Using 3D bio-printing for the development of human organs and tissues

Name: Kimberley Bakker
Student number: S2591170
Supervisor: Rob Coppes
University of Groningen, Department of medical cell biology, University Medical Centre Groningen
Date: 22-06-2017

Abstract
Since 1950 organ transplantation has been very helpful and live saving for millions of people worldwide. There is a growing shortage of donor organs and new alternatives for transplantation have been investigated already. 3D bio-printing of cell-laden scaffolds or even entire tissues and organs has been emerged over the last decade and there are already several applications for 3D bio-printing. To find out if 3D bio-printing of tissues or organs is a reasonable alternative to transplantation, the main question in this review is: "Is 3D bio-printing of tissues or organs a possible solution for patients who need a donor organ?" The 3D bio-printing procedure contains multiple steps to design a desired construct. Imaging can help to design the proper shape and structure of the produced artificial bio-tissue and computer-aided design (CAD) and mathematical modeling can be used to assemble and digitalize the information about structure and shape for reconstruction. Subsequently, the information can be converted into two dimensional slices and referred to the 3D bio-printer. Different cellular bio-printing methods use other bio-ink deposition mechanisms and in printing modality. Common cellular bio-printing methods are droplet based, extrusion based or stereolithography based techniques, which distinguish themselves in available bio-materials, resolution, printing speed and cell viability. Bio-inks contain biomaterials, biochemical agents and cells. Bio-inks can be deposited in different specific patterns and can be printed with or without scaffolds. Besides a specific deposition pattern to form a stable construct, the composition of bio-inks also is important for the strength and function of the 3D construct. Different cell types can respond differently to shear stress, therefore a scheme of optimal conditions of bio-inks, like printing speed, additional biomaterials, density, viscosity, for each cell type should be generated to optimize cell viability and unaltered cell function. Conclusion: 3D bio-printing is a fast developing technique, but has currently multiple limitations that have to be overcome, like problems with vascularization and innervation. Furthermore, 3D bio-printing techniques need more development to improve bio-print speed and resolution. So the answer to the main question for now is no. Simple tissues might be expected on short-term duration, but at this moment science is not that far that bio-printing an entire organ or tissue in a close future is realistic and organs being present on a list of organ shortages are usually complex organs and cannot be expected on short-term duration.

Key words: 3D bio-printing, tissue engineering, scaffolds, CAD, biomaterials, droplet bio-printing, extrusion bio-printing, stereolithography bio-printing, *in vivo* bio-printing

Index

Introduction

In the US, each day every 10 minutes another person is added to the waiting list for an organ transplant and every day 22 people die waiting. The number of organ donors is growing slowly, while the waiting list grows considerably every year[1,14]. Since 1950, organ transplantation has been very helpful and live saving for millions of people worldwide with incurable diseases or organ damages[9]. Unfortunately, not every donor organ can simply be matched to every person, because some common factors as immune-tolerance, body/organ size, age, health, survival of the organ outside a body and patient's waiting time on the donation-list play an important role in the matching process. Some of these factors are more organ dependent, for example organ size or duration of the organ's survival outside the body. Hearts and lungs have a relatively short survival in comparison with other organs, which is shown in figure 1[1]. Not only a shortage of donating people, but also acute or late immune rejection of the transplant is a big issue after the transplantation process[2,14], which makes immunosuppression necessary[3].

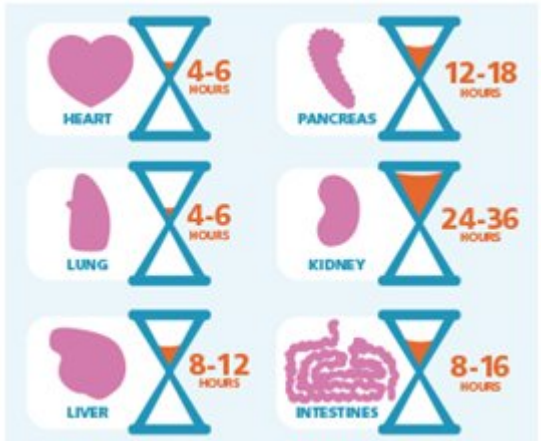

Fig 1. In the matching process of organ donation some factors are organ dependent. Hearts and lungs, for example can only survive for four to six hours outside the body. Also the size of an organ does matter here, because the organ has to fit comfortably in the rib cage of the receiver. Kidneys can survive much longer outside the body and therefore can cover a longer distance. Figure adapted from U.S. Government, Information on Organ Donation and Transplantation

Immunosuppression has improved last decades, but in general, there is no optimal regimen to prevent an acute or late rejection[3]. Besides, immunosuppression is not without risks or side effects[3,4] and there is no standard procedure for treatment, because of many individual differences. For example, factors such as human leukocyte antigen (HLA) difference, age and ancestry are influencing the balance between efficacy and risks of immunosuppression[3]. If a transplantation succeeds, the patient needs maintenance therapy for months afterwards[3]. Despite that the transplantation process has improved over the last decades, there might be other ways to shorten the waiting list and to increase the amount of donor organs. For example by education of the medical staff to improve the referral of potential donors[5]. Also the government plays an important role to inform and stimulate people to sign up for being a donor[1,6]. However, different approaches to overcome the organ shortage and this immune rejection problem have been conceived. One of these approaches is a personalized medicine approach, whereby individual treatment strategies are being induced[4]. In an interesting personalized medicine research from Andrew T. Sage and Shana O. Kelley *et all.* a novel class of chip-based sensors is investigated in lungs, which is able to perform a quick analysis of a lung biopsy, measuring multiple mRNA biomarkers correlated to the development of primary graft dysfunction (PGD). In this study, a model was developed to predict the chance of PGD incidence in a donor lung[7]. Decellularization and subsequent recellularization of donor organs has also been investigated[24]. Another upcoming approach is the idea of fabricating the patient's own organs by 3D bio-printing. 3D bio-printing could be used to recover or replace damaged tissue with healthy and functional tissue[14]. Repair and replacement of dysfunctional organs with 3D printed organs is promising[2,9,14], but is still in its earliest state and needs further investigation. In this review will be discussed if 3D printing can contribute to impair the shortage of donor organs and if 3D printing could be a realistic alternative for organ transplantations. Several topics concerning 3D printing such as use of scaffolds, the current possibilities of 3D bio-printing, the advantages and disadvantages of 3D bio-printing of bio-artificial organs, the main steps in the 3D printing process, different printing techniques, potential cell types and materials, their limitations and also applications will be highlighted. The research question in this review is therefore: "Is 3D bio-printing of tissues or organs a possible solution for patients who need a donor organ?"

Scaffolds

In the regenerative medicine field, scaffolds have widely been investigated the last decades as alternative for transplantation[14]. Scaffolds are bio-artificial cell supporting biological structures used for tissue engineering, which have an extra cellular matrix (ECM)-like function[8,12,14]. In the human body, the ECM has several functions, such as giving cells a structure to attach, proliferate, migrate and differentiate, as well providing nutrients and growth factors to maintain the surrounding cells[8,14]. The ECM also provides organ- or tissue specific mechanical functions, such as rigidity and elasticity[8]. A good working scaffold mimics the native ECM of the desired target tissue very precisely and should provide a good microenvironment for cells with the possibility to become a functional tissue[2,8,14]. However, the ECM is a dynamic and complex structure, because of its multiple components and diverse functions, and is therefore hard to mimic[8]. In figure 2 four major methods to fabricate scaffolds are shown[8]. The different types of scaffolds support three dimensional tissue formation[8,14] and can be implanted or injected to replace or repair defective tissues[8]. Pre-made porous scaffolds can exist of diverse natural or synthetic degradable biomaterials[8,15], which can be loaded with cells afterwards[8]. They can be made with different methods, including 3D bio-printing[8,11,14]. Porous scaffolds have advantages, such as large diversity of biomaterials and relatively precise designs of microstructure, which is important for mechanical properties. However, this technique also has some disadvantages. The cell loading process is time-consuming and therefore detrimental for the cell's viability[8,10] and because of limited penetration ability of cells into the pre-made scaffold, the cells do not become homogeneously spread, leading to different properties in different parts of the scaffold[8]. Decellularized ECM has this same problem of heterogeneous distribution of cells into the scaffolds. It also is difficult to keep all ECM during the removal of the cells and therewithal an immune response is not excluded when not all foreign cells are completely removed[8]. Nevertheless, decellularized ECM is the most true to nature type of scaffold and has a good biocompatibility[8,24]. In a 2008 study, the first decellularized heart from a rat was described. The organization of the vasculature was preserved and reseeding the heart with rat cells led to macroscopic contraction and visible pomp function[24]. Also decellularized human hearts have been investigated more recently and several other studies have investigated also kidneys, lungs and pancreas[24]. Though, this personalized tissue engineering technique has been extensively evaluated and discussed[24]. For clinical application, an entire decellularized organ needs besides recellularization also endothelialization for maintenance of the blood flow, because the endothelial protects blood from the thrombogenic properties of certain ECM proteins[24]. The number of different cell types to recellularize the decellularized scaffolds is also limited, furthermore there is no control of precise distribution of reseeded cells[24].

