Laser-based bioprinting for multilayer cell patterning in tissue engineering and cancer research

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1. Summary

- Bioprinting has shown great potential in the construction of biomimetic structures.
- Laser-based bioprinting can achieve both high cell viability and resolution, enabling the printing sophisticated models with high precision.
- Laser-based bioprinting faces many unsolved challenges associated with the preservation of cell function in processed cells.
- Laser-based bioprinting of structures containing one or more cell types has been used in recent years to create artificial tissues and in vitro cancer models.

2. Abstract

3D printing, or additive manufacturing, is a process for patterning functional materials based on the digital 3D model. A bioink that contains cells, growth factors, and biomaterials are utilized for assisting cells to develop into tissues and organs. As a promising technique in regenerative medicine, many kinds of bioprinting platforms have been utilized, including extrusion-based bioprinting, inkjet bioprinting, and laser-based bioprinting. Laser-based bioprinting, a kind of bioprinting technology using the laser as the energy source, has advantages over other methods. Compared with inkjet bioprinting and extrusion-based bioprinting, laser-based bioprinting is nozzle-free, which makes it a valid tool that can adapt to the viscosity of the bioink; the cell viability is also improved because of elimination of nozzle, which could cause cell damage when the bioinks flow through a nozzle. Accurate tuning of the laser source and bioink may provide a higher resolution for reconstruction of tissue that

may be transplanted used as an *in vitro* disease model. Here, we introduce the mechanism of this technology and the essential factors in the process of laser-based bioprinting. Then, the most potential applications are listed, including tissue engineering and cancer models. Finally, we present the challenges and opportunities faced by laser-based bioprinting.

3. Introduction

According to official statistics, 80,000 individuals in the United States required organ transplantation because of a disease or an accident, with less than a third receiving one from 2000 to 2001. Tissue engineering (TE) (2) was conceived to generate artificial tissue or organs in vitro. Guiding the self-organization of stem cells to normal tissue (3) and constructing a scaffold or environment for cells to develop through bioprinting are the most common methods.(4) As for bioprinting, there are three approaches: autonomous self-assembly, biomimetic processing, and mini-tissues that can be applied to bioprinting.(5) The principle of these approaches is to build a proper environment or scaffold for cells to develop. Collagen, growth factors, and glycoprotein are components of the extracellular matrix (ECM), which supports cell activity.(6, 7) The main steps in bioprinting include imaging, design, printing of the tissue, and use with patients as shown in Figure 1. Structural data collected from the human body through 3D imaging systems such as computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound (US) can be used to create a digital representation of tissue architecture.(8) Finally, cells and biomaterials are patterned in a layer by layer manner into a tissue-like structure.

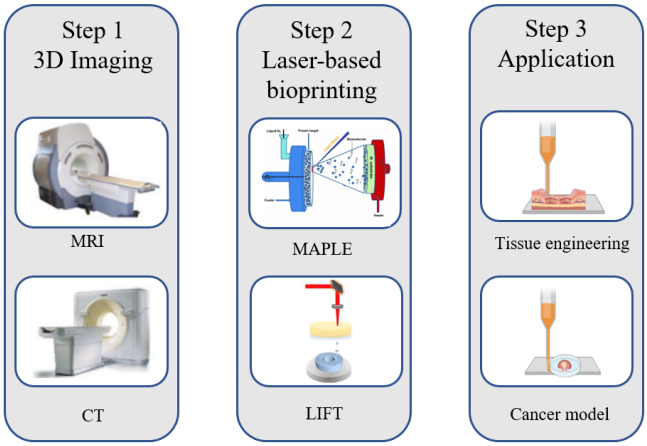

Fig 1. The main process of laser-based bioprinting. The first step is data collection; MRI and CT are used for imaging the targeted tissue and environment to guide the bioprinting. Then, bioink is patterned into the desired structure. There are two commonly used laser-based bioprinting methods, namely MAPLE-based and LIFT-based methods. After the 3D model is constructed, it can be used for tissue repair or as an in vitro cancer model. The LIFT image recreated from (9) tissue engineering and the cancer model image are generated using BioRender.com

After the concept of bioprinting was initially described, a variety of bioprinting techniques have emerged in recent years. Extrusion-based bioprinting, inkjet bioprinting, and laser-based bioprinting (LBB) have emerged as powerful technologies for biological reconstruction with micrometer or sub-micrometer scale resolution. (10)

