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Biofabrication: an overview of the approaches used for printing of living cells

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Keywords
used, cells, approaches, living, overview, biofabrication, printing

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Biofabrication: an overview of the approaches used for printing of living cells

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Abstract

The development of cell printing is vital for establishing biofabrication approaches as clinically relevant tools. Achieving this requires bio-inks which must not only be easily printable, but also allow controllable, reproducible printing of cells. This review outlines the general principles, current progress, and compares the advantages and challenges for the most widely used biofabrication techniques for printing cells: extrusion, laser, microvalve, inkjet and tissue fragment printing. It is expected that significant advances in cell printing will result from synergistic combinations of these techniques and lead to optimised resolution, throughput and the overall complexity of printed constructs.

Keywords: biofabrication; inkjet printing; cells; hydrogels; cell printing

Introduction

Biofabrication is defined as the production of complex living and non-living biological products from living cells, biomolecules and biomaterials (Mironov et al. 2009a). An interdisciplinary technological field drawing from cell and developmental biology, mechanical engineering and materials sciences, it encompasses a broad range of fabrication approaches. While some biofabrication techniques have been applied to tissue engineering (TE) since its inception, an increasing realisation of the importance of developing more sophisticated fabrication approaches has been reflected in a rapidly growing interest in the field (Derby 2012).

To understand the need for advanced biofabrication techniques, it is first important to outline the limitations of conventional approaches. The traditional approach to the fabrication of TE constructs has been to seed a solid, pre-formed, biodegradable polymeric scaffold with a cell suspension**.**. The scaffold is typically highly porous to facilitate mass transfer and cell incorporation through the bulk. Initially, conventional manufacturing processes including solution casting, particulate leaching, gas foaming, phase separation, melt molding and freeze drying were employed to fabricate these scaffolds (Leong et al. 2003; Yeong et al. 2004)**.** Electrospinning has also been widely adopted as a means to create porous scaffolds with nano-scale fibrous architecture (Pham et al. 2006). However these methods provide little control over pore size, geometry, interconnectivity and spatial distribution, i.e. placement of cells within scaffolds is essentially random.

There remain two key challenges in TE of functional constructs resulting from these limitations. Firstly, mass transfer and the development of a vascular network within

constructs is a significant issue (Kaully et al. 2009; Lovett et al. 2009; Novosel et al. 2011). *In vivo*, cells reside no more than 200 μm from a blood vessel or capillary that supplies the cells with oxygen and nutrients, and this supply must be reproduced in engineered constructs if cell viability and function is to be maintained long term (Rouwkema et al. 2008). Secondly, seeding a scaffold does not facilitate control over cell-cell contact and tissue architecture, which are primary determinants of cell behaviour and tissue function (Hurtley 2009). In order to address these challenges, biofabrication techniques must provide the means to accurately control cell position and tissue architecture in 3D constructs with microscale precision.

More recently, the *modus operandi* for solid scaffold biofabrication has shifted significantly as a result of the proliferation of additive manufacturing (AM). Often referred to as rapid prototyping or solid free-form fabrication, AM refers to a suite of techniques capable of layer-by-layer fabrication of 3D objects through computer-aided design (CAD) and/or computer-aided manufacturing (CAM). AM techniques including 3D printing, selective laser sintering, fused-deposition modelling (FDM) and stereolithography have all been applied extensively to the biofabrication of scaffolds for TE applications, as highlighted in several review papers (Hutmacher et al. 2004; Leong et al. 2003; Peltola et al. 2008; Tsang and Bhatia 2004; Yeong et al. 2004). These approaches provide precise control over both the external macrostructure and internal microstructure of scaffolds. Programmable micro-porosity can therefore be designed to aid in mass transfer throughout constructs and in the development of a vascular network (Miller et al. 2012). Also, the possibility of integration between CAD principles and 3D medical imaging (magnetic resonance imaging, computer tomography) means that scaffolds can potentially be designed to suit an individual patient (Sun et al. 2004).

Although the scaffolds can be fabricated with added complexity through these AM techniques, they are still secondarily seeded with cells.. As such, many of the problems associated with traditional solid scaffolds, particularly the inability to recapitulate a complex multi-cellular architecture, are not alleviated through the use of complex scaffolds produced by AM (Mironov et al. 2009a). One promising advance that offers the potential to overcome these limitations is cell printing technology.

Cell printing can be described as the use of material transfer processes to pattern and assemble cells and biomaterials with a defined organisation (Mironov et al. 2006). It is one of the most powerful combinations of TE with AM techniques that allows the production of 3D tissues constructs, where multiple cell types and biomaterials can be directly placed in specific spatial arrangements. Biomaterials have typically been hydrogels which, by virtue of their high water content, are generally biocompatible, and often utilise gentle crosslinking/gelation mechanisms that facilitate cell encapsulation with minimal adverse effects on cell viability. The design of hydrogel biomaterials for TE and biofabrication applications (Brandl et al. 2007; Drury and Mooney 2003; Hunt and Grover 2010; Lee and Mooney 2001; Nicodemus and Bryant 2008; Seliktar 2012; Slaughter et al. 2009; Ulijn et al. 2007) is a broad area of research that will not be covered extensively here. Printing can facilitate cell deposition either in organotypic architectures to engineer functional tissues, or in cellular microarrays where individually addressable micro-cultures contain cells in a physiologically relevant microenvironments (Fig. 1). The work of Klebe and co-workers (Klebe 1988) in ‘cytoscribing’ laid the foundation for the precise patterning of living cells on surfaces, but it was the more recent advances in AM and CAD/CAM that have seen the advent of several bioprinting technologies that can actually deposit living cells. Several reports outline progress in cell printing to date (Binder et al. 2011; Burg and Boland 2003;

Calvert 2007; Campbell and Weiss 2007; Derby 2012; Guillemot et al. 2010a; Guillotin and Guillemot 2011; Mironov et al. 2006; Wüst et al. 2011; Xu et al. 2011b).

In this review, we have outlined the general principles, current progress, and compared the advantages and challenges for the most widely used biofabrication techniques for printing cells: extrusion, laser, microvalve, inkjet and tissue fragment printing. We have particularly focussed on bio-inks used to suspend cells for printing and the methods employed for fabricating 3D structures.

Extrusion printing

General principle. Extrusion printing refers to techniques where continuous filaments of a material are forced through a nozzle in a controlled manner to construct a 3D structure. For extrusion printing of cells, the material usually consists of a highly viscous cell-laden hydrogel (Fedorovich et al. 2007) that can flow from the nozzle without the need for high temperatures. Once deposited, solidification of the hydrogel through physical or chemical means provides sufficient mechanical integrity to fabricate 3D structures**.** The printer design is generally simple, consisting of a 3-axis robot that controls the movement of either pneumatically or volumetrically driven displacement pens or syringes with a typical nozzle diameter of 150-300 μm. The utilisation of extrusion approaches for cell printing have recently been reviewed (Chang et al. 2011; Fedorovich et al. 2007; Wang et al. 2010; Wüst et al. 2011)**.**

Current progress. Williams and co-workers were the first to report on the use of an extrusion printer to deposit living cells (Smith et al. 2004)**.** Using a commercial printer, they deposited cold (2 - 10 °C) solutions of either human fibroblasts encapsulated in Pluronic-F127, or

bovine aortic endothelial cells (BAECs) in type I collagen, onto heated substrates where solidification of the printed structures was induced by thermal gelation of the biopolymers. The low temperatures used in this work and problems with dehydration of printed filaments have resulted in low cell viabilities, although BAECs printed in collagen from a 250 μm tip maintained 86% viability and were shown to proliferate over 24 hr. Importantly, this work showed that CAD/CAM technology could be used to deposit cell laden structures that mimicked an anatomical vascular structure. In follow-up work the viability of rat microvascular cells printed in collagen as a function of several process parameters was assessed (Smith et al. 2007).