Cell sheets with secreted ECM have high cell density and can be stapled to form a thicker layer of multiple cell sheets[8]. However, it is difficult to produce a thick implantable construct[8] and another disadvantage of this method is that not enough ECM could be formed for ECM-rich tissues[8]. The fourth method encapsulates cells in self-assembled hydrogels, which can be injected[8]. Hydrogel based scaffolds are mainly used in cartilage regeneration, because chondral tissue has good compatibility with the hydrogels[20].

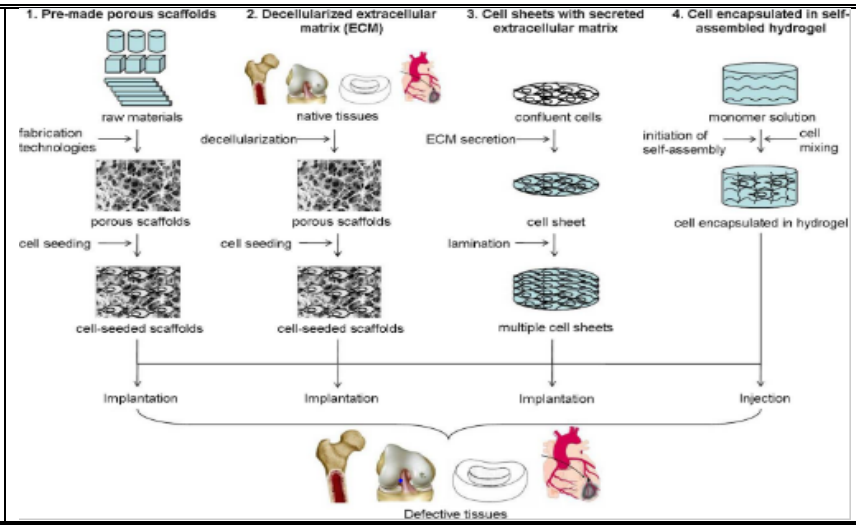

Fig. 2. Four major approaches to generate scaffolds for replacement or repair of defective tissues. Pre-made porous scaffolds and decellularized scaffolds can be loaded with cells afterwards. Porous scaffolds can also be 3D printed. Cell sheets can be used for thin tissue types with high cell density. Cells can also be encapsulated in hydrogels and then be injected. Figure adapted from B.P. Chan and K.W. Leong (2008)

However, current fabrication methods for scaffold generation are not ideal and have been doubtful[2,14]. Pore size, pore geometry, mechanical strength and high connectivity cannot efficiently be fabricated, because of limitations in conventional techniques[8,14]. As mentioned before, through unequal distribution over a scaffold, the cell seeding procedure is not very staunch[2,8,14]. Additionally, it is difficult to keep the vasculature of a certain tissue and remaining decellularization reagents can cause toxicity to growing cells on decellularized tissue scaffolds[2,22]. Therefore a growing interest in three dimensional printing of cell laden scaffolds or even entire tissues has been emerged, since it has several advantages[14,28]. The ability to print scaffolds or ECM simultaneously with cells may lead to the facility to fabricate a biomaterial with properties similar to native tissue[20,28]. 3D printing enables cells and scaffolds to be built in a layer-by-layer process and to have a complex structure with homogenously cell distribution[14,28]. Thence, the scaffold imitates the ECM better than scaffolds whereat the cells have to be loaded afterwards[14,20].

Current state of 3D bio-printing

In 1986, the 3D printing technique of stereolithography was first described by Charles Hull[2,22,24]. His method was originally designed to print non-living materials, such as molten plastics and metals, so applying this technique to bio-print living materials is therefore challenging[22]. Presently, 3D bio-printing techniques are an upcoming branch in tissue engineering and have already been widely explored and advanced over the last decade[9,22]. 3D bio-printing can also be used for scientific applications other than tissue engineering, like toxicology studies, *in vitro* disease models and also pharmacological purposes as drug delivery and discovery[9,14,21]. 3D bio-printing could be used for prosthesis production and for other personalized medicine purposes as well[11,24]. Multiple tissues have been investigated for 3D bio-printing, including some more simple tissues as cartilage, bone, bladder and skin, but also more complex tissues as nerves, muscle, heart, liver, kidneys and more[2,9,14,19,22,24]. Most studies are focused on strategies for regeneration of cartilage, bone and skin[9]. Poorly vascularized tissues with few different cell types are easier to engineer than well-perfused tissues with multiple cell types[28]. Simple 2D tissues, such as skin, and hollow organs, such as bladder, existing of one cell type can be produced already with use of scaffolds[24]. Cartilage has already been 3D printed and been investigated in multiple studies[19]. Methods to bio-print complex structures and shapes have been developed, for example to bio-print ear-shaped structures containing chondrocytes[19,23]. Chondrocytes were found to be capable to produce cartilage in *in vivo* experiments[19,23]. In an interesting study from Thomas Möller *et al.* 3D printed cellulose/alginate scaffolds containing chondrocytes were implanted subcutaneously (fig. 3) in nude female mice to investigate *in vivo* chondrogenesis[16], which is the formation of cartilage[17]. Cells were mixed in a hydrogel before printing. Four types of printed scaffolds were used, one cell-free scaffold for control, one scaffold consisting of human male nasal chondrocytes, one consisting of human female bone marrow-derived mesenchymal stem cells and one scaffold containing a mixture of these two types of cells. The implanted 3D printed scaffolds, especially the mixed construct, were concluded to be able to synthesize cartilage *in vivo*, so 3D bio-printing might be a potential appliance for cartilage reconstructions in the future[16]. However, at this moment science is not that far that bio-printing an entire organ or tissue in a close future is realistic[26,29], but developing more advanced techniques able to bio-print specific cell types simultaneously with biomaterials similar to native tissues will help to

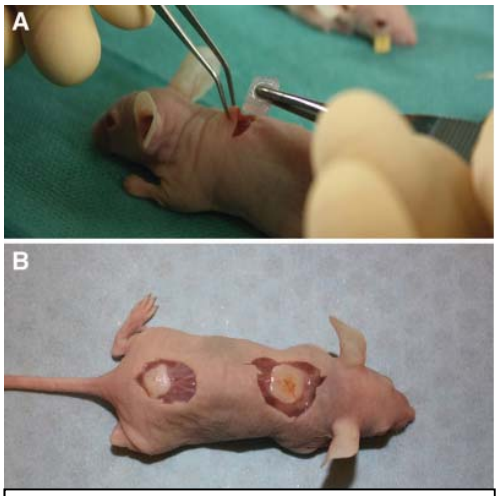

Fig. 3. A. 3D bio-printed scaffolds were implanted subcutaneously in nude female mice. B. 14 days after implantation the scaffold was integrated and surrounded with native tissue. Scaffolds containing a mixture of human male nasal chondrocytes and human female mesenchymal stem cells were able to synthesize cartilage *in vivo.* Figure adapted from Möller T *et al.* (2017)

realize the bio-printing process of tissues and organs[9,14,20,24,27]. 3D Printed bio-artificial tissues larger than 3,8 mm in length, high and width are classified as large constructs[23]. For now, cell viability and accurate control over cell dispersal are limiting factors to produce functional large tissues[9]. More advanced biomaterials capable of integrating in native tissues or organs have to be developed to make tissue regeneration possible[27]. Also, printing a thick tissue is still problematic, because bio-printing of vascularized tissues is not possible yet and thick tissues do not integrate (completely) with the vasculature of the receiver[9,18,19,21]. The size of the bio-printed tissue without vasculature or integration in tissue of the receiver is therefore limited to diffusional distances, which ranges approximately from 100 to 200 micrometer[9,21]. Biological and mechanical properties of native blood vessels are hard to mimic[9]. Hence, high cell viability and functionality of the 3D bio-printed product, regulated by oxygen and nutrient influx, is challenging[19,21]. Several ongoing studies are searching for new techniques to bio-print vascularized 3D structures[9,19]. For example, in an ongoing study from Richards *et al.*, a functional *in vitro* vascular channel was created to support the viability of tissues. Using this method, it might in the future be possible to bio-print vascularized tissues[18]. Despite much progression and improvements of 3D bio-printing techniques for producing more complex tissues the last decade, 3D printed tissues have not been clinically succesful yet[9]. But there are multiple ongoing studies with the intention to change this[9,19].

Advantages and disadvantages of 3D bio-printing

The possibility of 3D printing organs has multiple advantages. One advantage is the potential to bypass the intrinsic limitation of immune response and organ rejection, which is occurring in the transplantation process[2,9]. If 3D printing of organs becomes truly possible, this upcoming technique can in the future possibly solve the problem of donor organ-shortage[2,19]. 3D bio-printing also overcomes the limitations of scaffold-based tissue engineering, because it provides precise control of cell distribution and high cell deposition[19]. Another advantage is the possibility of custom-made constructions of tissues and organs when patients need one[11,24]. 3D bio-printers can be designed with completely digitalized systems. Fully digitalization of 3D printers will reduce costs, because 3D bio-products could automatically be produced with preset digital models, whereby less expenditure for labor-costs is required[11,19]. Furthermore, if techniques allow 3D bio-printing of organs someday, it might be a potential way to reduce animal testing[26,29]. Drugs are always tested on a rodent animal-type and a non-rodent animal-type before they are allowed to be used in human clinical trials. Animals can have other cytochrome P450 iso-enzymes to catalyze metabolic reactions and they can therefore react different on certain medications than humans[24,26]. 3D bio-printing can offer a way to test drugs and other therapeutic materials on 3D bio-printed personalized tissues containing the patient's own cells[26].

