Human Organ and Tissue Engineering: Advances and Challenges in Addressing the Medical Crisis of the 21st century

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ABSTRACT

Organ failure is the imminent health-care crisis of the 21st century. In the United States, the estimated cost of lung, kidney, and liver disease combined is upwards of $256B annually.[1, 2, 3] One in seven adult Americans is suffering from some form of progressive kidney disease, and currently, 660,000 adults in the US are suffering from end-stage renal failure.[4, 5, 6] Lung disease is the 3rd leading cause of death,[7] liver disease is the 12th leading cause of death,[4] and heart disease, the number one cause of death in the US, can be caused, or exacerbated by, loss of organ function.[7, 8] Endocrine diseases can also be thought of as a failure in an organ system; a notable example is Diabetes. Globally, Type 2 Diabetes affects over 400 million people,[9] while Type 1 Diabetes affects fewer patients, the numbers are in the millions. Type 1 Diabetes is autoimmune-driven and has an annual global increase in incidence of 10 percent year over year.[10, 11] Both Type 1 and Type 2 Diabetes can benefit from transplantation of tissue known as the Islets of Langerhans, an essentially curative procedure that leads independence from insulin injections.[12, 13] Transplantation is the only known curative option to address organ failure. Development of vascularized organs and tissues for transplantation is not feasible with current technology. Despite the development of numerous methods, none have broached what will ultimately become a multi-trillion-dollar global market that can address hundreds of millions of patient's needs: living human organ and tissue replacement. Here, we review current approaches in the race to produce functional human organs for transplantation, discuss roadblocks, and present novel technology that will pave the way to building human organs in the laboratory setting.

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INTRODUCTION

Organ and tissue transplantation can restore physiologic homeostasis, reduce stress on other organ systems, and improve the lives of the recipients. Donor-derived transplantation, however, is significantly limited by supply, with only 34,771 organ transplants being performed in the US in 2017, and well over a million potential recipients turned away from a waitlist, or waiting for an immunologically matched donor.[14] Efforts in the regenerative medicine space have advanced treatment options, and include work with patient-derived stem cells to promote tissue repair, as well as development of improved immunosuppression options. However, medical options to treat end-stage organ failure have not advanced for nearly 50 years, and still require human-to-human donation. This leads to a limited number of healthy organs available for transplantation and only select candidates are added to the waitlist to receive a life-saving organ. Tremendous advances in regenerative medicine have been reported in the last decade, but in terms of human organ engineering, the field remains stalled at thin sheets of cells that lack capillary blood flow, and therefore are not suitable transplantation.

In the United States, an estimated 150 million people are suffering from kidney, lung, or liver disease, the 3rd, 9th, and 12th leading causes of death, respectively.[15, 16, 17] Co-morbidities including heart failure precede palliative care, often for decades, taking a significant toll on patients, their families, and the health care system.[1, 8, 18, 19] Compounding the toll on our healthcare system, it has recently been reported that extended periods of time with low organ function are associated with progressive loss of cognitive function and physical abilities.[20, 21] Even in a reasonably well-managed disease such as Diabetes, it is reported that autonomic and central nervous system decline is accelerated, leading to increased incidence of dementia, cognitive decline, as well as an increased risk of heart attack and renal failure.[22, 23, 24] As we age, decline in organ systems may contribute to and precipitate neurological disorders, or induce significant stress on other organ systems including the heart.[21, 25, 26, 27]

Organ transplantation allows patients to reclaim normalcy, return to work, and in many cases, extends life by decades. However, lifesaving transplantation is limited by too few viable organs. Donation from 16,468 deceased individuals led to a record-breaking 34,771 transplants performed in 2017 (more than one organ can be harvested per donor).[14] The increase in healthy organs for transplantation is widely attributed to the current opiate addiction crisis,[28] and does not represent a sustained increase in donation which, until 2015, remained below 30,000 transplant surgeries per year in the US.[29] Typically, fewer than 16,000 organ donors give the gift of life each year, far fewer than the millions of patients who could benefit from an organ transplant.

Although significant efforts have been made to bridge the chasm between organ availability and need, increasing organ donor numbers and improving organ procurement procedures will not bridge the gap. Advances in stem cell biology and novel engineering approaches have brought scientists closer than ever before in medical history to producing a functional replacement human organ. The race between three technologies in the field will likely bring the capability to produce fully functional replacement human organs into medical reality: recellularization of tissue structures, xenograft transplantation (from animals), and 3D printing to manufacture organs. Here, we focus on the field of organ and tissue development in the laboratory setting using 3D printing, and provide a brief overview of other technologies, including recellularization, and the efforts to develop porcine breed-stock for xenograph transplantation.

MANUFACTURING 3D PRINTED HUMAN ORGANS AND TISSUES

There are four requirements for scalable 3D printing of functional human organs: Resolution, Speed, Complexity, and Biocompatibility.

