

Three-dimensional bio-printing

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Three-dimensional (3D) printing technology has been widely used in various manufacturing operations including automotive, defence and space industries. 3D printing has the advantages of personalization, flexibility and high resolution, and is therefore becoming increasingly visible in the high-tech fields. Three-dimensional bio-printing technology also holds promise for future use in medical applications. At present 3D bio-printing is mainly used for simulating and reconstructing some hard tissues or for preparing drug-delivery systems in the medical area. The fabrication of 3D structures with living cells and bioactive moieties spatially distributed throughout will be realisable. Fabrication of complex tissues and organs is still at the exploratory stage. This review summarizes the development of 3D bio-printing and its potential in medical applications, as well as discussing the current challenges faced by 3D bio-printing.

3D bio-printing, cell, function, fabrication

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Three-dimensional (3D) printing, also called rapid prototyping (RP), can be used to model and fabricate a three-dimensional object layer by layer. This involves computer or software aided design (CAD) and the printer transferring the signals into actions to fabricate the desired items with inkjet materials. Three-dimensional printing emerged from stereolithography (SLA) [1]. With SLA materials are sensitive to light or laser undergoing photon initiated solidification. The whole model is accomplished after all layers are formed [2]. Other 3D printing technologies are now available, including fused deposition modelling (FDM), selective laser sintering (SLS), digital light processing (DLP), three-dimensional printing (3DP), laminated object manufacturing (LOM), and polyjet (Table 1). Whatever the technology is, the basic theory is that the object consists of finite layers, and the greater the numbers of layers, the higher the resolution required [3]. There are different

methods to fix the layers, some materials can be melted and coagulated easily, some can be laser moulded, and some can be easily coagulated. A complete 3D printing system contains software, to design moulds and operate, and a printer, the main part of the system. The printer can achieve inkjet and fabrication [4–6]. In China, it has been reported that the house could be printed, so the time, cost and pollution would be reduced (<http://www.theguardian.com/>). Additionally, 3D printing technique has been used to print batteries to precisely control the morphology of electrodes and to improve the capacity of the battery (<http://3dprint.com/>). Compared to traditional fabrication technology, the advantage of 3D printing is high resolution and individuation and it has been widely used in various areas including consumer products, film making and games [7].

With the development of biomaterials and cell biology, bionic medicine and regenerative medicine are becoming important research fields with fast growth. Bionic bones and cochlear have come into the market already and bionic eyes

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Table 1 Types of 3D printing technology

| Names | Description | Materials | Ref. |
|---------|--|--|------|
| SLA | Stereolithography: light sensitive materials could be solidified to a thin layer. | Thermoplastics | [2] |
| FDM | Fused deposition modelling: a fundamental 3D printing technology and is used extensively. | Plastics and some foods | [8] |
| SLS | Selective laser sintering: similar to SLA, but solidify materials with the aid of infrared laser. | Plastics, wax, ceramic and metal materials | [9] |
| DLP | Digital light processing: similar to SLA, but is faster because of whole layer fabrication after laser scanning. | Photopolymer | [10] |
| 3DP | Three-dimensional printing: using binder, the powders are bond together to format a whole layer. | Plaster | [11] |
| LOM | Laminated object manufacturing: the materials are fused by heating roller. | Paper, ceramic and metal materials | [12] |
| Polyjet | Similar to SLA, many tips and ultraviolet lights work together. Inkjet and solidification could be accomplished simultaneously with higher resolution. | Thermoplastics | [13] |

and hearts are in development. 3D printing has been used in fabricating hard-tissues which suit the damaged sites individually. With the involvement of cells, more complex organ could be printed. Here we review the application of 3D printing in biological and medical fields, summarize printing strategies and analyze the current issues of 3D bio-printing. Finally, we discuss the challenges of 3D bio-printing for future research.