Unfortunately, the 3D bio-printing techniques also have some disadvantages[9,23]. As mentioned before, there are still several limiting factors, such as cell viability and vascularization[9]. Vascularization is essential for viability of a bio-printed 3D construct intended for implantation[22]. Therefore, only small tissues on millimeter scale can be fabricated yet and not human-sized tissues[9,23]. Production time to fabricate tissues might be a problem, because the more precise and complex the artificial tissue or organ becomes, the more fabrication time it will cost[14,15], which will be disadvantageous for cell viability[8,14,15,23]. Fabrication time required for production and maturation of a perfused vascular network through an entire tissue is probably longer than survival time of cells in the construct[22]. However, bioreactors, containing fluid with growth factors and other signaling molecules, can help to maintain tissue viability and provide extra time for post-printing processes, like tissue crosslinking, remodeling and maturation[22]. Besides maintenance of tissue viability, a bioreactor also can be used in combination with growth factors that promote angiogenesis and innervation, so the use of a bioreactor inures progress of research of the fabrication of implantable tissue constructs[22]. Another disadvantage is that the development of complex multicellular tissues and organs requires more complex and advanced bio-inks[9]. More complexity is associated with prolonged fabrication and residence time, which is not only detrimental for cell viability, but also for differentiation and proliferation-capability of cells[23]. Some cell printing methods use cell

encapsulating hydrogels. Temperature of these hydrogels is an important factor for cell viability and cannot be too high or too low[23]. Beside temperature, time also is an important factor for cell viability. Prolonged printing processes can lead to long exposure of previous printed layers, causing the hydrogels to evaporate. Thence cells can be exposed to a rough environment outside the hydrogels and subsequently cells could die or become contaminated, because the layers can fuse with each other[23]. Therefore, 3D printers have to be more fast and precise. Also, 3D bio-printers require specific properties of bio-inks to keep cells alive and functional[28]. So the variation of available biomaterials is often limited[28].

The 3D bio-printing process

In general, the 3D printing process contains three main steps; pre-bio-printing, bio-printing and post-bio-printing[2,28]. First, a computer model screens how to engineer the tissue layer-by-layer with a computer-aided design (CAD)[2,9,22,28]. Second, deposition of cells and other biomaterials is automatically positioned and processed by 3D bio-printers. Third, after the printing process the cell laden tissues are ready for implantation or have to maturate further into the desired tissue[28]. According to Hong N. *et al.* a 3D printed tissue can be incubated *in vitro* in a bioreactor and grow and maturate before implantation or tissues can be printed for *in situ* applications, which means that the human body itself acts as a bioreactor and helps with the integration of the construct into the body[9].

Biomimicry, autonomous self-assembly and mini-tissue building blocks are three different existing design approaches for 3D bio-printing tissues to create at long last functional and clinically usable tissues and organs[9,22]. In the biomimicry approach the native tissue or organ is imitated using similar cellular and extracellular components[9,22]. Autonomous self-assembly uses stem cells or embryonal stem cells that differentiate in a desired structure and function to replicate a biological tissue[9]. For example, earlier mentioned cell-encapsulating self-assembling hydrogels can be injected in damaged tissue and further polymerization of biomaterials can be initiated *in situ*[8]. Mini-tissue building blocks are made up of small functional building blocks, which can accrete together in a larger construct[22]. Applications of this last method include vascularized building blocks to create vascular networks or screening *in vitro* disease models[22]. A combination of these three approaches might be the best method to print a functional tissue with comparable structural and mechanical components and properties as native tissue[22]. In figure 4, six main steps of the three dimensional bio-printing process are shown, respectively imaging techniques, approaches for design, selection of materials, selection of cell type, bio-printing methods and some possible applications of 3D bio-printed tissues[22].

Imaging of damaged tissue and its surrounding tissue can help to design the shape and structure of the produced artificial bio-tissue[22,24,28]. The three dimensional structure at cellular, tissue, organ or organism levels can be elucidated and information about composition and organization of several components can be gathered[22,24,28]. For example, using a combination of high-resolution imaging, computer-aided design and 3D bio-printing a customized 3D printed bio-resorbable tracheal splint was implanted successfully in an infant with tracheobronchomalacia[25]. The infant suffered from compression of his left mainstem bronchus and 21 days after implantation, the infant was capable of breathing independently without respiratory support and 3 years after implantation, the splint was fully resorbed in the native tissue[25]. This shows that imaging is important to design personalized and anatomic implantable constructs[25]. Common used imaging techniques are X-ray, magnetic resonance imaging (MRI) and computed tomography (CT)[2,22,28]. Computer-aided design or computer-aided manufacturing (CAD/CAM) and mathematical modeling can be used to assemble and digitalize the tomographic information about structure and shape for reconstruction[2,22]. Computer models have also the capability to predict mechanical and biochemical properties of the artificial tissue[22]. The created three dimensional model has to be divided into two dimensional horizontal slices and subsequently imported into the 3D printer. The 3D printer receives via imaging techniques information from the CAD to build the desired tissue in a layer-by-layer process[2,22,27,28].

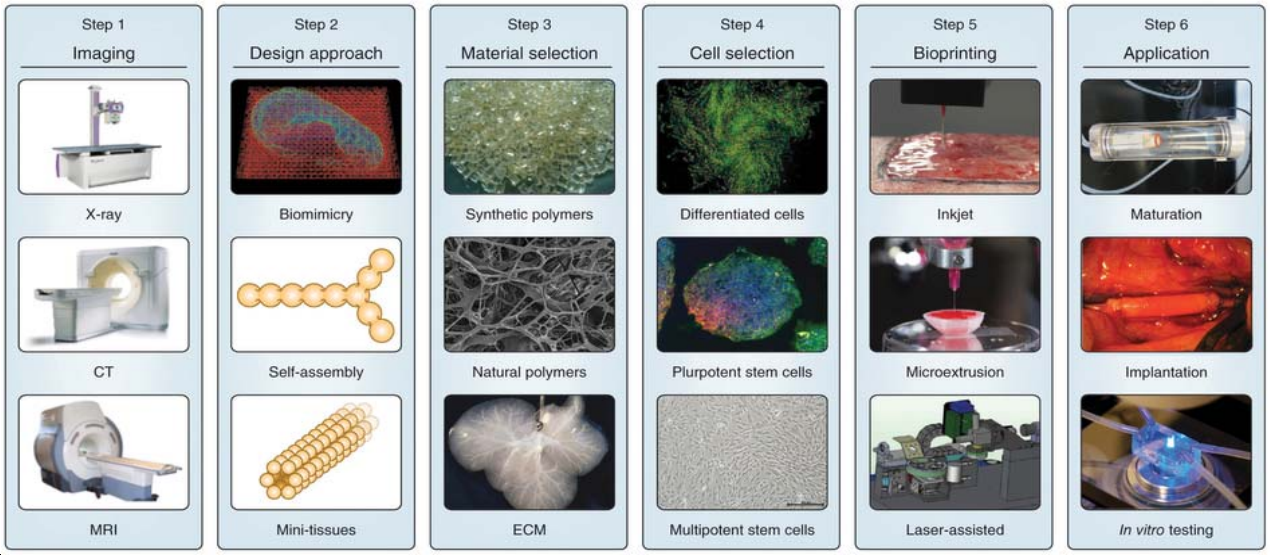

Fig. 4[22]. A representative 3D bio-printing process to fabricate tissues.
Damaged tissue and the surrounding tissue can be mapped with imaging techniques to construct an artificial tissue. Three different design approaches for 3D bio-printing tissues exist: biomimicry, autonomous self-assembly and mini-tissue building blocks. Material selection and cell selection is important to select the right components to mimic the structure and function of the desired tissue construct. Synthetic and natural biomaterials as well as decellularized ECM are common used materials, which provide oxygen and nutrients and other factors important for cell support. Cells can be allogenic or autologous and have to be mixed with polymers for integration in bio-printing systems. Several bio-printing techniques have been developed, such as inkjet, micro extrusion or laser assisted printers. To make 3D printed tissues suitable for implantation, sometimes maturation before the implantation in a bioreactor is necessary, but also direct implantation of the 3D bio-printed tissue could be possible. Another application for 3D bio-printed tissues is *in vitro* investigation, for example for drug testing and toxicology studies. Figure adapted from Murphy SV and Atala A. (2014).

