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RESEARCH ARTICLE

In vitro pre-vascularization strategies for tissue engineered constructs–Bioprinting and others

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Abstract: Tissue-engineered products commercially available today have been limited to thin avascular tissue such as skin and cartilage. The fabrication of thicker, more complex tissue still eludes scientists today. One reason for this is the lack of effective techniques to incorporate functional vascular networks within thick tissue constructs. Vascular networks provide cells throughout the tissue with adequate oxygen and nutrients; cells located within thick un-vascularized tissue implants eventually die due to oxygen and nutrient deficiency. Vascularization has been identified as one of the key components in the field of tissue engineering. In order to fabricate biomimetic tissue which accurately recapitulates our native tissue environment, *in vitro* pre-vascularization strategies need to be developed. In this review, we describe various *in vitro* vascularization techniques developed recently which employ different technologies such as bioprinting, microfluidics, micropatterning, wire molding, and cell sheet engineering. We describe the fabrication process and unique characteristics of each technique, as well as provide our perspective on the future of the field.

Keywords: vascularization, vasculogenesis, endothelial, microfluidics, micropatterning, cell-sheet engineering, wire-molding

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1. Introduction

There is a wide spectrum of human pathologies which plague mankind and affect our quality of life. These diseases often lead to organ failure and even death of the patient. The conventional treatment for organ failure would be an organ transplant, where the patient's damaged organ is replaced by a functional and compatible donor organ. Although organ transplantation has proven its efficacy over the years, thousands of people continue to lose their lives every year due to organ failure. The reason for this is the demand and supply disparity of donor organs. The demand for donor organs far exceeds the supply, and there are simply not enough donor organs to go around. Research by the U.S. Department of Health and Human Services found that in the United States alone, 22 people waiting for organ transplants die each day due to shortage of donor organs[1]. Moreover, the percentage gap between the number of patients on the waiting list and the number of organ donors has been steadily increasing every year. Tissue engineering has shown promising signs to be a solution to this problem.

The term *tissue engineering* was first coined in 1993 by Langer and Vacanti in their highly influential paper[2]. Tissue engineering is a cross-disciplinary field of research, comprising the principles of biology and engineering to create functional tissue in a lab in order to replace or restore damaged tissue in a patient. A patient suffering from tissue injury, such as skin burn,

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can be treated using this approach. A biopsy is first performed to extract healthy autologous (skin) cells from the patient. These cells are cultured and expanded *in vitro* to obtain a sufficient number of cells needed for the treatment. The cells are then seeded onto a biodegradable scaffold which will then be cultured to maturity. When the tissue-engineered graft is fully matured, it can then be transplanted onto the patient's injury site to enhance the wound healing process.

Research in this field is expanding quickly and has given rise to many tissue engineering companies which carry out research and manufacture human Tissue-Engineered Products (hTEPs) for clinical use today. Tissue engineering is still in its early initial phases, thus only few hTEPs have been successfully translated into commercial availability. These commercially available hTEPs mainly comprise skin products, followed by cartilage, and lastly bone products. By 2003, more than 20 skin replacement products were available in USA and Europe[3]. Tissue-engineered cartilage products have found widespread application today to treat traumatic knee joint damage by undergoing Autologous Chondrocyte Transplantation (ACT) surgery[3]. The application of tissue-engineered bone products is limited to the treatment of small bone lesions as larger defect sites still remain untreatable by this approach and autologous bone grafts still remain the preferred approach. With the successful translation of these hTEPs into clinical application, tissue engineering has proven its legitimacy as a promising candidate for the treatment of injured tissue, sparking more and more research in the field. Today, research groups all over the world are working to create hTEPs from various tissues such as cardiac[4,5], liver[6], cornea[7], trachea[8], artery[9] and many others.

Various research groups have published works reporting the successful engineering of functional tissue *in vitro*, such as bladder[10] tissue, although they have not yet been used in clinical trials due to its early stage of development. Many of these published works report the successful engineering of only very thin, microscale tissue constructs. One of the main reasons for this is the fact that cells encapsulated deep within a large tissue construct have limited access to oxygen and nutrients, causing them to die during long-term culture. We know that cells located more than 200 μm away from a nutrient source (blood vessels) do not receive sufficient nutrients for survival[11]. With the ultimate goal of tissue engineering in mind, which is to successfully engineer complete organs, new techniques need to be developed to allow the fabrication of larger tissue constructs which demonstrate long-term viability post implantation. A promising approach to this problem is the pre-vascularization of tissue implants, where techniques are used to incorporate functional vascular networks within a tissue construct *in vitro* before implantation. Compared to an un-vascularized tissue construct, a pre-vascularized tissue construct has shown enhanced anastomosis with host vasculature post implantation, thus providing adequate nutrients to encapsulated cells and improving viability. The vascularization of tissue engineered constructs is deemed to be vital to the progress of tissue engineering today and in the future[12]. In this review, we highlight the significance of pre-vascularization and its impact on tissue engineering. We also identify recently developed *in vitro* vascularization techniques which have shown promising results, categorized based on the technologies they employ, and describe each of their fabrication processes.

2. Significance of Pre-Vascularization

2.1 Tissue Engineering for Regenerative Medicine

With avascular tissue products such as skin and cartilage already made commercially available, tissue engineers are now looking to engineer larger, more complex tissue which could potentially be used as a viable treatment option for patients suffering from critical diseases. However, there are a host of crucial challenges to be addressed to meet this goal. Our native tissues possess highly specific architectures, and different tissues have their own unique structural organization. It is imperative that tissue engineers accurately emulate the intrinsic heterogeneous architecture of these complex tissues when fabricating their tissue engineered construct as there is a well-accepted correlation between tissue architecture and pathogenesis[13]. Tissue-engineered constructs that do not accurately mimic the heterogeneous nature of native tissues could lead to disease and be carcinogenic when implanted into a patient. The successful engineering of complex tissue also requires control of differentiated function of the cells within the tissue engineered construct, failing which could cause the tissue-engineered construct to be dysfunctional and malignant. There are many other challenges in the pursuit of engineering large, complex tissue constructs such as matrix stiffness, molecular gradients and hierarchical structure[14], but one of the main challenges is the one we

will be focusing on in this report—the problem of vascularization.