RESOLUTION

High resolution printing is critical for building single-cell walled capillaries and microcellular niches, necessary components of functional organs and tissues.

All living tissues and organs are perfused by 5-10 micron (in diameter) blood vessels that are known as capillaries. Capillaries are the smallest blood vessels in the body and serve as the primary exchange point for oxygen, nutrients, and cellular wastes. After nutrient and waste exchange capillaries recombine to form the veins that ferry blood back to the heart and then lungs. These small blood vessels are the critical functional unit in all tissues. Without a network of capillaries, tissues and organs starve for oxygen (hypoxia), and cells begin to die off in a matter of minutes leading to tissue necrosis (death). Therefore, the resolution of a given printing process must be about or near that of a single cell.

Organs need oxygen for cellular respiration, but also require thin-walled capillaries to function effectively. Within many organs, capillaries play additional functional roles. Kidneys, for example, require single-cell walled capillaries for blood filtration, lungs require single-cell walled capillaries for gas exchange, and the liver requires single-cell walled capillaries for waste processing. The functional networks of capillaries in an organ are extensive. A human kidney contains 12 miles of glomerular capillaries that are precisely

placed to filter blood and reabsorb salts and nutrients.[30] The human brain, which uses 20% of our inhaled oxygen, boasts over 400 miles of capillaries, where each neuron has a dedicated capillary.[31, 32]

Living tissue dependence on capillaries makes bioprinting at a high resolution a critical element in 3D printing and manufacturing of organs. Although large structures can be created with current 3D bioprinting technologies, the smallest blood vessels created to date are on the order of 50 to 1,500 microns in diameter, too large to act as functional capillary networks.[33, 34, 35, 36, 37] The average inner diameter of a capillary is 5 to 10 microns, about one one-thousandth of a centimeter. This inner diameter is so small, red blood cells pass through in single file. In organs and tissues, capillaries are spaced a maximum of 250-300 microns apart, the limit of diffusion for oxygen (O_2). Capillary spacing can vary significantly, and in tissues with high metabolic requirements, such as cardiac tissues, capillaries are much closer together, an average distance of 20 microns apart.[38, 39] Therefore, a printing technology must achieve both fine enough resolution to create a thin-walled capillary, while allowing for cells to be placed between capillaries. Without the ability to build a functional vascular system, human tissue engineering will not progress beyond thin sheets of cells.[40] The lowest reported resolution to date was achieved with two-photon raster-scanning of a laser.[41] Spray-based deposition printing can print the lowest resolution of the non-light based systems and allows for 50 micron resolution, still 5 to 10 fold to large to build functional tissues.[42]

Along with building vasculature, the ability to place single cells allows for the creation of microcellular niches, an important aspect of tissue engineering. Microcellular niches are small groupings of diverse cell types that create a self-sustaining, highly specialized micro-environment through chemical cross-talk and cell-cell interactions. Often, as with the crypts of the small intestine, these niches contain stem cells to replenish local cell populations during tissue remodeling, repair, and during the natural course of cell death.[43] In vitro cells can be induced to self-organize to create these niches, however often fail to achieve a fully organized system, this may be due to geometries and intracellular signaling factors.[44, 45, 46] However, the capacity for cells to organize and differentiated once placed in a larger tissue with circulation remains to be determined. It is likely that cells will have to have specific 3D organization to recapitulate functional microcellular niches. Therefore, the ability to place groups of cells with high resolution is likely to be an important component of tissue and organ engineering.

In sum, the physiologic requirements for organ structure and function require single cell placement to build capillaries and microcellular niches. Therefore, the resolution for creating tissues, organs, and extracellular matrix should fall within the range of a single cell, between 1 to 10 microns.

SPEED

Unlike a standard manufacturing process, human tissue engineering is constrained by the finite lifetime and health of the cells being used to create the tissue.

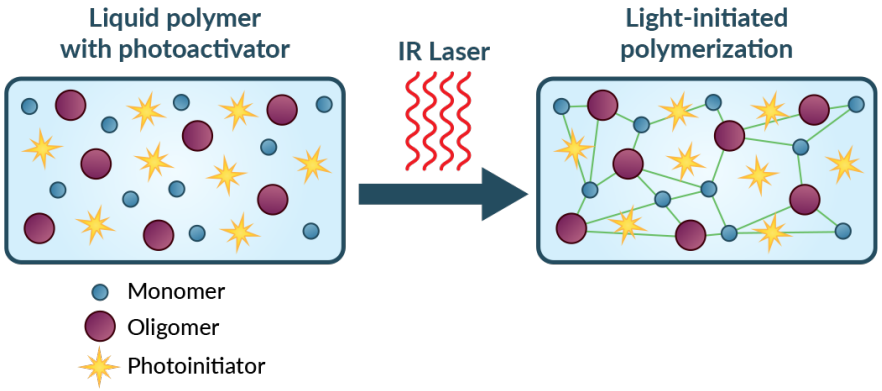
Complex organs contain cells at various stages of differentiation (or development). Extended periods of time in a sub-par environment may induce undesirable cell differentiation. While many cell types can survive in carefully-controlled culture conditions for up to two to three months, high pressure, speeds, sheer force, and heat created by extrusion or droplet-based printing are not well tolerated by cells.[47, 48, 49, 50]

