

3D Bioprinting and 3D Imaging for Stem Cell Engineering

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1 Introduction

Three-dimensional (3D) bio-printing, a rapid prototyping method to construct complex 3D structures through a layer-by-layer approach, allows depositing various types of cells and scaffold materials in the desired 3D pattern, and thus has a great potential in cell and tissue engineering applications. An important advantage of this technique is its capability to simultaneously deposit live cells and biochemical molecules (*e.g.* growth factors) along with biomaterial scaffolds at the desired location to mimic the native tissue architecture or to create a specially-designed 3D micro-environment.

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With its flexibility and power, the 3D bio-printing technology has been considered as a versatile tool for controlling stem cell fate and creating stem cell niche. 3D bio-printing system capable of precisely deposit biomaterials in desired 3D pattern, allows fine-adjustment of microenvironment where stem cells are embedded. A variety of bio-printing methodologies have been developed in order to generate different stem cell culture environments (*e.g.* stem cell patterning in single-cell level, controlling embryonic body formation) in an efficient and reproducible way.

Meanwhile, 3D printed structures often incorporate thick opaque scaffold, dense population of cells or cell aggregates. Therefore, there are significant difficulties in visualizing the 3D constructs with current imaging modalities. Biological understanding of stem cell differentiation and function has been mainly achieved in cell culture and tissues via destructive techniques such as western blots, immunohistochemistry or quantitative polymerase chain reaction (qPCR). However, to elucidate the interaction of stem cells with the microenvironment in tissue engineering applications, it is necessary to non-destructively monitor the spatio-temporal proliferation and/or differentiation of these cells in an unperturbed environment. To this end, developing novel molecular imaging techniques is critical to observe stem cell fate, cell-cell interactions, and/or structural features of an engineered tissue [1].

In this chapter, we first review the usage of bio-printing technologies for cell and tissue engineering applications, and discuss the potential of each bio-printing system for stem cell application. We also summarize the usage of commonly-used biomedical imaging modalities to tissue engineering and stem cell applications. Then, as optical imaging modalities are the most widely used imaging tools in stem cell studies, we cover the vast array of optical techniques developed to date. We address advantages and limitations of each bio-printer and imaging system, and suggest a perspective on integrating multiple bio-printing techniques and optical imaging for stem cell and tissue engineering applications.

2 Three-Dimensional Bio-Printing Techniques

2.1 Inkjet-Based Printing

The pioneer bio-printing group initially converted a commercial 2D inkjet printer into a bio-printing by replacing the printer ink with biological ink [2, 3]. While the first bio-printing approach is in use for many tissue engineering applications [4–6], numerous inkjet-based bio-printing systems are newly developed to handle a wide range of biomaterials at increasing resolution and speed. Inkjet-based printers commonly use thermal [7], piezoelectric [8–10], or microvalve-based [11, 12] drop-on-demand methods, in which picoliter or microliter volume of aqueous biological materials are dispensed in drops (Fig. 1a, 1b, 1c).