Several groups have printed cells encapsulated in a range of hydrogels that are solidified through either thermal processes or by post-print crosslinking, to engineer diverse tissues ranging from liver to bone. For example, Wang et al developed extrusion printing approaches for the biofabrication of liver constructs (reviewed in Wang et al. 2007). They used a custom-build printing system to deposit printed primary rat hepatocytes encapsulated in materials including gelatin (Wang et al. 2006), gelatin/chitosan (Cheng et al. 2008; Yan et al. 2005), gelatin/alginate (Yan 2005) and gelatin/fibrinogen (Xu et al. 2007). Structures were deposited at low temperature (< 10 °C) onto a warmer stage, with the thermal gelation of gelatin providing initial structural support, which was stabilised by immersion of the construct in a solution containing a crosslinking agent or polymerising enzyme: glutaraldehyde for gelatin; sodium tripolyphosphate for chitosan; calcium chloride for alginate; thrombin for fibrinogen. These approaches showed good cell survival and retention of metabolic function, although the use of harsh crosslinking conditions is a concern, and vascularisation of the constructs remained a challenge (Wang et al. 2010).

Subsequently this research group demonstrated that adipose-derived stem cells (ASCs) printed in gelatin/alginate/fibrinogen gels could be induced to differentiate into endothelial cells at the walls of printed channels (Xu et al. 2009a). They also showed that simultaneous deposition of hepatocytes in gelatin/alginate/chitosan and ASCs in gelatin/alginate/fibrinogen facilitated the fabrication of complex 3D structures mimicking the liver (Li et al. 2009) **(**Fig. 2).

The research of Fedorovich and co-workers has focussed on engineering bone and cartilage constructs using a commercial extrusion printer (EnvisionTec, Germany) (Fedorovich et al. 2011a)**.** In early studies, multi-potent stromal cells (MPSCs) from goat bone marrow were deposited in both Lutrol F127 and alginate hydrogels, with gelation induced by thermal changes and ionic crosslinking with calcium chloride, respectively (Fedorovich et al. 2008). Cell viability was shown to be unaffected by the printing process, and in alginate constructs (but not Lutrol F127 constructs) cells survived over time in culture with some evidence of osteogenic differentiation. Additional investigations of both human and goat MPSCs in printed alginate constructs showed that the enhanced diffusion of nutrients and oxygen to encapsulated cells, afforded by the engineered porosity, enhanced cell viability and reduced apoptosis and hypoxia in comparison with cells in solid alginate constructs when assessed both *in vitro* and *in vivo* (Fedorovich et al. 2011b)**.** However, only a fraction of cells in either construct differentiated towards an osteogenic lineage.

Multiple-cell-type constructs were then fabricated to incorporate goat endothelial progenitor cells (EPCs) in an effort to improve the engineered bone grafts and encourage vascularisation (Fedorovich et al. 2011d)**.** Both heterogeneous Matrigel/Matrigel constructs (EPCs and MPSCs both in Matrigel™), and Matrigel/alginate constructs (EPCs in Matrigel™, MPSCs in

alginate) were fabricated and implanted *in vivo*. In each case, the alginate or Matrigel component containing MPSCs was supplemented with osteo-inductive biphasic calcium phosphate (BCP) particles. After 6 weeks *in vivo*, Matrigel/BCP constructs demonstrated considerable bone formation in the MPSC-laden Matrigel that was enhanced by the BCP particles, and vascularisation in the EPC-laden Matrigel. There was little bone formation or vascularisation in alginate constructs, however, which was attributed to the lack of cell interaction and migration in alginate. A similar lack of cell interaction and function was observed for human MSCs and articular chondrocytes encapsulated in alginate in multi-cell fabricated osteochondral grafts (Fedorovich et al. 2011c).

In all of the work summarised above, a common problem was a lack of mechanical integrity in the printed hydrogel constructs. This limited the scale and fidelity of the constructs, and also posed challenges for implantation and the retention of the fabricated structure *in vivo*. Recently, several reports have introduced approaches where extrusion printing of soft, cell-laden hydrogels is combined with extrusion printing of stiffer, structural synthetic polymers. For example, Schuurman et al demonstrated sequential printing of polycaprolactone (PCL) and cell-laden alginate (Schuurman et al. 2011). The alginate component was crosslinked with calcium chloride following deposition. Only a slight decrease in viability of a human chondrocyte cell line deposited in the alginate was observed after 24 hr, and was likely due to heat shock from the PCL printed adjacent to it (deposited at 160 °C). A very similar approach was adopted by Kim et al, who used a custom multi-head dispensing system (Kim and Cho 2009) to deposit hybrid constructs containing a PCL/PLGA blend as the structural polymer alongside collagen containing pre-osteoblast cells (Shim et al. 2011). In contrast, He et al took a different approach to this problem with a 'cryo-printing' technique (He and Wang 2011). They printed ASCs in gelatin/alginate/fibrinogen containing a cryo-protectant (DMSO

or glycerol) alongside polyurethane in a cell-compatible organic solvent (tetraglycol), both at -20 °C. The constructs were then stored at -80 °C for one week before thawing and crosslinking the gel component. They observed high cell viability (up to 75%) and the cells proliferated over 14 days in culture.

Sun et al developed a novel bioprinting tool comprising four separate nozzles, each with a different mode of deposition (Khalil et al. 2005)**.** They used one of these, a pneumatic microvalve nozzle in extrusion mode, to fabricate constructs with rat heart endothelial cells encapsulated in alginate hydrogels by deposition of filaments directly into a thin layer of calcium chloride solution (Khalil and Sun 2009; Khalil and Sun 2007). Subsequent reports from this group have focused on developing mathematical models of the forces experienced by cells during cell printing (Yan et al. 2010)**,** and probing their effect on the viability of extruded hepatocytes (Chang et al. 2008) and endothelial cells (Nair et al. 2009)**.**

Laser-assisted bioprinting

General principle. Laser based approaches were among the pioneering works in cell printing. Odde et al developed the "laser guidance direct write technique" (Odde and Renn 1999)**,** where a weakly focused laser beam acts as an optical trap that can control the movement of particles in a solution. This was used to pattern living cells on a substrate (Odde and Renn 2000), and has been explored for layering multiple cell types (Nahmias et al. 2005) and positioning of cells in microarrays (Ma et al. 2011)**.** However, the technique is limited by low throughput, low cell viability and a narrow range of fabricated structures and thus has not been explored extensively as a biofabrication approach.

By far the most productive application of laser-based printing techniques has been approaches based on the principle of laser-induced forward transfer (LIFT). This technique was initially developed for the direct writing of metal features using a high-energy pulsed laser to deposit a metal film on an optically transparent support (Bohandy et al. 1986). Modifications of the LIFT technique to deposit biological materials, including cells, have collectively been described as laser-assisted bioprinting (LAB) techniques (Schiele et al. 2010b)**.** The three key components of any LAB technique are a pulsed laser source, a target plate usually made of quartz and coated with the ink to be printed (the ribbon), and a receiving substrate that faces the ribbon (Fig. 3A). There have been two widely employed variations of the LAB approach, which are distinguished by the nature of the ribbon. In matrix-assisted pulsed laser evaporation – direct write (MAPLE-DW), the ribbon is coated with a sacrificial biopolymer hydrogel (Fig. 3B) which acts as an attachment layer for cells and absorbs the laser, typically a low powered pulsed laser in the UV or near-UV region. Volatilisation at the ribbon-biopolymer interface induces cavitation which generates a high speed (20 - 100 ms^{-1}) jet that transfers a small volume of the biopolymer and cells to the substrate. In contrast, biological laser printing (BioLP™), which is also referred to as absorbing film assisted-LIFT (AFA-LIFT), uses a ribbon with a thin (1 - 100 nm) metal or metal oxide (usually Au, Ti or TiO_2) layer to absorb a high-powered laser pulse (Fig. 3C). Due to rapid thermal expansion of this layer, a small volume of the ink is propelled to the substrate as before, but with little transfer of the laser energy to the ink solution. In this approach, cells can be coated onto the ribbon in either cell culture media or a biopolymer hydrogel.