1 3D bio-printing

3D printing technology used in biomedical field is denoted 3D bio-printing and it has a great potential for future regenerative therapy. An ideal 3D bio-printing system is shown schematically in Figure 1. Firstly, accurate information of tissues and organs should be collected for designing the model. Secondly, the server should be able to transfer the information into electrical signal to control the printer to print the tissues, and the printer should be able to maintain the cell viability during the fabrication process (Figure 1B and C). Usually, a tissue is composed of many types of cells and the cells will be mixed with some substances to be better fused (Figure 1C) [14–16]. At present, some hard tissues can be fabricated with bionic materials by 3D printer [17], and have been used in clinical trials. A real complicated tissue cannot be constructed from 3D printer at the moment. In the future, 3D bio-printer could be used to print organs for repairing the damaged body part, and to simulate some functional tissues for research, therapy and drug screening. Furthermore, 3D bio-printing can be used for personalized therapy that could reduce the cost of therapy. Biocompatible and biodegradable materials can be combined with 3D bio-printing to reduce the incompatibilities caused by materials [15,18]. Therefore, 3D bio-printing will lead to a novel technology revolution.

1.1 History of 3D bio-printing and types of 3D bio-printing technology

3D printing is being acknowledged by more and more people, and that in turn will promote the development of the

technology. 3D printing can benefit the bio-therapy field as well. It has been reported that only 20% of patients who wait for organ donation for grafting therapy can have organs to transplant, and the other patients have to keep waiting or are treated by alternative and often less appropriate therapy (<http://www.unos.org/index.php>). The history of tissue and organ culture *in vitro* is nearly 30 years old [19]. Langer and Vacanti [20] invented the technology of combining cells and materials. Tissue engineered organs would be a source of organs for transplantation in the future, but at present there have been no acceptable artificial organs for clinical trial. There are some successful cases in skin allograft trials, and tissues simply constructed *in vitro*, such as bladder, myocardial membrane and trachea, have also been used clinically [21–24]. But these tissues are two-dimensional cell sheets rather than 3D structure of dermis or epidermis [25]. Current 3D tissue fabrications have been achieved by adding cells to the specially formatted scaffolds [26–29]. 3D scaffolds can provide extracellular matrix to improve cell growth and interactions. The bio-degradable and bio-compatible solid freeform fabrication (SFF) scaffolds have been used in tissue fabrication *in vitro*. Some SFF scaffolds can be built up with the help of computers to improve their accuracy and personalization [30]. Fusion of materials and cells can produce simple 3D dermis-like tissues, which, however, are only used in animal models [27]. With advanced equipment and formulation protocols, some tissues containing vessels can now be fabricated [31].

3D printing can do a better job than the above mentioned, with the printed-out scaffolds being able to accurately depict the complicated bio-tissues and the cells being planted on them after fabrication is completed. Cells and tissues can also be printed simultaneously after encapsulating cells in materials. 3D bio-printing is a super multi-discipline technology involving tissue biology, cell biology, computer technology, materials science and medical sciences. The printed organs should meet the requirements of all these disciplines. At present, there are four types of 3D bio-printing technology which are derived from basic 3D printing techniques for depositing cells: valve-based, direct inkjet, acoustic and laser-mediated (Figure 2).