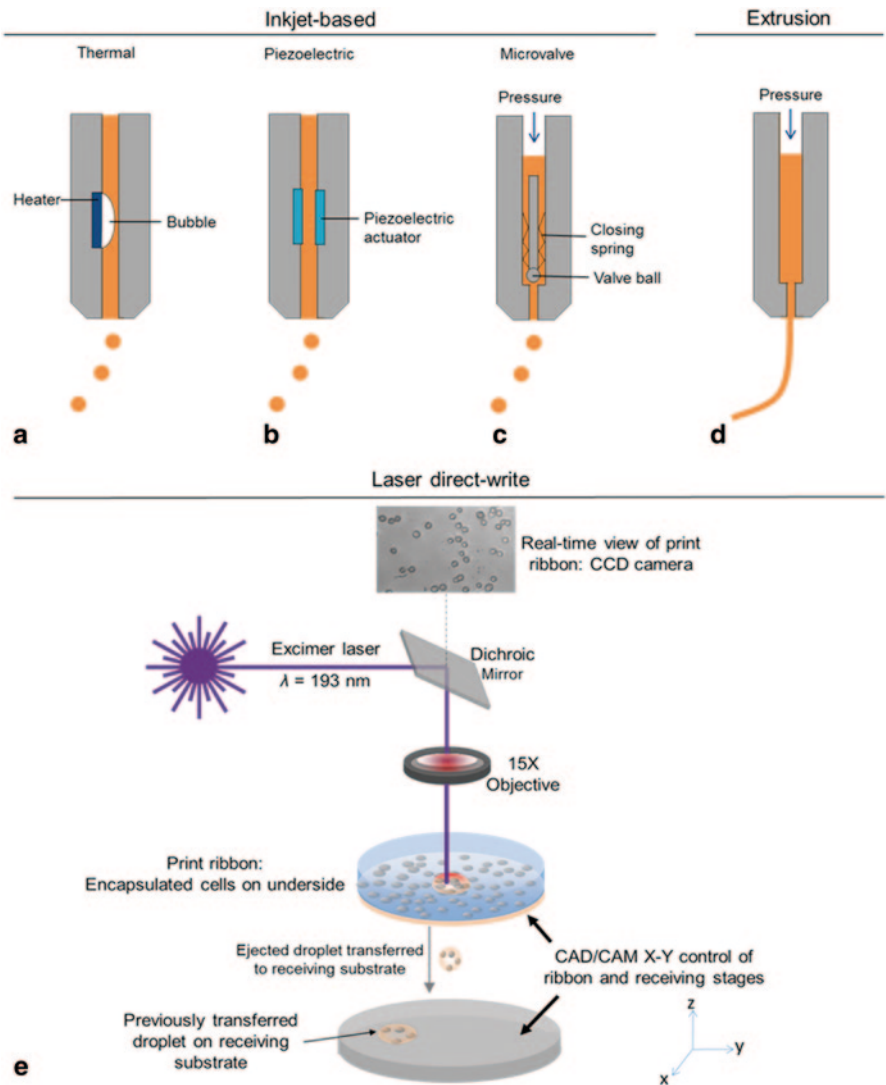


Fig. 1 Bioprinting Techniques. **a–c** Inkjet-based bioprinting technologies, **a** Thermal inkjet printing system, **b** Piezoelectric inkjet printing system, **c** Microvalve-based inkjet printing system, **d** Extrusion-based printing system, **e** Laser direct-write schematic for cell deposition

2.1.1 Printing Mechanism

Thermal bio-printers dispense droplets by increasing temperature of the heating element to produce pulses of pressure (Fig. 1a). The temperature the heater increases for short time, forming a bubble that forces the bioink out of the printer head. Although the temperature of the heating element reaches 200–300 °C during this procedure, it does not have a substantial influence on post-printing viability of

mammalian cells [7, 13]. Piezoelectric bio-printing systems utilize piezoelectric actuators or piezocrystals (Fig. 1b). Once electrical signals are applied on the crystals, they generate vibrations inside the printer head to break the bioink into droplets [8, 9, 14]. In both thermal and piezoelectric printing systems, size of nozzle (printer head) orifice and electrical pulse pattern (duration and amplitude) play main roles in determining the droplet size. The viscosity of bioink has an effect on the droplet size and reproducibility, as highly-viscous materials or media suspensions with high cell density often require higher force to be ejected and cause clogging problems.

The components of microvalve-based printers vary by design and application. Commonly-used printers include valve coils, a valve ball, and closing elements in the printer head (Fig. 1c). Unlike thermal or piezoelectric systems, additional pneumatic or piston-derived pressure is applied on the biomaterials loaded inside. The valve coil is triggered by electrical signal, lifting the valve ball, consequently dispensing a droplet. Then, valve orifice is quickly blocked by closing elements [12, 15, 16]. The droplet size is determined by the valve opening duration, the actuation frequency, material viscosity, and the pressure applied.