As previously mentioned, the incorporation of a mature, inter-connected vascular network within a tissue construct is vital in tissue engineering as it helps to prevent the development of a necrotic core due to nutrient deficiency of cells deep within the construct, and provides a readily perfusable network for nutrient perfusion throughout the construct during the fabrication stage, or after implantation in a patient[15]. The implantation of a pre-vascularized tissue construct minimizes the need for vasculogenic and angiogenic processes to occur after implantation, and has been shown to induce rapid vascularization and inosculation with host vasculature upon implantation into mice when compared to un-vascularized constructs[16]. These studies demonstrate the importance of pre-vascularizing a tissue construct *in vitro* before implantation. Today, success in the vascularization of tissue constructs has been limited to the vascularization of thin (2D) tissue slices, and the vascularization of large 3D tissue constructs has seen slower progress. The lack of viable fabrication techniques has been a prime hindrance to our progress in this area. Successful vascularization of large 3D tissue constructs would undoubtedly provide invaluable contribution in the field of tissue engineering and bring us a step closer to fabricating whole organs.

2.2 *In Xitro* Tissue Models

Besides its obvious applications in regenerative medicine, the ability to vascularize large 3D tissue constructs would also contribute to the development of *in vitro* tissue models which better replicate our native tissues. The use of animal models and *ex vivo* (cadaveric) human tissue models for various studies, such as pathophysiology and pharmacology, has resulted in groundbreaking findings over the years. However, there are inherent limitations with the use of these models. Cadaveric human tissue models offer exceptional replication of native tissue; however, they suffer from limited availability. The use of animal models brings rise to issues with species-specific tissue response[17], as well as ethical issues with regards to the well-being of lab animals. These shortcomings have driven researchers to develop more advanced *in vitro* tissue models which accurately mimic native tissue and are easily fabricated without the need for donor tissue and animal experiments. The most common *in vitro* model still being used in scientific research today is the cell monolayer, i.e., 2D cell culture[18]. As we know, the cells in our bodies are enclosed within a 3D extra-cellular matrix (ECM) environment where they attach and proliferate. The results of experiments utilizing these 2D *in vitro* models may not be an accurate representation as it is known that cells function differently when cultured in 2D vs 3D cellular environments, affecting cellular cues, differentiation, adhesion, and morphogenesis, among others[19–21]. 3D *in vitro* models are able to more closely replicate the cellular environment found in our native tissue, thus providing researchers with more reliable results compared to using 2D *in vitro* models[22]. However, the fabrication and application of 3D *in vitro* models are not as trivial as cell monolayers. A common problem faced by the application of 3D *in vitro* models is the loss of cell viability in long-term culture[23], thus driving research to improve the lifespan of these models through modifications to the system[24]. Vascularized 3D tissue models have the potential to remain viable for long periods while still able to accurately replicate native vascularized tissue. As we know, cell-cell interactions and signaling plays a vital role in determining cell functionality *in vivo*. As such, monoculture tissue models may not accurately depict and account for the cellular interactions between parenchymal cells (i.e., hepatocytes and cardiomyocytes) and endothelial cells (ECs) which occur repeatedly in our organs, given that blood vessels are found throughout our entire body. The development of vascularized 3D tissue models is crucial in this regard. Moreover, vascularized 3D tissue models could also be used to boost our understanding of vascular physiology such as vasculogenesis, angiogenesis, and the physiology involved in vascular pathogenesis which would help to improve treatment of patients suffering from arterial diseases such as thrombosis and atherosclerosis. Vascularized 3D tissue models could also be useful in pharmacology studies and drug screening.

3. Current *Kn Xitro* Vascularization Approaches

Different vascularization techniques have been recently reported in literature, including the *in vivo* approach where perforated, un-vascularized tissue constructs are implanted to allow the host's peripheral vascular system to naturally vascularize the tissue construct[25]. This method requires the timely invasion of the host vasculature into the un-vascularized tissue construct through angiogenic sprouting in order to provide the cells within the tissue construct with adequate nutrients to survive. Naturally, it would take a longer time to vascularize larger tissue constructs by this approach. Thus, this approach may not be viable

for large 3D tissue constructs as the time taken for *in vivo* vascularization may be too long causing necrosis before a functional vascular network is formed, leading to premature failure of the construct. This disadvantage has driven many researches to develop *in vitro* vascularization techniques to fabricate pre-vascularized tissue constructs before implantation, which has clear advantages over un-vascularized constructs. The use of *in vitro* pre-vascularized tissue constructs would speed up the process of anastomosis with host vasculature and provide cells with quick access to a nutrient supply[26]. We will now look at the current methods developed by various research groups to fabricate blood vessels *in vitro*, and discuss their advantages as well as disadvantages.

3.1 Bioprinting

The term bioprinting refers to any additive manufacturing technique which uses biological ink to produce living tissue constructs for a variety of applications including regenerative medicine and cellular studies[27]. There are numerous bioprinting techniques which rely on fundamentally different principles of fabrication such as extrusion, ink-jet, and laser-based approaches. Bioprinting technology has been a hot topic of research in recent years, given its potential advantages over other conventional techniques, with research groups striving to improve the performance of existing bioprinters as well as developing new bioprinting technologies. This pursuit has given rise to novel bioprinting technologies in recent years such as the development of the "freeform reversible embedding of suspended hydrogels" process, able to produce 3D constructs with complex architecture not achievable by conventional approaches[28]. Today, advanced bioprinters with state-of-the-art features such as temperature and viscosity are now commercially available in the market, and researchers have been utilizing these bioprinters to produce groundbreaking researches. Researchers have demonstrated the ability of bioprinting technology to fabricate hybrid constructs made of multiple hydrogel materials and cell types, offering control of the construct's mechanical stiffness and composition[29]. Scaffold-free, large diameter tubular tissue constructs have also been produced by bioprinting for vascular tissue engineering applications using an indirect agarose molding technique[30]. The technique offers control of the tube's shape, dimension and hierarchical branching. The same approach was utilized to fabricate fused toroid-shaped, scaffold-free tissue from an alginate-based mold produced by bioprinting, showcasing the ability to produce viable tissue with customizable architecture[31].

Novel laser-based bioprinting approaches have also been developed in recent years including the Laser-Induced-Forward-Transfer (LIFT) technique and stereolithography (SLA). The LIFT technique involves the focusing of a high powered laser beam onto a photo-absorbent material coated with biological ink. When the photo-absorbent material is exposed to sufficient laser intensity it vaporizes and causes a high-pressure zone which propels a small volume of biological ink onto a donor slide where the ink is collected. By controlling the laser intensity and axial motion, high resolution patterns of biological material can be printed[33,34]. Stereolithography was patented in the 1980's but only recently has the technology found applications in the field of tissue engineering as researchers demonstrated its ability to be used for cell encapsulation and the fabrication of 3D tissue scaffolds. Projection stereolithography (PSL) has been utilized to fabricate living tissue constructs with controllable, porous architecture and demonstrated that cell viability was improved due to enhanced nutrient delivery within the porous scaffolds compared to solid scaffolds[35]. Commercially available SLA systems have also been modified to improve and expand the system capabilities for tissue engineering applications such as the ability to fabricate 3D tissue constructs comprising distinct layers of different cell types and material composition, thus improving the long-term viability of encapsulated cells[36].