Ideally, printing an extracellular matrix that contains cells would take less than 12 hours before cells are returned to physiologic conditions; generally 37°C, and 5% CO_2 in a nutrient sufficient environment. Cell death during the tissue printing process is an important consideration as it can result in a 'dead' tissue or significantly alter the environment for the remaining cells.[51] The limit for the percentage of cells that can be lost during a printing process is unknown, and different cell types have different tolerances for environmental changes. Extrusion and electrospin based bio-printing causes significant cell death, while ink jet based bioprinting and other methods have reported higher cell viability.[52, 53, 42] Other potential methods for improving cell viability include cooling of materials, including cryogenic printing which offers an interesting method for maintain cells. However, the resolution of cryogenic printing is too low to create vasculature, and defects such as air-bubbles in the range of hundreds of microns in diameter are reported.[54]

To calculate three-dimensional printing times at a given resolution, the type of technology being used to print must be considered. Three dimensional (3D) light based printing at its fastest described, to date, is performed through layer-by-layer additive manufacturing through the deposition of a single line or 2D planar illumination, often via UV-light based excitation.[55, 56] UV light is known to be mutagenic, and thus, despite the demonstration of printing with bioinks that are both cell compatible and UV reactive, repeated UV light exposure limits the safety of these engineered tissues for use in transplantation. Extrusion printing of 5-10 micrometer layers has yet to be described and the speed of deposition at these resolutions is not rapid enough for the creation of microvasculature for transplantable tissues. Therefore, we will focus primarily on the light-based deposition of structures or photolithography, for assessing speeds of tissue engineering.

Laser or light-based polymerization of 3D printing materials can print polymerize biological proteins and soft non-toxic transplantable materials. The polymerization occurs much like the curing process of an epoxy resin with light. Light can be used outside of the visible range of wavelengths to induce photo-crosslinking **(Figure 1)**.

Figure 1. Photo-polymerization of a liquid polymer



This process is both fast and can reach the resolution necessary for single cell layer deposition. Current laser-based printing approaches utilize a one-dimensional line scan of a pinpoint laser, or 2D planar deposition to print 3D structures in layers. Scaling of the print volume in 3D leads to significant gains in speed without compromising resolution. To estimate time required to produce a structure with light-based polymerization of a material, we must account for both the time required to scan the laser through the sample and the time that must be spent to polymerize each voxel. A is the voxel cross section orthogonal to the scan direction, v is the scan velocity, f is the fill factor, n_{voxels} is the number of voxels in the print volume, t is the dwell time necessary for material polymerization, and R is the holographic exposure rate (voxels/s).[57, 58]

Equations 1-3

$$time_{galvo} = \frac{V}{Av} + f * n_{voxel} * t$$

$$time_{polygon} = n_{voxel} * t$$

$$time_{holography} = f * n_{voxel} * R$$

Using these equations, we present calculations comparing the printing times of a centimeter cubed with one micron resolution, a 100 µs dwell time, and two different fill factors **(Table 1)**. Note that polygon scanners are rate-limited by the dwell time, which leads to fill factor independence. 3D holographic printing can be scaled by scaling up optical components. The first two numbers are representative of the fastest technology built by Prellis Biologics prior to introducing custom optical components followed by the estimates for the next generation system which will print at a rate of 12 million voxels per second.

Table 1. Estimated fastest time to print 1 cm^3 with 1 micron resolution

Scan speeds (high/low)	1% fill factor	15% fill factor
Galvanometer (Scanner)		
10 mm/s	3.2 years	3.6 years
10 m/s	12.7 days	175 days
Polygon Scanner		
10 mm/s (limited by dwell time)	3.2 years	3.2 years
3D Multi-Photon Holography		
36,000 voxels/s	3.2 days	48 days
240,000 voxels/s	11.6 hrs	7 days
12 M voxels/s	14 minutes	3.4 hours

Even at 10 mm/s, the resolution is relatively low (10s of microns), and as much as 30-50% what would be structurally necessary to print is skipped in each illumination pass, severing limiting the cohesive structures that are constructed at this speed.[59] [60, 61] 3D Multi-Photon Holography developed by Prellis Biologics, Inc. rapidly prints 3D structures and is described in detail later. Holography is effectively fill-factor independent if sufficient laser power exists. Therefore, in holographic based laser printing speed not limited by resolution wherein speed is volume dependent, and the print volume is dictated by static optical components. Holography is achieved with multi-photon excitation through a combination of beam expansion wave-front shaping which can be thought of as beam-steering that allows for complete or near-complete structure deposition (**Figure 2**).