2.1.2 Resolution and Patterning Capability

Inkjet-based printers create patterns in drop-by-drop manner, in which a series of droplets are closely deposited to form line and surface patterns [17]. Therefore, the resolution of inkjet-based printer is determined by the minimum size of droplet that the printer can generate. The actual resolution of inkjet-based printer tends to be lower than the minimum droplet size because the contour of printed pattern often becomes enlarged by merging of closely printed droplets. Droplet size is depending on numerous parameters, including nozzle diameter, printing mechanism, material viscosity, and substrate properties (e.g. hydrophilicity). Droplet size and printing speed can be controlled electronically, and can range from picoliter to nanoliter in volume with dispensing frequency of 1–10,000 droplets/sec. The spatial resolution of inkjet-based bioprinters ranges from ~50 μm to 1 mm (Table 1). Despite this technique does not support comparable single-cell level of spatial resolution, printing of single cell can be achieved by adjusting cell suspension density and droplet size [18].

Table 1 Comparison of bioprinting techniques

	Inkjet-based [7–12, 18, 81, 149–151]	Extrusion [3, 36, 41, 44–50, 53, 54, 152]	Laser direct-write [58–63, 65, 67, 68, 70, 76–80]
Resolution/droplet size	50 μm–1 mm	5 μm–1 mm	> 10 μm
Material viscosity/density	Low	High	Low–High
(Post-printing) cell viability	High	Medium	High
Single cell control capability	Low	Medium	High
Printing speed (Total fabrication time)	Fast	Slow	Medium-fast

2.1.3 Available Materials

Materials used for bioprinting applications must be biocompatible and cytocompatible, and provide appropriate structural integrity and functional properties during post-printing maturation [19, 20]. Considerations on the viability and functionality of printed bio-structures, limit the range of available chemistries, operating temperature, mechanical and rheological properties. Naturally-derived hydrogel (including collagen, fibrin, chitosan, alginate) and synthetic polymer such as polyethylene glycol (PEG) are commonly-used scaffold materials in the field of tissue engineering [20]. The hydrogel polymers are printed in aqueous precursor form, and then solidified by post-printing crosslinking or gelation process (including enzymatic crosslinking, photo-crosslinking, pH- or temperature-sensitive phase transition). Naturally-derived hydrogels have advantages in supporting biological functions. On the other hand, synthetic polymers are more beneficial for functionalization or tailoring of scaffold material, cost efficiency, and reproducibility.

Hydrogel polymers serve as scaffolds to support 3D structure created via layer-by-layer approach. Thus, the hydrogels used for 3D bioprinting applications are required to have proper mechanical strength in order to maintain the structural integrity during and after printing procedure. The required mechanical strengths often correlate to the concentration and viscosity of polymer. Higher concentration of hydrogel provides more sturdy structure, increased stiffness, and in some cases, better resolution or patterning capability. However, this condition may not be beneficial for embedded cells since they need to degrade the matrix to migrate and proliferate. Hence, material properties need to be specifically optimized for each cell and tissue engineering applications to find balance between structure integrity and preferred cell culture condition.

Inkjet-based printing systems are capable of dispensing most of the hydrogel polymers stated above, but certain materials are not suitable for these printing systems due to the limitation of printing mechanism. The use of gentle ejecting force created by a bubble, vibrations, or pneumatic pressure (< 10 psi) minimizes post-printing cell death, phenotype alteration, or functionality loss, thus has a great advantage in viable cell printing. However, it has a limitation in dispensing highly-viscous materials or high-density cell suspensions that often cause printer head clogging issues and irregular printing patterns. In general, microvalve-based bioprinters have advantages in handling viscous materials compared to the thermal or piezoelectric bioprinters since the additional pressure applied on the loaded materials can be adjusted to create proper ejecting forces.

2.1.4 2D and 3D Cell Printing Applications

Inkjet-based bioprinting systems are more beneficial for dispensing aqueous materials such as cell suspensions (with low cell density) and soluble growth factors. The printing technique can conveniently introduce gradient patterns of cells and growth factors in 3D space by altering droplet size, spacing between droplets, or number of

printing times [21–23]. Inkjet-based bioprinters are also widely used for direct cell dispensing to generate 3D *in vitro* co-culture models [16, 24], vascular tissue models [25, 26], and cell aggregates/spheroids [14, 27] maintaining high cell viability.