Bio-printing techniques

Different 3D bio-printing techniques can affect tissue and organ design because of different approaches in building strategies of tissues[2,22]. Figure 5A shows a schematic illustration of different 3D bio-printing approaches. 3D bio-printing can be executed acellular to fabricate acellular functional scaffolds, or cellular to fabricate cell laden constructs in one step[2]. The acellular 3D printed constructs can be implanted directly or seeded with cells and maturate further before implantation[2]. In the cellular method, several methods with different advantages and disadvantages have been developed to generate artificial tissues and organs[2,22]. Different cellular bio-printing methods use other bio-ink deposition mechanisms and they therefore differ in printing modality[2]. Common cellular bio-printing methods are droplet based, extrusion based or stereolithography based techniques, which distinguish themselves in available bio-materials, resolution, printing speed and cell viability[2,22,27]. The 3D bio-printing systems have different properties and capabilities and therefore also have different limitations[22,27].

Droplet based bio-printing (figure 5B) includes inkjet (thermal and acoustic mechanisms), pneumatic pressure assisting mechanisms, electro-hydrodynamic mechanisms and laser assisted mechanisms[2]. The droplet-based bio-printer has no physical contact with the construct[2]. Overall, droplet-based bio-printers have multiple advantages, including high resolution, high speed, simple printing process and low costs[2]. The possibility to generate constructs with concentration gradients of cells or growth factors by controlling droplet size and cell density in a droplet is beneficial for the construct and more true to nature[2,22]. Inkjet bio-printing is applied to non-biological materials as well to biological materials[22]. This type of printer has the advantage of relative precise and repeatable cell deposition

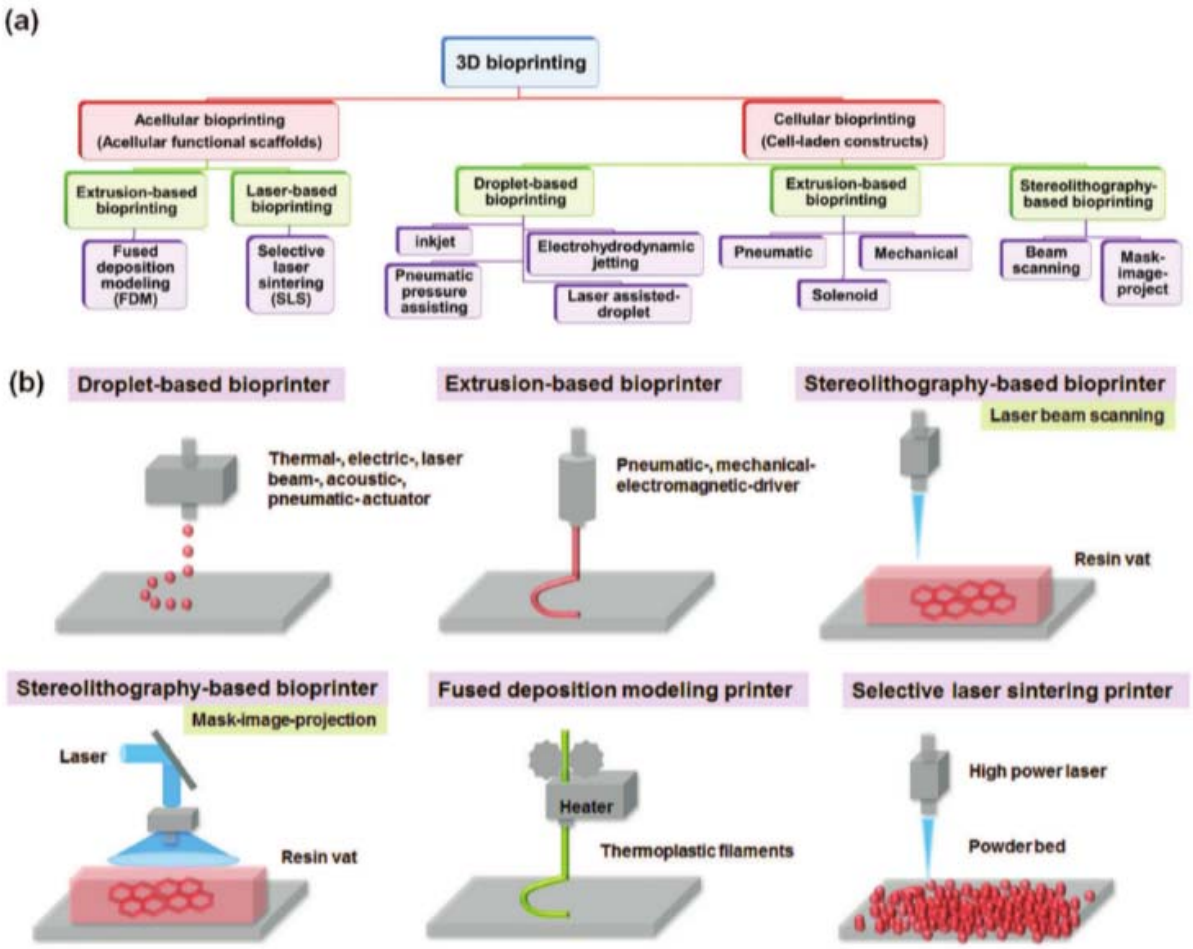

Fig. 5. Different 3D bio-printing techniques.
A. An overview of different acellular and cellular 3D bio-printing techniques is shown. The different bio-printing techniques are divided into several different approaches. B. Simplified illustrations of the 3D bio-printing techniques used for tissue- or organ regeneration. Figure adapted from Cui, H. *et al*. (2017).

capabilities, because it is capable to deposit controlled volumes with multiple cell types and biomaterials on established locations[2,22,27]. After the printing process the cells have also a high cell viability[2]. Multi-jet inkjets have already been developed to build organ prototypes containing multiple cell types and bio-inks[2]. Advancements concerning resolution, speed and precision of the printer and complexity of the construct are made[22]. Yet, bio-ink patterns of single droplets, containing one or two cells have been printed in lines with $50\mu m$ distance up to a speed of 10.000 droplets per second[22]. Inkjet printers operate alike 2D printers on a drop-on-command principle and are driven by thermal or by acoustic forces to drop a certain volume of bio-ink[2,22]. A bio-ink solution containing cells and biomaterials as growth factors and matrix components is stored in a reservoir, before it is transferred to an ink chamber for droplet ejection[2]. Thermal inkjets use heat to eject a droplet from the print head[2,22]. The print head is electrically heated to generate pressure pulses, which cause the droplets to be released[2,22]. The inkjet is locally heated up to 200-300^{o}C, causing the print head to be 4-10^{o}C warmer[2,22]. This heating is considered to be detrimental to stability of biological molecules, such as DNA, and to cell viability of the produced tissue, but several studies have contrarily shown that the higher temperature in the print head does minimally affect the materials that have to be printed[2,22]. Thermal inkjet printers are relatively fast, low in costs and are widely available[2,22]. Acoustic inkjet printers use piezoelectric or ultrasound pressure. A piezoelectric element creates an acoustic wave in the print head, which generates droplets of the same size. Changing the voltage in the piezoelectric element means a change of shape, which causes change of pressure on the bio-ink[22]. The ultrasound type is using parameters, such as amplitude and duration of exposure, to exactly control droplet size and ejection rate[2,22]. Also acoustic frequencies are not ideal

to use for bio-printing, because it can harm the cells in the bio-ink by inducing cell lysis and membrane damage[2,22]. In general, the bio-ink always has to be a liquid that turns into a solid 3D structure with an organized structure and function[22]. This problem could be solved with crosslinking of hydrogels together after droplet deposition, using chemicals, pH or UV methods, but this will slow down the printing process[22]. Additionally, the viscosity of the bio-ink cannot be too high, because the force to eject droplets from the print head has to be also high, which is not beneficial for cell vitality[2,22]. Another limitation of inkjet bio-printing is cell density in a droplet. The cell concentration in a droplet cannot be too high, because of clotting of cells in the nozzle of the print head and shear stress of the cells[2,22]. Though, inkjet bio-printers have been used already to print living cells[22,29]. For example, to generate bone constructs in mice. The printed bone was incubated *in vitro* and matured further *in situ*, resulting in a implanted bone tissue with a similar cell density as the native bone[22]. According to Murphy and Atala (2014), an inkjet bio-printer is that fast, that printing directly on skin or cartilage injuries could be possible[22].

Electrohydrodynamic jetting (EHDJ) is also a form of droplet-based bio-printing with a drop on command principle, but it is not driven by heat. The EHDJ contains a potential difference between a grounded electrode and a positively charged metallic needle, which leads to the generation of coulombic forces onto the surface of the bio-ink solution[2,29]. This coulombic forces cause deformation of the tip of the needle and cause droplet ejection in nanometer to micrometer scale[2,29]. Droplet size and distribution can be controlled by potential difference, flow rate of the solution to the needle, the distance of the electrodes and solution properties of the bio-ink[2,29]. The bio-ink covers a relative short distance and the cells remain undamaged when they reach the target substrate. EHDJ does not influence cell vitality while printing of the bio-ink occurs[2]. Cartilage constructs have already been made *in vitro* with a combination of inkjet bio-printing and EHDJ[22].

