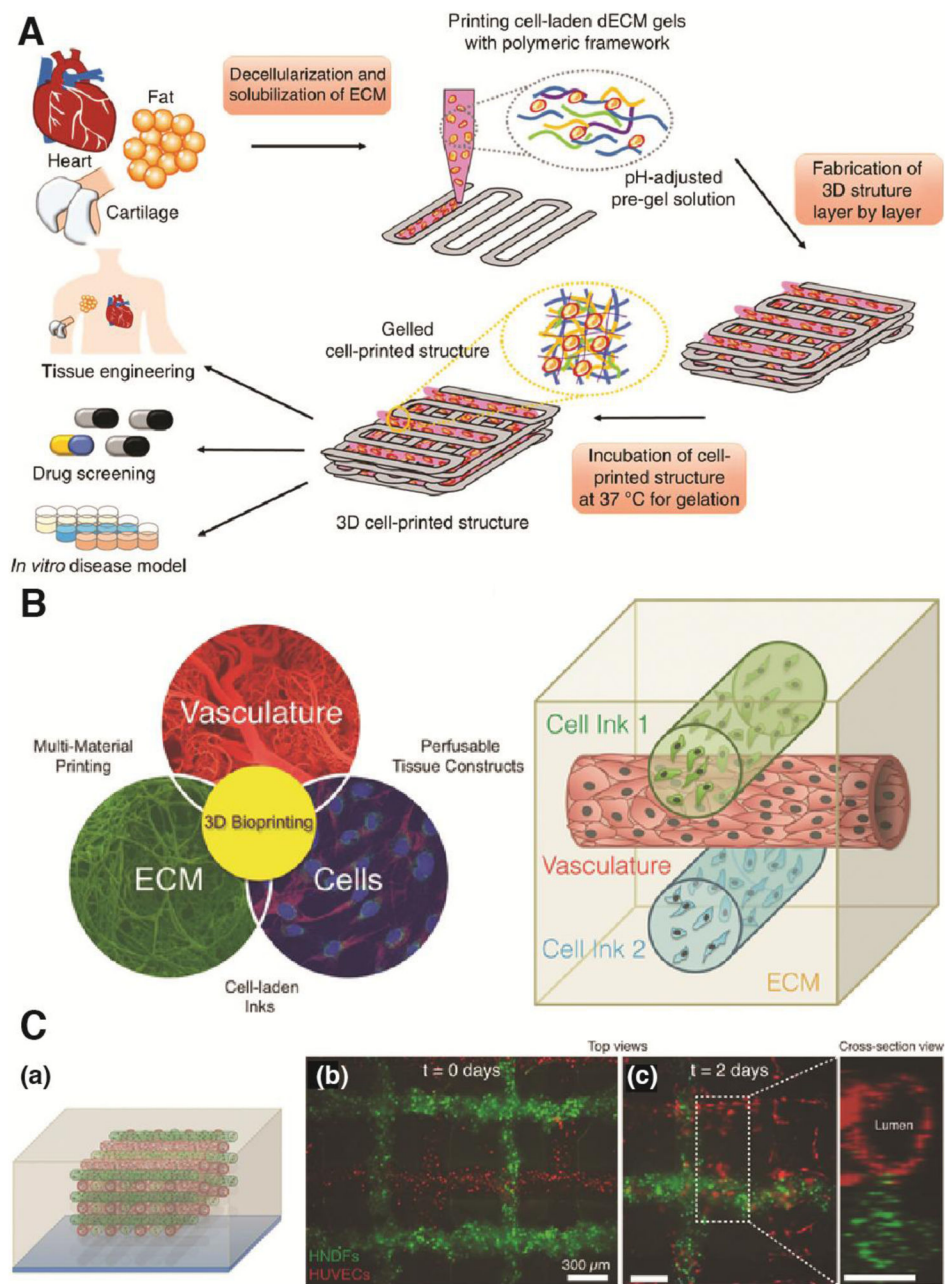


Figure 2.