Recent reviews provide both a broad overview of LAB in the context of other biofabrication technologies (Guillemot et al. 2011; Guillotin and Guillemot 2011), as well as detailed summaries of the development of each of these LAB techniques (Schiele et al. 2010b).

Current progress. Chrisey et al first demonstrated the MAPLE-DW technique and its ability to pattern biomaterials including poly(ethylene glycol), enzymes and Chinese hamster ovary (CHO) cells (Wu et al. 2001)**,** as well as bacteria (Ringeisen et al. 2002) and proteins. Further work enhanced the cell printing capabilities, showing that various cell types including human osteosarcoma and rat cardiac cells (Barron et al. 2004b, Ringeisen et al. 2004), could be printed with viability approaching 100% at near single-cell resolution using Matrigel™ as the absorptive layer. At the same time, this group developed the improved BioLP™ approach in an attempt to limit direct interaction between the laser and sensitive biomaterials (Barron et al. 2004a). This technique also proved capable of depositing highly viable human osteosarcoma cells (Barron et al. 2005) and olfactory ensheating cells (Othon et al. 2008) onto Matrigel™ substrates.

Since this initial work, both MAPLE-DW and BioLP™ approaches have been implemented with a range of ink materials and cell types, with increasing sophistication of printed constructs. For example, Guillemot et al demonstrated printing of human endothelial cells from an alginate ink as well as deposition of nano-particulate hydroxyapatite (nHAp) by BioLP™ (Guillemot et al. 2010b). They subsequently showed that sequential deposition of nHAp alongside human osteoprogenitors in alginate onto Matrigel™ substrates could be used to pattern these components in 2D and 3D with retention of cellular function for application in bone TE (Catros et al. 2011)**.** Deposition of nHAp alongside osteoblast-like cells has also been demonstrated by a MAPLE-DW approach (Doraiswamy et al. 2007).

Chichkov and co-workers demonstrated that BioLP™ can be applied to reactive printing approaches. For example, both human adipose-derived stem cells (ASCs) and endothelial colony-forming cells (ECFCs) were printed sequentially from an ink mixture containing hyaluronic acid (HA) and fibrinogen, onto a substrate sprayed with thrombin to crosslink the printed material (Gruene et al. 2011b). This process could be repeated to construct, layer-by-layer, 3D arrays of encapsulated cells for the study of cell-cell and cell-material interactions. Similarly, mesenchymal stem cells (MSCs) were encapsulated in hydrogels by printing them from an alginate ink onto substrates coated with $CaCl_2$, and the MSCs were subsequently differentiated towards osteogenic or chondrogenic lineages (Gruene et al. 2011a). Sequential layer-by-layer printing of fibroblasts and keratinocytes from collagen or alginate based inks onto a Matriderm™ (dermal membrane) substrate with or without $CaCl_2$ was also shown to allow BioLP fabrication of skin-like 3D constructs (Koch et al. 2012).

Several recent reports have demonstrated BioLP of cells onto scaffolds pre-formed via other fabrication techniques. For example, Chichkov et al used it to achieve selective seeding of acrylated PEG scaffolds with ovine vascular smooth muscle cells and endothelial cells printed from alginate-based inks (Ovsianikov et al. 2010). The same group also reported printing HUVEC and human MSCs onto polyester composite cardiac patches which they implanted in infracted rat hearts to yield functional improvement (Gaebel et al. 2011). Another approach has been to BioLP cells onto thin bio-papers, which are subsequently stacked to form 3D structures. For example, osteosarcoma cells were printed from an alginate-based ink onto electrospun polycaprolactone (PCL) mats which were stacked together (Catros et al. 2012). A parallel study outlined BioLP of HUVECs from a methyl-cellulose ink onto porous poly-lactide-co-glycolide films filled with collagen or Matrigel, and

subsequent stacking of cell-laden layers (Pirlo et al. 2012). In a novel approach, Corr and co-workers used a MAPLE-DW approach with a gelatin-based ink to print human dermal fibroblasts (Schiele et al. 2010a) as well as mouse embryonic stem cells that retained their pluripotency (Raof et al. 2011). The advantage of the gelatin ink is that it melts and is removed during culture so that the printed cells then have access to an application-specific growth substrate, which in these reports was demonstrated with poly-L-lysine (PLL) surfaces.

Microvalve printing

General principle. Microvalve printing is a simple droplet-based deposition mechanism where fluids under constant pneumatic pressure are dispensed from tips by opening and closing a small valve, which can be controlled mechanically, electrically or magnetically. This style of deposition has been implemented in extrusion-style printing as outlined above, where the microvalve remains open for extended periods, but finds most application in drop-on-demand deposition by fast actuation of the microvalve. The tips are usually 100-200 μm in diameter, and are capable of dispensing droplets with volumes ranging from tens of nano-litres to several micro-litres, from inks with relatively low viscosities (1-20 mPa.s).

Current progress. The deposition of living cells using microvalve dispensing systems was first explored by Demirci and Yoo (Demirci and Montesano 2007), who developed a custom printing tool where four of these dispensers were mounted above a three-axis robotic stage for use in cell printing. They demonstrated the fabrication of cell-laden collagen constructs by a modified reactive printing process (Lee et al. 2009b; Lee et al. 2009a). Two dispensers were used to print sequential layers of cells in culture media, and an acidic collagen solution. After printing each layer of collagen, gelation was induced through a pH change by spraying the construct with sodium bicarbonate using a nebuliser, before deposition of the cells onto the

gelled collagen. In this manner, skin constructs containing human dermal fibroblasts and epidermal keratinocytes (Lee et al. 2009a), as well as neural constructs containing embryonic rat astrocytes and neurons (Lee et al. 2009b)**,** were printed with high cell viability.

It was also shown that channels suitable for media perfusion could be included in these constructs by printing sacrificial gelatin channels, and that perfusion enhanced the viability of encapsulated fibroblasts (Lee et al. 2010). Since cells are suspended in culture medium in this approach, cell settling and aggregation led to variations in cell output and clogging of nozzles. In subsequent work, rat bladder smooth muscle cells were encapsulated in cold, pre-neutralised collagen solutions which could be microvalve printed and solidified by thermal gelation with only a slight decrease in cell viability (Xu et al. 2010). Three-dimensional patches containing encapsulated cells were fabricated in a layer-by-layer process, although 5 min equilibration at 37 °C was required after each printed layer to induce gelation (Moon et al. 2010)**.** Microvalve cell deposition has also been explored in the fabrication of constructs tailored for *in vitro* studies, for example, co-cultures were printed onto Matrigel surfaces to act as a model for the study of ovarian cancer (Xu et al. 2011a) and single mouse embryonic stem cells were also deposited in microarrays for RNA analysis (Moon et al. 2011)**.**