In addition, electrospinning is used to create nanofibers; scaffolds containing nanofibers, living cells, and signaling molecules for tissue repair and wound healing.(11) Nanofibers with core–sheath structures have been prepared; core–sheath nanofibers may possess better biological functionality or mechanical properties than single-phase nanofibers.(12) A pressurized gyration approach been recently developed to create nanofibers to produce scaffolds at a higher rate and with lower power consumption than electrospinning.(13)

Matrix-assisted pulsed-laser evaporation direct-write (MAPLE DW) and laser induced forward transfer (LIFT) are types of LBB for nozzle-free direct writing of the matter. MAPLE DW is a direct-write approach that can construct sophisticated tissue structures. The reliability and high precision of MAPLE DW permit its use for processing biomaterials and living cells. (14) LIFT was applied for metal deposition in 1986 (15); it has been successfully tested for the deposition of different materials, mainly metals (16-20), ceramics, and high-temperature superconductors (21). This technology has also been demonstrated to be able to be an efficient tool for liquid-phase or solid-phase material deposition. A wide range of biomaterials such as proteins and DNA can be patterned into 2D and 3D structures with high resolution through LBB.(22) A typical LIFT bioprinting apparatus consists of a laser source, donor ribbon, and collector substrate. The laser source provides the energy for the device to propel the bioink droplet toward a collector substrate. The donor ribbon is both the carrier and the protector of the bioink. An energy absorption layer (EAL) can be added in the middle of the bioink layer and the donor ribbon, which is used to absorb the laser energy and prevent negative effects on cell viability. The collector substrate contains a donor ribbon to receive the bioink droplets. For the sake of minimizing the influence of laser on cell viability, absorption film-assisted LIFT (AFA-LIFT) (23) and blister-actuated LIFT (BA-LIFT) (24) were proposed by adding an energy absorption layer (EAL).

The mechanism and application of LBB have been actively investigated by researchers; however, the complexity of LBB is a largely unexplored area of bioprinting research. This review considers the mechanism and factors

that have a deep influence on the final result in LBB. Furthermore, based on engineered tissues and cancer models, we summarize the applications of LBB. Finally, the challenges and prospects of LBB technology are addressed.

4. Printing mechanisms

The jetting dynamic of targeted material driven by laser printing has been studied by fast-imaging acquisition.(25-27) The process of laser printing can be considered in three parts: 1) application of a high energy laser pulse to a thin biomaterial layer, 2) high-pressure bubble generation, and 3) ejection of a bioink droplet (Figure 2). In the first stage, after the focused laser irradiates the interface between the donor ribbon and the bioink layer, the bioink or EAL absorbs the energy transferred from the laser source. As a result, a steep increase of temperature within a small volume of bioink is generated. Due to rapid vaporization of bioink, a tiny bubble with high pressure will be formed in the bioink layer.(28) Owing to the presence of donor ribbon, the bubble tends to expand toward the free surface of the bioink layer, pushing the bioink vertically. Consequently, a gradient of pressure appears between the bubble sides and its pole; a liquid stream converges at the pole along the bubble walls and increases the pressure at the pole. When the pressure reaches the critical point of bubble collapse, two jets moving in opposite directions are generated. (29) The jet moves from the donor layer at high speed, resulting in ink deposition.