Figure 2. Single photon absorption as compared to two photon absorption and holographic laser printing.

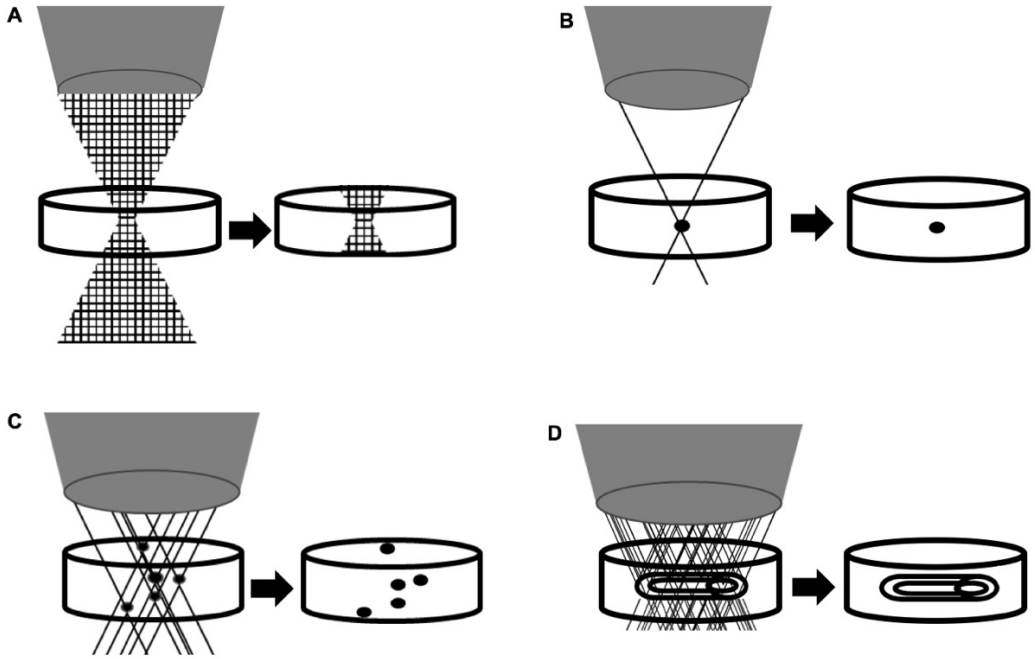


Figure 2. Graphics demonstrating laser printing output based on optics of single photon and multiphoton printing processes and the expected structural outcomes. (A) Graphic depicting single photon laser projection into a print media containing bath without masking or isolation of the intended plane of focus, which would be expected to leave a printed structure behind in the shape of the entire cone of light along the focal length. (B) Graphic depicting a multi-photon absorption process where the photon density is high enough only at the point of focus, leaving only a pinpoint structure behind in a photosensitive print media bath. (C) Representative graphic of wave front shaping to produce a hologram in which the multiphoton absorption process occurs at multiple points of focus in the x, y, and z planes. In this process, rapid switching between 3D projected portions of a complete structure may be used to build the complete structure. (D) Representative graphic showing a complete image projection in multiple planes allowing for holographic printing of a complex structure.

COMPLEXITY

Biological complexity in 3D organ printing can be defined as the close placement of structural components that work in concert to carry out the functional purpose of the organ.

For example, the primary functional purpose of the lung is gas exchange between the circulatory system and the environment. The majority, 90%, of gas exchange occurs across two cells thinly stretched cells in close association, one that comprises the capillary wall, one that makes up the alveolar cell wall; the end-point for all branches of the lung.[62] Alveolar spaces are structurally similar to a bunch of grapes, where each alveolus or 'grape' is the smallest subunit of lung, measuring a mere 200 microns across. To ferry air and blood to this membrane, airways divide an average of 23 times between the trachea and the alveoli where gas exchange occurs. Branched airways and a dual circulatory system create the structural and physiological support system of the lung which is comprised of 1,500 miles of airways and 700 million alveolar spaces.[63] The complexity of the lung is not unique: kidney, liver, and even skin each have structures of similar complexity that are required for physiological function. An engineered tissue must be able to match the complexity of these functional subunits, structural requirements, and nuances therein to be considered viable for human donor organ replacement.

Layer-by-layer extrusion printing can achieve some measure of complexity but cannot match the complexity necessary for creation of human tissue. Short wavelength light, much like that used in resin-based polymerization, can achieve quite a bit of complexity if controlled from multiple point sources; however, it still lacks biocompatibility and the ability to build true complexity.[64] This is reviewed further in biocompatibility of printing processes.