Robotic spotting technologies have been widely implemented in the fabrication of microarrays where antibodies, DNA, proteins or other biomaterials are typically deposited onto glass slides by contact pin-style printers (Anderson et al. 2005; Anderson et al. 2004; Flaim et al. 2005)**.** While this style of printing can be used to print microarrays of fixed cells (Hart et al. 2009)**,** it is not suitable for live-cell deposition. Dordick and co-workers have used non-contact spotters, which utilise microvalve dispensing tips, to produce live cell microarray platforms (Fernandes et al. 2009). Their approach is based on the deposition of individual

droplets of cells suspended in a hydrogel matrix onto functionalised glass slides to create stable pseudo-three dimensional arrays of encapsulated cells (Lee et al. 2008) (Fig. 4**)**. Glass slides were coated with poly(styrene-co-maleic anhydride) (PS-MA) to enhance hydrophobicity, and spots of poly-L-lysine (PLL) containing $BaCl_2$ were printed onto the surface and dried. Cells suspended in alginate are then deposited directly on these first spots. the PLL encourages adhesion of the alginate by ionic interaction, and the Ba^{2+} ions diffuse through the alginate solution to crosslink it, forming a cell encapsulating hydrogel spot. In the first demonstration of this approach, breast cancer and human hepatoma cell lines were deposited in alginate spot microarrays and interrogated with a complimentary array slide loaded with drugs or enzymes (Lee et al. 2008)**.**This method was also employed to develop an immunofluorescence based array to investigate the response of alginate-encapsulated human pancreatic tumour cells to chemically-induced hypoxia (Fernandes et al. 2008). The microarrays were also utilised to study the expansion and neural commitment of mouse embryonic stem cells (Fernandes et al. 2010).

Inkjet printing

General principle. Inkjet printing is a non-contact technique capable of reproducing digital image data on a substrate using picolitre ink droplets. The technique can be divided into two broad categories: continuous inkjet (CIJ), where a steady stream of small droplets produced by fluid instability on passage through a nozzle is either deflected by an electrostatic field onto a substrate or not deflected and collected for reuse and drop-on-demand (DOD) inkjet, were ink droplets are only produced when required. DOD inkjet is further categorised by the mechanism used to produce the ink droplets. In thermal inkjet printing, rapid local heating generates a bubble within the ink chamber that ejects a small droplet. Conversely, in piezoelectric inkjet printing, the voltage-mediated actuation of a piezo-crystal is used to

create a pressure pulse resulting in droplet ejection. Static electricity actuated print heads comprise a third ejection mechanism but are far less common. CIJ requires electrically conducting ink formulations, and contamination concerns on ink re-cycling all but rule out the technique for cell printing. Consequently only DOD inkjet has been utilised in cell printing to date.

The foundational work of Klebe et al. (Klebe 1988) 'cytoscribing' with inkjet printers in 1988 could be considered the birth of bioprinting. Later, inkjet was introduced as a non-contact alternative to traditional contact pin arrayers for the deposition of biological materials in microarray fabrication (Lemmo et al. 1998; Sumerel et al. 2006; Zaugg and Wagner 2003). These instruments were expensive and limited to commercial microarray fabrication. At the same time, however, the development of thermal inkjet technology by Hewlett-Packard and Canon enhanced the accessibility of the inkjet approach by producing cheap and readily available desktop printers. Research in cell printing has since utilised these readily available technologies, and several reviews have outlined the use of inkjet printing in tissue engineering and biofabrication applications (Binder et al. 2011; Boland et al. 2006; Burg et al. 2010; Calvert 2007; Campbell and Weiss 2007; Derby 2009; Derby 2008).

Current progress. The use of inkjet printing technology to deposit living cells was first explored by Wilson and Boland (Wilson and Boland 2003). Their printer was based on a standard Hewlett-Packard (HP) printer housing, but for cell deposition they utilised a specially designed print head containing 9 individual piezoelectric pumps connected to needles (~ 160 μm internal diameter) that deposited relatively large (~ 15 nL) droplets. It was demonstrated that bovine aortic endothelial cells (BAECs) and smooth muscle cells in cell culture media could be deposited onto collagen or Matrigel™ substrates (Wilson and Boland

2003). In follow-up research with Mironov and Markwald, aggregates of BAECs were printed onto collagen and thermo-reversible gels in a layer-by-layer fashion, and closely spaced aggregates showed evidence of tissue fusion (Boland et al. 2003). Although this approach lacked the resolution that true inkjet printing could offer, it became the foundation for the tissue fragment printing approach discussed in the next section.

Boland and co-workers were the first to show that viable mammalian cells could be deposited from standard commercial thermal inkjet print heads (Xu et al. 2005). Using HP print heads with a relatively large nozzle size (~ 50 μm) and drop volume (~ 80 pL), CHO cells and embryonic rat motor neurons were deposited onto soy agar and collagen substrates. Primary neural cells printed onto collagen were subsequently shown to exhibit normal electrophysiology (Xu et al. 2006a). In these reports, though, cells were suspended in a concentrated phosphate buffered saline (PBS) ink, which aided passage through the print head as a result of cell contraction, however a significant portion of cells were lysed (~ 15%). Later work investigated further the viability of CHO cells printed from a 1x PBS ink and indicated that relatively high cell viability was maintained (89%), with only a small number of apoptotic cells (~ 3.5%) (Cui et al. 2010). It was found that the thermal inkjet printing process generated small (~ 100 Å) transient pores in cell membranes that self-repaired within 2 hr of printing and, interestingly, this phenomenon could be applied to transfect plasmid DNA into CHO cells (Cui et al. 2010) and endothelial cells (Xu et al. 2009c) during printing.

Burg and co-workers have investigated some fundamental aspects of inkjet cell printing that are key checkpoints in the development of the technology towards becoming a clinically relevant biofabrication tool. Having recognised that printing salt-containing solutions through thermal inkjet heads can quickly lead to nozzle failure due to salt crystallisation, the chelating

agent ethylenediaminetetraacetic (EDTA) was included in a culture media ink in an attempt to extend print head lifetime (Parzel et al. 2009). This approach was successful in enhancing throughput, although there was some evidence of reduced cell viability after longer (~ 30 min) exposure times to the EDTA-containing ink. The group has also thoroughly characterised the adverse effects of cell settling and aggregation on cell output during printing, noting that the reproducibility of cell deposition is affected after ~ 10 mins (Pepper et al. 2012b; Pepper et al. 2011). Co-cultures of D1 murine mesenchymal stem cells and 4T07 murine mammary cancer cells were deposited on collagen hydrogel substrates (Burg et al. 2010) and the group has developed novel methods to achieve alignment of multiple print heads (Burg et al. 2010), retain printed pattern fidelity and viability through various post-processing methods (Pepper et al. 2010), and quantitatively analyse the fidelity of printed patterns (Pepper et al. 2012a).

Boland et al. explored reactive printing approaches to encapsulate inkjet printed cells in 3D hydrogel structures, using both alginate/calcium and fibrin/thrombin reactive systems. Cell-containing fibrin channels that mimicked simple vasculature were fabricated by suspending human microvascular endothelial cells in 1xPBS solution containing thrombin and calcium chloride, and printing onto thin layers of fibrinogen (Cui and Boland 2009). While cells were not directly printed in the following approach, alginate solutions containing cells were also selectively cross-linked by the inkjet deposition of calcium chloride to create cell-encapsulating hydrogels with defined three-dimensional structure (Boland et al. 2007; Xu et al. 2009b).