2.1.5 Advantages and Disadvantages

Advantages of inkjet-based bioprinting technology include wide availability, high cell viability, low cost, and high printing speed. The printing mechanism allows utilizing various biological materials, especially the materials with lower viscosity. The use of gentle pressure guarantees high post-printing viability, showing a great potential of the technique in handling delicate biostructures such as stem cells, progenitor cells, and embryonic bodies [10, 27, 28]. There are some concerns regarding the instant heat exposure in thermal inkjet printers, 15–25 kHz frequencies used in piezoelectric printers, and shear stress caused by microvalve dispensing. These factors may induce cell damages, phenotype alteration, or loss of functionality. However, the strength of these stresses is minimal in comparison with other commonly-used bioprinting technologies. Due to the material viscosity limitation, the aqueous form of hydrogel precursor is widely used for inkjet-based bioprinting platform. Post-printing crosslinking or gelation process is required in this case. These produces include UV irradiation, temperature changes, or the use of acidic/basic solutions, and may induce various cell damages. Another limitation of inkjet-based printers is the difficulties in achieving physiological cell density and matrix density. High concentration of hydrogel polymers or high density cell suspension often cause issues including nozzle clogging, irregular droplet size, irregular dispensing trajectory, and premature gelation.

2.2 Extrusion-Based Printing

Microextrusion is the most commonly-used technique for non-biological 3D printing. A heated printer head extrudes a filament of materials to build 3D shape in additive manufacturing manner. The technique has been successfully applied in bioengineering field, deriving numerous applications in hard tissue replacement/regeneration and porous scaffold designs [29–32].

2.2.1 Printing Mechanism

Extrusion-based bioprinters consist of 3-axis robotic stages and pneumatic [33–36] or mechanical (piston or screw-driven) [37, 38] dispensing system. Continuous pressure is applied on bioink to extrude filaments (Fig. 1d). This technique results continuous line of materials rather than aqueous droplets. Mechanical dispensing systems generally provide more direct control over the material extrusion through

the printer nozzle because it can avoid the delay of the gas compression occurred in pneumatic systems. Thus, mechanical dispensing methods are thought to be more suitable for printing highly-viscos materials than pneumatic system. Pneumatic systems support wider range of dispensing pressure thanks to simpler components, whereas mechanical systems that consist of more complex components often had limitations on maximum pressure capabilities.

2.2.2 Resolution and Patterning Capability

The resolution of extrusion-based bioprinter is determined by diameter of extruded filaments. Multiple parameters influence on filament dimension, including nozzle orifice size and geometry, the amount of pressure applied, deposition speed, and mechanical properties of bioink [35, 36, 38–41]. The spatial resolution of extrusion-based bioprinters ranges from $\sim 5\text{ }\mu\text{m}$ to 1 mm, showing its potential in various biomedical applications from single cell deposition to bulk scaffold fabrication (Table 1).

The deposition speed is determined by the moving capability of robotic motors, and is a critical factor to decide total printing time as well as the filament diameter. Current extrusion-based technologies provide 10–50 $\mu\text{m}/\text{sec}$ of printing speed [19]. The printing speed could be an obstacle for constructing millimeter- or centi-meter scale of biostructure because it is challenging to maintain cell viability during many hours of printing procedure.

2.2.3 Available Materials

Extrusion-based bioprinting systems support a wide range of viscoelastic properties, with a broad array of biocompatible materials including most of hydrogels described in previous section (2.1.3 Available Materials for inkjet-based bioprinters) and cell spheroids [42]. This type of bioprinters often lose its printing accuracy with low viscos materials as the pressing force of dispensing system may be excessive for those materials [43]. Thus, for direct cell printing, cells are encapsulated within hydrogel to achieve a proper level of viscosity. Materials with shear-thinning properties, which are not suitable for inkjet-based printers, are commonly used for extrusion-based bioprinting platforms.