Multi-photon excitation of a fluorophore, and its use in photolithography, has inherent advantages in that it yields high complexity. 3D control of structural polymerization and the ability to print behind and inside of already-printed structures allows for unprecedented complexity. Multi-photon excitation allows for a 3D hologram to be generated with ultra-fine, one-micron resolution, in a mask-less lithography process, while using 2P long wavelength light that is can pass through already polymerized structures or cells and be focused on the other side to continue printing. This is achievable because focal plane or voxels in the case of a holographic projection are the only place where polymerization of the print materials is induced. Therefore, complexity is easily created by printing behind and even within already existing structures using multi-photon polymerization.

BIOCOMPATIBILITY

To build an organ or tissue, biocompatibility, is of paramount importance.

First, the printing substrate or materials and process used to create the tissue must be compatible with living cells. Second, the tissue must be tolerated by the host's immune system. Third, the materials must be structurally sound; matching the properties of tissues such that it can operate within the physiologic requirements of an organ.

Printing materials that are highly biocompatible range from extracellular matrix proteins such as hyaluronic acid and the most abundant, collagen (60-80% of a given tissue), to biologically inert materials such as polyethylene glycol (PEG).[65, 66, 67] Collagen is highly evolutionarily conserved wherein human and animal collagen is often indistinguishable at the biochemical level, leading to cross-species compatability.[68] Furthermore, collagen is generally immunologically inert with only 2-4% of patients reacting to cosmetic injections of bovine collagen, a response that can be tested for well ahead of transplantation.[69] Furthermore, recombinant sources of pure collagen have been developed to bypass any complications with animal-based production of materials making this an ideal material for the scaffold of transplantable tissues.[70] Mild cellular toxicity can occur in some printing formulations that include high amounts of photo initiators; however, with protective formulations and low exposure time, these issues are not expected to hinder tissue and organ printing via light-induced polymerization.[71, 72, 73]

The printing process that creates a cell-containing extracellular matrix must also be biocompatible. Extrusion and spray-based printing of cell-containing bioinks creates significant sheer force, even at low resolution, and therefore is not scalable to a higher-speed lower resolution that could maintain cell viability at the speeds necessary to build whole organs. Light-based polymerization processes do not exert sheer force on the cells and can print at high resolutions; however, photo-damage and heat-induced damage can be significant issues. Short-wavelength light in the visible range, projected at a high intensity is photo-toxic to cells, and UV light sources cause single and double stranded DNA breaks in as many as 10% of the cells with potential to result in oncogenic transformation.[73, 74] Transplantation of a potentially cancerous cell population would be of prohibitive concern, making UV or short-wavelength light-based lithography for living bioinks a high-risk proposition.

PRELLIS BIOLOGICS 3D PRINTING TECHNOLOGY

Manufacturing methods have made significant progress towards meeting each of the four requirements for building human organs from scratch; Resolution, Speed, Complexity, and Biocompatibility. However, to date, no single technology has been reported to meet all four. Here we present our laser-based holographic printing process that provides a single platform technology that meets the resolution, speed, complexity, and biocompatibility, requirements necessary to bioprint human tissues and organs.

By using holographic 3D printing with a far-red laser, we effectively decouple speed from resolution, while introducing previously impossible complexity. This has allowed, for the first time, the creation of sub-cellular resolution structures using cell-laden bioink. The applications of this technology are numerous, ranging from ultra-fast single cell encapsulation to printing of replacement human organs and tissues.

Subcellular Tunable Resolution and Print Areas

In the lithography field, often used for computer chip manufacturing, the resolution obtained by printing with light can be sub-wavelength (on the order of tens of nanometers). Although the extracellular matrix that holds tissues and organs together is sub-cellular in resolution, on the order of one-hundredth of a human hair, these structures are far larger than the components of a silicon wafer, ranging from 200 nanometers to several microns in diameter.

At Prellis Biologics, we have built a photo-lithography-based system that prints within a given 3D field of view with simultaneous multi-voxel projection, much like the projection of a hologram (holography) with voxels (3D pixels) that maintain a print resolution of 0.5 x 0.5 x 3 microns in the x, y, and z dimensions. The resolution of the system is dependent upon the optical components used to guide and focus the light source.

Custom optical components can be introduced to decrease resolution or increase the speed of printing. Subcellular optical resolution (less than about 1 micron), however, may not be required or desired for printing vasculature embedded tissues. Because the holography process utilizes optics, rather than a print nozzle for material deposition, the width of the projected laser light can be can be altered on the order of milliseconds to increase or decrease structural dimensions of the printed materials. Optics-based holographic printing therefore offers both high resolution and near-immediate changes in resolution that can be applied to create ultrafine structures.