These reactive printing approaches were pursued further by Atala and Xu by suspending cells in a calcium chloride solution, followed by printing into an alginate/collagen mixture (Xu et

al. 2008b; Xu et al. 2006b)**.** In this way, hydrogels containing regions patterned with multiple cell types could be fabricated (Xu et al. 2006b). Printed cell-laden constructs were also implanted in mice and monitored by magnetic-resonance imaging (MRI) (Xu et al. 2008b; Xu et al. 2006a)**,** revealing changes in tissue microvasculature. The reactive process was also reversed, with cells suspended in low viscosity alginate solutions and printed in a calcium receiving bath; this was used to create single droplets of alginate hydrogels encapsulating pancreatic islet cells that continued to produce insulin (Xu et al. 2008a)**.**

The approach of printing low viscosity alginate solutions containing cells into calcium chloride receiving baths has also been investigated by Nakamura and co-workers. This group used inkjet print heads that eject ink droplets by actuation of the ink chamber via static electricity (SEAjet™, Seiko EPSON). Having demonstrated that the print heads could deposit cells (Nakamura et al. 2005), HeLa cells were encapsulated in 3D alginate hydrogels by this reactive printing approach (Nakamura et al. 2006). In further work, the quality of the printed structures was improved by including viscosity enhancers such as PVA into the receiving bath and employing a high calcium chloride concentration, although this reduced cell viability to ~ 70% (Nishiyama et al. 2009). In a more recent report, this reactive printing approach was reproduced with a custom piezoelectric inkjet head containing four separate nozzles (Arai et al. 2011)**,** although cell viability was not thoroughly investigated.

There have been only a few other examples of cell deposition by piezoelectric inkjet printing, and until recently these used single nozzle systems. Derby et al. printed human fibroblast cells using a commercial single nozzle (60 μm diameter) piezoelectric ejector (Microfab Inc., USA) (Saunders et al. 2008)**.** Importantly, this was the first report to conduct a comprehensive study on the viability of inkjet printed cells. Additionally, control over the

actuation waveform used to drive the piezoelectric deposition allowed analysis of the effect of forces applied to cells during printing on their subsequent behaviour. Cell survival was high (> 90%) in all cases, with a slight reduction in viability with increasing actuation voltage (98% at 40V, 94% at 80V), and was indistinguishable from control cells under optimal printing conditions. This printer was also used to deposit cells into micro-well compartments pre-fabricated by inkjet printing of a novel ink material combining thermal and photo-initiated crosslinking mechanisms (Di Biase et al. 2011)**.** Derby has also been instrumental in highlighting to the cell printing community the requirements for printable inkjet fluids (Derby 2010)**.**

Parsa et al. used single piezoelectric nozzle ejectors (60-100 μm diameters, Microdrop Technologies, Germany) to print hepatocytes from a surfactant-containing ink. They found that although initial cell viability was high, it decreased after 7 days in culture (Parsa et al. 2010). The authors were unable to determine of this was a result of the printing process, the added surfactant, or other factors. This work also employed gentle agitation of the print head in an effort to reduce cell settling and aggregation, although this led to reduced cell viability. Liberski et al used the same nozzle ejector to fabricate living cell microarrays by combining deposition of cell-laden droplets with novel water-in-oil emulsion cell culture (Liberski et al. 2011)**.**

Recently, two research groups have successfully addressed one of the main challenges of cell printing: preventing the settling and aggregation of cells, whilst meeting the stringent fluid property requirements. Chahal et al. successfully addressed this issue by using a surfactant (Ficoll-PM 400) to control the ink density resulting in reliable cell printing through a commercial single nozzle (MicroFab, Germany) over 90 min (Chahal et al. 2012). Ferris et al

showed that a bio-ink based on a novel microgel suspension in surfactant-containing tissue culture media could be used to prevent cell settling and aggregation (Ferris et al. 2013). The stable suspension and optimal fluid properties of the bio-ink allowed reproducible printing of several different cell types, from two different commercially available drop-on-demand printing systems, over long printing periods. They demonstrated (Fig. 5) that two cell types (C2C12 and PC12) could be printed simultaneously from two different commercial inkjet print heads (each with 126 nozzles) in defined two-dimensional patterns onto collagen hydrogel substrates. Printing multiple cell types from different print heads is a highly attractive feature of inkjet printing as a biofabrication tool, allowing the fabrication of more complex multi-cellular constructs.

Tissue fragment printing

General principle. The combination of bioprinting techniques with biological self-assembly is an approach to fabricating tissue structures that has been developed over the last decade. Founded on the dictum that 'nature knows best', and drawing from the principles of developmental biology, this approach exploits the intrinsic capacity of closely spaced tissue fragments to fuse together; otherwise known as 'tissue fluidity' (Forgacs et al. 1998). Tissue fragments, often spheroids containing several thousand cells, are deposited in close spatial organisation so that they fuse together to generate an organotypic structure. In many ways, this could be viewed as a distinct bioprinting concept, rather than just an alternate bioprinting method. It has been the subject of several topical reviews and opinion pieces (Jakab et al. 2010; Marga et al. 2012; Mironov et al. 2009b; Mironov et al. 2007; Mironov et al. 2006). The technique has historically been termed 'organ printing'.

Current progress. This bioprinting approach was proposed in 2003 (Mironov et al. 2003) following the development of the first cell printer by Boland and Wilson (Boland et al. 2003)**.** The following year saw the development of techniques used to obtain reproducible spherical aggregates of CHO cells (~ 500 μm in diameter) by controlled cutting of tissue cylinders (Jakab et al. 2004b)**,** and the fusion of these CHO aggregates after manually positioning within cell-responsive gels was demonstrated (Jakab et al. 2004b; Jakab et al. 2004a).

Concomitantly, various bioprinting tools were being developed for the extrusion printing of living cells. It was shown that CHO aggregates could be aspirated into a capillary and then printed into defined assemblies surrounded by collagen gel using a commercial mechanical bio-assembly tool (nScrypt, USA). This approach was also applied to the fabrication of cardiac constructs from aggregates of embryonic cardiac and endothelial cells (Jakab et al. 2008)**.** The process was refined further, through the development of a specifically designed bioprinter and alterations to existing printing methods (Norotte et al. 2009)**.** Specifically, it was recognised that the use of collagen gels limited the fabrication of 3D structures due to premature gelation and unwanted integration with the fusing aggregates. Collagen was therefore replaced with bio-inert agarose gels, and branched vascular structures were fabricated from spheroids of human skin fibroblasts (HSFs) (Fig. 6A-C). Without suitable methods to upscale the fabrication of tissue spheroids and reproducibly aspirate them into a capillary, however, this approach was cumbersome and limited to small structures. Furthermore, the fusion of tissue aggregates was a slow process and could lead to distortion of the printed constructs (Norotte et al. 2009). Consequently, an alternative approach was demonstrated where long cylindrical tissue aggregates were matured and strengthened in agarose moulds before aspiration into capillaries and deposition in 3D arrangements (Fig. 6D-F). This approach was utilised to fabricate vascular tubes from human umbilical vein

smooth muscle cells (HUVSMCs) and HSFs (Norotte et al. 2009), and preliminary results have been reported on the fabrication of nerve grafts containing Schwann cells (SCs) (Marga et al. 2012).

Comparison of approaches and remaining challenges

The selection of a particular biofabrication approach for assembling a cell-laden structure is dependent on the desired geometry (i.e. 2D or 3D). This in turn is largely dictated by the performance of each biofabrication method in terms of amount of material deposited and precision/repeatability of positioning. Based on these two criteria it is reasonable to assume that extrusion printing methods are more suitable for fabricating 3D structures, whereas drop-on-demand techniques (e.g. inkjet printing) are more amenable for fabricating 2D structures and for the precise placement of cells within engineered constructs. The suitability of each biofabrication method (as reviewed in this article) for assembling 2D and 3D structures has been outlined in more detail in what follows.