A valve-based 3D bio-printer controls the droplets size

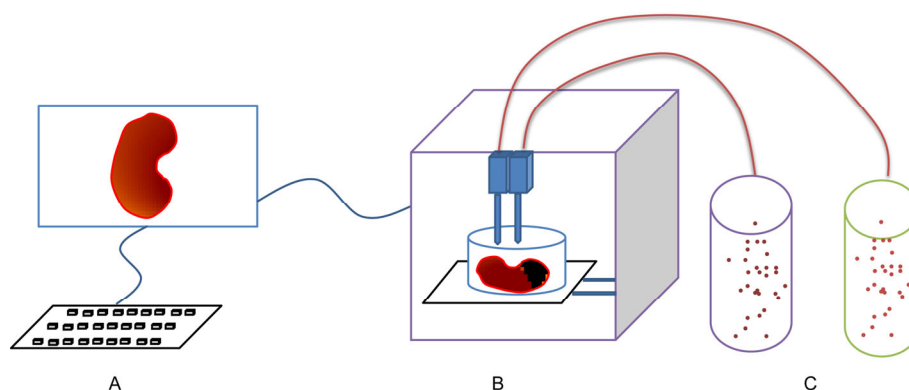


Figure 1 The diagram of ideal 3D bio-printing. A, Server, which is responsible for designing the model. B, 3D bio-printer, the main facility responsible for printing. C, The sources of different types of cells.

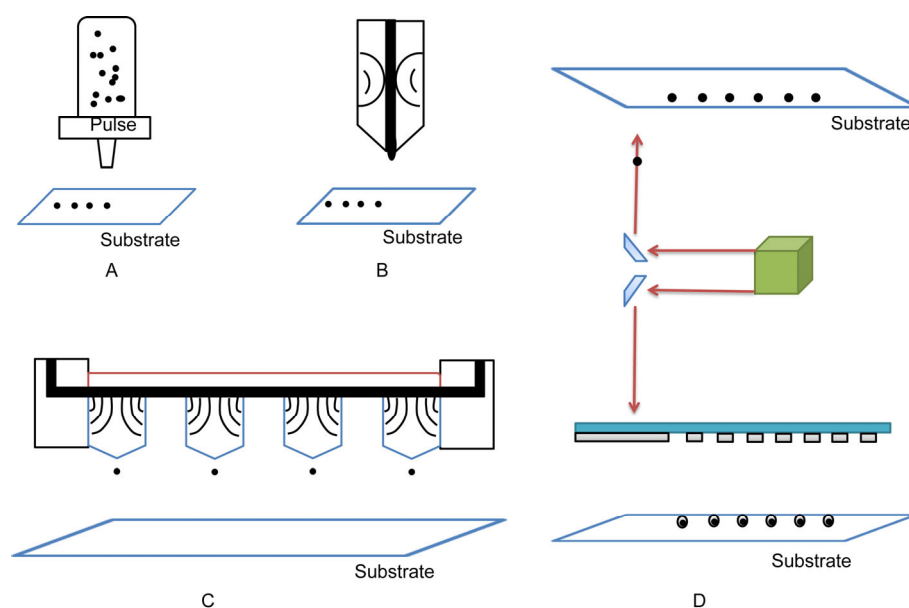


Figure 2 Four types of 3D bio-printing technology for depositing cells. A, Valve-controlled 3D bio-printing. B, Direct inkjet 3D bio-printing. C, Acoustic 3D bio-printing. D, Laser-mediated 3D bio-printing.

by modifying pulses (Figure 2A). Its resolution and throughput are both at moderate level. At present, it is mainly used in single cell sequencing and minute cell sample analysis [32].

Inkjet is a non-contact image reconstruction technology. The signals can reappear on the platform through ink droplets (Figure 2B). The technology has been widely used in electrical materials and integrated circuits [33]. Printing accuracy and specificity may be improved by manipulating ink droplets from microliter to picoliter. The technology has been applied to printing tissue-scaffolds and bio-materials. The difficulty is how to control the size and the flow continuity of the ink droplets [34,35]. Hydrodynamics needs to be considered here. Methods used by inkjet technology include piezoelectric ceramics and thermal conductivity. The technology can be used in high-throughput bio-printing but it is difficult to achieve control over single cells [36].