The bioprinting approach has also shown potential applications in the field of vascularization of tissue constructs. A key advantage of using bioprinting technology is the ability to fabricate truly three-dimensional microchannel networks which are perfusable and can be lined with ECs. These 3D networks can be fabricated into pre-designed patterns which could be useful in studying the effects of vascular network spatial organization. Using a newly developed extrusion-based bioprinting approach, 3D tissue constructs consisting of multiple cell types were successfully produced and Human Umbilical Vein Endothelial Cells (HUVECs) were observed to line the lumen of embedded microchannels simulating perfusable blood vessels[37]. Microchannel networks were incorporated into the bulk ECM through the bioprinting of fugitive ink which was later removed, leaving behind microchannels which were then seeded with HUVECs. A similar study, using the same prin-

ciples but slightly different methodology, was carried out where researchers were able to incorporate an interconnected vascular network within bulk hydrogel containing hepatocytes and showed that perfusion of the vascular network with cell medium was able to sustain metabolic activity of the surrounding hepatocytes[32] (**Figure 1**). Recently, a technique capable of printing cell-laden tubular hydrogel constructs was developed using a multilayered coaxial extrusion system[38]. The technique demonstrated high cell viability, tunable tube dimensions, perfusability and complex architecture.

Despite its obvious advantages, bioprinting does suffer from several drawbacks when applied to vascularization of tissue constructs. Firstly, potential problems arising from the use of fugitive ink include the biocompatibility of the fugitive ink, as well as the removal process. Many reports found in literature today utilize the same fugitive ink approach to tackle the problem of vascularization[39] (**Figure 2**). Fugitive ink may contain cytotoxic compounds which are detrimental to cell viability and affect cell phenotype. The process of removing the fugitive ink, such as chemical dissolution and heat treatment, may also affect cell phenotype and lead to abnormal cell function and necrosis. Secondly, 3D printing technologies, such as Selective Laser Melting (SLM) and Fused Deposition Modelling (FDM), are known for their ability to fabricate objects with complex architecture. However, bioprinting technology may be less capable in this aspect considering the fact that the bioprinted material is soft and gel-like, featuring high water content and incorporating live cells. This limits the level of structural complexity achieved in the printed tissue constructs.

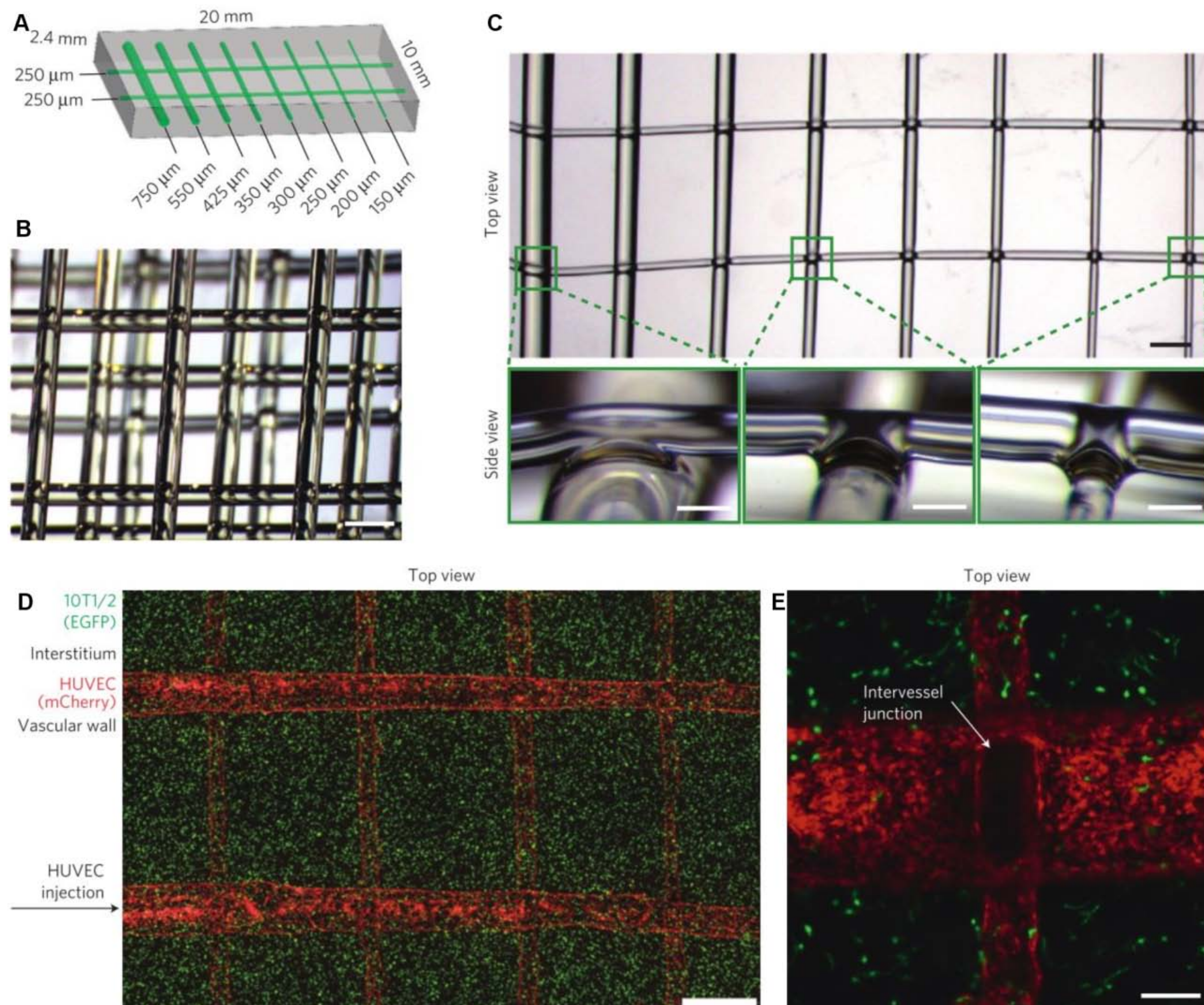

Figure 1. 3D printed carbohydrate-glass lattice used as sacrificial template in engineered tissues containing living cells to generate interconnected networks lined with endothelial cells and perfused with blood. Scale bars: (B, D, C top-view) 1 mm; (C side-view, E) 200 μm. (Adopted from Miller *et al*.[32])