Extrusion printing could be considered the simplest of all the approaches described here, and is arguably the most amenable to the production of 3D hydrogels given that cells can be encapsulated within a hydrogel ink prior to deposition. This could also be considered a detriment, however, because it means that cells must be confined to a printable matrix; this does not permit the separation of cell deposition and matrix deposition. Reliance on contact with the substrate is not ideal and printing resolution is generally poor. The primary drawback is that deposition is restricted to continuous filaments, which limits the scope of fabricated structures. The advantage is that 3D structures, whether bulk solids or porous architectures built by the familiar ‘log-pile’ stacking of filaments, can be fabricated relatively quickly and simply. Drop-on-demand techniques are advantageous in that they employee

digital fabrication capable of reproducing essentially any pattern or structure, although in comparison to extrusion printing the fabrication of 3D structures can be more difficult as it requires the coordinated coalescence and solidification of droplets.

Printing tissue fragments could be considered a drop-on-demand technique if cell spheroids are deposited. This approach is attractive because cells within the tissue fragments are in a physiologically relevant configuration with high densities and cell-cell contact, and because natural developmental mechanisms are elicited. There remain, however, significant challenges to this technique. The formation and processing of tissue spheroids is cumbersome, and the alternative of extruding cylindrical tissue fragments limits fabrication flexibility; it is no longer a drop-on-demand style approach and the scope of possible printed architecture is diminished. Removal of agarose supporting rods can also be difficult with more complex structures (Norotte et al. 2009). As with conventional extrusion printing, resolution is generally poor. Cell aggregates are 300-500 μm in diameter, larger than the diffusion limit of oxygen (100-200 μm), and therefore hypoxia is a concern, as evidenced by the presence of apoptotic cells within printed vascular walls (Norotte et al. 2009).

The drop-on-demand techniques including laser, microvalve and inkjet printing boast good resolution and flexibility. They are amenable to deposition of cells directly on a surface or, as demonstrated for each technique, 3D structures can be fabricated using reactive printing or layer-by-layer approaches. Each of these techniques incurs substantial technological hurdles.

The key advantage of LAB is that it is a nozzle-free approach, which allows printing of inks with a wide range of viscosities (1-300 mPa.s), at concentrations up to $1x10^8$ cells/mL, without issues with nozzle clogging (Guillotin and Guillemot 2011)**.** High resolution is

possible and only a very small amount of material is required. However, laser techniques are not as accessible or as well characterised as microvalve or inkjet printing. The process requires ribbon preparation prior to printing and therefore non-uniform ink coating can cause inconsistent cell output, and the thin layer of ink can dry quickly on the ribbon surface (Schiele et al. 2010b). Printing speed and throughput is also limited and scale-up is difficult (Guillotin and Guillemot 2011), which makes this technique more suitable for the fabrication of 2D films, rather than 3D structures. The use of intense laser irradiation and its long term effect on cells is a concern, although BioLP addresses this somewhat with the absorptive interface layer. This layer presents additional problems, however, as cytotoxic metal and metal oxide particles can be transferred to the printed material (Guillotin and Guillemot 2011), although this could potentially be avoided by replacing the metal layer with a polyimide (Brown et al. 2010).

Microvalve printing allows simple drop-on-demand deposition that, in comparison with inkjet printing, is not as dependent on the fluid properties of the ink (Derby 2010). Importantly, control over microvalve actuation and the applied pressure means that the deposited droplet volume can be easily adjusted across a wide range, which is not possible for the other drop-on-demand techniques. The minimum droplet size is large and thus printing resolution is poor compared to that of laser and inkjet printing, and this could prevent fabrication of some organotypic structures. The capability to dispense larger volumes could, however, allow faster fabrication of larger 3D structures. Although not as problematic as in inkjet printing, cell aggregation and settling within the printer can alter cell output and clog nozzles, especially when printing from CCM.

Inkjet printing is arguably the most attractive bioprinting technique for positioning cells within engineered constructs, providing a combination of non-contact, high-throughput deposition with single cell resolution. Widespread implementation of inkjet in the consumer graphics printing market has led to rapid development of the technology, which is now well understood and highly accessible. Relationships between droplet generation and fluid properties or printing parameters are thus well understood (Derby 2010)**.** Furthermore, the application of inkjet to colour printing, where several print heads are used to simultaneously deposit different coloured inks, should be easily transferrable to the deposition of multiple cell types, biomaterials and other biological factors; this has already been demonstrated to an extent (Burg et al. 2010; Xu et al. 2006b). However, efficient inkjet printing is heavily reliant upon the ink meeting a relatively stringent set of fluid property requirements (Derby 2010)**.** The issue of settling and aggregation of cells has recently been successfully addressed (Chahal et al. 2012; Ferris et al. 2013). Furthermore, work to date has utilised thermal inkjet , single-nozzle piezoelectric, and multiple nozzle piezoelectric printheads (Ferris et al. 2013). Piezoelectric heads are the industry standard for high end printing applications and could provide advantages over thermal heads; primarily, a greater control over droplet formation parameters (Derby 2010; Saunders et al. 2008). Long-term cell viability could be an issue due to the high shear forces imposed during deposition, and further work is required to characterise this further for both thermal and piezoelectric inkjet printing.

Conclusions

In conclusion, the development of cell printing is vital for establishing biofabrication approaches as clinically relevant tools. Central to this is the design of the bio-inks, which must be easily printable, allow controllable, reproducible printing of cells and have some solidification mechanism to enable the fabrication of 3D structures. Crucially, this must be

achieved without causing cell damage, and the final printing structure should support normal cell function and the development of 3D engineered constructs that mimic normal tissues.

The cell printing techniques presented in this review provide alternative routes to controlled cell deposition, with individual advantages and challenges which have been outlined. The different approaches should be regarded as complimentary rather than competing technologies. It is likely that significant advances in cell printing will be made through synergistic combinations of these techniques in order to optimise resolution, throughput and the overall complexity of printed constructs. For example, the use of printing approaches with different resolutions could be employed to mimic aspects of natural biological systems operating on different scales. Additionally, some work highlighted in this review has demonstrated the merging of cell printing with solid scaffolds produced via other biofabrication methods, and there is certainly scope to investigate these combinations further.

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Figure Captions

Fig. 1 Cartoon representing the concept of cell printing technology. A patient-specific computer model of a jaw bone instructs the deposition of cells and biomaterials from a cell printer to fabricate a tissue engineered replacement construct. Reproduced from (Campbell and Weiss 2007)**.**

Fig. 2 Sequential deposition of hepatocytes in gelatin/alginate/collagen (A, clear) and ASCs in gelatin/alginate/fibrinogen (B, red) to produce a 3D liver construct (C). Adapted from (Li et al. 2009).

Fig. 3 Schematic representation of the LAB approach (A), including the ribbon design utilised in both MAPLE-DW (B) and BioLP (C) variations.

Fig. 4 3D cell microarrays produced by microvalve deposition of cell-laden alginate droplets onto pre-printed $BaCl_2$/ poly-L-lysine (PLL) spots on poly(styrene-co-maleic anhydride) (PS-MA). Gelation of alginate droplets is mediated by Ba^{2+}, and adhesion of the gel to the PS-MA surface is enhanced through PLL. Adapted from (Fernandes et al. 2009).

Fig. 5 Patterning of two cell types printed simultaneously from two separate inkjet print heads onto collagen substrates. (a) Schematic representation of multiple head printing. (b, c) C2C12 (red) and PC12 (green) cells pre-stained with CellTracker™ dyes and printed in various patterns. Images were taken 1 hr after printing, following the addition of culture media. (d, e) Printed patterns of C2C12 and PC12 cells after 8 days under differentiation conditions. Cells were immunostained for desmin (C2C12, green) and β-III tubulin (PC12, red). Dotted lines represent the outline of the original print pattern. Scale bars represent 500 μm (b-d) and 200 μm (e). Reproduced from (Ferris et al. 2013).