Printing Speeds Compatible with Organ and Tissue Generation

The polymerization process is controlled by the localized absorption of light, lending flexibility on par with that of projecting a series of images on a movie screen. Indeed, the holograms utilized to recreate the structure are projected as a series of images at speeds well over video rate speeds, up to 250 Hz. Manufacturing of 3D-printed components is dependent upon the speed of layer deposition. In this case, the layers are three-dimensional and deposited by a light-induced chemical reaction (**see Figure 1**) that occurs on the order of 5 or fewer milliseconds. At these speeds, printing time shifts to be more dependent upon the chemistry of the printing formulation than the physical properties of laser projection. By removing the mechanical encumbrances of laser light scanning in the case of pinpoint laser printing, or z-step requirements of planar printing, while scaling the manufacturing technique from 1D or 2D, a significant reduction in print times occurs. This allows biologically complex structures to be created at unprecedented speeds. Increased print speeds decrease manufacturing times of complex tissues such that printing of human organs and organ structures is now in the range of possible.

3D Layering Allows for Organ Systems Level Complexity

Beyond meeting requirements of resolution and speed, Prellis Biologics can utilize far-red based light polymerization to introduce previously undescribed complexities into the 3D printing process. Printing a fully formed structure inside of another already formed structure is possible using multi-photon polymerization, where the structure is optically clear to the far-red excitation source. This process is demonstrated below in **Figure 3**, where a single sphere is deposited inside of a printed tube.

Figure 3. Demonstration of precision multiphoton printing inside an already formed structure.

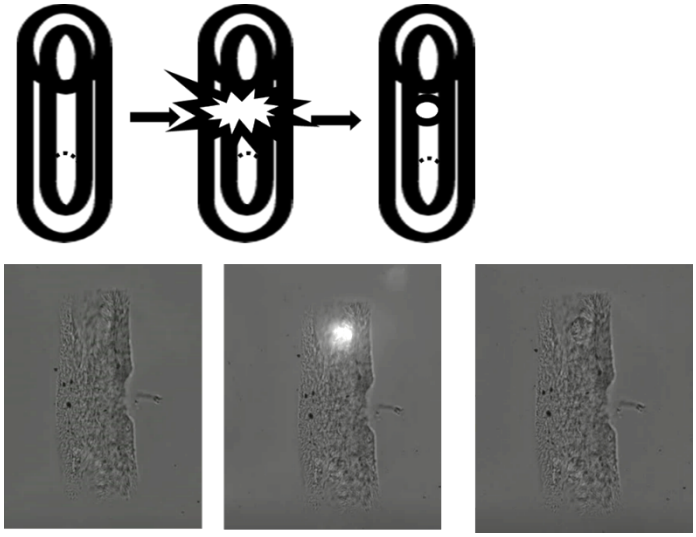


Figure 3. A representative graphic of printing holographically printing a sphere inside of an already formed three-dimensional tube (top row). A hollow tube structure printed with multiphoton holography allows for the projection of a spherical hologram into the center of the tube depositing the complete sphere inside of the tube without disrupting the structure. The sphere is printed in 5 or fewer milliseconds (bottom row).

To date, the ability to print behind or within another already deposited layer has not been described with any other 3D printing method. This feature is useful for adding additional tissue layers within or behind already deposited layers in a multi-layered cell printing process. This capability is critical for building complex tissues, such as the kidney, especially the glomerulus the primary point of blood filtration. The glomerulus is comprised of a fine networks of renal vasculature sit within the capsule like a ball and socket joint, or baseball inside of a baseball glove. Therefore, 3D layering with living cells allows for creation of structures around finely layered microvasculature and thus the development of fully functional tissues and in the future, organs.

Biocompatibility of Multi-Photon Holographic Printing

3D printing ink is of paramount importance for building complete tissues. Traditional cell-laden bioinks can be formulated for multiphoton-based printing through the addition of a photo-initiator. The photo-initiator undergoes a photoreaction on the absorption of far-red (infrared) light, catalyzing the polymerization of the bioink. There are a variety of bioink/photo-initiator combinations that are compatible with multiphoton printing, all of which have demonstrated high biocompatibility when subjected to cell viability studies.

Relative to UV and single wavelength polymerization, far-red multi-photon excitation, provided by a femtosecond (10×10^{-15} second) pulsed laser source, ensures that high energy is only absorbed at the focal plane, therefore the rest of the bioink only experiences brief pulses of far-red low energy light.