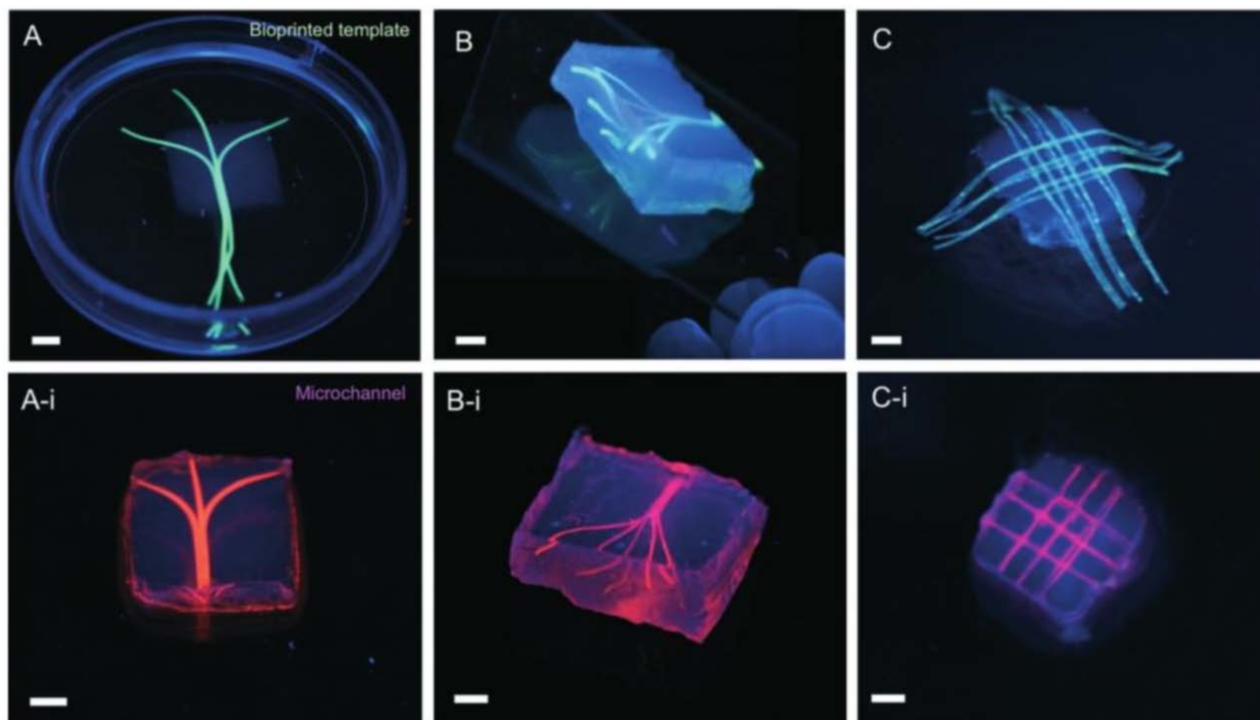

Figure 2. Bioprinted agarose template to fabricate microchannel networks within Gelatin Methacrylated (Gelma) hydrogel. Scale bars: 3 mm. (Adopted from Betassoni *et al*.[39])

3.2 Microfluidics (Lithography)

Microfluidic technology has been gaining popularity in research over the past two decades with more and more papers containing the keyword "microfluidic" being published[40]. This technology has found applications in many different fields of research, one of which being the vascularization of tissue constructs. Today, advanced lithographic technology allows us to fabricate complex microfluidic networks with ultra-high resolution, giving the user superb control over the networks' geometrical features. Its small scale minimizes the amount of consumables needed (such as cell medium) for each experimental run, thus reducing cost and increasing throughput. Microfluidic technology has been used in various ways to achieve vascularization. In one approach, microchannel networks were produced within bulk collagen matrix and seeded with HUVECs to simulate perfusable blood vessels[41] (**Figure 3**). The biofunctionality of the fabricated *in vitro* vessels was demonstrated including HUVEC interaction with pericytes which affected barrier function. In another approach, microfluidic channels were fabricated within bulk agarose hydrogel encapsulating murine fibroblasts. The microfluidic channels were not seeded with ECs but murine fibroblast viability was shown to improve by the perfusion of medium thorough the microchannel networks[42].

Another commonly used and exciting approach today involves the encapsulation of ECs within bulk hydrogel where they spontaneously self-assemble into perfusable vascular networks. Microfluidic technology is used in this method to fabricate the device, as well as to provide the encapsulated cells with medium and supplement perfusion with controlled parameters such as flow rate, flow direction, and pressure. Various microfluidic designs have been developed to suit the objectives of each research project including the replication of dynamic angiogenesis *in vitro*[43], the creation of a perfusable vascular network on a chip[44] (**Figure 4**) under physiologically relevant shear rates[45], the vascularization of cardiac tissue for improved functionality[46], and the controlled formation and characterization of capillary networks using a microfluidic device[47]. In these studies, directed angiogenic sprouting has been achieved and strong barrier function, as well as perfusable network interconnectivity has been demonstrated. The advantages of this approach include that it has high throughput, and the vascular networks are formed through natural vasculogenic and angiogenic processes which rely on self-assembly of the ECs, allowing the ECs to degrade and migrate