Another droplet-based approach is by pneumatic pressure on a bio-ink solution, which opens micro-valves and causes droplet ejection[2]. Droplet volume is controlled by adjusting the pressure on the bio-ink to flow distance and opening time of the micro-valves[2]. A benefit of this bio-printing type is the possibility to use bio-inks with higher viscosities. Still, there are several factors to take into account, concerning the structure of the final construct. Because the viscosity is still too low, crosslinking methods, like UV light, pH, temperature or chemical reagents, have to be applied to generate a solid and stable 3D structure[2]. As aforementioned, crosslinking can slow down the printing process, which is detrimental for cell viability and biological functionality[2].

Another existing droplet-based technique is laser-assisted 3D bio-printing. The laser-assisted technique is classified vaguely, because it is sometimes listed as an individual bio-printing technique and sometimes as part of a bio-printing technique[2,22,28]. In figure 5A the laser-assisted bio-printing (LAB) technique is included in the droplet-based bio-printing methods. This printer contains a laser source, substrate and in between a donor layer, consisting of a material that absorbs laser energy, such as gold or titanium, and of a bio-ink layer, which is prepared in a liquid solution[2,22]. The laser is beamed on the absorbing layer and causes the generation of a pressure bubble into this layer, pressuring a droplet out of the bio-ink layer. Subsequently, the droplet, with a volume in a range from 10 to 7000 Pico liter, is falling onto the substrate. The volume of a droplet is controlled by regulating the viscosity of the bio-ink and the thickness of the bio-ink layer, leading to a higher print resolution[2]. Beside thickness and viscosity of the bio-ink layer, the resolution is also influenced by the laser beam, surface tension of the bio-ink layer and wettability of the substrate[2]. There is no print head or needle and therefore droplets with high cell density and viscosity can be printed. However, this technique is relatively expensive, time-consuming, has a low efficacy and is limited in bio-ink choice[2].

Cells encapsulated in hydrogels can be directly printed in the **extrusion-based bio-printing** method[23]. A lot of different materials are suitable for extrusion-based bio-printers, including hydrogels, biocompatible copolymers and cell spheroids[22]. Micro-extrusion printing is the most common non-biological 3D printing method and is relatively low in costs[22]. The biological printer-type consists of a bio-ink dispenser for extrusion control and a controlled robotic system to control bio-printing[2]. The dispensing system of an extrusion bio-printer contains a stage and syringes or pens, that can be loaded with a bio-ink[2]. The extrusion head is capable of moving along the x, y and z axes and bio-ink beads can be continuously and exactly deposited into the desired 3D structure using CAD-CAM

software[2,22]. The following bio-ink layer can be deposited onto the earlier deposited bio-ink layer[22]. One advantage of continuous printing is a better structural integrity during a relatively fast bio-printing process[2]. Common extrusion bio-printer types are pneumatic-, solenoid- or mechanical-based dispensing systems[2,22]. Pneumatic-based bio-printers are driven by air pressure and can exist of a valve variant or a valve-free variant[2]. The valve containing printer is more precise, because air pressure and pulse frequency is more controllable than the valve-free variant[2]. Whereas pneumatic bio-printers have relatively simple compounds and mechanisms, mechanical bio-printers have more complex mechanisms and smaller compounds[22]. The mechanical-based printer has therefore a better spatial control, but can be limited in force capabilities because of its complexity[22]. Mechanical bio-printers can be divided into two systems; the screw and the piston system[2]. The screw system uses a higher pressure to deal with bio-inks with a higher viscosity, which can be disadvantageous for cell vitality, while the piston system contains needles, lower pressure and is used for bio-inks with lower viscosity[2]. Solenoid-based extrusion is electromagnetically driven and a very complex system. An electromagnetic force is generated by a ferro-magnetic plunger and a ferro-magnetic ring magnet. Electric pulses hinder the electromagnetic field and open the extrusion-valves for bio-ink ejection[2]. Overall, extrusion-based bio-printers are in comparison with droplet-based bio-printing very fast, have more cell deposition in shorter time and multiple bio-inks can be used heterogeneously, including cell-laden hydrogels, cell aggregates, micro-carriers, decellularized matrices and synthetic polymer fibers[2,22]. The properties of bio-inks and their interactions are important for 3D structure and function[2]. Solid bio-structures with high cell densities are printed directly, without any scaffolds and are crosslinked or fused together after printing[2,22]. However, high cell densities can cause shear stress and extrusion printing has lower resolutions. Therefore cell viability using micro-extrusion bio-printing is generally lower than cell viability using inkjet bio-printing, but regulating extrusion speed and pressure and the diameter of the nozzle can help to increase cell viability[2,22]. Also, the fabrication time needed to generate complex bio-structures with high resolution may be too slow to produce clinically relevant structures[22]. Therefore printing speed and nozzle, syringe and motor-control systems have to be improved[22]. Though, several tissue types have already been printed using micro-extrusion, including heart valves, branched vascular trees, *in vitro* pharmacokinetic models and *in vitro* tumor models[22].

Stereolithography-based bio-printing is capable of producing tissues with complex structures layer-by-layer at high speed[2,30]. The stereolithography appearance (SLA) technique has high resolution capabilities and the 3D bio-printing procedure is very accurate[2]. Liquid-based resins are solid or high viscous substances often existing of a mixture of compounds, which can be derived from woody plants or trees or can be synthetically derived[30]. The stereolithography bio-printing process is spatial controlled by CAD to solidify the liquid-based resins, using ultra-violet (UV) light in a specific pattern[2,30]. The UV light causes the formation of free radicals and other reactive species that lead to photo-polymerization of resins in a solid material[30]. SLA can be divided into beam scanning and mask-image projection. The beam scanning technique is also called laser direct writing (LDW) and contains a laser which solidifies the liquid-based resins in a bio-ink reservoir. The resolution is exposure-dependent, so the diameter and intensity of the laser beam, wavelength, exposure time and absorption affect the resolution[2]. The structure of the 3D bio-printed tissue is depending on concentration of bio-inks, intensity or power of the laser beam and scanning speed. Besides the high resolution of this technique, a disadvantage is that it may happen that earlier printed layers are repeatedly exposed to the laser, which causes uneven mechanical strength of the printed structure or undesired constructs[2]. The mask-image projection uses a digital-light procession technique (DLP) and is capable of solidifying a whole patterned layer simultaneously[2,30]. Therefore, this technique is very fast compared with the laser beam technique. At first, the pattern was projected using a mask and a light projector to illuminate the specific pattern. Later, a liquid crystal display (LCD) was added to prepare irradiation patterns and subsequently digital micro-mirror devices (DMD) are added to make the pattern more dynamic and to provide high resolution[30]. Overall, stereolithography can be used to produce very complex structures without supporting materials at high speed and with high resolution[2]. The main limitation of stereolithography bio-printing is that not every bio-ink can be photo-polymerized[2]. And as mentioned before, photo-polymerization gives free radicals and other

reactive species that can be harmful for cell membranes, nucleic acid and proteins and therefore for cell viability. Furthermore, this technique is very expensive[2].
Another approach of 3D bio-printing is ***in vivo* bio-printing**, which means that cells and bio-materials are directly deposited on or in a patient[22]. In mice is *in vivo* bio-printing already used for bio-printing skin directly on wounds and burns and bone is already 3D bio-printed into calvaria (skull) as well[22]. If printing speed and resolution will be good enough for *in vivo* bio-printing, it might in the future even be possible to bio-print viable tissues, possibly with computer driven robotic techniques, onto patients immediately after injury or during surgery[22].