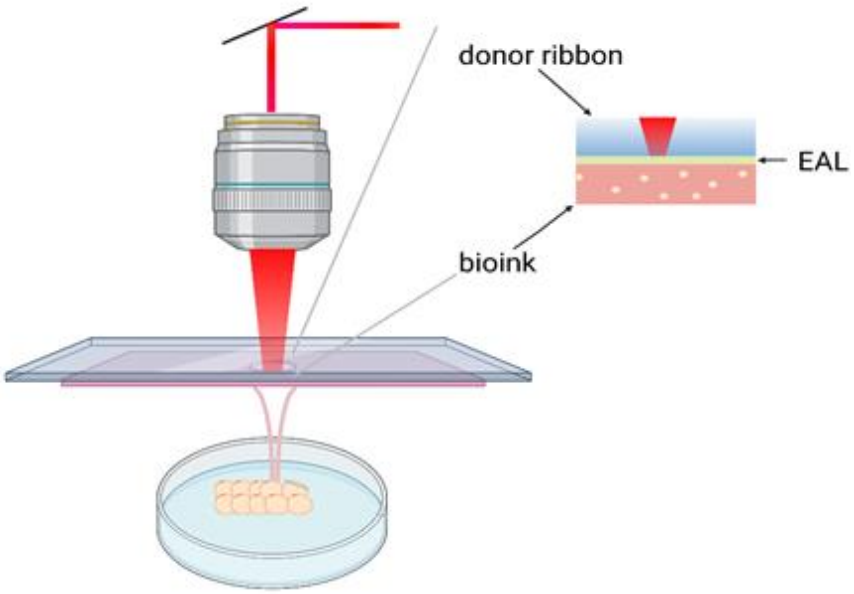

Fig 2. Schematic of LBB, the typical apparatus of LBB consists of three parts, laser source, donor ribbon, and collector substrate. The bioink layer is attached to transparent donor ribbon; an EAL can be added between the donor ribbon and the bioink layer. The laser is focused on the interface between EAL and bioink layer; a tiny bubble is generated by a steep temperature increase, and a jet of material is directed towards the collector substrate. With proper tuning of the laser and the bioink, a homogeneous voxel is printed. The image was created with BioRender.com.

Many improvements have been made to LIFT. For example, absorbing film-assisted laser induced forward transfer (AFA-LIFT), which uses a sacrificial layer of a metal on the ribbon to interact with the laser, improved the cell viability by preventing direct interaction between the bioink and the laser. In (30), silver films with 50–400 nm thickness were applied as absorbing layers. Blister-actuated LIFT (BA-LIFT) contains a thick layer that isolates the cavitation bubble from the bioink (31); a layer of polyimide tape is treated as a thick absorption layer where the blister is formed. The laser energy is absorbed by the thick layer completely; therefore, the shear force of the bubble has a limited effect on cells. The cell membrane may be negatively affected when it is exposed to the stress waves by bubble expansion, creating membrane permeability that enables either permanent rupture or detrimental influx of medium.(32) In the laser induced backward transfer (LIBT) setup, the collector is transparent to the laser wavelength; the laser energy moves through the collector plate and is focused on the donor substrate.(33) In (34), an infrared femtosecond laser is treated as an energy source, and the donor substrate consists of a flat glass slide where a washer has been glued, forming a tank recipient which contains bioink. No absorbing layer is used; the laser is focused on various depths of the bioink to determine the optimal processing parameters. Laser induced side transfer (LIST) utilizes low-energy laser pulses to create a transient micro-bubble at the distal region of a glass microcapillary that contains the bioink (Figure 3 (a)). This process leads to the ejection of the cell-containing micro-jet in a direction that is perpendicular to the irradiation axis.(35) LIST is similar inkjet bioprinting; it treats the glass microcapillary as a nozzle rather than a nozzle-free printing method like LIFT bioprinting. The laser replaces the microheater or a piezoelectric transducer as an energy source to generate bubbles to deposit bioink on the substrate. High-viscosity bioinks were noted to also be suitable for LIST; no

bioink clogging issues were noted.(35) Matrix-assisted pulsed laser evaporation-direct-write (MAPLE-DW) is a laser-based forward transfer direct writing approach that involves bioink processing in ambient air; this approach was introduced by Chrisey et al.(36) In this approach, the laser beam softly transfers a micrometer-dimension amount of bioink to the substrate.(37) The basic setup of MAPLE-DW, which is similar to that of AFA-LIFT, utilizes a low-powered pulsed laser in the ultraviolet or near-ultraviolet region (Figure 3 (b)).

Several significant strengths are associated with LIFT. The use of an EAL is associated with minimal effect on the cell viability or function; this approach is capable of positioning small droplets containing cells and/or biomaterials at high resolution (e.g., resolution at the single-cell level).(38) Patterned cells and biomaterials can be used to generate precise vitro models to explore intercellular communication and cell-environment interactions that can enable better understanding of disease and human development processes.(39) Furthermore, this approach provides precise control over the deposition of high-viscosity material and deposition of cells with high densities.(40)