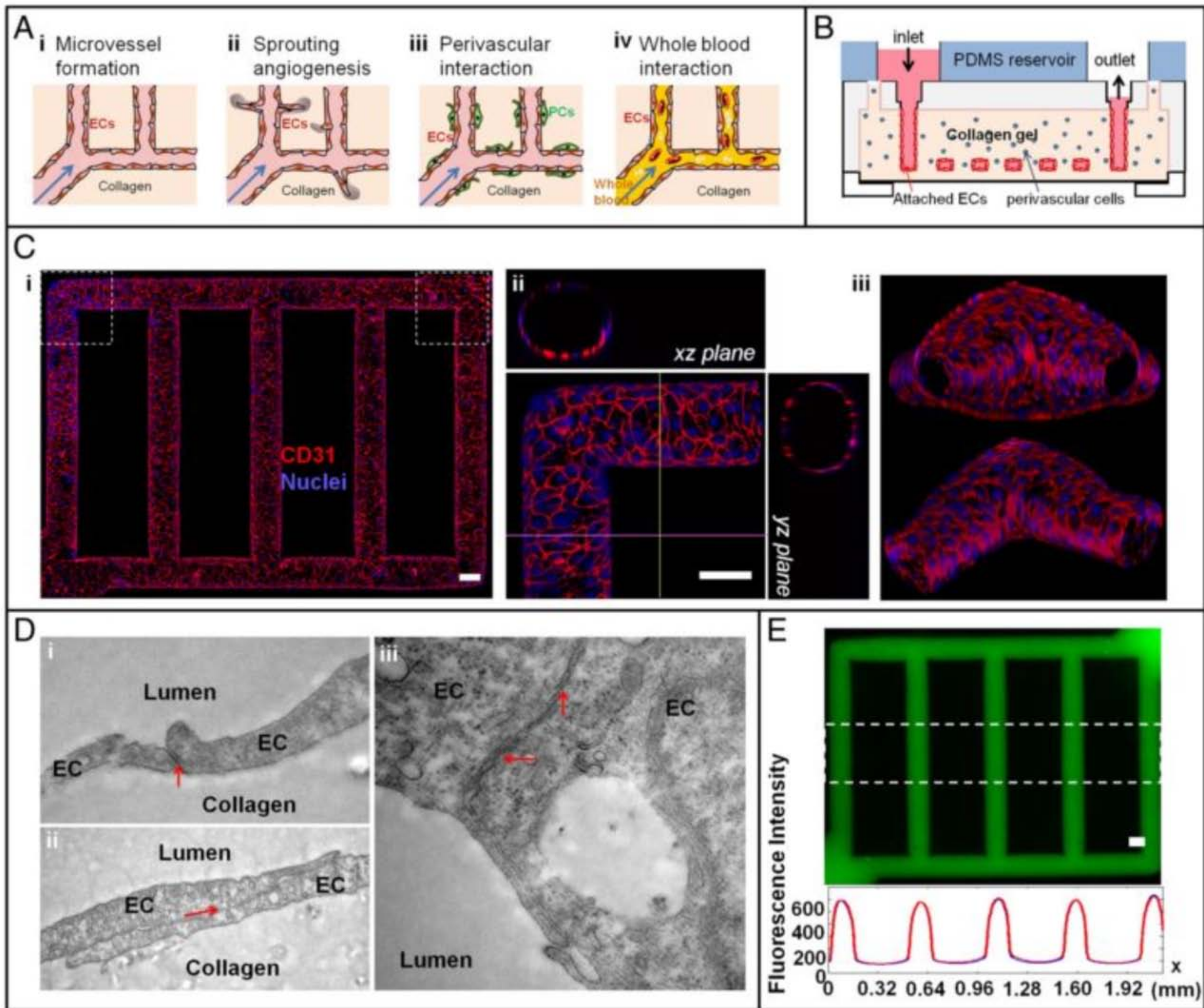

Figure 3. Microfluidic technology used to engineer microvascular networks within 3D tissue scaffolds for applications in vascular tissue modelling. Scale bars: 100 µm. (Adopted from Zheng *et al*.[41])

through their surrounding Extra-Cellular Matrix (ECM) freely which is beneficial compared to mechanically constraining the ECs into their lumenized structures[48]. However, the vascular networks formed using this approach are limited to a thin tissue, unlike the networks formed using the bioprinting approach, which may not accurately recapitulate our native vascular networks which are arranged in 3D. Another limitation would be that the formed vascular networks are confined within the microfluidic system and cannot be easily separated for implantation, thus its application for regenerative medicine is diminished.

3.3 Micropatterning

The micro-patterning approach to vascularization involves the patterning of biological material or adhesive proteins on a substrate to induce vasculogenesis with controlled spatial organization. In one study, researchers used standard photolithographic techniques to produce PDMS stamps, which were subsequently used to pattern Fibronectin (Fn) strips on glass coverslips following the procedure shown in **Figure 5A**[49]. Human Endothelial Progenitor Cells (hEPCs) showed preferential adhesion on the Fn surface compared to the non-adhesive PEG surface while also demonstrating directed elongation along the Fn strips after 24 hours post seeding (**Figure 5B**). Optimal strip width for directed cell elongation was found to be 50 µm. After 5 days in culture, immunostaining was performed to show confinement of hEPCs within the Fn strips with sparse migration to neighbouring strips (**Figure 5C**). Another common approach is the use of

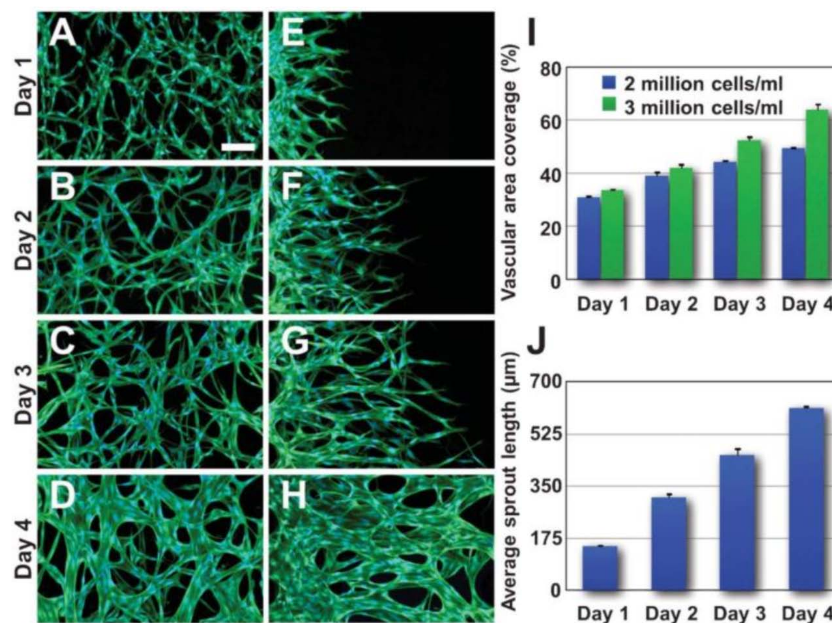

Figure 4. Microfluidic technology used to form perfusable 3D vascular networks along with tumor vasculature by the spatially controlled co-culture of endothelial cells with stromal fibroblasts, pericytes or cancer cells. Scale bars: 100 µm. (Adopted from Kim *et al*.[44])

photolithographic techniques to produce substrates with design-specific microgrooves which can be filled with cellular material and cultured *in vitro*. Raghavan *et al*. utilized this technique in their work which successfully produced lumenized vascular tubes with controlled diameters by varying the dimensions of their microgrooves. By culturing cellular material within branched microgrooves with varying designs, lumenized vascular tubes were also observed to branch into multiple tubes while maintaining their lumenized structure[50]. The branching patterns could be controlled by fabricating microgrooved structures with different designs. Using a similar technique, Chaturvedi *et al*. developed a technique to successfully produce vascular tubes within microgrooved structures which could be harvested and encapsulated within bulk fibrin hydrogel to produce vascularized tissue used for *in vivo* implantation to study the impact of various design parameters on the vascularization of tissue engineered constructs upon implantation in rats[51]. This is an advantage over the closed microfluidic systems where vascularized tissue could not be harvested for subsequent *in vivo* implantation. However, the harvesting process needs to be further developed to increase throughput and achieve 3D vascularized tissue.