Bio-ink selection
Currently, a lot of studies have been focussing on the development of new bio-printing techniques, applications of 3D printed bio-constructs and improvement of printer parameters, like speed and resolution. Development and improvement of (new) bio-inks has been a bit out of focus lately, therefore a lot of research remains to find the ideal bio-ink[2]. However, finding the ideal bio-ink is not that simple, because different printing techniques require suitable bio-ink properties[2,22]. Depending on their composition, bio-inks differ in viscosity and density and have to support cell-survival, motility and differentiation[2,28]. Bio-inks can be deposited in different specific patterns and can be printed with or without scaffolds[2]. Besides a specific deposition pattern to form a stable construct, the composition of bio-inks, including cells, biomaterials and biochemical signals (like growth factors, chemokines, adhesion factors, signaling proteins), also is important for the strength and function of the 3D construct[2,28]. Selection of appropriate biomaterials for 3D bio-printing and their use in specific applications is depending on several features, like printability, biocompatibility, degradation kinetics and byproducts, structural and mechanical properties and material biomimicry[2,22]. Printability means that the bio-ink has suitable conditions for deposition requirements, such as spatial and temporal control, of a specific bio-printing technique and biocompatibility is the interaction between biomaterials and their environment[2,22]. Biomaterials in a bio-ink have to support cell kinetics, like degradation and formation of byproducts, which have to be non-toxic[22]. The biomaterials should be capable to interact with endogenous tissues and give biochemical cues, without triggering an immune response[2,22]. Biomaterials also have to provide physicochemical properties, including mechanical strength, structural stability and degradation kinetics[2,22]. Viscosity is important for flexibility in deposition and stability of the 3D printed construct[28]. Bio-inks can contain hard or soft biomaterials and can both be naturally or synthetically derived[2,22]. Hard biomaterials are for example metals, ceramics or polymers[2]. Several metals have already been studied for 3d bio-printing and some have shown to be promising for clinical trials as well, but the use of metals in bio-inks has also limitations, like little suitable 3D bio-printing techniques, toxicity and erosion[2]. Therefore bio-degradable metals should be explored for long-term implantation[2]. Ceramics are used in dental-, joint- and bone implantations, because of their mineralization abilities. Bone scaffolds with osteogenesis-promoting capabilities have already been printed with ceramic substances, like hydroxyapatite, which is a major component in bone and teeth[2]. Polymers can be easily synthesized, have low fabrication-costs, wide variation and give usually no immunogenicity[2]. Soft biomaterials are commonly hydrogels. Temperature control and viscosity of hydrogels is important for cell viability and function[23]. For example, some naturally-derived hydrogels are alginate, gelatin, collagen, fibrin and agarose and some synthetically-based hydrogels are pluronic or polyethylene glycol (PEG)[2,22,28]. Pluronic-, gelatin-, PEG-based, and combinations of this compounds with other hydrogels, are suitable biomaterials for extrusion bio-printing, because they are fluidic enough for bio-printing and after printing they quickly recover their gel state to hold the printed structure together[2,28]. Hydrogel-based constructs are often used in cartilage regeneration using extrusion or inkjet bio-printing, because hydrogels have high biocompatibility with chondrocytes[20]. Benefits of naturally-derived hydrogels are their intrinsic bioactivity and similarity to human ECM, but one disadvantage is that they also can excite an immune response and be harmful to cells[2,22,23]. Synthetic polymers are very adaptable in their physical properties, like chemical structure and functional groups, molecular weight and composition[2,22]. However, synthetic polymers often have low biocompatibility, toxic degradation products and loss of mechanical function over time[22]. To keep the tissue construct stable

and together, the biomaterials have to be crosslinked after bio-printing. There are different crosslinking methods; for example physical crosslinking, which uses non-covalent ionic interactions or thermally induced non-covalent interactions. Covalent bonds are irreversibly formed in chemical crosslinking[28]. For example, long-term stable constructs can be generated when an alginate solution reacts with calcium-ions and forms a solid hydrogel. In contrast with chemical crosslinking, physical crosslinking is reversible and is only able to support the construct temporarily, which for example is required at bio-printed vasculature constructs[28]. Another upcoming type of bio-ink method is the solubilization of decellularized-ECM (dECM) from a certain tissue or organ that can be loaded subsequently with cells and materials from the same organ or tissue and then can be bio-printed in a frame/scaffold with a layer-by-layer approach to produce comparable tissue-analogues (figure 6)[22,28].

Cell selection

One of the main important compounds for functionality and clinical applications of bio-inks are cells[2,22]. Multi-potent stem cells, pluripotent stem cells and differentiated cells can be used in bio-inks (figure 7)[2]. Use of stem cells will be promising for tissue engineering applications, due to their capability of self-renewal and long-term vitality and their capability to differentiate into several tissue-specific phenotypes[22]. More differentiated stem cells, like bone marrow adult stem cells and stem cells from fat or placenta, are considered to be more save for clinical transplantation, due to their limited multipotent differentiation capabilities[22]. Mesenchymal stromal cells (MSC) have already been generated for 3D bio-printing related *in vitro* tests and if cell-culture techniques improve in the future, use of other stem cell populations for 3D bio-printing would be a realistic possibility for clinical applications[22]. But first, more research has to be done to ensure safety of this cells in clinical or even therapeutic applications. Progresses have been made lately in several studies to control proliferation and differentiation of cells with use of small molecules[22]. Precise control of proliferation *in vitro* and *in vivo* is important for the bio-printed construct, because cell viability will be too low if too little proliferation occurs and too much proliferation will result in too much cells[22]. Tough, high cell density is important for a bio-ink to give the 3D construct post-printing functionality[28]. However, bio-printing high dense and viscous bio-inks can cause shear stress, which can directly affect cell-vitality and at long terms can cause proliferation alterations and subsequently can affect post- printing functionality[28]. Cells can be encapsulated individually in bio-inks or encapsulated in spheroids. Encapsulating cells in spheroids can reduce bio-printing time and reduce shear stress, which both is beneficial for cell viability[28]. Besides the cells have to survive different 3D bio-printing processes, the cells also have to survive the post-printing crosslinking steps[28]. As mentioned before, there are different cross-linking methods, including the use of chemicals and UV light, which can possibly alter proliferation[28]. Matured somatic cell types are expected to be more resistant to harsh circumstances than stem cells, so they might better survive a 3D bio-printing procedure[28]. Because different cell types can respond differently to shear stress, a scheme of optimal conditions of bio-inks, like printing speed, additional biomaterials, density, viscosity, for each cell type should be generated to optimize cell viability and unaltered function[28]. Bio-inks containing multiple cells require more preparation than single cell bio-inks, but are more close to *in vivo* circumstances[28]. Besides primary cells, tissues exist of other cell types that provide supportive-, barrier- and structural functions. This cells are for example also involved in vascularization and providing a niche for stem cell maintenance and proliferation[22,28]. Therefore simultaneous deposition of multiple cell types is necessary to precisely mimic tissue- or organ function (figure 7)[24,28].

Additionally, bio-printed 3D constructs have to be able to maintain long-term function, even after implantation. Therefore, ability to maintain cellular homeostasis, self-renewal and response to tissue damage or injury is necessary for post-printing functionality[22]. So, more knowledge about nature and composition of stem cells and stem cell-niches will inure long-term functionality of the 3D bio-printed construct[22]. However, tissues intended for implantation cannot contain just a random cell type, because it may result in an immune rejection. Therefore the patient's own autologous cells could be obtained from biopsies or the patient's stem cells could be differentiated into the desired cell type. But, it could still be possible that the isolated cells due to intrinsic genetic reasons do not have the desired function in the printed construct[22].

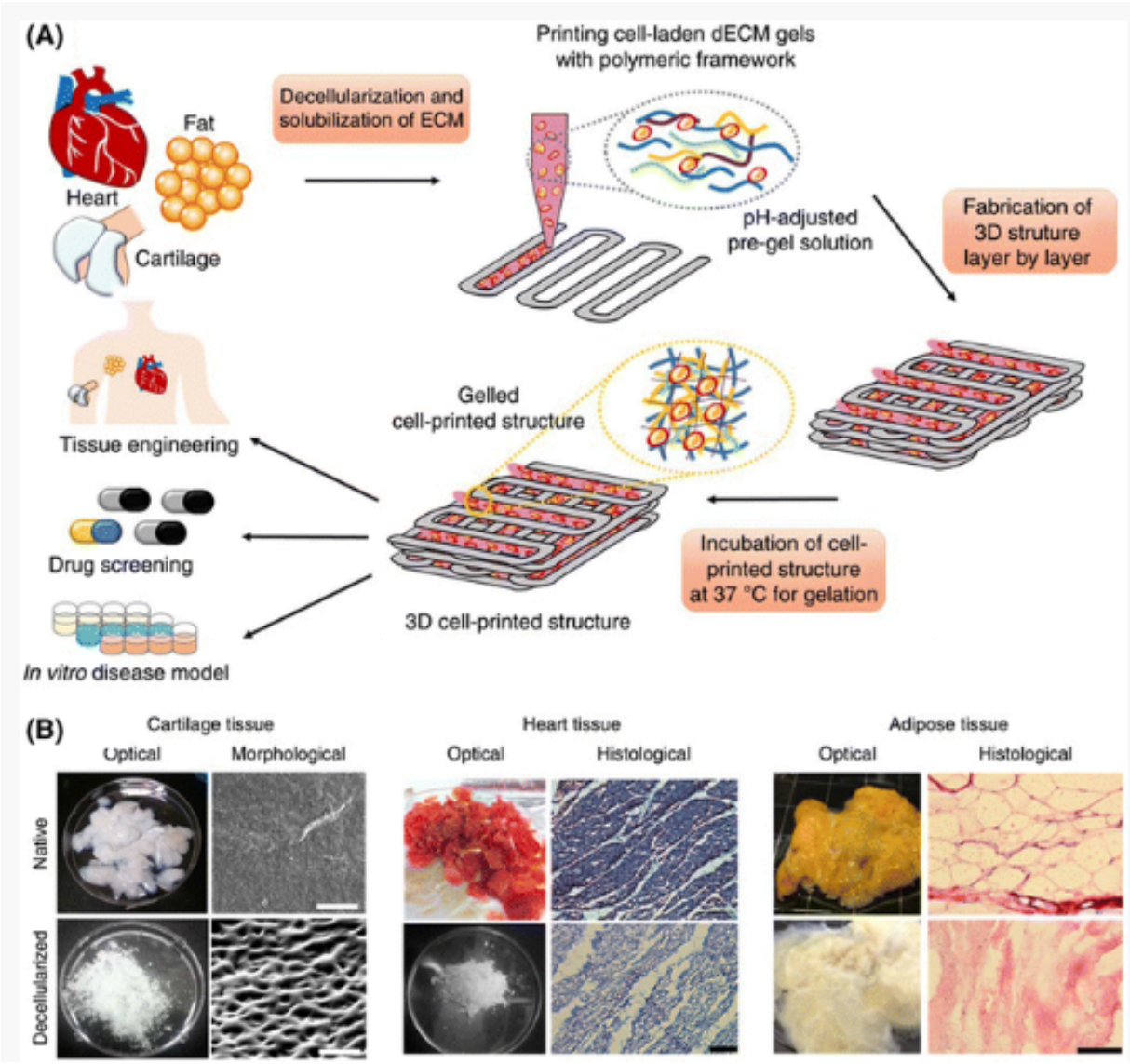

Figure 6. 3D bio-printing with dECM bio-inks from different tissue types. A. Solubilized dECM bio-inks originate from different tissues or organs, can be mixed with stem cells and subsequently be 3D bio-printed in a layer-by-layer approach into a polymeric frame. The 3D printed construct can be used for several applications, like tissue engineering, drug screening and *in vitro* disease models. B. Native tissues and dECM 3D bio-printed structures have comparable histological- and morphological appearances. Figure adapted from Zhang, YS. *et al*. (2017).