Studies using far-red light at various wavelengths have demonstrated far-red laser light to be non-oncogenic and protective from oxidative stress in cell culture.[75] Accumulated cell damage is pulse-length, power, and dwell-time dependent; therefore rapid printing and short laser exposure minimizes the risk of cell death. Furthermore, laser irradiation at low powers has no measurable effects on cell division, at infrared wavelengths roughly above 1 nm.[76, 77]

Summary of 3D-Bioprinting Technology

Prellis Biologics is poised to move into the human organ and tissue market rapidly and with a unique approach that can be scaled to generate whole organs in timeframes that will allow for transplantation in high-need patient groups. 3D printing approaches that can meet all four requirements of resolution, speed, complexity and biocompatibility will enable a myriad of functional applications of tissue and organ engineering. Examples of applications that Prellis Biologics' technology is uniquely suited for include rapid single cell encapsulation, organoid printing for disease models, and tissue and organ printing for transplant.

Prellis Biologics has demonstrated biocompatible single cell encapsulation capabilities at estimated rates of up to 20,000 cells per second. Using a deterministic, or targeted approach, Prellis Biologics' holographic projection of encapsulation spheres is being developed with computer vision capabilities to enable selection and rapid encapsulation of specific cells based on label or morphology, while preventing accidental encapsulation of multiple cells at once. Single cell encapsulation enables single cell precision during the engineering of complex tissues, and will facilitate delivery of cells in vivo for therapeutic uses. The single cell encapsulation total addressable market is estimated at $1.67B in 2017.

Vascularized organoids for detailed study of human disease models has numerous advantages over studying cells in a dish and animal model use. The discrepancy between in vitro efficacy and clinical outcomes can be attributed to limitations of 2D cell culture models. With print resolutions in the sub-micron range, Prellis Biologics will have the capacity to recreate complex 3D tumor microenvironments that combine tumor cells with extracellular matrix. This will allow for improved screening of drug responses and the study of tumor-stroma molecular interactions**.** The total addressable market for 3D cell culture systems was estimated at $559M in 2017.

Scalable Human Tissue and Organ Engineering

More than 2 million people die prematurely every year for lack of access to treatment for kidney failure.[92] Numerous chronic illnesses are managed by therapeutic interventions that are both costly with numerous repeat interventions required and lead to high patient morbidity and mortality. Replacement human organs and tissues will both help to alleviate sky-rocketing medical costs and allow for patients to regain functional independence from dialysis, oxygen tanks, multiple daily insulin injections, and other life-altering therapeutic interventions. Scalable human and organ engineering, using a patient's own cells to maximize compatibility is a medical intervention with the true hallmarks of a disruptive technology as it can be applied to nearly every major disease state. In solving the final hurdle in organ manufacturing, the ability to rapidly produce a vascular system, holographic 3D printing can support for the first-time large-scale tissue manufacturing that will allow for significant advances and pave the way to functional organ replacements. The size of the human organ and tissue market when estimated by the number of patients in need, multiplied by the standard procurement cost of a donated organ, totals well over $3T world-wide.

Other approaches to solving the human organ shortage and the impending medical crisis including recellularization of existing tissue structures and development of animals for xenografts that would allow for easy transplantation due to removal of immune-system triggering elements. Both technologies offer promise in specific areas, however both face significant technical hurdles that may be cost and energy prohibitive in development of full human organ transplantation.

Recellularization of tissue scaffolds involves dissolving cells with detergents, washing the remaining extracellular matrix, then re-introducing the desired cells by bathing the tissue structure in a cell-containing media. Recellularization is one approach that has been developed with moderate success in the laboratory setting. Indeed, visually stunning examples of cellular behavior in decellularized structures such as cardiomyocyte repopulation of a spinach leaf [78] have inspired the field in the direction of replacement tissue engineering. To-date one animal transplant with a rat lung lobe has demonstrated partial success, wherein the lung retained at least partial function for 6 hours but the tissue function ultimately failed, and the rats required intubation.[79] Furthermore, this process has yet to produce working blood vessels and microvasculature, however numerous promising studies especially in the area of lung recellularization are underway. In some skin transplant studies, blood vessels begin to grow into the transplanted tissues, however thicker portions of tissue rapidly become necrotic due to lack of oxygen. Another hurdle faced by recellularization is that it does not solve the critical problem of single cell resolution or placement of cells within finite functional cell niches. For example, airway endothelial cells

and vascular endothelial cells, along with alveolar macrophages must have the correct relative placement for the lung tissue to differentiate and function properly. In one re-cellularized lung studies, scientists observed incomplete differentiation of the engrafted cells, noting a lack of mature ciliated or secretory cells in the conducting airways.[79] Recellularization is effective in painting layers of cells on a tissue scaffold which works for thin tissues. Ultimately sourcing of the organ scaffold may become a significant bottleneck that can be alleviated by effective 3D printing of identical cell scaffolds.