2.2.4 2D and 3D Cell Printing Applications

Extrusion-based bioprinters have been actively used in the field of tissue engineering, some examples include the fabrication of cardiovascular tissue structure [44–46], *in vitro* multi-layer tissue models [36, 41, 47–49], and 3D cancer co-culture models [50]. The technique has strengths in depositing highly-viscos materials, thus allows achieving physiological cell and matrix density by direct printing. The

printed structure with high density experiences higher level of diffusion limit thus requires interconnected hollow structures for sufficient oxygen and nutrient supply. Various bioprinting methodologies have been developed in order to address the perfusion issue including fabrication of porous scaffolds [32], interconnected channels [51], and vascular networks [26, 44, 52].

The ability to print cell- or tissue-spheroid is one of distinctive features of extrusion-based printer. The technique is capable of creating highly-condensed population of cells and matrices and depositing cell spheroids in desired 3D structure for later tissue merging and maturation process [53–55]. The self-assembling spheroid strategy has a potential in tissue organization by direct printing as the multi-cellular spheroid can serve as a biological unit of much complex tissue or organ structures.

2.2.5 Advantages and Disadvantages

The major advantage of extrusion-based bioprinting technique is the ability to dispense high-viscosity and high-density biomaterials. The mechanical properties and cell densities matching with physiological condition is generally beyond the capability of current bioprinting technologies. Extrusion-based bioprinters have a definite advantage in addressing the density issue. Cell-hydrogel mixture with dense cell population, and cell- or tissue-spheroids are deposited through extrusion approach and allowed to fuse and self-assemble into the desired 3D biostructure [46, 53, 54].

Post-printing cell death is the most critical limitation of current extrusion bioprinting. 40–80 % of cell viability [35] is significantly lower than that of inkjet-based printers (>80 %). The major cell death during the deposition procedure is mainly due to the high dispensing pressure and increase shear stress [35, 39, 56]. The mechanical stresses applied on printed cells may also induce other types of cell damages such as phenotype alterations of stem cells and progenitor cells and loss of cell functionality.

2.3 Laser Direct-Write

2.3.1 Printing Mechanism

Laser Direct-Write (LDW) is a non-contacting method of material deposition that utilizes laser energy absorption to propel a cell-suspended hydrogel droplet to a growth surface. This technique is comprised of two major components: a laser-transparent print ribbon and a receiving substrate. The print ribbon contains both a sacrificial and a transfer layer of material. The laser is pulsed with a configurable energy and repetition rate through the transparent ribbon. The sacrificial layer absorbs the transmitted laser energy, volatilizes, and forms a vapor pocket at the ribbon-material interface. This vapor pocket rapidly expands and ejects a droplet of

the transfer layer to a receiving substrate. The amount of transferred material can be adjusted with modifications to the laser energy profile. Notably, the rate of mass transfer exceeds the transfer of heat and thereby only negligible amounts of laser thermal energy is transmitted to the deposited transfer material.

For print ribbon preparation, a sacrificial and a transfer layer of material are coated onto the ribbon. The ribbon is first thinly coated with a sacrificial layer. This sacrificial layer will be the only material that will interact with the laser during the transfer step and needs to be able to adhere to the transfer layer material. For recent studies involving transfer of viable cells, a cellular suspension is prepared through the use of cultured mammalian cells resuspended in media or a non-cytotoxic hydrogel. The cellular suspension is then distributed evenly onto the sacrificial layer. The receiving substrate is initially prepared with a hydrogel layer to dampen the kinetic energy of the falling droplet of transferred material. This method allows for the deposition of a high-resolution, 2D pattern of cells, or other bio-payload, on the receiving substrate. A recent study involving alginate deposition and a calcium chloride-coated receiving substrate has also demonstrated *in situ* cross-linking of the hydrogel cellular suspension into a 3D microbead [57]. This envelops cells within a 3D isolated microenvironment, and allows custom placement of cells or other bio-payloads on a planar surface. Typically, this surface is a controlled hydrogel microenvironment, which enables delivery, release, or sequestration of the bio-payload. Direct-written microbead fabrication allows media, growth factors, and waste products to diffuse in and out of each microenvironment on the receiving substrate.