Laser-mediated bio-printing is a technology mediated by laser to position the cells of cell suspensions and tissue suspensions (Figure 2C). It is a commonly used 3D printing method and can be divided into the following sub-types: laser-guided bio-printing (LGB), biological laser processing (BLoLP), laser-induced forward transfer (LIFT), and matrix-assisted pulsed laser evaporation direct writing (MAPLEDW). LGB has low throughput but high resolution, and can control single cells. LIFT has higher throughput than LGB but a poor control of single cells. Heat generated by laser pulse is transmitted to the thin films that coat the cells and cut out cell droplets of particular sizes and shapes, which attach to the movable basement. The heat might affect the viability of cells [37].

There is no nozzle on the acoustic bio-printer (Figure 2C). The pressure of sound radiation can shake the cell ink and controls the droplet size by adjusting acoustic frequency and

amplitude. It generates little energy thus does not harm cells [38]. At present, it has been used with many types of cells and cell suspensions, and has been used in some other fields such as manipulation of single cell DNA and RNA [39].

1.2 Bio-active scaffolds from 3D printing

Extracellular matrix (ECM) provides structural and biochemical support for cell growth and is composed of many types of proteins and glycans. The alteration of ECM would influence cell state and function [40,41]. With high resolution, 3D printing could be used to print scaffolds that mimic *in vivo* structure and environment of tissues, and there are many reports that scaffolds from 3D printing have been used in drug delivery, tissue engineering and cell viability tests [14,15]. Model fabrication is the first 3D printing application, with optimization of the technique and material modification, some bio-active hard tissues have been printed [42]. In 2012, the first clinical trial of 3D bio-printing was performed at a hospital in the Netherlands. A bionic jaw printed using 3D printer was transplanted into a patient (<http://www.bbc.com/news/technology-16907104>). Since then, more trials have been performed in Japan, Poland and some other countries (<http://3dprint.com/19617/osteo3d-3d-print-mouth/> and <http://www.3ders.org/>). The grafted materials can specifically interact with vocal organs without affecting the hearing and the vocality of patients [43].

To directly print the live tissues, many printed scaffolds were used for cell culture. Cells obtain higher viability and function when cultured on 3D scaffolds compared to two-dimensional environments [44,45]. Ploy caprolactone (PCL) scaffolds from printing have been used in culturing different types of cells, as shown in Table 2. However, this is just the combination of scaffolds with cells at the two-dimensional level, and is therefore called indirect 3D bio-printing. Actually, the interaction between cells and materials in the indirect 3D printing is still two-dimensional because only one side of the cell surround is scaffolds. Table 2 shows progress of application of 3D printing scaffolds on cell culture and another bio-printing called direct 3D bio-printing compared to indirect 3D bio-printing and the direct 3D bio-printing shows that the cells are encapsulated in materials and then are used as bio-ink.

1.3 Cell 3D bio-printing

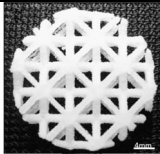
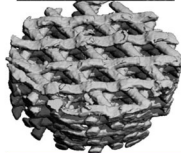
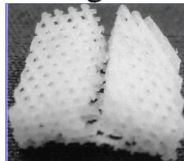
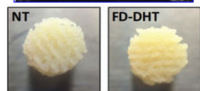
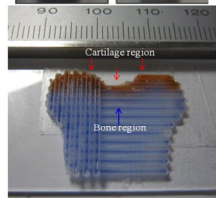
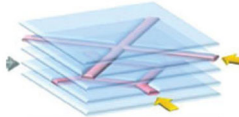
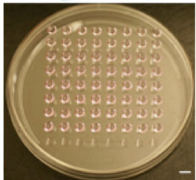
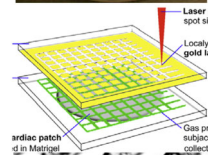
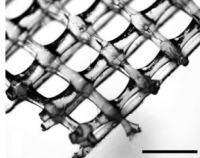
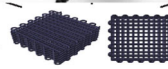
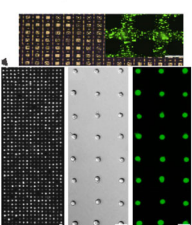
There are more than 200 cell types in a human body, and these cells form different and complicated tissues and organs. That makes it difficult to replicate complex and functional 3D tissues and organs *in vitro*. 3D bio-printing may solve this problem. The ink droplets can deposit live cells and fabricate the tissues and organs as desired. Despite a promising future for organ transplantation, at the moment there has been no biologically active organ fabricated by 3D bio-printing. Each tissue and organ of the human body is