Xenograph Transplantation

Xenograft models are particularly attractive source of organs for transplant. Heart valves and ligaments from porcine, bovine, and ovine sources have been used successfully for decades. These larger structural elements are chemically treated prior to remove cellular components prior to transplantation to remove cellular components and there are few reported cases of rejection. Chemical pretreatment is not compatible with the transplantation of more complex function organs, however, leaving two significant hurdles preventing the widespread adaptation of xenotransplantation: transmission of zoonotic diseases and immunological rejection.

Recent advances in gene editing approaches have catalyzed efforts to breed and develop genetically modified pigs that will produce organs suitable for human transplantation. Much like humans, mammals carry diseases and viruses that could be transmitted to xenograft recipients. Of concern are porcine endogenous retroviruses (PERVs), which are inserted into the genome of every pig and can infect human cells. Recently, scientists have used the genome editing approach, CRISPR, to reportedly eliminate all 62 known PERVS from their genetically modified porcine population.[80] While significant additional work, particularly in immunological compatibility will be required to optimize this approach in pigs developed for organ transplant, this represents a positive step in the development of organs for transplantation.

The second significant barrier is rejection due to immune response, or graft v. host disease where the organ graft brings enough of its own immune cells along to mount an attack against the host. Our immune systems are highly evolved to reject any foreign material; organs from a porcine source breed stock that exists today could provoke a significant immune response. Scientists have begun efforts to use gene editing to modify porcine specific-genes to develop pigs with organs that have improved bio-compatibility. However, this will be a difficult approach. First, to manipulate the porcine genome to be accepted by the human immune system numerous genes must be altered. Second, there are thousands of protein

differences between humans and porcine species, and many more are likely to become recognized as immunogenic. Third, and perhaps the most prohibitive, many of the genes in the immune system and similar proteins play a significant role in the developmental process of the animal during gestation, mutations and alterations to these may be a non-starter in developing a breeding population. Finally, even if most of the factors that cause rejection were eliminated, a patient would likely still require life-long immunosuppression. Immunosuppression drugs average $20,000 per year and increases the likelihood of death from aggressive cancer by three-fold across all age groups.[81]

An alternative approach would be to grow human organs in host animals using human-pig chimeras. In recent studies, scientists used gene editing to remove the genes responsible for pancreatic development in a pig embryo. With the introduction of human stem cells during the gestational period, scientists are determining if a functional human pancreas will develop inside of the porcine host. While this approach was successful in mouse-rat chimeras,[82] recent attempts to create human-pig chimeras showed extremely low rates of human cell engraftment.[83]

The xenograft approach is beneficial in that tissue will not be as supply limited as re-cellularization approaches using human organs and combining the two approaches may prove fruitful for the development of many tissues for transplantation.

SUMMARY

Fewer than 20,000 organ transplants are carried out each year in the United States. Every year less than one-sixth of the people on the organ transplant waitlist receive an organ. An estimated 330 people die every day in the United States due to organ failure. Requirements for being on a transplantation list are strict; the over 120,000 people waiting are only a fraction of over 90 million people in the United States who are suffering from some form of progressive organ failure and could benefit from an organ transplant. Progressive organ failure, specifically kidney failure, is a silent disease that will affect one in seven adults in the United States, and is a contributor to heart disease,[8, 84, 85] dementia,[86, 87, 88] loss of bone density and catastrophic fractures,[86, 89] and Parkinson's disease.[90, 91]

Advances in immunotherapeutics, vaccines, and treatments for communicable disease have demonstrated significant impact in health and life-span; however, few advancements have occurred in the field of organ replacement. As these causes of death are minimized, a new challenge is on the horizon: how does our health care system cope with the tremendous costs associated with organ failure?

Building human organs with 3D printing not only has tremendous potential for improvement of therapeutics development but in developing an interventional protocol that will minimize the progression of the major points of health failure and cost.

Significant issues exist in technological approaches to organ engineering, such as re-cellularization, and the development of animals for xenotransplantation. Standard 3D printing methods are hindered by the trade-off between speed and resolution in manufacturing. The slow speed at necessarily high resolutions does not allow for cell-containing native tissue structures, complete with blood vessels, to be printed fast enough to keep cells, or a patient, alive.

At Prellis Biologics we have developed a laser-based technology that decouples speed from resolution, in a biocompatible printing process with capacity to build highly complex structures. Our mission is to develop this platform technology to build fully functional human organs and tissues on demand. These organs will represent a disease-free, fully-human, cell-based alternative to donor organ transplantation. In addition, this technology can create perfectly matched organs that will eliminate the need for immunosuppression and offer the possibility of removing disease causing genetic mutations. The vision of Prellis Biologics is to give the millions of patients suffering from progressive organ failure their lives back.

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Prellis Biologics, Inc. was founded in San Francisco, CA in October of 2016, has filed 2 patent applications and has raised a total of 3.1M in pre-seed, seed funding from lead investors IndieBio and True Ventures.

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