2.3.2 Resolution and Patterning Capability

The LDW system has a camera lens that is coincident with the path of the laser. This setup allows direct visualization of either the transfer layer or receiving substrate. The visualization capability also allows control of which regions of the transfer layer are deposited and placed on the substrate. Furthermore, the size of transfer material that is deposited can be precisely controlled through laser energy adjustment via the beam size. In other bioprinting techniques (e.g. inkjet printing), this step is dictated by the limiting size of the nozzle. For LDW, the printed droplet size is controlled by the selected level of transmitted laser energy. To achieve high spatial patterning resolution, the LDW system has the capacity to independently automate movements of the receiving substrate and ribbon platforms. Thus, highly specific and precise spatial patterning can be achieved and programmed through the use of computer-aided design and computer-aided manufacturing (CAD-CAM) technology. Specifically, high resolution spatial patterning can be attained through the extreme precision and accuracy of the LDW deposition technique, coupled with the CAD-CAM-controlled stages. LDW resolution and patterning capability are therefore within the microscopic tolerance scale for the precise spatial patterning of cells.

2.3.3 Available Materials for LDW

For LDW deposition, a variety of biological materials have been successfully printed. These biomaterials include proteins [58], nucleic acids [59], polymer biomaterials [60], and live cells [61]. To enable material deposition, a sacrificial layer is used to absorb energy from the laser. A vapor pocket forms and ejects the desired material (i.e. transfer material) to the receiving substrate. Sacrificial layers can be created from several different biomaterials such as metals [61, 62] and hydrogels such as Matrigel and gelatin [63, 64]. Each has been successful for multiple cell types. Typical transfer materials include media, glycerol, and various hydrogels (e.g. alginate, gelatin, etc.). Notably for the printing of cells, cytocompatible materials are needed. For the substrate, a large range of materials can be used. However, a softer hydrogel layer is often desirable to cushion cells during bioprinting. Matrigel has been a typical selection for a substrate coating, because it meets this requirement and provides a growth surface [61, 62, 65].

2.3.4 2D Cell Printing Applications

Utilizing the full capability of LDW, live mammalian cells can be deposited in prescribed patterns. LDW has demonstrated with a variety of cell types, including epithelial cells [66], endothelial cells [62, 63, 67, 68], fibroblasts [63, 64] neuroblasts [69], and more. LDW can also be a useful platform in studying stem cells, where spatial control of cell location is needed. Specifically, MSCs, mESCs and hESCs have also been successfully printed (Figs. 2 and 3).

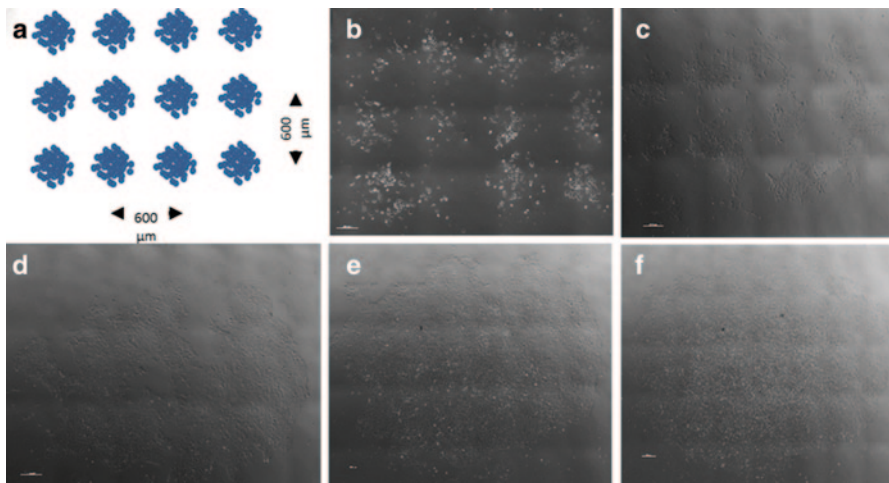


Fig. 2 Grid pattern of hESCs in MEF-CM and bFGF for maintenance of pluripotency, and ROCK inhibitor for survival as single cells, shown **a** schematically and **b–f** under phase contrast microscopy. Cells maintained registry to the initial pattern **b** immediately after printing, but formed a single larger colony over time, showing morphology expected of pluripotent hESCs after **c** 24 h, **d** 48 h, **e** 72 h, and **f** 96 h

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