Photolithographic techniques have also been used directly to pattern photo-crosslinkable cellular material onto adhesive substrates for vascularization applications. In one study, a UV source, a photomask, and photo-crosslinkable Gelma hydrogel were used to pattern cell-laden Gelma strips, containing ECs and other cells self-aligning cells, onto treated glass slides to demonstrate the ability to control cell alignment and elongation orientation by mechanically confining the cells within a 3D architecture[52]. In another study using a similar approach, strips of Gelma micro-constructs containing ECs and of varying dimensions were patterned onto a treated glass slide where after culture endothelial tubes formed within the patterned strips[53]. They found that optimal tube formation was only achieved at a given micro-construct size. A variety of other micropatterning techniques have also been used for vascularization applications such as soft lithography[54] and laser-assisted micropatterning[55]. In all the abovementioned papers, successful engineering of lumenized endothelial tubes were reported with controlled spatial organization.

3.4 Wire Molding

The incorporation of microchannels within a tissue engineered construct allows immediate perfusion of medium throughout the tissue construct to supply cells with adequate nutrients for survival. The wire molding technique is a simple and effective method of producing microchannels within a tissue construct which

can be perfused with medium to enhance the viability of cells within the surrounding polymerized gel[56]. The fabrication procedure used previously by another research group is shown schematically in **Figure 6**[57]. A pre-polymer solution (with or without cells) is cast and polymerized around a wire held in suspension by mechanical supports. After complete polymerization, the wire is then manually withdrawn from the polymerized material, leaving behind a perfusable microchannel which provides nutrients to encapsulated cells thus simulating our native microvessels. In their report, the generation of microporous cell-laden hydrogel constructs through sucrose crystal leaching was shown to enhance diffusivity through the construct and increase viability of encapsulated cells. A clear advantage of this approach is the ability to incorporate

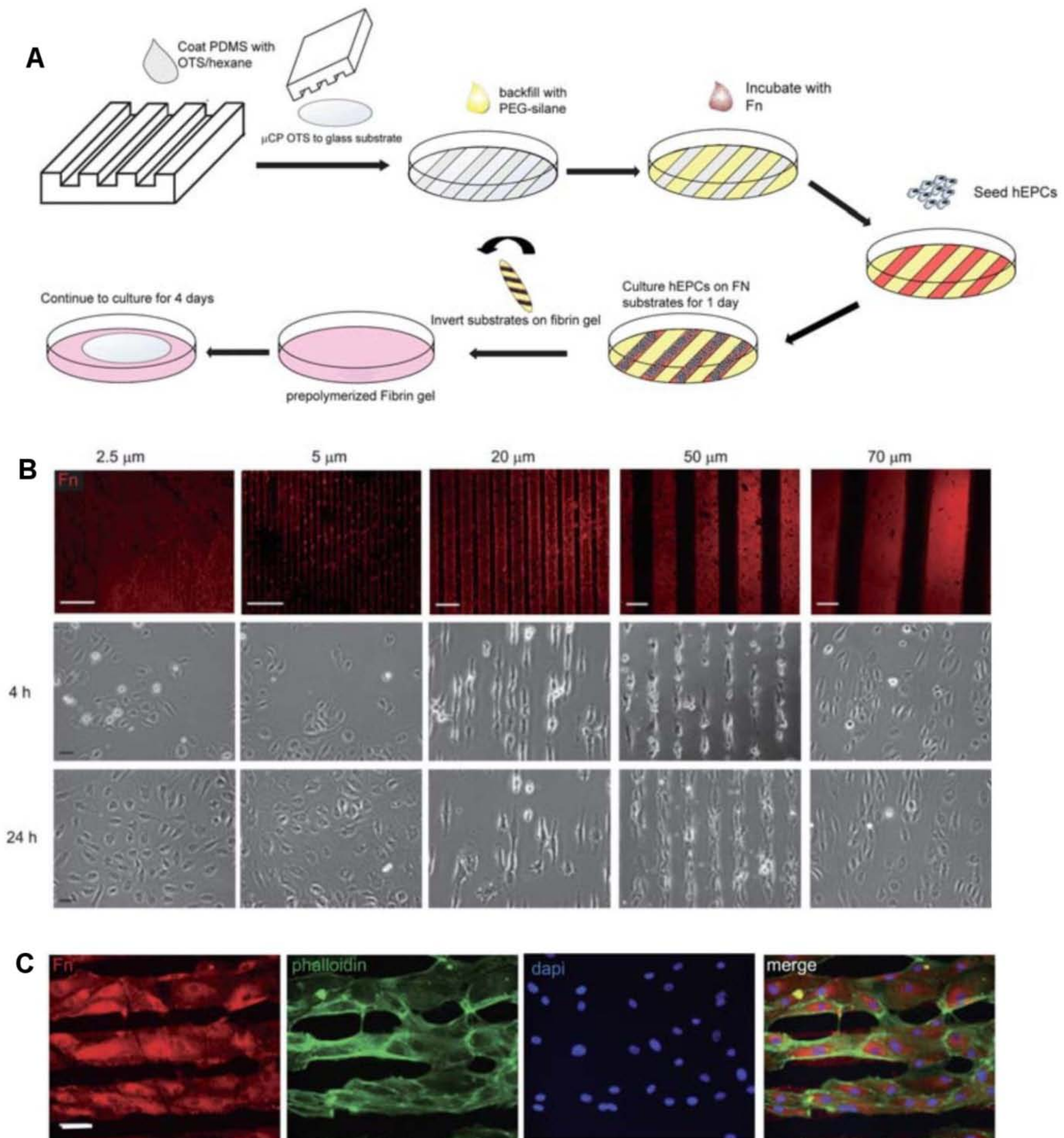

Figure 5. PDMS stamping technique used for Fn micropatterning on coverslips. hEPCs were confined within Fn strips and demonstrated controlled elongation along strip direction. Scale bars are 50μm. (Adopted from Raghavan *et al*.[49])