sophisticated in construction, and the combinations of various types of cells require not only *in vitro* tests of viability and functionality but also *in vivo* tests of interactions with other tissues and organs. 3D printing has been widely used in the manufacturing industry. It has also been used in the bio-therapy field with hard and vessel-free structures. Gabor Forgacs and colleagues [46] printed short blood vessels and beating cardiac valves, demonstrating that cell-printing is possible. They later established a company named Organovo, dedicated to bio-printer development and marketing. Fibroblasts have been successfully printed by improving 3D printers [47]. Cell sources have been extended to adult stem cells and endothelial cells, and after printing, some cell-cell interactions can be observed. These pave the way for future 3D organ-printing [48].

When the cells are encapsulated into the materials and used for ink, we call this 3D bio-printing as direct 3D bio-printing as shown in Table 2. One disadvantage of cell encapsulated in materials is the loss of cell viability. Hydrogel is the first candidate for cell printing. To maintain cell viability and cell-cell interactions, some hydrogel materials can be added into the ink. Hydrogels can also work as substrates and scaffoldings [49]. Hydrogels have special chemical and physical properties, and can be degraded *in vivo* sometime after grafting. The initial materials used as scaffoldings were electrospun fibers, for substituting blood vessels [50]. Now collagen, ployanhydride and fibronectin have been widely used. It is still a hot field nowadays [51,52]. Below are some hydrogels having been used successfully in 2D fabrication via 3D printing technology and the functions of the fabricated products could also be detected: culture medium [53], agarose [54], alginate [55], collagen [56], matrigel [57–59], fibrin [60], k-70 series [61] and polyvinyl alcohol (PVA) [62]. After extruded from the printer tips, hydrogels would be polymerized by cross-linker [63]. It is a challenge to protect cells and to maintain the resolution during hydrogel's stabilization. Therefore, many new modified-hydrogels and methods are being explored [64–66].

Hydrogel is a type of polymer that could contain much water in the 3D structure. The mechanical properties and bio-properties of hydrogel need to be modified for printing and cell survival. At present, during printing and crosslinking there will be some cell death with a wide range (~2%–40%) because the cells are outside various natural environment [65,67,68]. There are chemical and physical ways to crosslink hydrogels. Gelatin is water soluble protein and has high biocompatibility [69]. With methacrylamide group modified, the gelatin (gelMA) could be cross-linked using ultraviolet rays [70]. The adding of hyaluronic acid (HA) improves gelMA printable properties [65]. Calcium could crosslink alginate and alginate-gelatin blends have been reported as potential materials for extrusion printing living cells [71,72]. Single cell printing has high resolution and could be used for cell patterning and material-cell blends precise control. Present technique “Block-Cell-

Table 2 Examples of 3D bio-printing

| Types | Materials | Cells | Morphology | Ref. |
|----------|--|---|---|------|
| Indirect | PCL | Myoblast cell line C2C12 |  | [74] |
| Indirect | Calcium phosphate modified PCL (PCL-CaP) treated with fibrinogen | Mesenchymal stem cells (MSCs) |  | [75] |
| Indirect | PCL | MSCs |  | [76] |
| Indirect | Glycerol with soy protein | MSCs |  | [77] |
| Indirect | PCL and alginate | Osteoblast cells and chondrocyte |  | [78] |
| Indirect | Collagen and gelatin | There is no cell |  | [79] |
| Direct | Cell culture medium | Mouse embryonic stem cells (mESCs) |  | [80] |
| Direct | Cells printed on PEUU immersed in Matrigel | Human umbilical vein endothelial cells (hUVECs), MSCs |  | [81] |
| Direct | HA and gelMA | Chondrocyte |  | [65] |
| Direct | gelMA | Live cancer cell line, HepG2 |  | [82] |
| Direct | Single cell pattern | A few breast cancer cell lines |  | [73] |

Printing” even makes cell 100% living after printing [73].