Fig. 6 Representative structures printed from tissue fragments, adapted from (Norotte et al. 2009). (**A**) Deposition scheme for a branched vascular structure from tissue spheroids. (**B-C**) Branched structure built from 300 μm HSF spheroids initially (B), and fused 6 days after deposition (C). **(D)** Deposition scheme for tube fabrication using tissue cylinders. **(E-F)** Printed tubes from agarose cylinders and pig SMCs initially (E), and after fusion and removl from agarose 3 days after deposition (F).

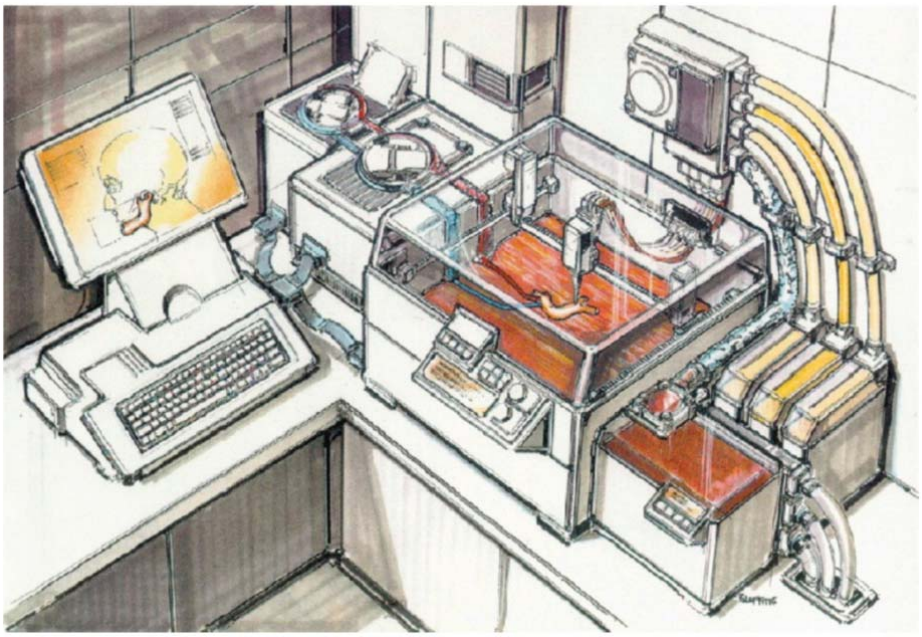

Figure 1

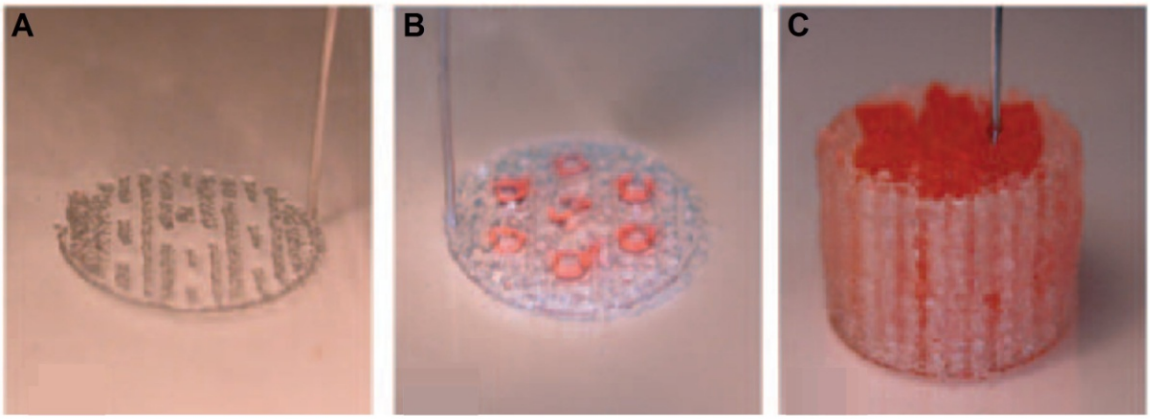

Figure 2

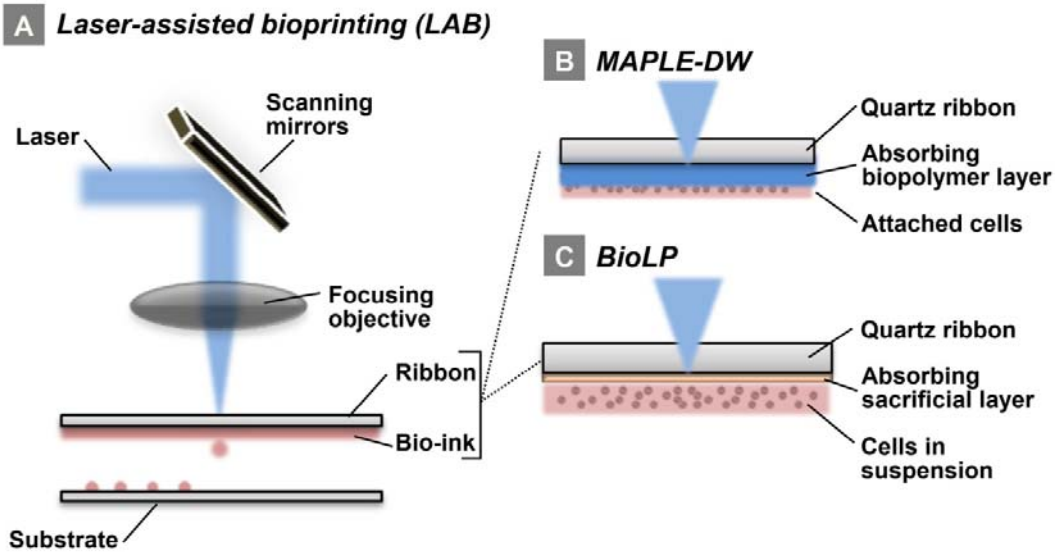
A Laser-assisted bioprinting (LAB)
Laser
Scanning mirrors
Focusing objective
Ribbon
Bio-ink
Substrate
B MAPLE-DW
Quartz ribbon
Absorbing biopolymer layer
Attached cells
C BioLP
Quartz ribbon
Absorbing sacrificial layer
Cells in suspension

Figure 3

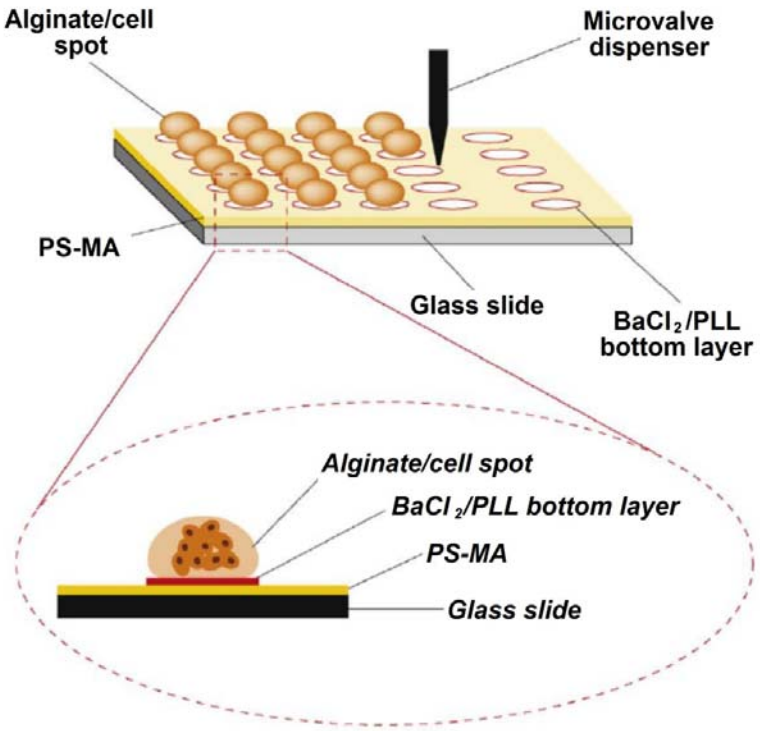
Alginate/cell spot
Microvalve dispenser
PS-MA
Glass slide
BaCl2/PLL bottom layer
Alginate/cell spot
BaCl2/PLL bottom layer
PS-MA
Glass slide