Applications of 3D bio-printing

Depending on the destination, 3D bio-printed tissues require different post-printing processes. Some tissues require a maturation period in a bio-reactor before they can be implanted[22]. 3D bio-printed tissues could also directly be implanted in patients. Implantation of 3D bio-printed tissue constructs containing autologous cells has the benefit of overcoming an immune rejection[2,26]. The perfect organ or tissue size and shape for the patient's requirements could be fabricated as well[26]. However, increasing complexity of organs means that more knowledge and new approaches are essential to overcome limitations to build the desired construct[22]. Tissues can be ranked from simple to complex and can be divided into 4 main types (figure 8); relatively 2D tissues, hollow tubes, hollow organs and solid organs. 2D tissues, like skin, have already been bio-printed and clinically tested and will probably be available on short-term for transplantation in patients[22]. Hollow tubes are for example blood vessels, trachea and urethra. Because much progress in development has been made,

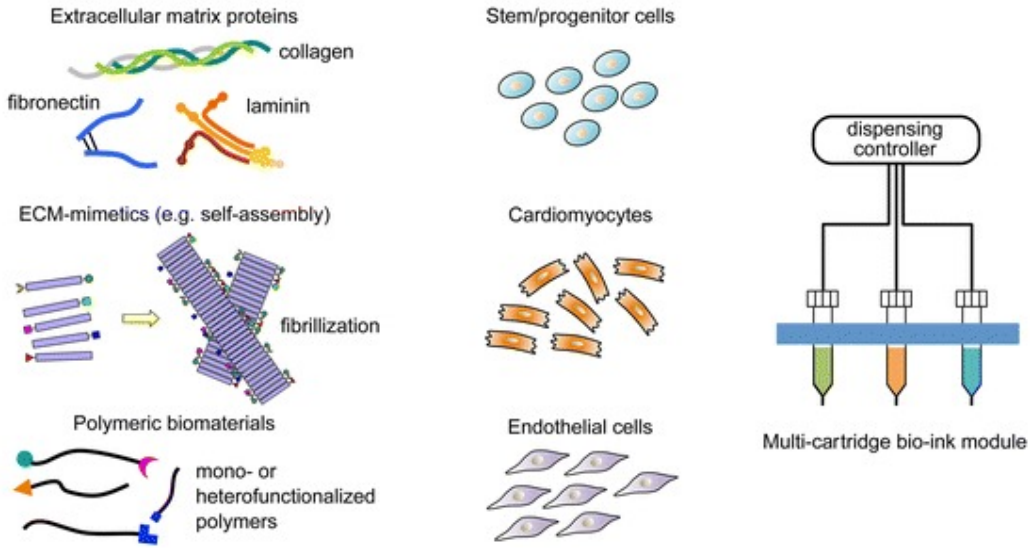

Figure 7. Bio-inks exist of cells, biomaterials and biochemical signals. Bio-materials contain ECM proteins (collagen, laminin, fibronectin), ECM protein-analogues or synthetic materials, like polymers. Stem or progenitor cells, or fully differentiated and matured cells can be used in bio-inks. Tissues exist of multiple cell types and a multi-cartridge bio-ink module provides deposition of multiple cell types on precise locations in tissues. Figure adapted and adjusted from Jung, JP. *et al*. (2016)

it is likely that hollow tubes closely follow 2D tissues for clinical applications. Hollow organs are already more complex, so hollow organs are not expected to be ready for transplantations very soon. And solid organs, for example kidneys, are very complex and have long-term expectations for clinical applications, because there are multiple challenges to overcome, like vascularization and innervation[22].

Otherwise, 3D printed tissues can also be used for *in vitro* testing[22]. Use of 3D bio-printed constructs for *in vitro* testing would have multiple benefits. A realistic and short-term application of 3D bio-printing is to provide alternatives or even totally replace animal testing[26]. Animal testing is not an optimal research method, because animals are not humans and they can respond different on drugs and illnesses. Therefore, 3D bio-printed constructs could be used for toxicology studies, disease models and other pharmacological approaches, such as drug delivery and even drug discovery[11,24].

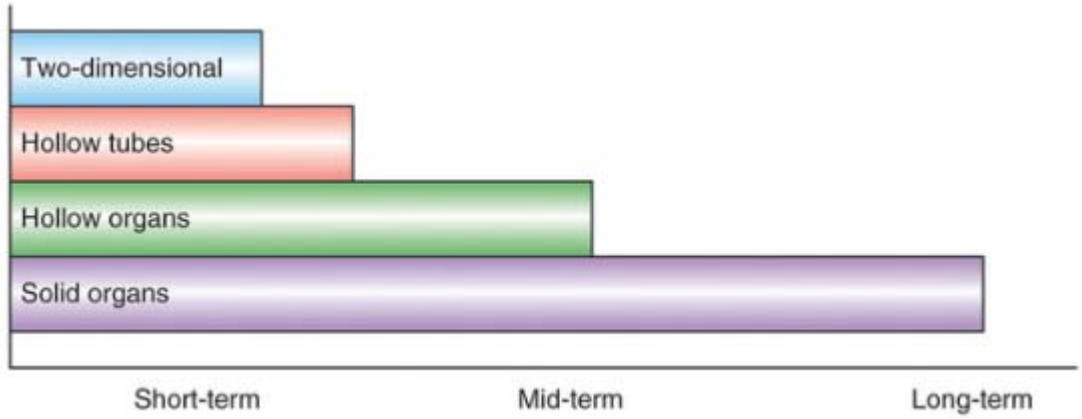

Figure 8. Timeframe for the development of 3D bio-printed tissues, ranked from simple to complex. Relatively from simple to complex, 4 main tissue types for bio-printing are 2D organs, hollow tubes, hollow organs or solid organs. Hollow tubes are following 2D tissues for short-term clinical applications. Hollow organs are more complex and expected to be ready for clinical transplantation on mid-term duration. Solid organs are very complex, so clinical applications are expected on long-time duration. Figure adapted from Murphy SV. and Atala A. (2014).

Discussion

Many studies have made much progress in the development of 3D bio-printed constructs. But, more knowledge about biomaterials, biochemical agents and cells is necessary to build more complex tissues. Development and improvement of CAD and bio-printing techniques will also help to overcome multiple limitations. However, many studies currently use robust or transformed cell lines for the bio-printing process[22]. That is fine for early studies of 3D bio-printing, but when the methods are refined enough for clinical trials, use of cell lines more close to endogenous cells is essential. These cell types are more sensitive than cell lines that are being used now, so they experience more stress in similar conditions. Complex tissues contain multiple cell types. Multipotent or pluripotent stem cells and already differentiated cells can be used for 3D bio-printing and precise control of differentiation and proliferation is necessary to prevent hyperplasia and eventual a subsequent carcinoma. More complex tissues require more fabrication time than simple tissues and more time is detrimental for cell- and therefore tissue viability. All of the 3D bio-printing techniques have multiple disadvantages and can print only a few biomaterials and cell types or adjusted cell types, so the idea of taking a biopsy from a patient that needs a transplant and use it to print for example his kidney or lung would be a long-long-term goal. When 3D bio-printing of (simple) organs works, transplantation into patients could give also new challenges that have to be overcome. For example, knowledge of the interaction between the environment and the 3D bio-printed transplant is also essential to estimate the chance that kinetic effects, like an immune response, occur. However, some limiting factors might be overcome on short-term duration. Limitations like printing speed, resolution, functioning of bioreactors and development of printable bio-inks and usable cell lines could be improved and possibly overcome on short-term duration. Several reviews mention the potential of 3D bio-printing to replace animal testing, but it is necessary to realize that drugs are differently metabolized in different organs, so testing a drug on one 3D bio-printed organ is not enough to fully replace animal testing.