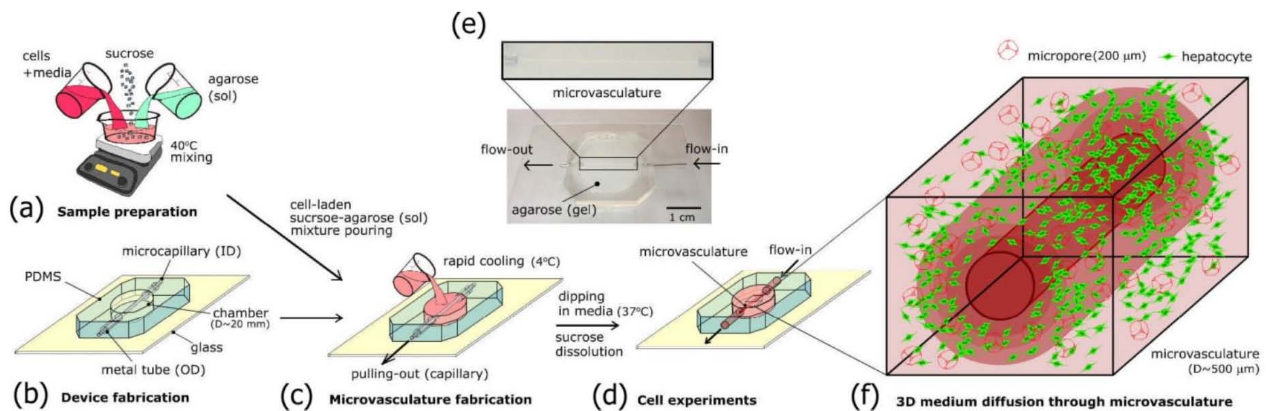

Figure 6. Wire molding technique employed to fabricate perfusable 3D microvascular tubes within microporous cell-laden hydrogels to produce biomimetic tissue constructs. (Adopted from Yao *et al*.[57])

evenly distributed microchannels within large tissue constructs in true 3D form where microchannels are stacked on various Z-planes. This was demonstrated by Yao *et al*. in their work where multichannel (up to 7) collagen conduits were fabricated using this technique to demonstrate the potential of using multichannel nerve guide conduits, instead of commercially available single channel conduits, to minimize dispersion of regenerating axons[58]. The same approach was utilized for vascularization applications where ECs were seeded onto the inner walls of microchannels within their tissue construct, and after implantation *in vivo*, demonstrated quicker vascular infiltration compared to tissue constructs without microchannels[25]. In a different paper, Chrobak *et al*. used the wire molding technique to create a single microchannel within bulk collagen gel, after which ECs were seeded and grown to confluence on the inner walls of the microchannel[59] (**Figure 6**). The relationship between channel diameter and gelling temperature was established, as well as the relationship between EC invasion into the surrounding matrix and collagen concentration. The vascular tubes also demonstrated appropriate response to inflammatory stimuli such as Histamine and Thrombin, which showed that they were functioning as native vessels would *in vivo*. Our native vessels are composed of more complex architectures than just a monolayer of ECs. Capillaries mostly comprise a bilayer structure of ECs surrounding by a Smooth Muscle Cell (SMC) layer responsible for defining vascular tone. By utilizing a modified wire molding technique in combination with other unique procedures, the ability to recapitulate this bilayer structure *in vitro* was demonstrated in recent reports, specifically using SAM-based cell transfer mechanisms[60] and hierarchical cell manipulation techniques[61]. In a separate report, a cell-seeded microchannel fabricated by wire molding was also used to determine the impact of mechanical signals on the stability and barrier function of engineered microvasculature simulating native vessels[62]. These works show the variety of research applications where the wire molding technique could be used to help us increase our understanding of vascular biology through the use of *in vitro* models.

Although the vascular network architecture cannot be precisely controlled using this technique, such as network branching and interconnectivity, wire molding has proven to be a promising technique for the vascularization of large 3D tissue constructs. It is elegantly simple and offers immediate perfusability as well as precise control over microvascular diameter. Endothelial layers seeded onto microchannel walls demonstrated healthy phenotype similar to our native vessels. The ability to vascularize thick 3D constructs could also prove advantageous over other techniques which are confined to thin sheets of tissue.

3.5 Cell Sheet Engineering

A novel approach to tissue engineering is the use of cell sheet technology. Cell sheet technology allows the user to harvest confluent cell monolayers from culture dishes with the use of thermo-responsive polymers which allow easy detachment without chemical treatment. The harvested cell sheets remain viable and intact with their naturally deposited ECM which allow for easy reattachment onto another substrate after harvest. Cell sheet engineering has been used to engineer biomimetic tissue *in vitro* such as corneal epithelium[63], skin[64], and myocardial tissue[65].

Asakawa *et al*. applied cell sheet technology to fa-

bricate vascularized tissue constructs made of stacked cell sheet layers[66]. EC monoculture sheets were stacked with fibroblast monoculture sheets in multiple different configurations to study the effect of EC positioning within the tissue construct on vascularization. After 3 days in culture, *in vitro* vascular networks were formed within the cell sheet stacks, and these pre-vascularized constructs showed enhanced vasculogenesis upon implantation *in vivo*. Subsequent reports managed to control the alignment of endothelial networks through cell-cell interactions with surrounding focally oriented fibroblast sheets[67]. In another study, a thick (30 cell sheets, close to 1 mm thick) myocardial tissue stack with an interconnected, perfusable vascular network was fabricated using cell sheet technology in tandem with *in vivo* vascularization achieved by subcutaneous poly-surgery (up to 10 cycles) implantation into nude rats[68]. This approach may not be feasible for clinical translation as it would require the patient to undergo repetitive surgical procedures. A technique able to vascularize thick cell sheet stacks *in vitro* would negate the need for poly-surgery. Sakaguchi *et al*. proposed a strategy for thick cardiac tissue vascularization *in vitro* using cell sheet technology in combination with a perfusion bioreactor and microfluidics[4,69] (**Figure 7**). Stacks of cell sheets consisting of cardiac and ECs were layered on top of a collagen construct containing microchannels and cultured in a bioreactor. ECs within the cell sheets were seen migrating through the collagen ECM to form vascular networks which were connected to the pre-fabricated perfusable microchannels, thus allowing medium perfusion throughout the layered tissue construct. As more sheets were stacked on the layered construct (12 sheets, more than 100 μm in thickness), ECs continued to form new vessels and connect with pre-existing microvessels to form an interconnected vascular network. Using a similar method, Sekine *et al*. demonstrated in a study that stacked layers of cell sheets composed of neonatal rat cardiomyocytes and ECs improved cardiac function when implanted into infarcted rat hearts with increasing EC density, and showed higher capillary density and inosculation[70].