Figure 4

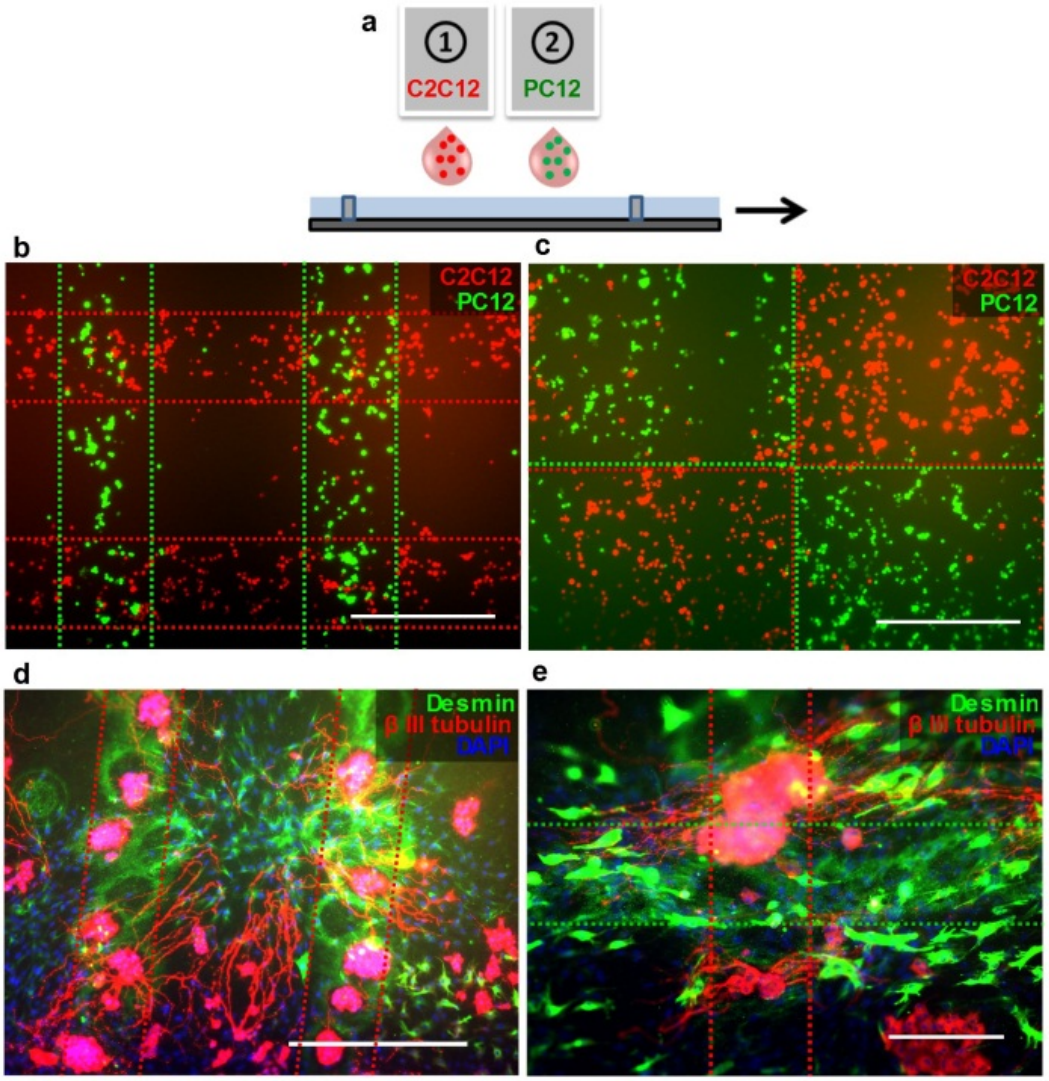
a
1
C2C12
2
PC12
b
C2C12
PC12
c
C2C12
PC12
d
Desmin
β III tubulin
DAPI
e
Desmin
β III tubulin
DAPI

Figure 5

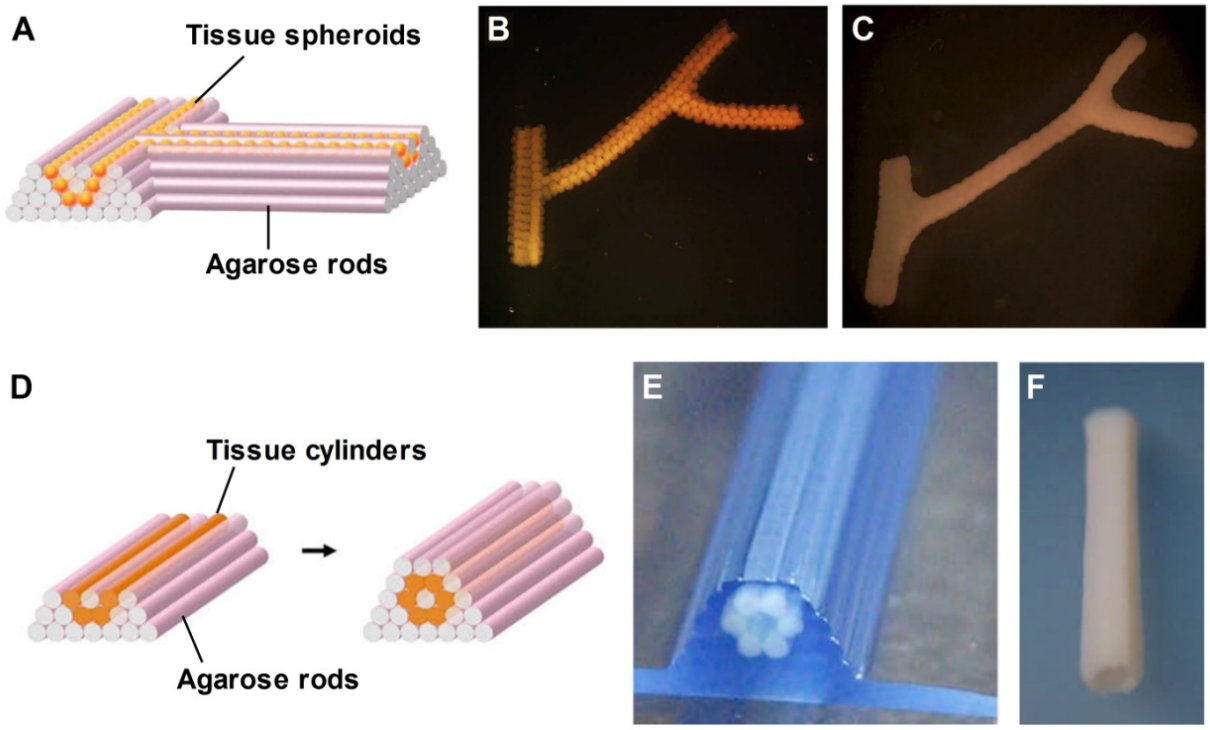
A
Tissue spheroids
Agarose rods
B
C
D
Tissue cylinders
Agarose rods
E
F

Figure 6