2 The future of 3D bio-printing

It takes a long time and needs lots of cells when accomplishing an organ-printing [83]. During such a long period of time, how to ensure the accuracy of printing and the cell viability have become the crucial questions. The jet nozzle can be enveloped with aluminium alloy to prevent exposure to the radiations from laser [84]. At present, complicated tissue scaffoldings can be fabricated using hydrogels by laser-mediated 3D bio-printer and inkjet 3D bio-printer [79,85–88]. Yet in the process of 3D fusion, the use of high temperature and high pressure and some solvents may lead to the death of cells [89]. Three-dimensional tissue printed from a mixture of cells and hydrogels [78,90] possesses mechanical properties similar to natural structures but with limited cell expansion capabilities and function. This is one of the major limitations faced by 3D bio-printing at the moment [91]. Although many reports demonstrated the cells could be printed, there is still no report about the function of printed cells. At present, we could keep cells living in materials but we cannot direct the future state of cells [92]. Cell states are dependent on the varied substrates, many materials are developed to mimic the ECM [93–95], native tissue derived bio-ink is also proposed to simulated the *in vivo* environment [96]. Therefore, how to optimize the materials for cells’ customization is one of future work.

Another issue of bio-printing is how to print organs containing blood vessels. Every organ needs the network of vessels and capillaries to supply oxygen, cytokines and nutrients, as well as to remove the wastes which are noxious to cells. It has been reported that cardiac muscle tissue containing vessel-like structure was obtained from endothelial cells through tissue culture [31]. Fabrication of functional 3D tissues containing blood vessels is still a great challenge faced by 3D bio-printing [97].

The key problem faced by 3D bio-printing is cell sources, as cells are the basic units of an organ. Stem cells may become the primary source for bio-printing. Stem cells have high viability and short cell cycle time, and can differentiate into other cell types in defined conditions. This may reduce the number of cells required in bio-printing, save printing time, and create conditions for printed organs to function. At present, initial trials have been performed in 3D bio-printing with stem cells. Embryonic stem cells were used in 3D printing to form embryoid bodies (EBs) [80]. Human mesenchymal stem cells (hMSCs) and human umbilical vein endothelial cells (hUVECs) were utilized to print fabricated patches to repair cardiac muscles [81]. However, there is still a long way to go in choosing the right stem cells for bio-printing the right organs.

Ideally 3D bio-printing involves *in situ* printing of cells at the site of injury. This requires rapid model construction,

rapid printing and sufficient cell sources. Fulfilment of such requirements would lead to a revolution in regenerative medicine. For example, a quick repair of the skin wound with 3D bio-printing would increase the recovery rate and reduce permanent scarring.

In the quest for optimised 3D bio-printing of tissues and organs, how we characterize and evaluate the functions of the printed organs is also an area that requires development [98]. Relevant analyses shall include tests of cell viability, cell tracking, functional marker detection and animal model experiments. To accomplish 3D bio-printing of larger tissues structural scaffolds need to be utilised. Materials such as degradable poly caprolactone (PCL) have been used [74–76,78].

Establishment of safety evaluation systems for testing 3D bio-printed structures is another issue to be considered [99,100]. Appropriate regulatory frameworks are yet to be developed. 3D bio-printing is a new and complicated technology, and the process involves cell preparation, cell expansion, graft, materials and observation after grafting, and safety and efficiency evaluation.

In summary, 3D bio-printing holds a great promising future. Real clinical advances require parallel advance in many fields to occur.

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