Conclusion

In the search for alternatives for organ transplantation, the idea of 3D bio-printing the patient's organ with autologous cells arose. Overall, 3D bio-printing to fabricate tissue constructs for transplantation is investigated very much over the last decade. This field of tissue engineering is in its early stages and is developing very soon. Scaffolds are bio-artificial cell supporting biological structures used for tissue engineering, which have an ECM-like function. There are several disadvantages of decellularized tissues and pre-made porous scaffolds, like little control over distribution, vascularization and the decellularization reagents can be toxic. Therefore a growing interest in 3D bio-printing of cell laden scaffolds or entire tissues has been emerged. 3D printing is originally developed for non-living materials, so using 3D printing techniques for generation of tissues and organs have to be adapted to living materials or new bio-printing techniques have to be developed. 3D bio-printing with autologous cells has the advantage of bypassing an immune rejection. It is also a personalized medicine approach and has the potential to replace animal testing. Currently, 3D bio-printing has also multiple disadvantages, like the problem with vascularization and innervation. 3D bio-printing techniques need more development to improve bio-print speed and resolution. A 3D bio-printing process contains generally 6 main steps; imaging, design, material selection, cell selection, actual 3D bio-printing and the different applications. There are droplet-based, extrusion based, stereolithography-based and *in vivo* 3D bio-printing techniques. Different 3D bio-printers have different properties and therefore also several limitations. Poorly vascularized tissues with few different cell types are easier to engineer than well-perfused tissues with multiple cell types. Therefore, only simple tissues on millimeter scale can be printed for now. Simple tissues, like skin and cartilage, have already been bio-printed and tested in clinical trials. More complexity of tissues means more fabrication time, which is not beneficial for tissue viability. Fabrication time can be shortened, when printing speed, resolution and functioning of a bioreactor can be improved. Development of a scheme with good matching cell types, biomaterials and bio-printing techniques has to be assorted to create ideal conditions for the bio-printing procedure. 3D bio-printing of entire vascularized and innervated 3D tissues is investigated in multiple studies, but to answer the question if 3D bio-printing is a possible solution for patients who need a donor organ, the answer is no for now. Simple tissues might be expected on short-term duration, but at this moment science is not that far that bio-printing an entire organ or tissue in a close future is realistic and organs that are present on a list of organ shortages are usually complex organs and cannot be expected on short-term durations. Therefore a lot of research is still remaining.

References

1. U.S. Government, Information on Organ Donation and Transplantation. Available via https://organdonor.gov/index.html. Consulted at 28-03-2017.
2. 3D Bioprinting for Organ Regeneration. Cui H, Nowicki M, Fisher JP, Zhang LG. Adv Healthc Mater. 2017 Jan;6(1).
3. Immunosuppression for kidney transplantation: Where are we now and where are we going? Lim MA, Kohli J, Bloom RD. Transplant Rev (Orlando). 2017 Jan;31(1):10-17.
4. Personalized treatment in heart transplantation. Khush KK. Curr Opin Organ Transplant. 2017 Mar 6.
5. Improving Donor Conversion Rates at a Level One Trauma Center: Impact of Best Practice Guidelines. Alban RF, Gibbons BL, Bershad VL. Cureus. 2016 Nov 22;8(11):e891.
6. Rijksoverheid, Orgaandonatie en weefseldonatie. Available via https://www.rijksoverheid.nl/onderwerpen/orgaandonatie-en-weefseldonatie. Consulted at 28-03-2017
7. Fractal circuit sensors enable rapid quantification of biomarkers for donor lung assessment for transplantation. Andrew T. Sage, Justin D. Besant, Laili Mahmoudian, Mahla Poudineh, Xiaohui Bai, Ricardo Zamel, Michael Hsin, Edward H. Sargent, Marcelo Cypel, Mingyao Liu, Shaf Keshavjee, and Shana O. Kelley. Sci Adv. 2015 Aug; 1(7): e1500417
8. Scaffolding in tissue engineering: general approaches and tissue-specific considerations. B. P. Chan and K. W. Leong. Eur Spine J. 2008 Dec; 17(Suppl 4): 467–479.
9. 3D bioprinting and its in vivo applications. Hong N, Yang GH, Lee J, Kim G. J Biomed Mater Res B Appl Biomater. 2017 Jan 20.
10. Direct-write bioprinting of cell-laden methacrylated gelatin hydrogels. Bertassoni LE, Cardoso JC, Manoharan V, Cristino AL, Bhise NS, Araujo WA, Zorlutuna P, Vrana NE, Ghaemmaghami AM, Dokmeci MR, Khademhosseini A. Biofabrication. 2014 Jun;6(2):024105.
11. 3D printing of medicinal products and the challenge of personalized therapy. Zema L, Melocchi A, Maroni A, Gazzaniga A. J Pharm Sci. 2017 Mar 24. pii: S0022-3549(17)30207-1.
12. Generation and transplantation of an autologous vascularized bioartificial human tissue. Mertsching H, Schanz J, Steger V, Schandar M, Schenk M, Hansmann J, Dally I, Friedel G, Walles T. Transplantation. 2009; 88: 203-10.
13. Host-integration of a tissue-engineered airway patch: two-year follow-up in a single patient. Steinke M, Dally I, Friedel G, Walles H, Walles T. Tissue Eng Part A. 2015 Feb;21(3-4):573-9.
14. 3D Printing of Scaffolds for Tissue Regeneration Applications. Do AV, Khorsand B, Geary SM, Salem AK. Adv Healthc Mater. 2015 Aug 26;4(12):1742-62.
15. Light-assisted direct-write of 3D functional biomaterials. Hribar KC, Soman P, Warner J, Chung P, Chen S. Lab Chip. 2014 Jan 21;14(2):268-75.
16. In Vivo Chondrogenesis in 3D Bioprinted Human Cell-laden Hydrogel Constructs. Möller T, Amoroso M, Hägg D, Brantsing C, Rotter N, Apelgren P, Lindahl A, Kölby L, Gatenholm P. Plast Reconstr Surg Glob Open. 2017 Feb 15;5(2):e1227.
17. U.S. National Library of Medicine. Available via https://meshb.nlm.nih.gov/record/ui?name=Chondrogenesis. Consulted at 06-04-2017.
18. Creating perfused functional vascular channels using 3D bio-printing technology. Lee VK1, Kim DY1, Ngo H1, Lee Y2, Seo L3, Yoo SS4, Vincent PA5, Dai G6. Biomaterials. 2014 Sep;35(28):8092-102.
19. 3D bioprinting for engineering complex tissues. Mandrycky C, Wang Z, Kim K, Kim DH. Biotechnol Adv. 2016 Jul-Aug;34(4):422-34.
20. 3D Bioprinting of Cartilage for Orthopedic Surgeons: Reading between the Lines. Claudia Di Bella, Amanda Fosang, Davide M. Donati, Gordon G. Wallace, and Peter F. M. Choong. Front Surg. 2015; 2: 39.
21. 3D Bioprinting for Vascularized Tissue Fabrication. Richards D, Jia J, Yost M, Markwald R, Mei Y. Ann Biomed Eng. 2017 Jan;45(1):132-147.
22. 3D bioprinting of tissues and organs. Murphy SV, Atala A. Nature Biotechnology 2014 Aug;32(8):773-85.

23. Three-Dimensional Cell Printing of Large-Volume Tissues: Application to Ear Regeneration. Lee JS, Kim BS, Seo D, Park JH, Cho DW. Tissue Eng Part C Methods. 2017 Mar;23(3):136-145.
24. Solid organ fabrication: comparison of decellularization to 3D bioprinting. Jung JP, Bhuiyan DB, Ogle BM. Biomater Res. 2016 Aug 31;20(1):27.
25. Bioresorbable airway splint created with a three-dimensional printer. Zopf DA, Hollister SJ, Nelson ME, Ohye RG, Green GE. N Engl J Med. 2013 May 23;368(21):2043-5.
26. 3D bioprint me: a socioethical view of bioprinting human organs and tissues. Vermeulen N, Haddow G, Seymour T, Faulkner-Jones A, Shu W. J Med Ethics. 2017 Mar 20. pii: medethics-2015-103347.
27. Three-dimensional printing of nanomaterial scaffolds for complex tissue regeneration. O'Brien CM, Holmes B, Faucett S, Zhang LG. Tissue Eng Part B Rev. 2015 Feb;21(1):103-14.
28. 3D Bioprinting for Tissue and Organ Fabrication. Zhang YS, Yue K, Aleman J, Mollazadeh-Moghaddam K, Bakht SM, Yang J, Jia W, Dell'Erba V, Assawes P, Shin SR, Dokmeci MR, Oklu R, Khademhosseini A. Ann Biomed Eng. 2017 Jan;45(1):148-163.
29. An Electrohydrodynamic Bioprinter for Alginate Hydrogels Containing Living Cells. Gasperini L, Maniglio D, Motta A, Migliaresi C. Tissue Eng Part C Methods. 2015 Feb;21(2):123-32.
30. Stereolithography in tissue engineering. Skoog SA, Goering PL, Narayan RJ. J Mater Sci Mater Med. 2014 Mar;25(3):845-56.