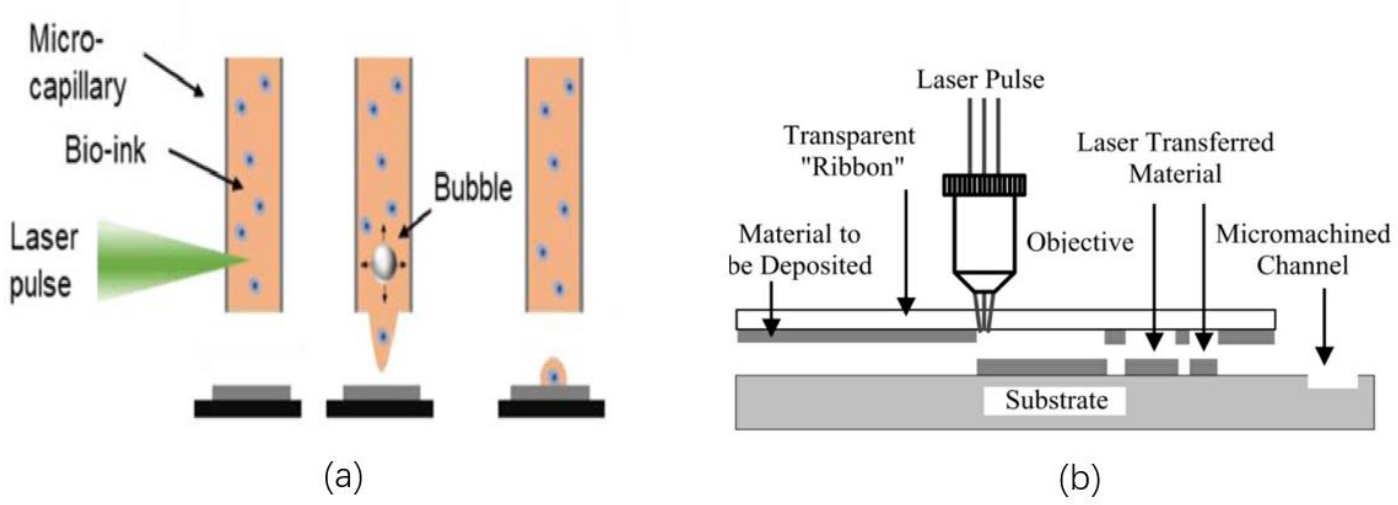

Fig 3. (a) Overview of LIST. The laser is focused on the capillary distal end to generate a small bubble; a small volume of bioink is pushed towards the substrate.(35) (b) Overview of MAPLE DW system.(41)

Optimization of the bioink viscosity, laser pulse energy, and other processing parameters can minimize cell or DNA damage for tissue engineering applications. (42) For example, the laser source, the optical focusing scheme,

the pulse duration, and the laser wavelength are important parameters.(43) 1064 nm (44-46) and 193 nm (47-49) laser source is used by most researchers for bioprinting applications.(50)

It was shown that infrared lasers do not affect the physicochemical characteristics of bioinks.(51) The energy of the 2940 nm laser is mostly absorbed by water, enabling bioprinting with hydrogel EALs or the avoidance of the use of EALs.(52) Ultraviolet lasers emit light with a higher energy density; in the absence of an EAL, ultraviolet light-bioink interactions may be associated with DNA double-strand breaks. (50) The 193 nm wavelength laser energy is absorbed by many hydrogels; this laser is compatible with hydrogel EALs or the avoidance of the use of EALs.(47) The pulse duration of the laser also defines the LBB transfer dynamics.(51) Nanosecond and femtosecond laser have been applied for LIFT bioprinting; femtosecond laser sources may also be useful for LIFT bioprinting.(54)

In addition to the laser source, the transfer process is also affected by the bioink thickness on the ribbon and the rheological behavior of the bioink (50); altering the rheological behavior of the bioink changes the jetting dynamics, droplet formation, and optimal processing regime. Generally, bioinks consist of cells as well as materials such as hydrogels; alginate, gelatin, Matrigel®, and collagen are commonly used in laser-based bioprinting; the cellular viability rate is affected by the type of material.(53) The hydrogel concentrations could affect the formation of droplets of the bioink. The viscosity of hydrogel is highly related to its concentration.(40) For example, higher alginate concentrations resulted in lower cell viability levels; this phenomenon was associated with nutrient transport limitations at higher alginate concentrations.(55)