Overall, cell sheet technology offers a unique method for the vascularization of tissue constructs with distinct advantages. Firstly, the ECM material is deposited naturally by the cells themselves, thus negating the need to fabricate a biodegradable scaffold which may require the use of cytotoxic chemicals. Secondly, the high cell-density and homogeneous distribution achieved in a cell sheet leads to higher regenerative function[4], and the method of cell sheet harvesting

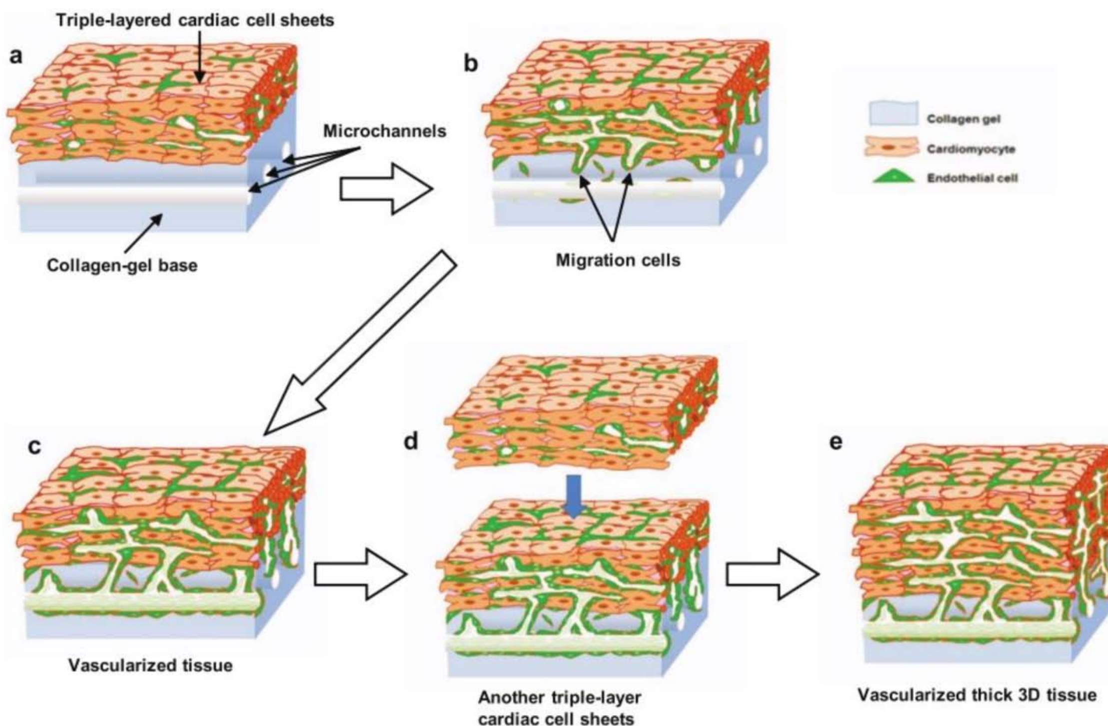

Figure 7. Cell sheet technology combined with a collagen based perfusion bioreactor for the preservation of cell viability by the vascularization of 3D tissues. (Adopted from Sakaguchi *et al*.[69])

Table 1. Characteristics possessed by each of the five techniques described

	Bioprinting	Microfluidics	Micropatterning	Wire molding	Cell-sheet
Vascularization of thick 3D tissue	✓			✓	✓
Control of tube dimensions	✓	✓	✓	✓	
Control of network architecture	✓		✓		
Perfusable networks	✓	✓		✓	✓
Multicellular vascularized tissue	✓	✓	✓	✓	✓
Suitability for *in vitro* models	✓	✓	✓	✓	
Tubulogenesis through self-assembly		✓	✓		✓
Ability for vascularized tissue to be harvested for downstream experiments	✓			✓	✓

and stacking only requires slight thermal treatment which does not significantly harm the cells. Thirdly, the ability to form thick layers of vascularized tissue and control over the orientation of vascular networks has been demonstrated using cell sheet technology.

4. Conclusion

In vitro vascularization techniques play a critical role in the advancement of tissue engineering. In the field of regenerative medicine, scientists have identified vascularization as a key hurdle that needs to be overcome. To date, the variety of tissue-engineered products successfully translated for clinical use has been limited to thin avascular tissue due to the inability of current technology to incorporate functional vascular networks into thick tissue constructs. The ability to fabricate physiologically accurate *in vitro* tissue models has also been hindered by the lack of effective *in vitro* vascularization techniques. Although 2D vascular models have been successfully fabricated and proven their efficacy, thick 3D vascular models remain elusive. Today, biologists and engineers are working hand in hand to develop working techniques for *in vitro* vascularization. We have described several enabling techniques being developed today which show promising signs of being able to achieve this goal. Each of these techniques has its own unique capabilities which make it particularly suitable for certain applications, such as for fabricating 3D perfusable networks within a tissue construct (bioprinting, cell sheet engineering), for controlled branching patterns and vessel diameter (micropatterning, wire molding), and for fabricating 2D *in vitro* vascular models (microfluidics, wire molding). **Table 1** shows a compiled checklist of characteristics possessed by the 5 techniques covered in this review. Admittedly, there are other approaches being applied to achieve *in vitro* vascularization such as electrospinning[71] and cell-accumulation[72] which were not covered in this review but also demonstrate potential for future development.

With the increasing flow of research into bioprinting technology, it is not surprising that the technology has experienced a rapid boost in development. Bioprinting technology now allows us to print multicellular constructs with high precision which mimics the hierarchal architecture of native tissue. It also possesses the ability to fabricate perfusable 3D microchannel networks within bulk tissue which is particularly useful in our efforts to achieve *in vitro* vascularization. Compared to other technologies like photolithography, bioprinting is young in terms of its development, thus it has the potential to be improved significantly and to find new applications in the years ahead. We believe that bioprinting represents the future of tissue engineering and could potentially evolve into becoming the gold-standard of biofabrication technology.

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