3D bioprinting of biomimetic extracellular matrices (ECMs). (A) Respective tissues were decellularized after harvesting with a combination of physical, chemical and enzymatic processes, solubilized in acidic condition, and adjusted to physiological pH. Tissue printing was performed with the dECM bioink encapsulating living stem cells via a layer-by-layer approach followed by gelation at 37 °C. The 3D cell-printed structure has applications in various border areas including tissue engineering, in vitro drug screening and tissue/cancer model. Reproduced from Pati et al.⁶⁵ (B) Schematic views of the vascularized 3D bioprinting approach (left), in which vasculature, cells, and ECM are co-printed to yield

engineered tissue constructs composed of heterogeneous subunits (right). Reproduced from Kolesky et al.⁵⁰ (C) Schematic view (a) and fluorescence images (b,c) of an engineered tissue construct cultured for 0 and 2 days, respectively, in which red and green filaments correspond to channels lined with RFP HUVECs and GFP HNF-1 α -laden GelMA ink respectively. The cross-sectional view in (c) shows that endothelial cells line the lumens within the embedded 3D microvascular network. Reproduced from Kolesky et al.⁵⁰

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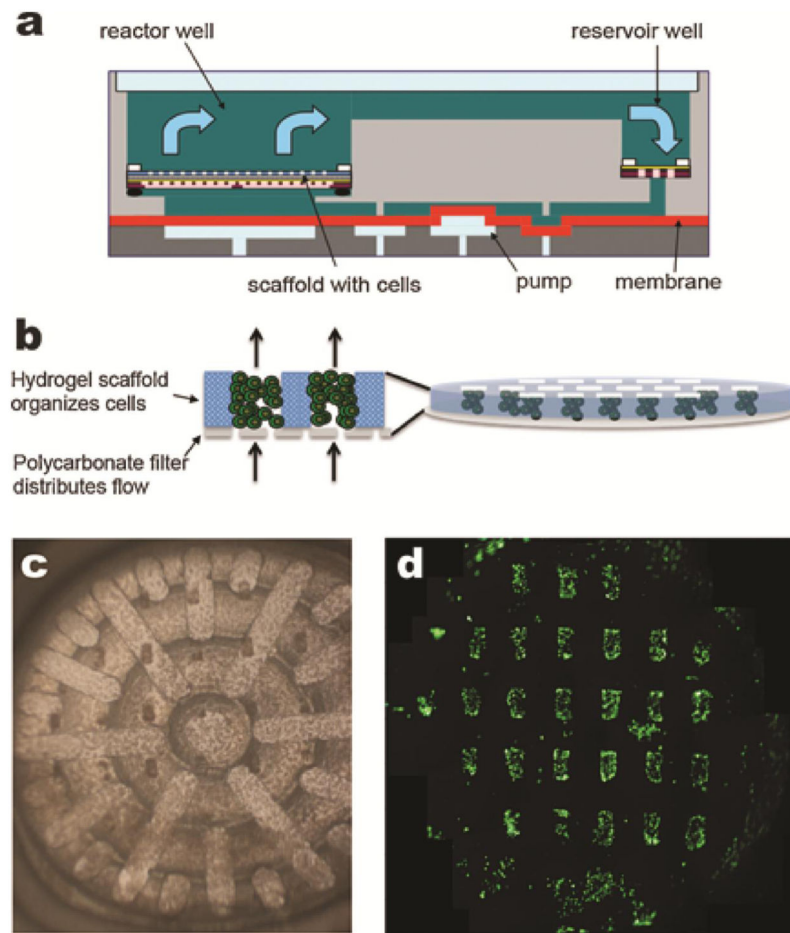


Figure 3. Hydrogel scaffold organizes liver cells in a perfused bioreactor. (A) A perfused bioreactor houses the fabricated hydrogel scaffold and recirculates media through the open channels of the scaffold in direct contact with seeded cells. (B) Hydrogel scaffolds organize the cells and tissue formation, while the chemically bound filter distributes flow to the cells in the perfused bioreactor due to its high impedance. (C) Tissue formation within the open channels can be observed *in situ* with phase microscopy on day 3. (D) Calcein AM staining on day 3 demonstrates the majority of cells are localized within the channels exposed to perfusion flow, while minimal cells reside on top of the hydrogel scaffolds where conditions more closely resemble static culture. Reproduced from Neiman et al.⁶³