Cells are another crucial component of bioinks. For tissue engineering, cell type depends on the study goals and cell availability. Animal-derived cells such as mouse fibroblasts (NIH3T3) and mouse bone marrow-derived mesenchymal stromal cells (mBE-MSC) are used in many LIFT bioprinting studies. The concentration and distribution of cells in bioinks also affect the process of bioprinting; avoiding local variations in the homogeneity of cell-laden bioinks is an important parameter in bioink development.(47)

5. Applications

Tissue engineering

LBB has the capability for printing a combination of cells with other biological compounds in a layer-by-layer fashion to generate living structures for the replacement of diseased or defective biological tissues.(56) For example, Sorkio et al. used a system with a 2940 nm laser and hydrogel EAL containing glycerol and Matrigel® to fabricate 3D cornea-mimicking structures.(45) Like building blocks, Pirlo et al. stacked 2D bio-papers to form a 3D structure.(57) The stacking structures supported the formation of human umbilical vein endothelial cell (HUVEC) networks. Wu and Ringeisen fabricated 3D branch structures through the co-culture of human umbilical vein smooth muscle cells (HUVSMCs) and HUVECs.(58) Tissue patches are also promising structures for use in tissue engineering. Gaebel et al. fabricated a cardiac patch containing HUVECs and hMSCs; they printed the cells onto a defined-pattern scaffold made from polyester urethane urea; the patch was transplanted for healing infracted rat hearts.(59) Laser-based bioprinting has also been applied to bone regeneration. Enhancement of infarcted heart function after application of the patches was demonstrated. Keriquel et al. showed that LIFT can be used as an in situ surgical therapy. Mesenchymal stromal cells were be printed along with nanoscale hydroxyapatite and collagen to facilitate bone regeneration in a murine calvaria defect model; this in situ approach shows great potential for tissue repair.(60)

Cancer models

The mechanisms associated with the development and growth of tumors have not been fully understood. 3D bioprinting can also be used to build disease models such as cancer models to study cell-cell interactions and cell-environment interactions.(39). Bioprinting can be used to create cancer models with an appropriate of cells and biomaterials in various regions. This approach be used to build an in vitro replica of a tumor. Furthermore, the characteristics of the tumor microenvironment that affect the biological function of the tumor may be replicated.(61) In addition, the high resolution of LBB makes it a useful tool to build a cancer model that enables the evaluation of cancer cell interactions with normal cells.

In (62), a single layer of mouse cancer cells was printed using LBB to verify the feasibility of a heterogeneous multilayer cancer model; this approach showed that the embryonal carcinoma cells retained their differentiated features after printing. In (63), human osteosarcoma (MG-63) cells were patterned into a three-dimensional structure. The cells retained high viability rates after processing; more than 95% of printed cells exhibited normal function. Based on LBB, a miniaturized spheroids array model of pancreatic ductal adenocarcinoma was constructed to study the factors that contribute to cancer progression.(64) This cancer model in vitro could also be used to understand the effect of chemotherapy on cancer cells. In (61), a human breast cancer model was prepared using LBB to investigate the relationship between tumor size and drug action. Core-shell structures containing self-aggregating embryonic stem cells and breast cancer cells were demonstrated. Variations in the uptake of transferrin, a ligand for receptor-mediated delivery, were associated with differences in spheroid size.

6. Conclusions

Current limitations

1. The long-term impact on cell function after the LBB process needs to be addressed. Gene expression and other function-specific markers can be used to evaluate cells after printing to demonstrate the functionality of LBB.
2. Efforts have been made to optimize the laser and bioink for each LBB application. The laser and bioink are two important factors that define cell viability and printing resolution associated with the LBB process.

Prospects

1. As mentioned above, the development of complex multicellular systems using LBB is a focus of recently published and ongoing research efforts. Many natural tissues contain several cell types that form a complex microenvironment; cell-cell interactions enable the normal function of these tissues. Since each cell type may require a different culture environment, balancing the environmental parameters in systems containing multiple cell types remains a challenge.
2. Computer-aid cell kinetics modeling is essential for understanding the function of LBB-processed structures. For example, cells will migrate, proliferate, and undergo apoptosis after the LBB process. In addition, the

LBB-processed structure will undergo structural changes due to cell metabolism over time. Predicting the structure and function of LBB-processed structures over time will enable the development of structures with enhanced functionality.

LBB is an emerging approach for bioprinting; although LBB technology is associated with several limitations, it shows significant promise for use in both tissue engineering and as a manufacturing approach for in vitro models.

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Author Contributions

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